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MOLECULAR MECHANISMS OF THE REGULATION OF EXPRESSION OF THE ANGIOTENSINOGEN GENE IN THE KIDNEY: EFFECT OF CATECHOLAMINES (*IN VITRO*)

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To my baby, she made me realize miracles could happen;

To my husband, he made me realize what I had been looking for for so long.

RÉSUMÉ

Le système rénine-angiotensin-aldostérone (RAS) est reconnu pour coordonner stimultanément la régulation de sodium et de l'homéostasie du volume sanguin, de la pression artérielle, et de la balance en potassium. La présence d'un système RAS intra-rénal est maintenant acceptée et les tubules proximaux du rein constituent le site de production de l'angiotensinogène (ANG, précurseur de l'angiotensine II (Ang II)). Les tubules proximaux de rein constituent une zone hautement innervée.

Le but de cette étude est d'examiner si l'expression du gène de l'ANG est modulée par les catécholamines et d'examiner le mécanisme moléculaire de l'effet des catécholamines en utilisant un système cellulaire *in vitro* .

Premièrement, nous avons introduit le gène de fusion composé d'un fragment d'ANG de rat d'approximativement 1500 paires de bases, fragment situé sur la partie 5' flanquante d'ANG en amont du site de transcription, relié au gène rapporteur de l'hormone de croissance (hGH), pOGH (ANG N-1498/+18) dans les cellules de tubules proximaux du rein d'opossum (OK). La lignée cellulaire transformée, OK 27, a été employée pour l'étude actuelle et l'expression du gène de fusion ANG-GH a été évaluée par la présence de l'hGH immuno-réactive secrétée dans le milieu.

L'iodoclonidine, un agoniste des récepteurs α_2-adrénergiques (α_2-AR) a un effet de stimulation sur l'expression du gène de l'ANG. Cet effet peut être complètement bloqué par le yohimbine (antagoniste α_2-AR) et aussi être bloqué par le staurosporine (un inhibiteur de voie de protéine kinase C). Nos résultats ont aussi montré que la dopamine et les agonistes des récepteurs dopaminergiques DA_1 (SKF-82958) et DA_2 (PPHT) peuvent stimuler l'expression du gène de fusion ANG-GH et que ces effets peuvent être bloqués par leurs antagonistes (SCH-23390, spiperone) respectifs. L'effet du SKF-82958 peut aussi être bloqué par le Rp-cAMP (un inhibiteur des voies

des protéines kinases A I et II AMPc-dépendants). L'effet du PPHT peut aussi être bloqué par la staurosporine. Nos résultats ont aussi montré que la Norépinephrine (NE) peut stimuler l'expression du gène de fusion ANG-GH et que cet effet peut être bloqué par les antagonistes des récepteurs adrénergiques α_2 et β_1 (Atenolol). L'effet de NE peut aussi être bloqué par le Rp-cAMP et le staurosporine. .

Tous ces résultats tendent à confirmer la présence des récepteurs adrénergiques α_2 et β_1 sur les cellules OK, ainsi que des récepteurs dopaminergiques DA_1 et DA_2. La régulation du gène de l'ANG implique aussi l'action via les voies des deux protéines kinases A et C. Nos résultats ont aussi montré que les catécholamines ont un effet de stimulation direct sur l'expression du gène de l'ANG dans les cellules OK 27.

Deuxièmement, nous avons obtenu plusieurs lignées cellulaires transformées par introduction des gènes de fusions composés des fragments d'ANG de rat relié au gène rapporteur de l'hormone de croissance dans les cellules OK. Ce sont les lignées OK 960 (pOGH (ANG N-960/+18)), OK 280 (pOGH (ANG N-280/+18)) et OK 53 (pOGH (ANG N-53/+18)). Nos résultats ont montré que la NE peut stimuler l'expression du ANG-GH dans les cellules OK 960.

Troisièmement, nous avons obtenu plusieurs lignées cellulaires transformées qui sont OK 95 (pOGH (ANG/CRE/-53/+18)), OK 95/M1 (pOGH (ANG/CREM1/-53/+18)), OK 95/M2 (pOGH (ANG/CREM2/-53/+18)) et OK 95/M3 (pOGH (ANG/CREM3/-53/+18)). Elles sont composées du gène de fusion du CRE (cyclic AMP responsive element) putatif du gène de l'ANG (ANG-CRE, N-806/-779) ou des mutants du CRE (M1, M2, M3) insérés en amont du promoteur fondamental du gène de l'ANG (N-53/+18) relié au gène rapporteur de l'hormone de croissance dans les cellules OK. Nous avons montré que l'isoproterenol (ISO) (agoniste des ß-ARs) peut stimuler l'expression du ANG-GH dans les cellules OK 95 et les cellules OK 95/M1. Ces résultats suggèrent

que l'ANG N-806/-779 est le ANG-CRE.

L'expérience de transfection de pRSV-CREB (le plasmide d'expression contenant le ANDc de 43-kDa-CREB, CRE binding protein) en les cellules OK 27 améliore davantage l'effet de l'ISO sur l'expression de pOGH (ANG N-1498/+18).

Finalement, nous avons introduit les deux plasmides, le pRSV/CREB et le pOGH (ANG/CRE/-53/+18) ou pOGH (ANG/CREM1/-53/+18) ou pOGH (ANG/CREM2/-53/+18) ou pOGH (ANG/CREM3/-53/+18) dans les cellules OK. Les transformants stables sont OK 96, OK 96/M1, OK 96/M2 et OK 96/M3 respectivement. Nous avons montré que l'ISO peut stimuler l'expression du ANG-GH dans les cellules OK 96 et les cellules OK 96/M1. Ces résultats suggèrent que l'effet du CREB sur l'expression du gène de l'ANG est médiatisé par le lien de CREB au CRE du gène de l'ANG.

Les études de transfection ont démontré que le plasmide de la sous-unité catalytique de PKA (protein kinase A), pRSV/Catß, stimule directement l'expression des gènes de fusion de l'ANG dans les cellules OK 27, OK 95 et OK 96. Ces résultats indiquent que le PKA est impliquée dans la régulation d'expression du gène de l'ANG et l'effet de ß-AR est médié par la phosphorylation de CREB et le CRE du gène de l'ANG.

L'essai de Mobility Shift a montré que l'extrait nucléaire de cellule OK communique avec le ^{32}P-ANG-CRE. Les expériences de Southwestern Blot ont montré que le ^{32}P-ANG-CRE se lie aux protéines nucléaires des cellules OK avec un poids moléculaire apparent de 43-kDa. L'anticorps polyclonal contre le 43 kDa-CREB interagit avec le 43 kDa espèce moléculaire. Ces données démontrent que l'effet des ß-ARs est probablement médié par la phosphorylation de protéine nucléaire CREB. Le CREB activé se lie alors au ANG-CRE pour déclencher l'expression du gène.

Nos études suggèrent que l'activation des nerfs au niveau rénal peut stimuler l'expression du gène de l'ANG localement via la voie de PKA-AMPc-dépendante et via la phosphorylation de CREB. L'activé CREB alors déclenche l'expression du gène. La production accrue de l'ANG active le RAS rénal qui lui meme augmente le niveau de l'Ang II. La production d'Ang II rénale pourrait alors moduler la physiologie des cellules de tubules proximaux (i.e. réabsorption de sodium et de fluides extracellulaires).

ABSTRACT

The renin-angiotensin-aldosterone system (RAS) simultaneously coordinates the regulation of sodium and volume homeostasis, blood pressure, and potassium balance. In the kidney, the presence of a local RAS has been accepted and renal proximal tubules are found to be the site of angiotensinogen (ANG, the precursor of Angiotensin II (Ang II)) production. The renal proximal tubules are richly innervated by the sympathetic nerve endings.

The objective of our present study is to investigate whether the expression of renal ANG gene is regulated by catecholamines and the molecular mechanism(s) of the effect of catecholamines on the ANG gene expression by using an *in vitro* cell system.

Firstly, we introduced permanently a fusion gene containing ~1500 base pairs of the 5'-flanking sequence upstream of the transcription starting site of the rat ANG gene linked to a human growth hormone (hGH) reporter gene (pOGH (ANG N-1498/+18)) into opossum kidney (OK) proximal tubular cells. This permanent cell line, OK 27, was used for the present studies and the expression of the ANG-GH fusion gene was detected by immuno-reactive-hGH secreted into the medium.

We found that iodoclonidine, the agonist of α_2-adrenoceptors (α_2-AR), has a stimulatory effect on the expression of the ANG gene, and this effect can be blocked by yohimbine (α_2-AR antagonist) and by staurosporine (an inhibitor of protein kinase C). We also found that both DA_1 and DA_2 receptor (dopaminergic receptor subtypes) agonists (SKF-82958, PPHT, respectively) and dopamine stimulate the expression of ANG-GH gene, and the effect can be blocked by their antagonists, SCH-23390 and spiperone, respectively. Moreover, the effect of DA_1 and DA_2 receptor agonists can be blocked by Rp-cAMP (an inhibitor of cAMP-dependent protein kinase AI and II) and staurosporine, respectively. We also demonstrated that norepinephrine (NE) stimulates

the expression of ANG-GH gene in OK 27 cells and the stimulatory effect can be blocked by the presence of β_1- and α_2-adrenoceptor antagonists, as well as Rp-cAMP and staurosporine.

These data confirm the presence of β_1- and α_2-ARs, DA_1 and DA_2 receptors in the OK cells. Both cAMP-dependent protein kinase A (PKA) and protein kinase C (PKC) pathways are involved in the regulation of the ANG gene. The addition of catecholamines stimulated the expression of the ANG gene in OK 27 cells.

Secondly, we obtained several other permanent cell lines with different fragments of the ANG 5'-flanking sequence fused with a growth hormone gene as reporter, these are OK 960 (pOGH (ANG N-960/+18)), OK 280 (pOGH (ANG N-280/+18)), and OK 53 (pOGH (ANG N-53/+18)). We demonstrated that NE stimulates the expression of the ANG-GH fusion gene in OK 960 cells, but not in OK 280 and OK 53 cells, respectively.

Thirdly, we obtained more permanent cell lines, these are OK 95 (pOGH (ANG/CRE/-53/+18)), OK 95/M1 (pOGH (ANG/CREM1/-53/+18)), OK 95/M2 (pOGH (ANG/CREM2/-53/+18)) and OK 95/M3 (pOGH (ANG/CREM3/-53/+18)) with the plasmid containing the ANG putative CRE (cyclic AMP responsive element) (ANG-CRE, N-806/-779) or mutants of the ANG-CRE (M1, M2, M3) inserted upstream of the basal promoter of the ANG gene (N-53/+18) fused with a growth hormone reporter gene integrated into the cell genome. We found that isoproterenol stimulates the expression of the ANG-CRE in OK 95 and OK 95/M1 cells but not in OK 95/M2 or OK 95/M3 cells. These results suggest that ANG N-806 to N-779 is the ANG-CRE.

Transfection of pRSV-CREB (the expression plasmid containing the cDNA for 43-kDa-CREB, CRE binding protein) into OK 27 cells further enhances the effect of isoproterenol on the expression of pOGH (ANG N-1498/+18).

Finally, we integrated both pRSV/CREB and pOGH (ANG/CRE/-53/+18) or pOGH (ANG/CREM1/-53/+18), or pOGH (ANG/CREM2/-53/+18), or pOGH (ANG/CREM3/-53/+18) into the OK cells, and obtained the permanent cell lines, OK 96, OK 96/M1, OK 96/M2 and OK 96/M3, respectively. We found that isoproterenol stimulates the expression of ANG-GH fusion genes in OK 96 and OK 96/M1 cells but not in OK 96/M2 or OK 96/M3 cells. These results suggest that the effect of CREB on the ANG gene expression is mediated via the binding of the CREB to the ANG-CRE.

Transfection studies demonstrated that the expression plasmid of Protein Kinase A (PKA) catalytic subunit, pRSV/Catß, directly stimulates the ANG gene expression in OK 27, OK 95 or OK 96 cell lines. These results indicated that cAMP-dependent PKA is involved in the expression of the ANG gene and the effect of ß-AR is mediated via the phosphorylation of the CREB and the CRE of the ANG gene.

Mobility shift assay showed that the OK cell nuclear extract interacted with the ^{32}P-ANG-CRE. Southwestern blot experiments showed that ^{32}P-ANG-CRE binds to OK cellular nuclear proteins with an apparent molecular weight of 43 kDa and the polyclonal antibodies against the 43 kDa-CREB interacts with the 43 kDa molecular species. These data demonstrate that the effect of ß-ARs is probably mediated via the phosphorylation of nuclear protein CREB. The activated CREB then bind to ANG-CRE to trigger the gene expression.

Our studies raise the possibility that the activation of renal nerves in proximal tubular cells may stimulate the expression of the renal ANG gene via the cAMP dependent PKA signal transduction pathway and the phosphorylation of CREB. The phosphorylated CREB then binds to the ANG-CRE and triggers the gene transcription. The increased production of the ANG will activate the renal RAS to augment the local level of Ang II. This local formation of renal Ang II might then modulate the physiology of the renal proximal tubular cells (i.e. sodium and fluid reabsorption).

ABBREVIATIONS

AC	adenylyl cyclase
AGE	angiotensinogen gene-activating element
AGF	angiotensinogen gene-activating factor
ANG	angiotensinogen
Ang	Angiotensin
APR	acute-phase response
APRE	acute-phase response element
AR	adrenergic receptor
AT_X	Ang II receptor subtype X
bZIP	basic region-Lucine zipper
CaM	Ca^{2+}-calmodulin-dependent protein
CBP	CREB binding protein
CRE	cAMP responsive element
CREB	cAMP responsive element binding protein
CREM	CRE modulator
DAG	diacylglycerol
GR	glucocorticoid receptor
GRE	glucocorticoid responsive element
IBMX	3-isobutyl-1-methylxanthine
ICER	inducible cAMP early repressor
IP_3	inositol 1,4,5-trisphosphate
JG	juxtaglomerular
MAP	mitogen-activated protein
NE	Norepinephrine
PI	phosphoinositides
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLX	phospholipase X
PT	proximal tubule
RAS	Renin-angiotensin system
SHR	spontaneous hypertensive rat
SNS	sympathetic nervous system
ßARK	ß-adrenergic receptor kinase
TPA	12-O-tetradecanoyl phorbol-13-acetate
TRE	TPA-inducible promoter element

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INTRODUCTION

I. KIDNEY AND RENAL FUNCTION

The cells of our body live in an internal environment which provides the medium necessary for the normal functioning of the cells. Maintenances of the constancy of this internal environment, so-called "homeostasis", is ensured by several body systems, in which, the kidneys play a vital role.

In fact, the kidneys can reasonably be regarded as the most important regulatory organs for controlling the internal environment, since they control not only the concentration of waste products of metabolism, but also the osmolality, volume, acid-base status and ionic composition of the extracellular fluid and, indirectly, regulate these same variables within the cells (Lote, 1994).

I.1. Structure and Function of the Kidney

The functional unit of the kidney is the nephron. The essential components of the nephron are the renal corpuscle (glomerulus and Bowman's capsule), the proximal tubule, the loop of Henle, the distal tubule, and the collecting duct.

The glomerulus is composed of a capillary network lined by a thin layer of endothelial cells; a central region of mesangial cells with surrounding mesangial matrix material; the visceral epithelial cells and the associated basement membrane; and the parietal layer of Bowman's capsule with its basement membrane. The function of the glomerulus is to ultrafiltrate the plasma which enters the nephrons.

Ultrafiltration occurs from the glomerulus into the Bowman's capsule. An almost protein-free ultrafiltrate passes into Bowman's capsule from the glomerular capillaries. Since the glomerular filter permits the free passage of molecules of molecular weight less than 7000, the initial glomerular filtrate will contain small molecules and ions (e.g. glucose, amino acids, urea, sodium, potassium) in almost exactly the same concentra-

tions as the afferent arteriolar concentrations and, similarly, the efferent arteriolar concentrations of such substances will not have been significantly altered by the filtration process (Lote, 1994).

The mesangial cells and their surrounding matrix material constitute the mesangium, which is separated from the capillary lumen by the endothelium. In addition to the usual organelles, the mesangial cell contains numerous filaments, some of which resemble myosin filaments. The mesangial matrix contains sulfated glycosaminoglycans as well as fibronectin and laminin (Pease, 1968; Farquhar, 1981; Madri, 1980).

The juxtaglomerular (JG) apparatus is located at the vascular pole of the glomerulus, where a portion of the distal nephron comes into contact with its parent glomerulus. It has a vascular and a tubule component. The vascular component is composed of the terminal portion of the afferent arteriole, the initial portion of the efferent arteriole, and the extraglomerular mesangial region. The tubule component is the macula densa, which is the portion of the thick ascending limb that is in contact with the vascular component (Barajas, 1989).

The JG apparatus represents a major structural component of the renin-angiotensin system (RAS). According to the macula densa theory, changes in the sodium or chloride concentration at the macula densa can control the intrarenal release of renin; according to the stretch-receptor theory, alterations in the volume and stretch of the afferent arteriole influence the control of renin release (Schnermann, 1985; Fray, 1976).

The loop of Henle is generally considered to begin at the transition from thick-walled tubule to thin-walled tubule. The descending thin limb is permeable to water but has a low permeability to sodium chloride which allows water to be extracted from the tubular fluid as the thin limb descends through the hypertonic interstitium of the medulla. In

contrast, the ascending thin limb is largely impermeable to water, but highly permeable to sodium chloride, which causes salt to diffuse out of the tubule. Thus, the thin limb plays an important role in the maintenance of a hypertonic medullary interstitium and in the delivery of a dilute fluid to the distal tubule. The fluid entering the loop of Henle is isotonic to plasma, but after traversing the loop, fluid entering the distal tubule is hypotonic to plasma.

The distal tubule contributes importantly to renal sodium and chloride reabsorption and potassium secretion. The thick ascending limb of the distal tubule has a higher urea permeability in the cortex and in the outer stripe of the outer medulla than in the inner stripe of the outer medulla, suggesting a correlation between the increase in apical surface area, lateral intercellular space, and urea permeability (Knepper, 1983). The distal convoluted tubule has a higher Na^+-K^+-ATPase activity than any other segment of the nephron (Katz, 1979). There are at least four different pathways mediate Na, K and Cl transport across the luminal membrane of distal tubule cells. Two pathways permit diffusive movement of cations, one an amiloride inhibitable channel for sodium, the other a barium inhibitable channel for potassium. Two others are mediate coupled K-Cl secretion and Na-Cl absorption across the apical membrane (Ellison,1987).

The collecting tubules are relatively impermeable to water, urea and NaCl, but the water permeability is increased by antidiuretic hormone. Thus, antidiuretic hormone leads to urine concentration by permitting the osmotic abstraction of water into the interstitium (Lote, 1994).

I.2. Proximal Tubule

The proximal tubule (PT) consists of an initial convoluted portion, the pars convoluta, and a straight portion, the pars recta.

The proximal convoluted tubule plays a major role in the reabsorption of sodium, bicarbonate, chloride, potassium, calcium, phosphate, water, and organic solutes such as glucose and amino acids. Approximately half of the ultrafiltrate is reabsorbed in the PT. Sodium reabsorption by the PT is an active process driven by Na^+-K^+-ATPase, which is located in the basolateral plasma membrane (Kinne, 1971).

The PT reabsorbs approximately 50 to 60% of the solutes and water filtered at the glomerulus. The active extrusion of Na^+ from the tubular cells into the lateral intercellular spaces provides the driving force for this reabsorption of water, much of which passes through the cells (Lote, 1994).

I.3. Renal Function and Control

The renal vasculature and glomerular mesangium respond to a number of endogenous hormones and vasoactive peptides by undergoing vasoconstriction and reductions in the glomerular ultrafiltration coefficient. Among these compounds are angiotensin II (Ang II), norepinephrine (NE), leukotrienes C_4 and D_4, platelet activating factor, endothelin, vasopressin and epidermal growth factor.

II. RENAL NERVOUS SYSTEM AND RENAL FUNCTION

II.1. Renal Nerve Distribution

The renal nerves are the communication link between the central nervous system and the kidney. In response to multiple peripheral and central inputs, efferent renal sympathetic nerve activity is altered so as to convey information to the major structural and functional components of the kidney, the vessels, glomeruli, and tubules, each of which is innervated. In response to normal physiological stimuli, changes in efferent renal sympathetic nerve activity contribute importantly to homeostatic regulation of renal blood flow, glomerular filtration rate, renal tubular epithelial cell solute and water

transport, and hormonal release. Whereas afferent input from sensory receptors located in the kidney participates in this reflex control system via renorenal reflexes that enable total renal function to be self-regulated and balanced between the two kidneys (DiBona, 1997).

The adrenergic innervation of the renal vasculature is distributed throughout the renal cortex and outer stripe of the renal medulla, with the greatest density being seen in the juxtamedullary region of the inner cortex (Moffat, 1967; Niewstead, 1969). NE is the important neurotransmitter since decreasing renal sympathetic nerve activity to zero by chronic renal denervation reduced renal tissue NE concentration by 95% or more (Bello-Reuss, 1975).

As to distribution, the greatest number of neuroeffector junctions are found in the proximal tubule, followed by the thick ascending limb of Henle's loop, the distal convoluted tubule, and the collecting duct. As to density, this is greatest in the thick ascending limb of Henle's loop, followed by the distal convoluted tubule, the proximal tubule, and the collecting duct (Barajas, 1984).

Dopamine is present in all adrenergic sympathetic postganglionic nerve terminals as a precursor of the neurotransmitter NE (Von Euler, 1966). Dopamine is an endogenous catecholamine that has a variety of actions in the kidney and is produced *in situ* by dopaminergic nerves as well as glomeruli and renal proximal tubules (Felder, 1984). In the dog, renal tissue dopamine concentration is substantially reduced following renal denervation, suggesting that renal nerves are the major source of renal dopamine content (Petrovic, 1986).

II.2. Neural Control of Renal Function

NE, released from renal sympathetic nerve terminals into the synaptic cleft, stimulates predominantly postjunctional α_1-adrenoceptors (ARs) on the renal vasculature (Wolff, 1987). Studies in isolated perfused rabbit blood vessels have revealed a vasoconstrictive effect of NE on interlobular arteries and both afferent and efferent arterioles (Edwards, 1985; Bührle, 1986). NE activation of α-receptors contracts mesangial cells as well (Pfeilschifter, 1986). NE acts on two ARs in the kidney, the α_1- and ß-AR subtypes. The activation of ß-ARs increases renin secretion by afferent arterioles (Baumbach, 1986).

Epinephrine, released from the adrenal medulla, together with NE, both circulating and that which overflows from the synaptic cleft into the renal interstitium, acts on extrajunctional ARs on the renal vasculature. Increases in efferent renal sympathetic nerve activity produces decreases in renal blood flow and increases in renal vascular resistance, which results in decreases in urinary sodium excretion and subsequently, increases in renin secretion (La Grange, 1973; Holmer, 1994).

The renal sympathetic nerves have a direct effect on renal tubular sodium reabsorption in the proximal tubule (Bencsath, 1971). NE, when added to the peritubular side of the proximal convoluted tubule, either *in vitro* or *in vivo*, increases sodium, bicarbonate and water reabsorption (Beach, 1987; Chan, 1980,a, b). Evidence showed that α_1- but not α_2-ARs mediate the tonic effect of basal efferent renal sympathetic nerve activity to increase chloride reabsorption in the rat proximal convoluted tubule (Wong, 1996).

II.3. The Renal Nerves in Pathophysiological States

In hypertension, it is established that the majority of the input to the kidney from the sympathetic nervous system (SNS) derives from the efferent renal sympathetic nerves and not from circulating catecholamines (Folkow, 1983). There is compelling evidence

that efferent renal sympathetic nerve activity is increased in human subjects with essential hypertension. It was demonstrated that the increase in renal blood flow in response to the renal arterial administration of the α-AR antagonist is significantly greater in human subjects with essential hypertension than in normotensive control subjects. This finding indicates that, in human essential hypertension, there is increased sympathetic vasoconstrictor influence on the renal vasculature (Hollenberg, 1975).

Studies have shown that SNS activity is increased in human subjects with congestive heart failure (Dzau, 1987-b), nephrotic syndrome (Humphreys, 1994), and hepatic cirrhosis (DiBona, 1984). In patients with congestive heart failure and hepatic cirrhosis, there are increases in renal NE spillover (Esler, 1990). In patients with nephrotic syndrome, plasma NE concentrations are increased. In addition to NE, plasma concentration of renin is also increased (Tulassay, 1987). It is possible that the chronic sustained increases in efferent renal sympathetic nerve activity and SNS activity that characterize chronic congestive heart failure may be derived from Ang II, since Ang II enhances the release of NE from renal sympathetic nerve terminals (Brooks, 1995).

In patients with diabetes mellitus, of short duration and without complications, there is evidence of sodium retention and increased intravascular volume. Such patients exhibit an attenuated natriuretic response to headout water immersion, a stimulus for cardiopulmonary baroreflex-mediated reductions in efferent renal sympathetic nerve activity (O'Hare, 1986).

III. ADRENERGIC RECEPTORS

The ARs are the sites of initiation of tissue response to catecholamines. The cloning and sequencing of cDNAs for α-, and ß-ARs has revealed that although each has a unique molecular structure, they appear to share several common features, including

an extracellular amino terminus, seven plasma membrane spanning domains, and an intracellular carboxyl terminus (Insel, 1989).

Each subtype of AR has been identified as an integral membrane glycoprotein whose molecular weight is 60,000 - 90,000 daltons. Each of the receptors is between 410 and 450 amino acids in length. Based largely on analyses of the hydrophobicity of the different amino acids comprising the receptors, it has been suggested that all of the receptor subtypes are proteins that extend from extracellular amino termini and then cross the plasma membrane seven times, ending up with their carboxyl termini intracellularly. Thus, each of the receptors has three extracellular loops and three intracellular loops plus seven membrane spanning domains. Amino acid sequences that represent putative sites of glycosylation have been identified near the amino termini. In addition, all of the receptor subtypes show substantial sequence identity (Dohlman, 1987).

The receptors appear to be relatively stable membrane proteins, having half-lives under basal conditions of several hours. Exposure of target cells to agonists markedly shortens these half-lives and promotes a variety of different feedback mechanisms to dampen target cell responsively. These include AR-specific and nonspecific forms of desensitization, receptor uncoupling from second messenger systems, receptor redistribution, and receptor depletion from the plasma membrane and presumably ultimately from the cell itself (Mahan, 1987; Clark, 1986; Sibley, 1985).

All of these receptors trigger appropriate effector molecules (and, in turn, second messenger production) via an interaction of the receptors with coupling proteins. These coupling proteins have been termed G proteins because they bind and hydrolyze the guanine nucleotide, GTP.

III.1. ß-Adrenoceptors

III.1.1. Classification, Signal Transduction and Distribution

ß-AR mediates most of the inhibitory functions of epinephrine and NE. $ß_1$-adrenoceptor stimulation causes tachycardia and increases the force of cardiac contraction. Stimulation of the $ß_2$ adrenoceptor causes skeletal muscle vasodilation. On the basis of different relative potencies of epinephrine and NE in cardiac and smooth muscles, ß-AR was initially subdivided into $ß_1$- and $ß_2$-subtypes (Aghajanian, 1982). Although NE binds to $ß_1$-AR with a considerably higher affinity than to $ß_2$-AR, epinephrine binds to the two receptors with comparable affinity. Pharmacological studies showed that a third ß-AR subtype exists, termed "atypical ß-AR" or $ß_3$-AR (Zaagsma, 1990).

Stimulation of the ß-AR results in the activation of adenylyl cyclase (AC) which converts ATP to cAMP, which then functions as the intracellular second messenger, stimulating cAMP-dependent protein kinases. The stimulated protein kinase is capable of phosphorylating a wide variety of protein substrates, thus accounting for the diversity of cellular responses (Carlson, 1979).

The $ß_1$-AR are regulated by NE released from adrenergic nerves, and $ß_2$-AR are controlled by circulating epinephrine.

$ß_1$- and $ß_2$-ARs have been localized in rat kidney slices by an *in vitro* autoradiography technique (Healy, 1985). In the rabbit kidney, functional ß-ARs are present in the proximal straight tubules (Kudo, 1991). The polymerase chain reaction experiment, combined with reverse transcription has detected the distribution of $ß_2$-AR mRNA in the glomerulus and early proximal convoluted tubule of the hamster nephron segments (Taniguchi, 1993).

Evidence has been obtained for the presence of ß-adrenoceptors on the proximal tubules of the rat kidney using enriched tubule suspensions prepared by Percoll centrifugation (Jacobs, 1986).

III.1.2. Desensitization

Interaction of a stimulus with a G protein coupled receptor invariably leads to two opposite consequences. First, activation of the receptor leads to interaction with G proteins and stimulation of specific biochemical effectors leading to physiological responses. However, the very same stimuli which promote receptor activation, also promote interaction of the receptor with other molecules which lead to its functional deactivation, a process generally referred to as desensitization (Lefkowitz, 1993).

Recently, much progress has been made in understanding a pathway which seems to function importantly to rapidly desensitize G protein coupled receptors. As is the case for activation, the first step in desensitization is the agonist-induced conformational change in the receptor protein. The activated conformation of the receptor is specifically recognized by a cytosolic receptor kinase termed ß-adrenergic receptor kinase (ßARK), which includes $ßARK_1$ and $ßARK_2$. The kinases appear to recognize only the activated conformations of the receptors as suitable substrates. Upon activation of the receptors the kinases translocate from the cytosol to the plasma membrane. The ßγ subunit interacts with ßARK, binding tightly to it. This interaction serves to facilitate and enhance the interaction of the enzyme with the lipid bilayer and its receptor substrate (Lefkowitz, 1993).

ßARK also phosphorylates its receptor substrates on serine and threonine residues located close to the carboxyl terminus of the protein. This phosphorylation facilitates the binding of a second protein known as ßarrestin. The binding of the arrestin molecules sterically prevents coupling of the receptor to G proteins thus leading to

functional desensitization. The entire process appears to be reversed by receptor dephosphorylation carried out by phosphatases which have been, as yet, only very poorly characterized (Lefkowitz, 1993).

The ßARKs and ßarrestins appear to be very widely distributed throughout the central nervous system, suggesting that they function to regulate a wide variety of neurotransmitter receptors and not just the ß-ARs. Moreover, nothing is known of the receptor specificities of either the two forms of ßARK or the two forms of ßarrestin (Lefkowitz, 1993).

III.2. α-Adrenoceptors

In relation to physiologic function, the α-ARs have been divided into two groups, α_1- and α_2-ARs. While the α_1-AR mediates muscle contraction, the α_2-AR mediates inhibition of various functions (Berthelsen, 1977). However, it is most effective to classify α-ARs in terms of agonist and antagonist affinity. α_1-AR is one that is activated by methoxamine, cirazoline or phenylephrine and is blocked by low concentrations of agents such as prazosin or WB 4101. The response of α_2-AR could be elicited by BHT-933, UK 14304 or BHT-920 and antagonized by selective concentrations of yohimbine, rauwolscine or idazoxan (Ruffolo, 1991).

III.2.1. α_1-Adrenoceptor Subtypes

III.2.1.1. Classification

Binding studies showed that the displacement curves for a series of agonists and antagonists to compete for [^{3}H]prazosin sites were biphasic in nature. The site at which ligands such as WB 4101, phentolamine, (-)-epinephrine, (-)-norepinephrine and phenylephrine interacted with high affinity was termed the α_{1A}-AR. The low-affinity site for these ligands was termed the α_{1B}-AR (Morrow, 1986).

Molecular cloning techniques enabled the further understanding of the classification of the α_1-ARs. Table 1. summarizes the cloning and classification of α_1-ARs.

TABLE 1. CLONING AND DISTRIBUTION OF α_1-ADRENERGIC RECEPTOR.

Cloned name	Classification	Distribution	References
α_{1b}-AR	α_{1B}-AR	liver, spleen, heart	Cotecchia, 1988
α_{1c}-AR	α_{1A}-AR	aorta, heart, kidney	Forray, 1994
	α_{1D}-AR		Lomasney, 1991

III.2.1.2. Signal Transduction of α_1-AR Subtypes

The contraction of vascular smooth muscle by the α_1-AR family is mediated by the well-studied and characterized second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Tanaka, 1994). These signaling molecules initiate contraction by activating different components of the cytoplasmic calcium regulatory system, leading to increases in myoplasmic calcium. IP_3 binds to a family of IP_3 receptor-operated Ca^{2+} channels to promote the release of intracellular non-mitochondrial calcium stores. DAG activates a family of membrane-associated protein kinase C (PKC) that can regulate several intracellular processes.

The exact nature of the G-protein(s) involved in the coupling of α_1-ARs to these vital second messenger systems has not been clearly elucidated. Wu (1992) has shown that all three cloned receptors can couple to pertussis toxin-insensitive G-proteins of the $G_{q/11}$ family. Luttrell (1993) has probed regions of the α_{1B}-AR that interface with the G-protein. Co-expression of the third intracellular loop of the α_{1B}-AR inhibits the ability of the native receptor to activate phospholipase C (PLC) in HEK293 cells, indicating that this region of the receptor is involved in G-protein interactions.

Recently, a high molecular weight G-protein, G_h, distinctly different from the lower molecular weight heterotrimeric class, has been shown to couple the α_{1B}-AR to the activation of a PLC (Nakaoka, 1994).

Experiments with cloned expressed receptors have shown that all three subtypes can increase intracellular calcium and promote calcium influx through voltage-sensitive Ca^{2+} channels (Perez, 1993). In addition, all known α_1-AR subtypes activate not only PLC, but also PLA_1 enzymes (Perez, 1993).

III.2.2. α_2-Adrenoceptor Subtypes

III.2.2.1. Classification

The α_2-AR family is heterogeneous, with several pharmacologically defined and cloned subtypes. Epinephrine and NE activate all of the known α_2-AR subtypes without subtype selectivity. Table 2. summarizes the classification of α_2-AR.

TABLE 2. CLASSIFICATION OF α_2-ADRENERGIC RECEPTOR.

Subtype	Ligand binding	1st identified in	References
α_{2A}-AR	Low to prazosin	human platelet	Bylund, 1985
α_{2B}-AR	high to prazosin	neonatal rat lung	Bylund, 1985
α_{2C}-AR	high to rauwolscine	opossum kidney	Murphy, 1988-a
α_{2D}-AR	low to rauwolscine	bovine pineal gland	Simmoneaux, 1991

To date, only three α_2-AR genes have been cloned -- α_{2A}, α_{2B}, and α_{2C}, which are summarized in Table 3.

TABLE 3. α_2-ADRENERGIC RECEPTOR SUBTYPES AND CLONED GENES

Subtype	Species	Clone Name	Selective Agonists & Antagonists	References
α_{2A}	Human	α_2-C10	Oxymetazoline, BRL44408	Kobilka, 1987
	Pig			Guyer, 1990
	Rat	RG20		
		cA_2-47		
	Mouse	Mα_2-10H		
α_{2B}	Human	α_2-C2	BRL42992, Imiloxan,	Lomasney, 1990
	Rat	RNG	Prazosin, Spiroxatrine	Zeng, 1990
	Mouse	Mα_2-2H	ARC-239	Chruscinski, 1992
α_{2C}	Human	α_2-C4	Prazosin, ARC-234,	Regan, 1988
	Rat	RG10	Rauwolsine, WB4101	Lanier, 1991
		pA2d	BAM1303	
	Mouse	Mα_2-4H		

III.2.2.2. Signal Transduction of α_2-AR Subtypes

The α_2-ARs were first categorized as a receptor population capable of inhibiting AC activity and thus, attenuating cAMP production in target cells. In many target cells, particularly electrically excitable cells, α_2-ARs have been demonstrated to couple to the activation of receptor-operated K^+ currents (Egan, 1983), as well as to the inhibition of voltage-gated Ca^{2+} channels (Horn, 1980). These electrical events could cause attenuation of secretion from neuroendocrine and neuronal cells.

Additional signaling pathways have also been demonstrated for the α_2-ARs in native cells and in heterologous cells expressing transfected α_2-ARs. These signaling pathways include acceleration of Na^+/H^+ exchange, activation of PLA_2, PLD, PLC and activation of ras (Sweatt, 1985; 1986; MacNulty, 1992; Cotecchia, 1990; Alblas, 1993).

In vascular smooth muscle, where postsynaptic α_2-ARs mediate contraction, the α_2-AR may be linked to a Ca^{2+} channel to allow translocation of extracellular calcium (Cooke, 1985). In addition, recent studies have demonstrated that α_2-AR activation stimulates Ca^{2+} channel currents in rat portal vein myocytes (Leprêtre, 1994). In all cases where

data are obtainable, α_2-AR activation of these pathways is pertussis toxin sensitive, suggesting that GTP-binding proteins of the G_i/G_o subfamily are responsible for eliciting these effects.

Epinephrine induces platelet aggregation via activation of PLA_2 independent of the PLC pathway and it was found that this effect was via the α_{2A}-AR. Despite the absence of DAG formation there is evidence for increased PKC activity, since the PKC inhibitor staurosporine prevents glycoprotein complex IIB/IIA exposure by epinephrine. It suggested that the α_{2A}-AR activates PKC via a mechanism that does not depend on DAG formation, probably via a pertussis toxin-sensitive G-protein (Siess, 1984; Saitoh, 1989; Nieuwland, 1993; Nieuwland, 1994).

IV. DOPAMINERGIC RECEPTORS

Dopamine receptors also belong to the G-protein coupled receptor family. With the advent of molecular cloning techniques, five pharmacologically distinct dopamine receptors have been defined through molecular cloning methods. Two of these cloned receptors exhibit the functional and pharmacological properties expected for a classical D_1 receptor, while the other three receptors exhibit the pharmacological characteristics of a D_2 receptor. So that the families of D_1 and D_2 receptors exist rather than singular receptor subtypes (Sibley, 1993).

IV.1. Classification and Distribution

IV.1.1. The D_1 Receptor Family

IV.1.1.1. The D_{1A} Receptor

The D_{1A} receptor has a small third cytoplasmic loop and a long C-terminus. This seems to be a characteristic of receptors which are coupled to G_S and activate AC (Strader, 1989). In addition, there is one consensus site for cAMP-dependent phospho-

rylation in the third cytoplasmic loop (Strader, 1989). The D_{1A} receptors were also shown to mediate dopaminergic stimulation of AC activity with a pharmacology identical to that seen in endogenous receptor-expressing tissue systems (Dearry, 1990). The localization of D_{1A} receptor includes the caudate putamen, nucleus accumbens, and olfactory tubercle, where it has the highest expression (Gerfen, 1990; Meador-Woodruff, 1991; Weiner, 1991).

The 5' flanking region of the human D_{1A} receptor gene has been characterized. The promoter region was characterized as lacking a TATA box and a CAAT box. In addition, consensus sequences for a putative cAMP responsive element were identified (Minowa, 1992).

IV.1.1.2. The D_5 Receptor

When expressed in mammalian cells, the D_5 receptor exhibits linkage to stimulating AC activity with a D_1-like pharmacology (Grandy, 1991). Further pharmacological analysis using a radioligand binding method shows that various agonist and antagonist ligands exhibit similar affinities for the D_{1A} and D_5 receptors, with the notable exception of dopamine which has about 5- to 10-fold higher affinity for D_5 than D_{1A}. This has led to the hypothesis that the D_5 receptor may be important in maintaining dopaminergic tone and arousal (Weinshank, 1991).

The areas of highest expression are in the limbic system and include the hippocampus and hypothalamus, with lower amounts being found in the frontal and temporal cortices. In general, the D_5 receptor is expressed at lower levels and with a more restricted distribution in comparison to the D_{1A} receptor (Weinshank, 1991).

IV.1.1.3. The D_{1B} Receptor

The D_{1B} receptor was cloned from the rat and it is a homolog of the human D_5 receptor (Tiberi, 1991). Expression of the rat D_{1B} receptor exhibits a similar pharmacology to the D_5 receptor, including a relatively high affinity for dopamine and linkage to stimulating AC (Tiberi, 1991).

Mosma (1991) demonstrated that both the D_{1A} and D_{1B} receptors are expressed in various kidney regions, including the inner and outer medulla, the glomeruli, and the proximal convoluted tubules. In all regions examined, the D_{1B} receptor mRNA was found to predominate over that for the D_{1A} receptor, with both transcripts being expressed in highest abundance in the proximal convoluted tubules.

IV.1.2. The D_2 Receptor Family

IV.1.2.1. The D_2 Receptor and Its Isoforms

The first cloned dopamine receptor was D_2 receptor (Bunzow, 1988). In contrast to the structures of the D_1 receptors, the predicted size of the C-terminus of the D_2 receptor is rather small, while the third cytoplasmic loop between transmembrane regions 5 and 6 is quite large. This feature of having a large third cytoplasmic loop and short C-terminus is a characteristic of many receptors which inhibit AC activity.

When expressed in stably transfected LtK^- fibroblasts, the D_2 receptor was shown to exhibit guanine nucleotide-sensitive agonist binding as well as pharmacologically specific inhibition of AC activity (Neve, 1989). The D_2 receptor in these cells has also been reported to stimulate phosphoinositide hydrolysis and Ca^{2+} mobilization (Vallar, 1990).

The D_2 receptor has two isoforms that differ in length by 29 amino acids and are derived from the same gene by alternative RNA splicing (Dal Toso, 1989). These two iso-

forms have been designated as D_{2S} (for short) and D_{2L} (for long). The D_{2L} isoform appears to express predominantly in all brain regions and tissues examined. However, no pharmacological differences have been observed between these two isoforms (Dal Toso, 1989). Both the D_2 receptor isoforms have been shown to inhibit AC as well as activate K^+ channels (Dal Toso, 1989; Einhorn, 1991).

Biochemical and functional studies have demonstrated the presence of D_2-like receptors in the kidney (Lokhandwala, 1991; Ricci, 1991). Radioligand binding studies have identified D_2-like receptors in the adventitia and intima of renal blood vessels and in the glomerulus, as well as cortical and medullary tissue.

IV.1.2.2. The D_3 & D_4 Receptors

The amino acid sequence, as well as the proposed membrane topography of the D_3 receptor, is very similar to that of the D_2 receptor. The human gene and cDNAs for the D_3 receptor have also been characterized (Giros, 1989).

Expression of the D_3 receptor in CHO cells indicates that its pharmacology is similar to that of the D_2 receptor (Sokoloff, 1990). Regional analysis of D_3 receptor mRNA in the brain has indicated that it is much less abundant and more narrowly distributed than D_2 receptor mRNA (Sokoloff, 1990).

The human D_4 receptor has a similar membrane topography to that for the D_2 and D_3 receptors, which include the existence of a putative large third cytoplasmic loop and short C-terminus (Van Tol, 1991).

As with the D_3 receptor, the D_4 receptor appears to be expressed at a lower level than the D_2 receptor. The areas of highest D_4 mRNA expression include the frontal cortex,

midbrain, amygdala, and medulla, with lower levels observed in the striatum and olfactory tubercle (O'Malley, 1992).

TABLE 4. SUMMARY OF DOPAMINERGIC RECEPTOR SUBTYPES

Receptor Subtypes	Effector Pathways	mRNA Distribution	Selective agonists
D_{1A}	↑ cAMP	Gaudate putamen, Nucleus accumbens	SKF38393, fenoldopam
D_5	↑ cAMP	Hippocampus, hypothalamus	SKF38393, fenoldopam
D_{1B}	↑ cAMP	Hippocampus, kidney	SKF38393, fenoldopam
D_2	↓ cAMP ↑ K^+ channel ↓ Ca^{2+} channel ↑PLC/PKC	Gaudate putamen, Nucleus accumbens	N-0437, bromocriptine
D_3	?	Olfactory tubercle, Hypothalamus	Quinpirole, 7-OH-DPAT
D_4	↓ cAMP	Frontal cortex, heart	?

IV.2. Signal Transduction Pathways

Signaling through dopamine receptors involves different second messenger systems, and each receptor subtype generally regulates multiple effectors. D_1-like receptors essentially stimulate AC, leading to increased PKA activity. The D_1 isoform is coupled to this enzyme mainly through G_{olf} and G_s (Kebabian, 1979; Hervé, 1993). D_2-like receptors which belong to the family of G_i-linked receptors, inhibit AC with a rapid decrease of cAMP levels. D_2 receptors are coupled to AC through $G_{i\alpha 2}$ (Strange, 1993; Senogles, 1990). cAMP regulates the expression of genes bearing the cAMP response element (CRE) in their promoter regions. These effects are mediated through the transcription factor CRE binding protein (CREB) (Karin, 1992).

D_1-like receptors also stimulate PLC, but PLC coupling to D_1 and D_5 receptors through G-proteins has not yet been clearly established (Mahan, 1990).

D_2-like receptors regulate PLC activity in a complex manner. Their activation stimulates PIP_2 hydrolysis, resulting in the production of IP_3 as well as enhanced DAG levels with PKC activation. IP_3 mediated Ca^{2+} release is under permissive control of cAMP (Vallar, 1988; Nakade, 1994). Dopamine, through D_2-like receptors which lower cAMP levels, circumvents this stimulatory influence leading to a late and indirect inhibition of IP_3-dependent Ca^{2+} transients, thereby inhibiting Ca^{2+}-dependent PLC activity (Vallar, 1988). D_2-like receptors also reduce $[Ca^{2+}]_i$ via K^+ channels. Two types of voltage-insensitive K^+-currents can be activated by D_2-like receptors (Einhorn, 1991).

Ang II stimulates renal brush-boarder membrane Na^+ uptake and activates brush-boarder membrane PLA_2 in rabbits. In the presence of dopamine, Ang II failed to stimulate brush-boarder membrane Na^+ uptake and PLA_2. Both DA_1 and DA_2 agonists similarly abrogate the actions of Ang II and both DA_1 and DA_2 antagonists are required to restore Ang II actions in the presence of dopamine (Sheikh-Hamad, 1993).

Immediate-early genes are responsive to dopaminergic stimulation in the brain. Injections of D_2-like receptor antagonists, haloperidol or supiride, exert a dose-dependent increase on the expression of c-fos and jun-B in the rat caudato-putamen (striatum) (Rogue, 1994). Acute treatment by D_2 antagonists also induces AP-1 binding activity and the expression of Fos-like proteins in the dorsolateral striatum (Nguyen, 1992; Dilts, 1993).

V. MOLECULAR MECHANISMS OF SIGNAL TRANSDUCTION PATHWAYS

V.1. G-Proteins

The family of GTP-binding proteins, called G-proteins, provides a signal transduction coupling mechanism for many cell surface receptors (including angiotensin II receptors and catecholaminergic receptors). The receptors act catalytically to mediate guanine nucleotide exchange at the GDP-GTP binding site of G-proteins. This process is referred to as activation and results in the displacement of bound GDP for GTP. The GTP-bound form of the G-protein then initiates a cellular response by altering the activity of specific enzymes (Johnson, 1984).

The receptors for many hormones (such as catecholamines, gonadotropins, parathyroid hormone, and glucagon), odorants, as well as light, span the membrane seven times (Dohlman, 1991). Stimulation of these receptors activates a group of coupling proteins that regulate a variety of enzymes and ion channels. Many receptors can stimulate a single G protein. G_s, for example, can be activated by 30 or more receptors. Conversely, individual receptors can activate more than one G protein (Cerione, 1985).

G proteins are made up of three polypeptides: an α subuit that binds and hydrolyzes GTP, a ß subunit, and a γ subunit. The ß and γ subunits form a dimer that only dissociates when it is denatured and is, therefore, a functional monomer. When GDP is bound, the α subunit associates with the ßγ subunit to form an inactive heterotrimer that binds to the receptor. Both α and ßγ subunits can bind to the receptor. Monomeric, GDP-liganded α subunits can interact with receptors, but the association is greatly enhanced by ßγ. Once GTP is bound, the α subunit assumes its activated conformation and dissociates both from the receptor and from ßγ. Until now, four subfamilies of G protein α subunits have been defined, and multiple G protein ß and γ subunits have been identified. All isoforms of α subunits are GTPases, although the intrinsic rate of

GTP hydrolysis varies greatly from one type of α subunit to another. Once GTP is cleaved to GDP, the α and βγ subunits reassociate, become inactive, and return to the receptor (Dohlman, 1991, Carty, 1990). The free α and βγ subunits each activate target effectors (Dohlman, 1991; Pitcher, 1992). The βγ-subunit complex has also been viewed as a regulatory component for α, which stabilizes its GDP-bound form and presents it to the receptor. The βγ entity serves as a membrane anchor for the oligomeric G-protein as well (Hepler, 1992).

The α-subunit currently is used to define the individual G-protein oligomers. The family of G-protein α-subunits can be subclassified according to functional and structural relationships with reasonable congruence. To date, cDNAs that encode 21 distinct G-protein α-subunits have been cloned; these can be divided into four major subfamilies according to their amino acid sequence relationships, i.e. those represented by G_s, G_i, G_q and G_{12}. In addition, at least four distinct β- and six γ-subunits have been described (Birnbaumer, 1990; Spiegel, 1992; Hepler, 1992).

Hormone and odorant receptors interact with members of the G_s family (G_s and G_{olf}) to stimulate AC, and thus enhance the rate of cAMP synthesis (Spiegel, 1992). Members of the G_q family have been identified as the regulators of the PKC pathway (Hepler, 1992). The effects of G_i subfamily include AC inhibition and modulation of several ion channels (Simonds, 1989).

Another feature of virtually all G-protein-coupled receptors is their susceptibility to desensitization and down-regulation upon prolonged or repeated exposure to the ligand. Such desensitization involves receptor phosphorylation which occurs within minutes and results in a rapid uncoupling of the receptor from its G-protein (Sibley, 1985).

V.2. Adenylyl Cyclase (AC)

ACs have molecular weights of roughly 120,000 daltons (range of 1064-1248 amino acid residues) and a complex topology within the membrane. There are at least eight isoforms of AC cloned so far. Although these enzymes share structural characteristics, such as tandem repetition of a hydrophobic domain that contains six putative transmembrane spans followed by a large hydrophilic cytoplasmic domain, emerging evidence indicates that each may have distinct tissue distribution and regulatory properties (Tang, 1992). For example, type I and III adenylyl cyclases are stimulated by Ca^{2+} in the presence of calmodulin (Tang, 1991-b), and the type VI enzyme is inhibited by Ca^{2+}, independently of calmodulin (Yoshimura, 1992), whereas types II and IV are insensitive to Ca^{2+} (Gao, 1991).

All isoforms of AC appear to be expressed in the brain, apparently in region-specific patterns. The type I AC appears to be present only in the brain; mRNA for the type II enzyme has been detected in brain and lung; type IV enzyme appears to be widely distributed; the mRNA for the type VII enzyme is found in S49 lymphoma cells, and in rat brain and heart; the type III enzyme was cloned from an olfactory epithelial cDNA library; both the type V and VI mRNAs are found at high levels in brain and heart, and at lower levels in several other tissues, including kidney, liver, lung and testes; the type VIII enzyme appears to be a brain-specific species (Iyengar, 1993-a; Feinstein, 1991; Bakalyar, 1990; Ishikawa, 1992; Premont, 1992; Katsushika, 1992; Yoshimura, 1992; Gao, 1991).

Most, if not all, ACs are multiply regulated. All of the cloned mammalian ACs that have been expressed are stimulated by the GTP-bound α subunit of the stimulatory G protein G_s and forskolin, a PKA stimulator (Feinstein, 1991; Gao, 1991). Examination of the sequences of the cloned ACs shows that all of the enzymes except the type IV contain one or two putative PKA phosphorylation sites. An interesting observation is

that the positions of putative PKA phosphorylation sites are not conserved among the various ACs (Iyengar, 1993a). All isoforms of AC are inhibited by certain adenosine analogs termed P-site inhibitors, 2'-deoxy-3'-AMP is particularly potent. However, they are further regulated in type-specific patterns by other inputs, particularly including those that are dependent on Ca^{2+} or that arise from other (non-$G_s\alpha$) G protein subunits (Taussig, 1995). Inhibition of ACs by $\beta\gamma$ is confined for now to the type I enzyme. However, when the effects of $\beta\gamma$ on other isoforms were tested, surprising stimulatory effects were observed with type II and type IV ACs (Tang, 1991-a, -b; Gao, 1991).

Changes in intracellular Ca^{2+} can have profound effects on cellular concentrations of cAMP if appropriate isoforms of AC are present. Types I and VIII AC (and type III to a lesser extent) are markedly stimulated by nanomolar concentrations of Ca^{2+}/calmodulin, and intracellular cAMP concentrations rise dramatically when transfected cells expressing these isoforms are exposed to agonists that elevate intracellular Ca^{2+}. The other isoforms of AC (II, IV, V, and VI) are insensitive to calmodulin. All ACs are inhibited by high (100-1000 μM) concentrations of Ca^{2+} as a result of competition for Mg^{2+}, which is required for catalysis. By contrast, types V and VI ACs are inhibited by low micromolar concentrations of Ca^{2+}. This effect is independent of calmodulin and is presumably mediated directly (Katsushika, 1992; Yoshimura, 1992). Phorbol esters elicit double the stimulation of transfected type II AC than does $G_s\alpha$ (Jacobowitz, 1993).

Taken together, the $G\alpha$ subunits, PKC, Ca^{2+} and $\beta\gamma$ subunits of G proteins can stimulate or inhibit particular ACs (Tang, 1992; Iyengar, 1993-b).

V.3. Protein Kinase A (PKA)

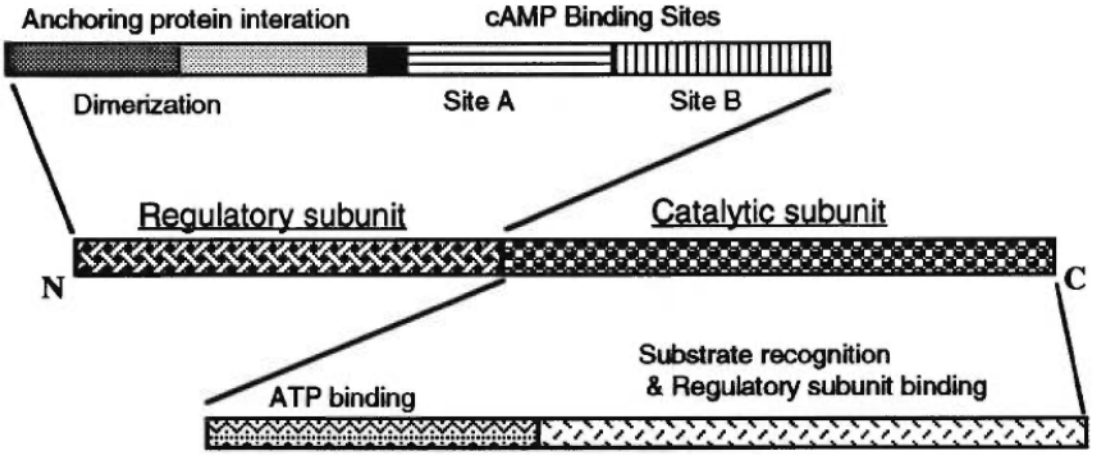
Since the discovery of cAMP as an intracellular second messenger, considerable research has focused on its action. The major intracellular "receptor" for cAMP is the

cAMP-dependent PKA, which controls many biochemical events through phosphorylation of target proteins.

V.3.1. Structure

PKA, in common with all protein kinases, contains conserved structural regions (Fig. 1). It composes a catalytic core of approximately 250 amino acids. The catalytic core contains the sequences responsible for substrate binding and catalysis of the phosphoryl transfer reaction (Corbin, 1990). The catalytic core is maintained in an inactive state by interaction with specific inhibitory sequences located on regulatory units. The structural organization consists of several subdomains which are responsible for inhibition of catalytic activity and other modulatory functions, e.g. dimer formation, cyclic-nucleotide binding and subcellular localization (Scott, 1991).

Figure 1. The Catalytic and Regulatory Units of PKA. (Modified from Scott, 1991)



The PKA holoenzyme is a tetramer consisting of two catalytic subunits and a regulatory subunit dimer. The regulatory subunit dimer is a stable complex which remains intact after release of the catalytic subunits (Scott, 1990). The regulatory subunit, when saturated with cAMP, is particularly sensitive to trypsin cleavage at the hinge region (Weber, 1979).

Each regulatory subunit monomer contains two high-affinity binding sites for cAMP which are located downstream from the dimerization and pseudosubstrate regions (the black box in Fig. 1). When both sites are occupied, the regulatory subunit adopts a conformation with lower affinity for the catalytic subunit (Rannels, 1979; Takio, 1984). A single molecule of cAMP binds to either site A or B in each regulatory subunit, causing conformational rearrangement of the inactive holoenzyme, priming it for activation. This creates a ternary complex with a heightened sensitivity for cAMP.

Upon binding of additional cAMP to the remaining binding sites, other conformational changes occur, which are transduced to the pseudosubstrate site causing a release of the active catalytic subunit (Wolfe, 1990). The sole function of the catalytic subuit is to catalyze the transfer of phosphate from the γ-position of ATP onto a target serine or threonine recognized in the substrate. Phosphorylation of substrate proteins by the catalytic subunit of PKA triggers the wide variety of physiological responses (Fischer, 1983).

V.3.2. Activation

Phosphorylation of target proteins alters their activity, which in turn, promotes changes in cellular function and metabolism. Furthermore, protein phosphorylation is a reversible process, with the dephosphorylation step catalyzed by the phosphoprotein phosphatases.

The generation of cAMP is catalyzed by AC. Ligand-receptor interactions, coupled to G proteins, transduce extracellular signals through the membrane stimulating AC on the inner membrane surface (Krupinski, 1989). Thus, newly synthesized cAMP is released from the membrane and diffuses into the cell to activate PKA. cAMP binding to the re-

gulatory unit activates the kinase by causing the displacement of the inhibitory sequences from the catalytic core.

Studies on the regulation of neuropeptide gene expression focused on cAMP showed that somatostatin mRNA levels increase in primary diencephalic cultures after exposure to forskolin, a potent activator of AC (Montminy, 1986a). The DNA element responsible for cAMP-regulated expression was characterized by deletion analysis of a somatostatin-chloramphenocol acetyl transferase reporter gene construct (Montminy, 1986b). The sequence was identified between 29 and 60 bp upstream from the transcriptional start site, which could confer "cAMP-responsiveness" when ligated onto a heterologous promoter. This sequence was designated the "cAMP-responsive element" (CRE).

The somatostatin CRE contains an 8 base palindrome, 5'-TGACGTCA-3', and is conserved in many other genes regulated by cAMP (Goodman, 1990). Using the somatostatin CRE as an affinity ligand, a binding protein, CRE-binding protein (CREB), was purified and shown to be a substrate for PKA (Montminy, 1987). Characterization of cDNAs encoding CREB provided its primary structure and identified the consensus phosphorylation site for PKA (Hoeffler, 1988).

V.4. Cyclic AMP Responsive Element Binding Protein (CREB)

V.4.1. The CREB Protein

cAMP regulates a striking number of physiological processes, including intermediary metabolism, cellular proliferation, and neuronal signaling, by altering basic patterns of gene expression. The transcriptional induction by cAMP is rapid, peaking at 30 minutes and declining gradually over 24 hours. This burst in transcription does not depend on new protein synthesis and suggests that transcriptional modulation by cAMP involves the covalent modification of a pre-existing nuclear factor (Lamers, 1982).

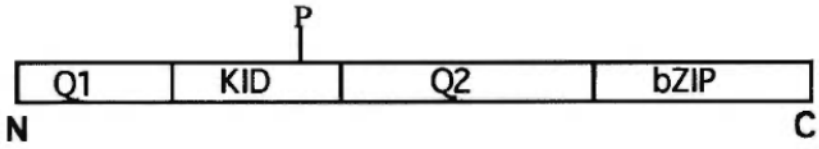
Previous studies showed that the CRE sequence is highly conserved among several cAMP-inducible promoters, including those for c-fos, somatostatin, and vasoactive intestinal peptide (Montminy, 1990). The CRE displayed properties of a classical enhancer sequence, stimulating transcription in a distance and orientation independent manner. Moreover, the CRE also conferred cAMP inducibility when placed upstream of a nonresponsive gene, indicating that CRE binding factors could interact with components of the transcriptional machinery (e.g. TATA factors) (Montminy, 1990).

Using a DNAse I protection assay, Montminy et al (1987) detected CREB activity in nuclear extracts of PC12 cells. By using DNA sequence-specific affinity chromatography, they purified a 43-KD protein to apparent homogeneity from PC12 cells and brain tissue. This 43K nuclear protein binds selectively to the CRE. cAMP appears to stimulate the phosphorylation of this protein *in vivo*. Moreover, the purified protein is a substrate for phosphorylation by the catalytic subunit of cAMP-dependent protein kinase *in vitro*. When added to nuclear extracts, purified CREB specifically stimulated transcription of CRE-containing genes like somatostatin, suggesting that CREB was a transcription factor as well as a DNA-binding protein. Further study revealed that CREB stimulates transcription of these genes by binding to DNA as a dimer. In addition, the transcriptional efficacy of CREB is regulated by PKA phosphorylation (Montminy,1987; Yamamoto, 1988).

CREB consists of an NH_2-terminal activation domain and a smaller, COOH-terminal bZIP DNA-binding and dimerization domain (Fig. 2). The activation domain can be further divided into several subdomains of various activation and regulatory functions. Among the subdomains, two glutamine-rich regions Q1 and Q2 are important to basal activity. The kinase-inducible domain (KID) includes several phosphorylation sites and confers the phosphorylation-induced activity of CREB (Richards, 1996). The mutation

activity analysis suggested that the phosphorylation of a serine residue at 133 amino acid by PKA is sufficient for transcriptional induction of CREB (Montminy, 1990).

Figure 2. Schematic Diagram of CREB. (Modified from Richards, 1996).



bZIP, basic leucine zipper domain; Q1 and Q2, glutamine-rich regions; KID, kinase-inducible domain; P, PKA phosphorylation site at ser-133.

The CREB gene consists of at least 11 exons, of which 10 are coding. The two Q domains are encoded by two separate exons (4 and 9). The KID is encoded by two exons (7 and 8), while the bZIP domain is encoded by the 3'-exons (10a and 11) (Ruppert , 1992). A differential splicing event was first described by Berkowitz (1990) describing that a 14-amino acid domain named α, encoded by exon 5 could be either absent (CREBΔ/327) or present (CREBα/341) in the CREB protein (Berkowitz, 1990). The significance of this splicing event is not completely clear. Yamamoto et al (1990) showed that the α domain enhances trans-activation by CREB, whereas other reports failed to demonstrate an effect (Ruppert , 1992; Berkowitz, 1990). Ruppert et al (1992) also identified four novel isoforms of CREB (γ, αγ, Ω, and Ψ), which are all characterized by insertion of exons containing inframe stop codons. These isoforms are predicted to encode possibly nonfunctional truncated proteins lacking a DNA-binding domain, although the proteins encoded by these isoforms have not yet been detected.

V.4.2. The Leucine Zipper

A stretch of regulatory spaced leucine residues, characteristic of a potential "leucine-zipper" dimerization domain (Fig. 2) is located near the C-terminus of the CREB molecule (Landshultz, 1988). A gel retardation assay to detect protein-DNA complexes in

crude nuclear extracts of PC12 cells and brain tissue using a ^{32}P-labeled CRE oligonucleotide showed two complexes of high relative molecular mass. Further studies revealed that the higher relative molecular mass represents the dimer form of CREB, whereas the lower complex represents the monomer form (Yamamoto, 1988). The same group also found that the phosphorylation of CREB by an appropriate kinase (PKC) should conversely promote the formation of the CREB dimer. Although CREB binding activity was unchanged following treatment with PKA, PKC caused a marked induction of dimerization *in vitro*. The results suggest dual regulation of CREB binding activity and transcriptional efficacy.

The leucine zipper domain of the CREB protein contains four leucines spaced seven residues apart and is located near the COOH-terminus of the protein. Mutations of the first three individual leucines quantitatively reduces the ability of the protein to form homodimers while mutation of the fourth leucine has no appreciable effect when compared to the wild-type protein. Mutations in the basic region of the CREB protein drastically reduce its ability to bind to CRE but do not significantly decrease the ability to form homodimers. These results imply that the role of the leucine zipper domain is to bring the two polypeptide chains of CREB protein in association as dimers to facilitate binding to CRE sites (Dwarki, 1990).

In the "leucine-zipper" region, CREB shares substantial homology with other "leucine-zipper" proteins including c-*fos*, c-*myc*, and c-*jun*, implicating the potential for formation of heterodimers with these factors (Landshultz, 1988). A computer search for sequence similarities between CREB and c-Jun revealed a single region of 61% identity of amino acids (Hoeffler, 1988).

V.4.3. Phosphorylation of CREB

Experiments showing that the dimerization and transcriptional efficacy of CREB are each stimulated by phosphorylation at distinct sites suggest that CREB is regulated by multiple kinases *in vivo*. Upon CREB cDNA cloning, Gonzalez et al (1989-b) discovered a cluster of PKA, PKC and casein kinase II consensus recognition sites near the N-terminus of CREB. The close proximity of these sites to one another suggests that they may interact to regulate CREB activity.

In response to cAMP elevation, the catalytic subunit of PKA dissociates from the regulatory subunit and migrates into the nucleus where it phosphorylates the cAMP-responsive transcription factor CREB at Ser-133 (Gonzalez, 1989-a). Mutagenesis studies have revealed that the 60-amino acid modulatory domain, KID, is critical for the effect (Foulkes, 1992-a). KID does not act alone to regulate transcription of target genes but rather functions synergistically with an adjacent Q2 that extends from amino acids 160 to 280 of the CREB protein (Brindle, 1993). PKA-mediated induction of CREB requires two activation domains, KID and Q2, functioning synergistically to stimulate transcription. Q2 recruits TFIID to cAMP-responsive promoters, probably by interacting with TAF110. Thus, the CREM proteins α, β and ε, which lack Q2, may function as repressors because they are unable to form productive interactions with proteins in the basal transcription complex (Ferreri, 1994).

Phosphorylation can alter protein function either by introducing an allosteric conformational change in the protein or, more commonly, by allowing (or blocking) specific electrostatic interactions with other molecules. Both of these mechanisms are thought to occur in the regulation of transcription. Models of CREB activation by PKA include structural changes following phosphorylation. The original model proposed that an allosteric conformational change in CREB enables a site distal to Ser^{133}, the Q2 region, to interact directly with the preinitiation complex (Brindle, 1993). A related model sug-

gested that PKA phosphorylation induces α-helix or ß-sheet secondary structures in the regions neighboring the Ser^{133} site, thus allowing specific protein-protein interaction (Meyer, 1993). Another model proposed that phosphorylation leads to an increase in DNA binding to CRE (Nichols, 1992). However, recent data suggests that phosphorylation of CREB simply creates a high-affinity binding site for other coactivating proteins. In particular, phosphorylation of Ser^{133} allows the high-affinity binding of CREB to the co-activator CBP (CREB-binding protein) (Lundblad, 1995). The most recent study by Richards' group (1996) observed that PKA phosphorylation does not induce either a conformational change in CREB or the DNA binding affinity of CREB for CRE sequences. However, the PKA activation of CREB occurs by the production of specific, complementary interactions with transcriptional co-activator proteins.

V.4.4. CREB Related Transactivating Proteins

The cloning of CCAAT box/enhancer binding protein (C/EBP), AP1, CREB, and the subsequent cloning of cDNAs for other CREB/ATF proteins, and analyses of their predicted encoded proteins have revealed a common structural motif, the basic region-leucine zipper (bZIP) domain. The bZIP domain is essential for binding to specific enhancer sequences. The bZIP family of proteins binds to specific enhancer sequences as dimers to their target DNA enhancer sequences. The bZIP domain governs whether these proteins form homo or heterodimers, and thereby also regulates the target DNA sequence to which the dimers bind .

Comparison of the cDNAs from humans, rats, and mice reveals the presence of two primary CREB protein isoforms, CREB327 (327 amino acids) and CREB341 (341 amino acids), that are encoded from the same gene by two alternatively spliced messenger RNAs (mRNAs) (Hoeffler, 1988; Gonzalez, 1989; Ruppert, 1992). In addition to the cAMP-responsive proteins, CREB, CREM, and ATF-1, members of the family of early response proteins Jun and Fos, and of the C/EBP proteins expressed during, or

at the time of terminal differentiation of cells, can bind CREs, although generally with lesser affinity than that of CREB, CRE modulator (CREM), and ATF-1.

V.4.4.1. CREB-2

CREB involved in negative regulation of transcription is CREB-2. CREB-2 has 351-amino acids which contain a COOH-terminal leucine-zipper motif and an adjacent basic domain. Its mRNA is expressed ubiquitously in human tumor cell lines and mouse organs suggesting that it is involved in regulating transcription in a wide variety of cell types (Kaprinski, 1992).

CREB-2 lacks the PKA and PKC phosphorylation sites and the α-helical transcriptional activator domain found in the NH2 terminus of CREB. It counteracts the PKA-induced activation of the human enkephalin promoter. The mechanisms of repression could be that CREB-2 can form heterodimers with other ATF/CREB activator proteins thereby preventing DNA binding and/or transcriptional activation; or CREB-2 monomers or homodimers can bind to CRE sites thereby displacing CREB activator protein. Deletion studies show that the bZIP domain is sufficient for full antagonism, as observed for the transcriptional antagonist CREM (Kaprinski, 1992).

V.4.4.2. ATF-1

ATF-1 is one of the CRE-binding proteins cloned from MG 63 osteosarcoma cells on the basis of its ability to bind to the CRE-like sequence in the adenovirus E4 enhancer (Hai, 1989). Rehfuss et al (1991) determined the complete nucleotide sequence of ATF-1 and found that the protein has extensive regions of homology and are 67% identical in the region extending from the PKA consensus site to the carboxyl terminus comparing to CREB. The DNA binding and leucine zipper regions of the two factors are 91% identical. The two factors recognize the same CRE sequences, suggesting that

they can form heterodimers. Indeed, they found that ATF-1 homodimers and CREB/ATF-1 heterodimers bind specifically to CRE sequences.

In addition, several of the domains which have been shown to be important in allowing CREB to respond to cAMP are conserved in ATF-1, including the consensus phosphorylation site for PKA. However, ATF-1 is unable to stimulate expression of the CRE reporter gene unless it is combined (co-expression) with PKA. The combination of ATF-1 and PKA produces a response that is significantly greater than that of CREB and PKA. The results suggest that ATF-1 is as active as CREB in its ability to mediate the transcriptional effects of PKA, and because ATF-1 has a smaller effect on basal expression, it is actually more responsive than CREB to cAMP.

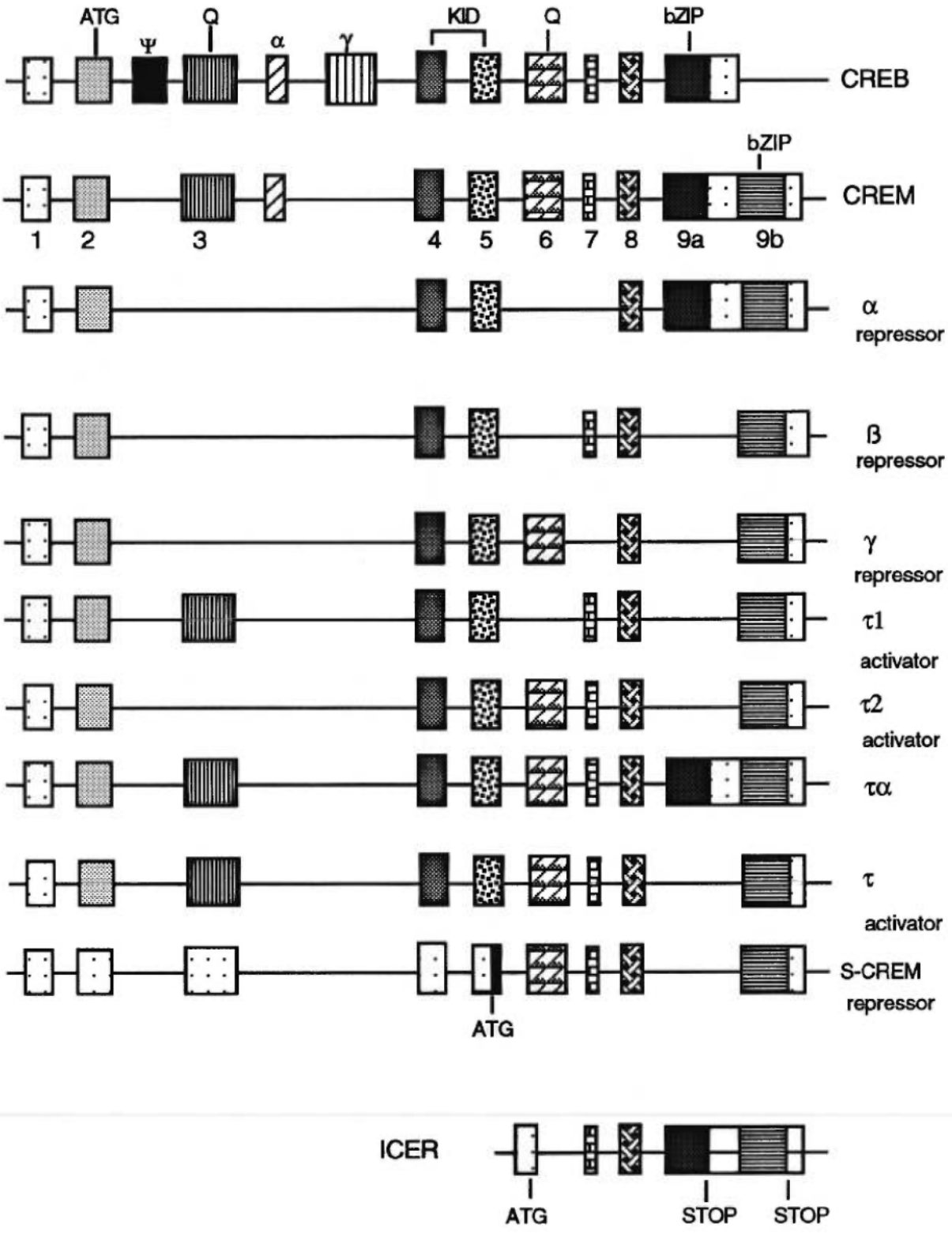
V.4.4.3. CREM

The exonic structure of CREM is highly homologous to that of CREB (Laoide, 1993). Exon boundaries occur at almost identical positions to CREB, dividing the coding sequence into functional domains (Foulkes, 1992-b).

There are several differences between the two genes (Fig. 3). One is the presence of a second DNA-binding domain in CREM, which is absent in CREB. Another is that CREM contains a short exon, named γ, which has no counterpart in CREB. The third difference between CREB and CREM is that in CREM, besides differential splicing, internal translation initiation is used to generate a unique variant CREM protein, S-CREM (Delmas, 1992).

While CREB is expressed ubiquitously in several cell types, CREM shows cell-specific expression (Foulkes, 1991-a).

Figure 3. Structures of CREM & ICER Comparing with CREB.



About eleven different CREM isoforms have been identified, which, differently from CREB, all appear to be functional. Two isoforms (α and τα) incorporate the first bZIP region, while the rest contain the second bZIP region. Three isoforms (α, ß and γ) lack both the Q-rich domains and encode transcriptional antagonists (Foulkes, 1991-a, -b).

Insertion of one or both Q-rich domains in CREM generates transcriptional activators (τ, τ1, τ2 and τα) (Fig. 3). The four novel forms whose reading frames were blocked upstream of the DNA-binding domain. Two of those forms contained a novel exon Ψ, which resides downstream of the first protein-coding exon of the CREM gene, and introduces an early in-frame stop codon (Gellersen, 1997).

The activator isoform of CREM, CREMτ, can also mediate cAMP-induced transcription (Foulkes, 1992-a). CREMτ is phosphorylated by PKA *in vitro* as well as *in vivo* on serine-117, the counterpart of serine-133 in CREB.

The antagonistic CREM products repress, either by competing with CREB or CREMτ activators for binding to CRE sites or by complexing with the activators to generate nonfunctional heterodimers. Since substoichiometric amounts of CREM antagonist are sufficient to elicit repression, and since a truncated CREM form containing only the bZIP domain is able to repress as efficiently as full-length CREM antagonists, it appears clear that heterodimerization is the most likely mechanism by which antagonism is obtained (Laoide, 1993).

V.4.4.4. ICER

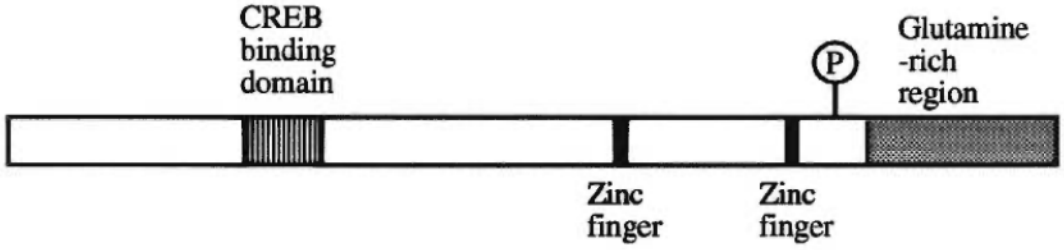
Inducible cAMP early repressor (ICER) is a product of alternative splicing of the CREM gene (Fig. 3). From an intronic second promoter (P2) in the CREM gene, the inhibitor ICER is transcribed in a cAMP-regulated fashion due to a cluster of four CREs in the P2 control region. The protein consists of no other functional domain than a DBD and acts

as a repressor on cAMP-responsive promoters (Molina, 1993). There are four isoforms of ICER, which differ by the presence or absence of the small exon γ and the presence of either DBD I or II (Gellersen, 1997).

V.5. CREB-Binding Protein (CBP)

Transcription factors bind to the CRE element, including CREB, which is activated as a result of phosphorylation by protein kinase A. This modification stimulates interaction with one or more of the general transcription factors or, alternatively, allows recruitment of a co-activator (Chrivia, 1993). Chrivia's group (1993) reported that CREB phosphorylated by protein kinase A binds specifically to a nuclear protein of 265 kd which they termed CBP. The cDNA of CBP was cloned from mouse brain cDNA library. The protein contains several calmodulin kinase II phosphorylation sites, a single PKA phosphorylation site, two regions resembling C_4 zinc-fingers, and a carboxy-terminal glutamine-rich domain (Fig. 4).

Figure 4. Schematic Representation of the CBP Molecule. (Modified from Kwok, 1994)



Indirect immunofluorescence using an affinity-purified antipeptide antibody, which showed that CBP expression is largely restricted to the nucleus, although there was also a small amount of cytoplamic staining (Chrivia, 1993). The binding between CREB and CBP occurs only if CREB is phosphorylated (between the amino acids 117-661 of CBP and amino acids 101-255 of phosphoCREB). They also detected that CBP does not function by recruiting endogenous CREB to the promoter. These findings fit a mo-

del in which phosphorylation by PKA enables CREB to serve as a scaffold for CBP, which then activates the processes responsible for inducing expression of cAMP induced genes.

CBP can activate transcription through a region in its carboxy terminus (Berger, 1992). CBP did not interact with non-phosphorylated CREB, additionally, CBP did not affect the ability of CREB or phosphoCREB to bind to the somatostatin CRE. Consistent with its role as a coactivator, CBP augments the activity of phosphorylated CREB to activate transcription of cAMP-responsive genes (Kwok, 1994).

The affinity of CBP for phosphoCREB was similar to that of other physiologically important protein:protein interactions (Heyduk, 1993), but considerably less than that between CREB and the CRE (Williams, 1993). This suggests that CREB may bind constitutively to the CRE, and that the phosphorylation-induced change in affinity of CREB for CBP may be a critical regulatory step for transcriptional activation (Kwok, 1994).

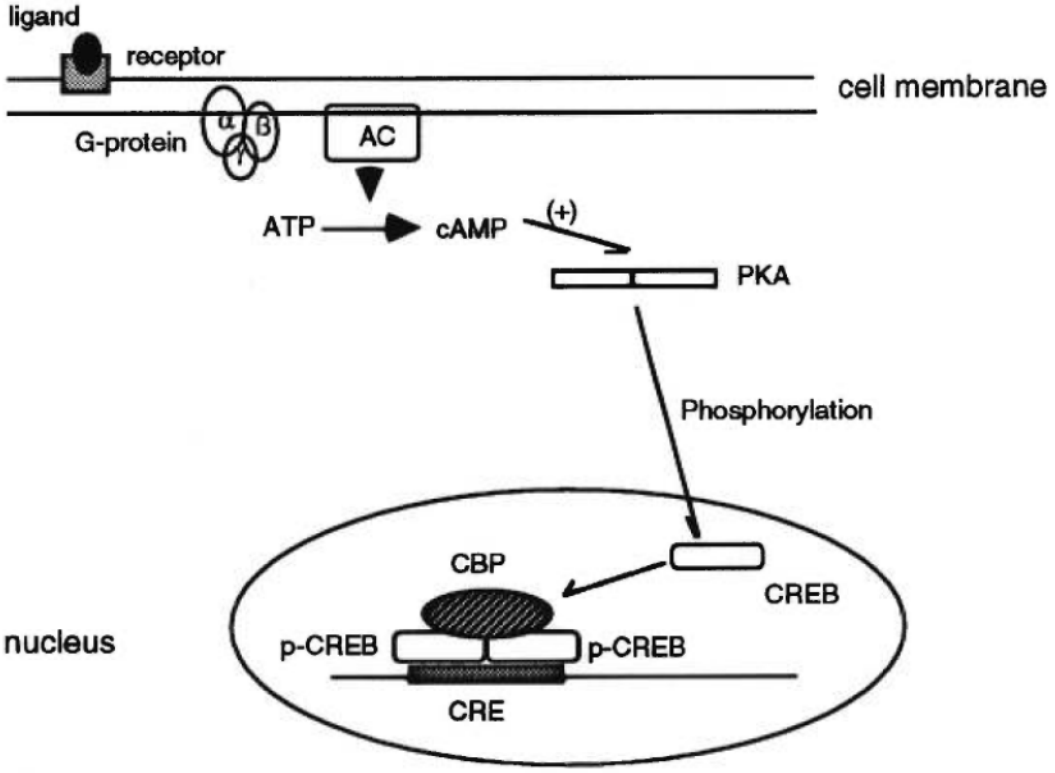
In the absence of exogenous CBP, the combination of CREB and PKA activates expression of the CRE-reporter gene by 15-fold; in the presence of exogenous CBP, CREB and PKA increase transcription in a dose-dependent manner up to a level of about 90-fold. CBP could not activate gene expression in the absence of CREB, even in the presence of PKA (Kwok, 1994).

***P300**

The domains in CBP that are involved in CREB binding and transcriptional activation are highly related to the adenoviral E1A-associated cellular protein p300 (Lundblad, 1995). The same group showed that CBP and p300 have similar binding affinity for the PKA-phosphorylated form of CREB, and that p300 can substitute for CBP in potentiating CREB-activated gene expression. The authors also found that E1A binds to CBP

through a domain conserved with p300 and represses the CREB-dependent co-activator functions of both CBP and p300. So that adenoviral E1A-associated protein p300 is a functional homologue of the transcriptional co-activator CBP.

Figure 5. Summary of PKA Pathway



AC: adenylyl cyclase; CRE: cAMP-responsive element; CREB: cAMP-responsive element binding protein; p-CREB: phosphorylated CREB; CBP: CREB binding protein; PKA: protein kinase A.

V.6. Phospholipase C (PLC)

A variety of hormones, neurotransmitters, and growth factors induce the rapid hydrolysis of phosphoinositides (PI) via a receptor-mediated process. The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) results in the generation of two second messengers, IP_3 and DAG (Berridge, 1987). IP_3 mobilizes Ca^{2+} by binding to specific intracellular receptors that promote the opening of calcium channels in vesicular storage sites associated with the endoplasmic reticulum (Berridge, 1987). DAG binds to and

activates PKC, resulting in the phosphorylation of a number of intracellular proteins (Nishizuka, 1986). The production of the two second messengers is catalyzed by a phosphatidylinositol-specific PLC, which is one of the most commonly used transduction and transmembrane signaling mechanisms in a wide range of tissues and cells (Rana, 1990).

The PLCs can be divided into three types -- PLC-ß, PLC-γ, and PLC-δ, each of which has more subtypes. All three forms are single-polypeptide enzymes and are discrete gene products (Rhee, 1989). The three types of PLC enzymes are dissimilar not only in molecular size but also in amino acid sequence. This lack of sequence similarity is consistent with the absence of immunological cross-reactivity between the three enzymes (Rhee, 1992-b).

Numerous studies have shown that G-protein is the transducer of PLC, where GTP has been shown to cause the breakdown of PIP_2 in the absence of agonist or to potentiate the receptor-mediated PLC activation. There are at least two G_s-proteins, pertussis toxin-sensitive and -insensitive, that mediate the selective coupling of G-protein receptors to PI hydrolysis (Rhee, 1992-a). Immunological studies have indicated that G_q protein activates PLC-ß1, but not PLC-γ or -δ (Taylor, 1991).

EGF and PDGF bind to and stimulate PLC by tyrosine kinases resulting in hydrolysis of PI turnover (Wahl, 1987). Treatment of cells with epithelial growth factor (EGF) or platelet-derived growth factor (PDGF) also promotes the association of PLC-γ_1 with their receptors (Wahl, 1989).

V.7. Protein Kinase C (PKC)

PKC is a monomeric polypeptide of molecular weight 79-82 KDa. PKC covers a family of serine- and threonine-specific protein kinases which have been identified functio-

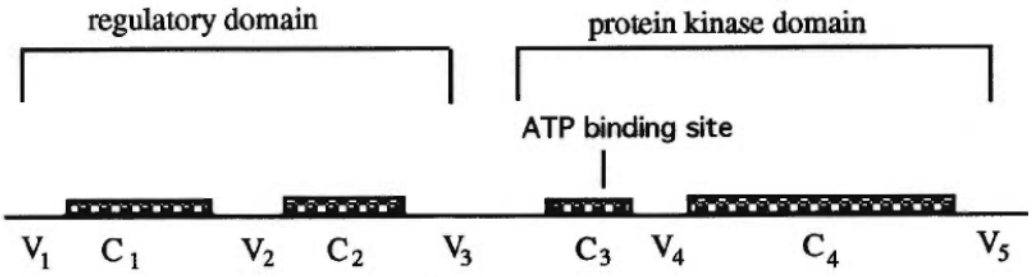
nally by common enzymatic properties, like phorbol ester binding, phospholipid-dependent kinase activity or by common structural features (Nishizuka, 1984). The PKC family is comprised of at least 12 (mammalian) isoforms (Dekker, 1994) which differ in structure and enzymatic properties.

PKC isoforms are involved in the transduction of a wide variety of cellular signals. Much of the interest that has been focused on PKC initially stemmed from the identification of PKC as the high affinity intracellular receptor for phorbol esters, a class of potent tumor promoters (Ashendel, 1985), which replace the DAG requirement for activity. The effects of many biological agonists are mimicked by phorbol esters, implicating a role for PKC in the transduction of such signals.

V.7.1. Structure

The single polypeptide chains of all PKC isoforms contain conserved (C_1-C_4) and variable (V_1-V_5) regions, and are composed of a catalytic domain and a regulatory domain (Coussens, 1986) (Fig. 6).

Figure 6. Structure of PKC



The regulatory domain, responsible for the cofactor dependence, contains an autoinhibitory pseudosubstrate region and sequences mediating the interactions with phospholipid and the activator DAG. The conserved region C_1 contains a tandem repeat of a cysteine-rich sequence. This sequence is similar to the consensus sequence of a

cysteine-zinc-DNA-binding finger that is found in many metallo-proteins and DNA-binding proteins that are related to transcriptional regulation (Berg, 1986), but there is no evidence that PKC binds to DNA.

The carboxy-terminal half of each enzyme containing the regions C_3 and C_4, forms the catalytic domain, as it shows large clusters of sequences that resemble many other protein kinases. It contains typical protein kinase sequences, e.g. the glycine-rich ATP-binding motif present in the cAMP-dependent protein kinase and many other kinases (Nishizuka, 1988).

Based on their structure, the members of the PKC family can be classified into three groups. The conventional or classical isoforms (cPKCs α, $\beta_{I/II}$ and γ) meet the original definition of PKC as Ca^{2+}- and phospholipid-dependent protein kinase. The Ca^{2+} dependence of these isoforms is mediated by the C_2 region. The novel isoforms [nPKCs δ, ε, η(L) and μ] lack this region, resulting in Ca^{2+}-independence. The atypical forms (aPKCs ζ, ι and λ) are characterized by lacking one of the two cysteine-rich zinc-finger regions present in the other isoforms. These isoforms are not able to bind and cannot be activated by phorbol esters. The novel and the atypical PKC isoforms can also be combined under the term 'nonconventional' isoforms (Nishizuka, 1988, Stabel, 1991).

V.7.2. Signal Transduction

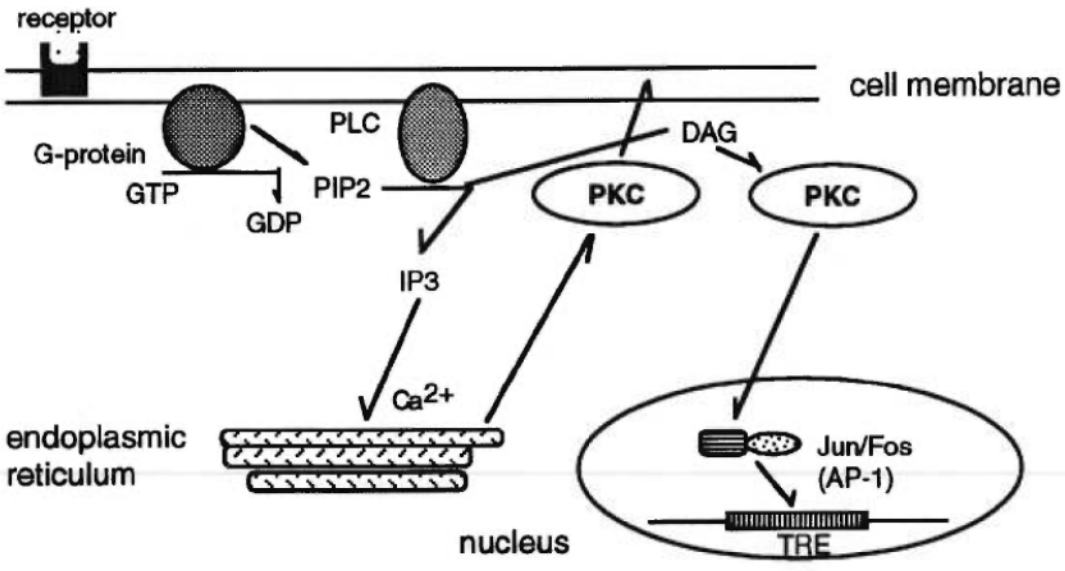
V.7.2.1. IP_3 and DAG

PKC is a ubiquitous protein that plays a crucial role in the transduction of extracellular signals provoking phospholipid turnover. PLC is responsible for cleaving the lipid precursor (Michell, 1981). Phosphatidylinositol bisphosphate serves as a substrate for PLC to generate IP_3 and DAG (Berridge, 1984).

IP_3 mobilizes intracellular Ca^{2+} from the endoplasmic reticulum, thereby controlling its intracellular concentration (Berridge, 1987), causing cytosolic PKC to translocate to the plasma membrane. Calcium is a major second messenger which stimulates cellular processes through the activation of a variety of enzymes such as calcium-dependent kinases and calcium-dependent proteases (Rasmussen, 1986-a, -b). The IP_3/Calcium pathway plays a major and direct role in initiating cellular responses.

The trigger for the activation of the signal transduction pathway in which PKC lies, is the production of DAG. DAG activates PKC by increasing its affinity for calcium. Although the DAG/PKC pathway may also contribute directly to the final cellular response, its predominant role appears to be that of a modulator of either the calcium signaling pathway or other signal pathways (Berridge, 1987). DAG is also produced via the combined action of a phospholipase D (PLD) and phosphatidic acid phosphohydrolase (Billah, 1990).

Figure 7. Summary of PKC Pathway



Activation of PKC can have effects on transcription factor transactivation function. Evidence has been presented that, in resting epithelial cells and fibroblasts, activation

of PKC results in site-specific dephosphorylation of the c-Jun protein which coincides with increased AP-1 binding activity and transactivation by c-Jun (Boyle, 1991). The data suggest that induction of TPA-inducible promoter element (TRE) function by PKC activators (Angel, 1987) is mediated by enhanced binding and transcriptional activity of a dephosphorylated AP-1 complex (c-Jun and c-Fos). The region identified, adjacent to the DNA-binding domain on the c-Jun protein, coincides with a region previously identified to be essential for activation of c-jun *in vitro* and *in vivo* and had been termed activation domain A2 (Baichwal, 1990).

PKC activation by phorbol esters is direct and bypasses the usual cascade triggered by normal, physiological stimuli. Phorbol esters dramatically increase the affinity of PKC for calcium, resulting in its full activation at physiological calcium concentrations (Castagna, 1982). However, prolonged incubation with phorbol esters depletes cells of PKC by down-regulation of the membrane-bound enzyme (Darbon, 1987). In addition, evidence has been provided for increased synthesis as well as increased degradation of PKC after treatment of cells with the phorbol ester (Krug, 1987).

PKC activity also depends on phosphatidylserine, an acidic aminophospholipid located exclusively on the inner leaflet of the plasma membrane. The activated kinase phosphorylates various proteins, in addition to catalyzing its autophosphorylation by an intrapeptide reaction (Newton, 1993).

V.7.2.2. Other pathways

The direct activation of PKC by phorbol esters illustrates that PKC is critically involved in growth control and it is widely accepted that PKC plays a pivotal role in the regulation of proliferation and differentiation (Clemens, 1992; Nishizuka, 1992). As these processes are dependent on the control of nuclear events, it is obvious that to

enable PKC growth-regulatory actions, some signal has to reach the nucleus after activation of PKC.

The framework of the receptor tyrosine kinase pathway toward the cell nucleus including Ras, Raf-1 and the mitogen-activated protein kinase (MAP-kinase) cascade has been established (Avruch, 1994). The regulation of MAP-kinases is a complex signaling network. Stimulation of PKC with phorbol ester leads to phosphorylation and activation of MAP-kinase kinase or erk-kinase, probably caused by activation of Raf-1 (Alessandrini, 1992). Activation of Raf-1 by phosphorylation by PKC was shown both *in vivo* and *in vitro* (Sözeri, 1992, Kolch, 1993). Thus, PKC-mediated signals can join in the pathway which initiates the MAP-kinase cascade and results in activation and nuclear translocation of MAP-kinase (Lenormand, 1993).

V.7.3. Actions

There have been several suggestions of possible functions for PKC, including involvement in secretion and exocytosis, modulation of ion conductance, regulation of receptor interaction with components of the signal transduction apparatus, smooth muscle contraction, gene expression and cell proliferation (Nishizuka, 1986).

There are a variety of signals known to trigger the translocation of PKC toward the cell nucleus. In several cell lines, the artificial PKC activators phorbol ester and bryostatin were shown to be effective. In HL-60 cells, bryostatin, which has growth stimulatory effects, leads to a translocation of PKC β_{II} but not of PKCα toward the cell nucleus. In contrast, phorbol ester, which induces differentiation in these cells, results in translocation to the plasma membrane and not the nucleus (Hocevar, 1992).

In B cells, analogs of cAMP and reagents inducing a rise in intracellular cAMP levels, lead to translocation of PKC toward the cell nucleus (Cambier, 1987).

Physiological messengers reported to trigger nuclear translocation of PKC are insulin-like growth factor-1 (IGF-1) (Divecha, 1991), interleukin-3 (Fields, 1989), platelet-derived growth factor (PDGF) (Fields, 1990), α-thrombin (Leach, 1992), Ang II (Haller, 1994) and vitamin D_3 (Simboli-Campbell, 1994). All of these signals have mitogenic effects in the cell types in which they are investigated concerning their ability to induce nuclear translocation of PKC. This suggests a role for translocated PKC in the mitogenic response. The physiological stimuli inducing nuclear translocation of PKC comprise signals binding to phosphotyrosine kinase receptors (IGF-1, PDGF) and to guanosine-nucleotide-binding-protein-coupled receptors (α-thrombin, Ang II). However, all of them, by activating different isoforms of PLC or PLD, can lead to formation of DAG, the most important endogenous activator of PKC.

VI. THE CROSS-TALK BETWEEN THE SIGNAL TRANSDUCTION PATHWAYS

Receptor-mediated activation of both AC and PI hydrolysis systems occurs through G-proteins and ultimately leads to specific activation of either cyclic AMP-dependent PKA or Ca^{2+}/phospholipid-dependent PKC. Given the remarkable diversity of agents that influence cellular metabolism through these pathways and the similarities of their components, interactions between the two signalling systems could occur.

VI.1. Phorbol Esters to cAMP Signal Transduction Pathway

Stimulation of cells with 12-O-tetradecanoyl phorbol-13-acetate (TPA), a phorbol ester that activates PKC, influences hormone-sensitive adenylate cyclase. In some cells TPA induces desensitization of receptor-mediated stimulation of AC (Sibley, 1984); whereas in others, such as frog erythrocytes, TPA treatment results in increased agonist-stimulated AC activities (Sibley, 1986). The diverse response of cAMP production to phorbol esters may be due in part to the multiple isozymes of both the PKC and the

components of the cAMP cascade (heterotrimeric G-protein, AC and phosphodiesterase).

Yoshimasa et al (1987) showed that TPA produces phosphorylation of the catalytic unit of adenylate cyclase in frog erythrocytes. They also showed that PKC can directly phosphorylate *in vitro* the catalytic unit of AC purified from bovine brain. Bell et al (1987) showed that addition of TPA to S49 lymphoma cells has little effect alone but doubles accumulation of cyclic AMP in response to isoproterenol. They discovered that TPA facilitates the interaction of the α subunit of the protein G_s with the catalytic unit of AC via PKC (Bell, 1985). Further studies found that phorbol ester increases cAMP synthesis by the Ca^{2+}-insensitive type II AC more than 9-fold within 10 min, while the treatment has no effect on the other types of AC (Yoshimura, 1992). The TPA treatment of cells expressing AC II increases basal activity and this increase is blocked by staurosporine, a PKC inhibitor (Jacobowitz, 1994).

The possibility exists that AC forms heterologous multimers. For example, a cell which expresses both types I and VI cyclase may be subjected to regulation by phorbol esters, whereas the expression of either individual isozyme alone is not responsive to phorbol esters. However, although phorbol esters have no effect on basal or forskolin-stimulated type I or VI cyclase activity, it increases receptor-, cholera toxin-, or GTPγS-stimulated cAMP production from these two AC isozymes. This indicates that cross-talk between cAMP and PKC signal transduction pathways not only requires the expression of specific isozymes of AC, but it also requires co-activation of the two pathways (Morimoto, 1994).

Investigation of the mechanism by which Ca^{2+} mediates gene induction in response to membrane depolarization found that, CREB functions as a Ca^{2+}-regulated transcription factor and as a substrate for depolarization-activated Ca^{2+}-calmodulin-dependent

protein kinases (CaM kinases) I and II. CREB residue Ser^{133} is the major site of phosphorylation by the CaM kinases *in vitro* and or phosphorylation after membrane depolarization *in vivo* (Sheng, 1991). Furthermore, Enslen's group (1995) found that activated CaM kinase IV (by CaM Kinase IV kinase) can activate CREB-dependent transcription about 30-fold, and the activated kinase also shows specificity for phosphorylation of Ser^{133}.

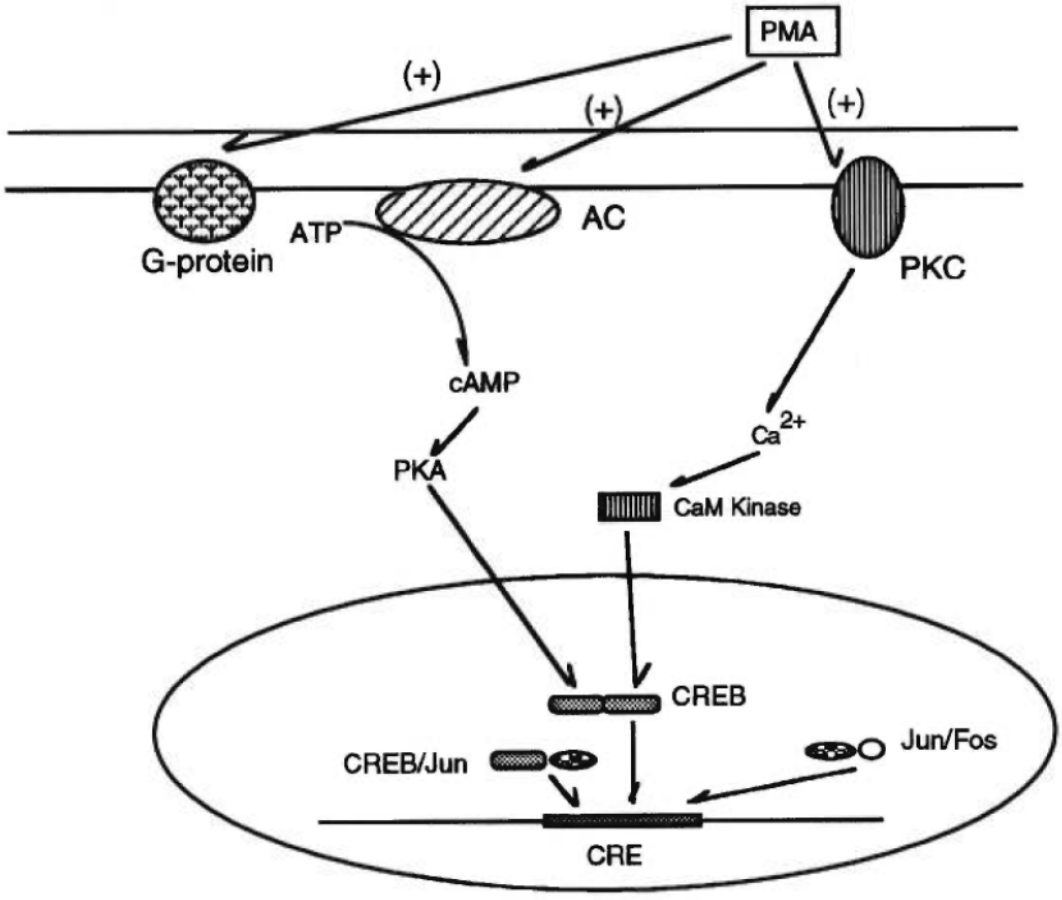
The fos/jun heterodimer binds to and activates transcription from TRE, which represent the final target of the PKC pathway. Sassone-Corsi (1988) showed that jun efficiently trans-activates CRE sequences and that fos and jun efficiently bind and cooperate in activating CRE promoter elements. Transfection studies of Benbrook's group (1990) also showed that CREB can form a heterodimer with cJun protein *in vivo*, but that the complex does not activate transcription.

The above possibilities are summarized in Fig. 8.

VI.2. cAMP to PKC Signal Transduction Pathway

The heterotrimeric G proteins mediate a variety of cellular processes by coupling transmembrane receptors to different effector molecules including inositol-phospholipid-specific PLC (Neer, 1995). The various PLC isoforms appear to be activated by different receptors, and in some cases by different G-protein components (Rhee, 1992-a). There are four well-characterized forms of PLC-ß and all of them are activated to various extents by the $G\alpha_q$ family of G proteins (Jiang, 1994). Liu et al (1996) reported that PKA specifically inhibits $G\beta\gamma$-activated PLC-ß2 activity (by directly phosphorylating serine residues of the PLC-ß2 protein both *in vivo* and *in vitro*), but not that of the Gα-activated PLC isoforms, and that the effect of PKA is not mimicked by PKC isozymes.

Figure 8. Cross-Talk. PMA to cAMP Signal Transduction Pathway



AC: adenylyl cyclase; CaM: Ca^{2+}-calmodulin kinase; CRE: cAMP responsive element; CREB: cAMP responsive element binding protein; PKC: protein kinase C; PKA: protein kinase A.

Ligand binding to receptor initiates the hydrolysis of polyphosphoinositides by PLC yielding DAG in IP_3. DAG, phosphatidylserine, and Ca^{2+} which is mobilized from the endoplasmic reticulum in response to IP_3, mediate the high-affinity association of PKC, which is normally cytosolic or loosely associated with the membrane, with the plasma membrane (Kraft, 1983). Altered subcellular distribution and activity of PKC is associated with transmembrane signaling in a variety of systems in which receptor occupancy leads to increased hydrolysis of polyphosphoinositides.

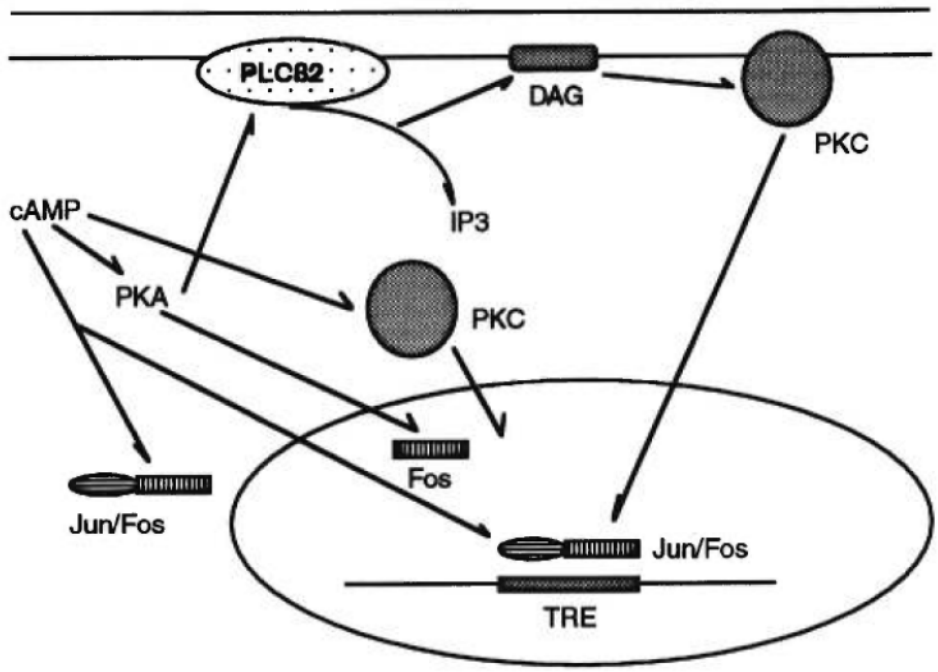
In B lymphocytes, cAMP-generating signal transduction pathways can activate translocation of PKC from the cytosol to the nucleus. Elevated cAMP levels and translocation of PKC to the nucleus are induced by antibodies against Ia antigens in normal B lymphocytes. Furthermore, the differentiation of B cells was induced, suggesting that the nuclear PKC may function in the regulation of gene expression (Cambier, 1987).

Examining changes in the activity of the atypical PKC isoforms, Wooten's group (1996) found that increases in intracellular cAMP leads to rapid stimulation of atypical PKC activity, 40-70% above control, for a sustained period of time. Further study indicated that the specific PKC isoform is PKCδ, which is redistributed to the cytoplasm with a concomitant increase in the phosphorylation state of the enzyme.

Muller et al (1989) demonstrated that the transcription factor AP-1 is modestly induced by cAMP in S49 cells, and cytoplasmic levels of c-fos and junB mRNAs are rapidly increased by cAMP. Studies on the phosphorylation of the nuclear oncoprotein Fos, Tratner (1992) demonstrated that the human c-Fos protein, phosphorylated either *in vitro* with purified PKA or *in vivo* in JEG-3 cells following treatment with forskolin, implying that phosphorylation of Fos by PKA is an important regulatory step in controlling its activity in normal cell growth and differentiation. Further studies of Sassone-Corsi (1988) characterized a CRE on the c-fos promoter region, the nuclear factor binding to the c-fos CRE is likely to be transcription factor CREB.

The above possibilities are summarized in Fig. 9.

Figure 9. cAMP to PKC Signal Transduction Pathway.



DAG: diacylglycerol; PKC: protein kinase C; PKA: protein kinase A; TRE: TPA-inducible promoter element.

VI.3. Enhancer Elements

The conserved sequence of CRE is a palindromic octamer TGACGTCA, whereas TRE has a very similar symmetrical heptameric motif, TGACTCA. Transfection studies in the JEG-3 placental cell line observed that, the reporter gene fused with CRE mediated responsiveness to cAMP but not to phorbol esters, whereas the TRE is responsive to phorbol esters but also imparts submaximal sensitivity to cAMP in the JEG-3 cells. The transcriptional activities of CRE and TRE are markedly influenced by the composition of the neighboring bases, but different sequences are permissive for the activity of the CRE versus TRE. Gel mobility-shift and UV cross-linking analyses showed that distinct proteins bind to the two control elements (Deutsch, 1988).

Other studies have also found that transcription of the human vasoactive intestinal peptide (VIP) gene is regulated by both cAMP and phorbol esters. A 17-nucleotide en-

hancer element within the human vasoactive intestinal peptide gene mediates transcriptional activation by both phorbol esters and forskolin. Mutations of this element decrease responses to both agents, suggesting that the trans-acting proteins that mediate both modes of transcriptional regulation have similar DNA-binding characteristics. However, the response of the vasoactive intestinal peptide enhancer element to forskolin, but not to TPA, is attenuated by treatment with a recombinant inhibitor of the cAMP-dependent protein kinase, suggesting that the PKA and PKC second messenger are distinct. Since the transcriptional factors CREB and AP-1 both interact with vasoactive intestinal peptide enhancer, the dual second messenger responses of the vasoactive intestinal peptide gene may result from the interaction of this second messenger enhancer with different transcriptional activator proteins (Fink, 1991).

VII. RENAL SYMPATHETIC NERVOUS SYSTEM AND THE RENIN-ANGIOTENSIN SYSTEM

Previous studies have shown that renal renin mRNA levels are lower in denervated than innervated kidneys (Page, 1992). Incubation with isoproterenol for a minimum of one hour causes significant increases in both renin secretion and renin mRNA (Bruna, 1993).

Nakamura & Johns (1994) studied the influence of renal nerve activity on renal function, plasma renin activity, and the corresponding expression of renin and angiotensinogen (ANG) genes in rat kidneys. They observed that renal nerve stimulation causes a reduction of the renal blood flow and GFR and an increase in plasma renin activity and angiotensin levels. Renal renin mRNA is increased due to the decrease in blood flow. ANG mRNA is increased in the kidney subjected to a low level of nerve stimulation but not the decrease of renal blood flow.

Further studies showed that basal plasma renin activity and responses to nerve stimulation in sponteneous hypertensive rat (SHR) are approximately half those of Wistar rats. SHR renal ANG mRNA is one quarter that of the Wistar rats and is unaffected by nerve stimulation, whereas in the Wistar rats it is increased threefold by a low but not high level of nerve stimulation (Nakamura, 1995-a).

In two kidney-one clip (2K-1C) hypertensive rats, it was found that plasma Ang I and Ang II are initially increased. The levels of striatum dopamine are higher than those in normotensive sham-operated rats. There were significant positive relationships between these monoamines and systolic blood pressure. It was suggested that in two kidney-one clip rat, both dopamine and RAS participate in the development of hypertension (Sawamura, 1996).

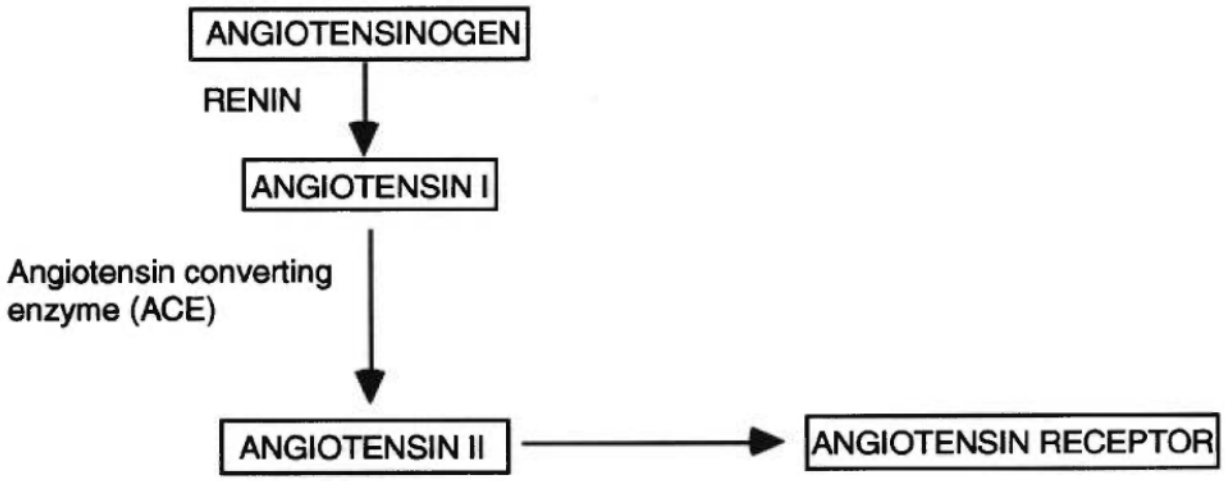
VIII. THE RENIN-ANGIOTENSIN SYSTEM

The renin-angiotensin aldosterone system (RAS) is one of the major factors involved in blood pressure and volume regulation. It participates in the control of systemic blood pressure and in the regulation of salt and water homeostasis; it modulates several neuroendocrine functions and the activity of the autonomic system. Its effector peptide Ang II increases peripheral arterial resistance by vasoconstriction, enhances renal sodium retention, and exerts growth-promoting effects. Malfunction of this system has been implicated in the pathogenesis of hypertension, cardiac failure, and progressive chronic renal failure.

Renin is an enzyme synthesized in the JG cells of the kidney. Following its release into the circulation, renin cleaves ANG to release the decapeptide angiotensin I (Ang I). Ang I is biologically inactive but is rapidly converted in the lungs and other organs to form the biologically active octapeptide Ang II. Ang II is an octapeptide resulting from

angiotensin converting enzyme (ACE) mediated cleavage of the His-Leu carboxy-terminal residue of Ang I. Ang II is a short-lived molecule ($t^{1/2}$ is 15 seconds). Ang II may increase blood pressure by the pressor effect on central nervous system accompanied by a stimulation of the sympathetic nervous system (Mann, 1982). Ang II is in turn degraded to several smaller peptides, including Ang III, which is also biologically active (Reid, 1985). The RAS components are shown in Figure 10. below.

Figure 10. The Components of Renin-Angiotensin System



VIII.1. Angiotensinogen

ANG is a 56-60 kilodalton (kDa) glycoprotein produced mainly in the liver and released into the blood. It is the only known prohormone of angiotensin and is the only known substrate for renin. The cDNA for rat ANG consists of a coding region of 1455 nucleotides encoding for 485 amino acid residues. ANG synthesis in the liver is regulated by glucocorticoids, estrogen, thyroid hormone and Ang II. These hormones cause parallel changes in ANG mRNA and protein levels in the liver (Menard, 1993).

ANG mRNA is distributed widely in a variety of tissues including vascular wall, renal proximal tubules, neuronal and astroglial cells (Menard, 1993).

The generation of transgenic rats carrying the human renin or human angiotensinogen gene allows the study of the regulation and expression of the human genes. Transgenic rats, which carry an additional rat renin gene (Ren-2) in their genome, present with severe hypertension in presence of a low plasma RAS (Mullins, 1990). The highest expression of the Ren-2 gene is found in the adrenal gland, whereas in the kidney it is rather low, probably due to feedback inhibition. The hypertension found in the transgenic mice is dependent on Ang II. However, transgenic rats carrying the human renin or human ANG genes are normotensive, although they have higher plasma levels of renin or ANG, respectively. These findings indicate that human ANG is not cleaved by mouse renin, suggesting species-specificity of the human renin substrate reaction (Ganten, 1993).

VIII.2. Renin

Renin belongs to a family of aspartic proteases (Morris, 1984). It is a glycoprotein with a molecular weight of 37,000 -- 40,000 daltons. The cDNA and genes from mice (Mullins, 1982), humans (Hardman, 1984), and rats (Burnham, 1987), were cloned and sequenced. The renin-producing (granulated) cells in the JG apparatus of the kidney are the most important part of systemic hormonal RAS.

Renin secretion is predominently influenced by the pressure in the renal artery (via vascular baroreceptor) (Tobian, 1962), by the activity of the SNS (via ß-AR) (Ehmke, 1989, Keeton, 1980), and by macula densa signal (Taugner, 1982), as well as by a number of humoral factors.

ß-adrenergic agonists stimulate renin release in virtually all experimental models of renin secretion (Campbell, 1979), in conjunction with the autoradiogaphic localization of $ß_1$-adrenoceptors to the JG apparatus and the glomerulus (Barajas, 1978). DiBona's group (1984) showed that when there is a very low frequency of electrical stimulation

on renal nerves in the anesthetized dog, it results in an increase in renin release without any change in renal blood flow, GFR or sodium reabsorption.

Among the humoral factors, Ang II inhibits renin release by a direct action on JG cells (Vander, 1967). The evidence suggests that this negative feedback mechanism operates in a tonic fashion.

VIII.3 Angiotensin Converting Enzyme

Angiotensin Converting Enzyme is localized in the vascular bed, particularly in the lung, and it is a membrane-bound ectoenzyme mainly localized on the surface of vascular endothelial cells (Yang, 1970). The relative molecular mass of human Angiotensin Converting Enzyme ranges from 140 to 170 kDa. It consists of two large homologous domains, surrounded by short non-homologous regions, each molecule having two enzymatically active sites, one at the C-terminus and one at the N-terminus (Soubrier, 1988). Each site functions independently and can cleave either Ang I or bradykinin.

Several agents, such as cAMP analogues and calcium ionophore A23187 have been shown to induce Angiotensin Converting Enzyme secretion from cultured bovine endothelial cells. Angiotensin Converting Enzyme secretion in cultured endothelial cells is also increased by glucocorticoid hormones. Glucocorticoid-responsive elements are indeed present in the somatic promoter of the Angiotensin Converting Enzyme gene and an increase in Angiotensin Converting Enzyme gene expression might be responsible for the increase in Angiotensin Converting Enzyme secretion. Angiotensin Converting Enzyme secretion is also increased in cultured endothelial cells by thyroid hormones, an experimental result which is in agreement with the observation that plasma Angiotensin Converting Enzyme is increased during hyperthyroidism in hu-

mans (Krulewitz, 1986; Dasarathy, 1991; Friedland, 1977; Hubert, 1991; Yotsumoto, 1982).

VIII.4. Angiotensin II (Ang II)

Ang II, the biologically active component of the RAS, acts throughout the body to produce an impressive number of cardiovascular, endocrine, metabolic, and behavioral effects. Ang II was first recognized for its potent vasoconstriction properties *in vivo* and *in vitro*. Another very important action of Ang II is to stimulate the release of aldosterone from the adrenals. Aldosterone causes sodium retention by the kidney and this dual action of Ang II is considered to be the unique feature of Ang II in initiating and maintaining hypertension. All of the actions exerted by Ang II are mediated by specific receptors located on the plasma membrane of the target cells, a location that is consistent with the rapid onset of the various actions of the peptide (Reid, 1985).

VIII.4.1. Biological Functions

There are several components to the pressor response to Ang II. The major component results from direct vasoconstriction in several vascular beds with the splanchnic, renal, and skin beds being the most sensitive. Ang II can also increase blood pressure indirectly through actions on the brain and SNS (Page, 1968). The centrally mediated pressor response results from a combination of an increase in total peripheral resistance secondary to increased sympathetic discharge and an increase in cardiac output secondary to withdrawal of vagal tone to the heart (Reid, 1984). Finally, Ang II can increase blood pressure by increasing the release and by decreasing the reuptake of NE at adrenergic nerve terminals, by increasing the release of catecholamines from the adrenal medulla, and by stimulating autonomic ganglia (Page, 1968).

Ang II is a potent stimulus to aldosterone secretion, and the increased activity of the RAS that occurs in a variety of situations, including sodium deficiency, renovascular

hypertension, and congestive heart failure is accompanied by parallel increases in the plasma aldosterone concentration. Administration of agents that inhibit the secretion of renin or that block the formation or actions of Ang II decreases aldosterone secretion in these situations (McCaa, 1980; 1977).

In the kidney, Ang II inhibits the secretion of renin. It also elicits a dose-dependent reduction in renal blood flow and a smaller decrease in glomerular filtration rate with the result that the filtration fraction usually increases (Navar, 1984). The increase in filtration fraction may indicate that Ang II acts preferentially on the efferent arteriole. Ang II also increases proximal tubular sodium reabsorption (to be discussed later).

VIII.4.2. Signal Transduction Pathways

Most of the responses exerted by Ang II can be explained by increased cytosolic Ca^{2+} released from either intracellular stores by transiently increased inositol trisphosphate owing to the action of PLC, a process mediated by G_q-protein, or by influx of the extracellular Ca^{2+} through various types of Ca^{2+} channels (mediated by G_o-protein). In certain cells, a decrease in cAMP production has been noted (mediated by G_i-protein). A chain of events follows these initial responses, i.e. stimulation of PKC, PLA_2 and PLD, and initiation of mitogenesis and/or protein synthesis (cellular hypertrophy). All of these receptor functions are mediated by the Ang II receptor subtype I (AT_1) receptor isoform through its interaction with G_q, G_i or G_o (Catt, 1993).

Ang II is also involved in cell growth processes. *In vivo* and *in vitro* studies showed that Ang II modulates vascular smooth muscle cell and mesangial cell growth through the AT_1 receptor, this action is probably via a direct nuclear action. Ang II actions appear to be mediated by the activation of other genes, such as platelet-derived growth factor, transforming growth factor-β_1 (TGF-β_1), *c-fos*, *c-myc*, and *c-jun*. These mediators may allow Ang II to act as a bifunctional modulator capable of activating hypertrophy or

hyperplasia of vascular smooth muscle cells depending on the local milieu (Gibbons, 1992; Hsueh, 1991; Owens, 1985; Chiu, 1991).

VIII.4.3. The Tubular Effects of Angiotensin II

Ang II (Lote, 1994) elicits vasoconstriction in efferent arterioles at lower concentrations (10^{-9} --10^{-11} M) than those needed to vasoconstrict afferent arterioles (10^{-7}--10^{-9} M).

Physiological concentrations of Ang II are in the range of 10^{-11} to 10^{-10} M (10-100 pg/ml) in plasma, and between 10^{-10} and 10^{-9} M (100-1000 pg/ml) in renal proximal tubular fluid (Semple, 1976). Since renal tissue concentrations are generally higher than those found in plasma, it is likely that the Ang II influences tubular reabsorption by prevailing upon the surrounding interstitial environment.

Ang II has a direct stimulatory action on tubular sodium reabsorption. Evidence for this has been obtained from studies in the kidney *in vivo*, and in isolated renal and nonrenal tissues. Barraclough et al (1967) showed that low doses of Ang II administered to conscious rats decreases urine flow and sodium excretion without significantly altering the glomerular filtration rate. Ang II receptors are present on apical and basolateral surfaces of PT cells. *In vitro* study showed that the basolateral Na flux was 23 to 56% greater than that of apical when equimolar Ang II was applied. In addition, the apical Ang II-dependent Na flux was mediated by AT_1 receptors, transcellular transport pathways and receptor-mediated endocytosis (Schelling, 1994).

A stimulatory effect of Ang II on net absorption of sodium and water is observed with low doses (10^{-12} - 10^{-11} M) and an inhibitory effect occurs at high doses (10^{-9} - 10^{-8} M).

The *in vivo* and *in vitro* studies provide substantial support for the contention that endogenous Ang II provides a stimulatory influence on the proximal tubule reabsorptive

rate. This effect may be rather subtle in normal animals maintained at moderate levels of sodium intake, but may be increased substantially under conditions of enhanced activity of the renin-angiotensin system (e.g. salt-restriction diet) (Navar, 1987).

VIII.4.4. Effect on Glomeruli

Ang II regulates the glomerular filtration rate by contracting the mesangial cells and thereby contracting the glomerular capillaries and decreasing glomerular size (Ausiello, 1980; Caldicott, 1981; Scharschmidt, 1986). The mesangial cell is the only one that is known as a contractile cell responding to Ang II and which contains much more actinomyosin than other cell types (Andrews, 1981) in the glomerulus. Cultured mesangial cells bear Ang II receptors and contract in response to Ang II (Foidart, 1980). Moreover, Ang II provokes the effect via the PKC signal transduction pathway, resulting in release of intracellular calcium, $[Ca^{2+}]_i$. Fujiwara (1989)'s group observed that the initial effect of Ang II on GFR is completely inhibited by depletion of extracellular calcium or treatment with calcium channel blockers, indicating that the Ang II effect is dependent on $[Ca^{2+}]_i$. However, the sustained effect can be mimicked by TPA, an activator for PKC. They concluded that the initial effect of Ang II on GFR is dependent on calcium released from the intracellular calcium store and the sustained effect is dependent on extracellular calcium influx and PKC activation.

VIII.5. The Angiotensin Receptors

The actions of angiotensin are all mediated by specific receptors located on the plasma membrane of the target cells, a location that is consistent with the rapid onset of the various actions of Ang II. Several angiotensin receptors are considered to be responsible for mediating the diverse actions of Ang II. By using the subtype inhibitors, it has been determined that the AT_1 receptor is the predominate receptor subtype in the vasculature, liver, and kidney. It appears that adult renal Ang II receptors are prin-

cipally of the AT_1 class, whereas fetal kidney Ang II receptors are of the AT_2 subtype (de Gasparo, 1990).

In addition to the classical angiotensin receptors, Hanley (1991) has observed that the mammalian proto-oncogene *mas* is an angiotensin receptor which is preferentially sensitive to Ang III over Ang II in brain tissue. The MAS receptor also belongs to the G-protein family. Sandberg (1992) has cloned the AT_3 receptor from pituitary. Most recently, another receptor has been discovered in brain which is specific for Ang II (3-8) and has been named the AT_4 receptor (Swanson, 1992).

VIII.5.1. The Signal Transduction Pathways

AT_1 receptor appears to be coupled to well-defined signalling mechanisms, including inhibition of cAMP generation via G_i, and activation of phospholipase C (PLC) and calcium mobilization (Timmermans, 1991).

The *mas* protein is regarded as angiotensin-sensitive receptors that are coupled in turn to the endogenous signaling cascade of PLC, IP_3, and $[Ca^{2+}]_i$ (Hanley, 1991). The expression of c-*mas* in proximal tubular cells can modulate cell proliferation.

In liver, adrenal, and kidney tubules, the AT_{1A} and AT_{1B} receptors are coupled by G_i to AC and inhibit AC (Pobiner, 1985). In these tissues, the inhibition of cyclase by Ang II is blocked by pretreatment with pertussis toxin. The levels of cAMP appear to be inversely related to Na and bicarbonate reabsorption in microdissected nephron segments and microperfused tubules. Thus, Ang II inhibition of AC activity appears to be the primary signal transduction mechanism that mediates salt reabsorption via regulation of Na/H exchange.

The neuronal AT_2 receptors inhibit cyclic GMP (cGMP) (Rydzewski, 1992) and this effect may be mediated via activation of a phosphodiesterase.

VIII.5.2. Regulation of the Angiotensin Receptor Gene

A variety of hormones independently modulate Ang II receptors. These include glucocorticoids, aldosterone, estradiol and peptides (e.g. insulin) (Douglas, 1987; Ballermann, 1985). Glucocorticoid increases the number of AT_1 receptors and their gene expression in cultured vascular smooth muscle cells.

Ang II down-regulates the receptor levels in a dose-dependent manner (Pujol, 1989). In neuronal cultures, both TPA or forskolin (PKA activator) stimulate levels of AT_1 receptor mRNA. The activation of PKC in response to angiotensin stimulation of the *mas*-oncogene may provide a negative feed-back loop by which the activity of this receptor is regulated (Jackson, 1989).

The mechanism by which the AT_2 receptor is regulated appears to be different to that of the AT_1 receptor. The human AT_2 receptor is down-regulated during pregnancy. This may be related to the high hormonal content of the environment induced by gestation (de Gasparo, 1994).

IX. LOCAL RAS

Ang II was long thought exclusively to be an endocrine, peripheral blood borne hormone (Campbell, 1987). However, studies in the brain have led to the concept that Ang II is a locally formed peptide which is present in many organs including brain, heart, liver, adrenal, kidney, blood vessels, gonads and skin. The widespread presence of all the components of RAS in the same tissues, has led to the assumption

that Ang II is a local paracrine hormone with paracrine functions of physiological importance to the tissue in which it is expressed.

IX.1. Vascular and Heart

In the vasculature, renin mRNA is found in the endothelium and the medial and advential layers (Iwao, 1988). Angiotensin Converting Enzyme, as well as its mRNA, are also found in the endothelium but do not appear in the deeper layers of the vessel wall (Iwao, 1988). ANG mRNA is expressed only in adventitia, and in the fatty tissue surrounding the blood vessels (Cassis, 1988; Campbell, 1987-a). Cultured vascular endothelial cells synthesize and secrete Ang II (Kifor, 1987). Ang II has been shown to cause hypertrophy in cultured, quiescent, rat aortic smooth muscle cells. This effect is mediated by the induction of the proto-oncogenes c-fos and c-jun by Ang II (Naftilan, 1990).

ANG mRNA is expressed in the atria, ventricles and whole heart (Dzau, 1987-b) in rat and mouse. Ang II levels, when measured in the rhesus monkey heart, are highest in the right atrium with decreasing levels in the right ventricle, left atrium, interventricular septum and left ventricle (Lindpainter, 1990). Renin mRNA has been isolated from heart tissue of both mice and rats (Dzau, 1987-b). Renin activity has been detected in isolated left ventricular rat cardiomyocytes and in cardiac tissue of mice (Re, 1987, Dzau, 1987-b).

mRNA for Angiotensin Converting Enzyme has been located in the left ventricular tissue of rats (Schunkert, 1990) while Angiotensin Converting Enzyme activity has been reported to be present in atria and ventricles of rats (Fabris, 1990).

Both the AT_1 and AT_2 receptors are equally distributed in the atria and ventricular myocardium of the rat and the rabbit (Rogg, 1990). Autoradiographic data from the

human heart shows that these receptors are localized in the myocardium, coronary vessels and sympathetic nerves (Urata, 1989).

Therefore, the mRNAs for all the components for RAS synthesis have been identified in mammalian heart tissue and there is evidence that these mRNAs are translated to proteins and these components of the RNA found in the heart interact to form Ang II.

IX.2. Kidney

In the adult kidney, the components of the RAS have been anatomically localized. Under normal conditions, renin is localized in the JG cells, modified smooth-muscle cells in the distal arteriole. Faraj et al (1992) discovered a small number of renin-containing cells in the glomerulus and efferent arteriole. Using in situ hybridization, Ingelfinger et al (1990) found that ANG mRNA is localized predominantly in the proximal tubule of rat kidney, with lesser quantities in glomeruli, distal tubules, and intra-renal vessels. Angiotensin Converting Enzyme is found in greatest concentrations in the proximal tubule, with smaller amounts in arterioles and glomerulus (Ikemoto, 1987). Autoradiography has indicated that Ang II receptors are found in highest concentrations on the arterioles, glomerulus, and vasa recta (Yamada, 1990). Binding studies using isolated nephron segments have also resulted in the specific binding of tubule receptors found in the proximal tubule (Mujais, 1986).

X.2.1. Proximal Tubule

In the proximal tubule, Ang II is an important regulator of NaCl and $NaHCO_3$ absorption (Liu, 1988, Harris, 1977). Although part of this effect is secondary to modulation of glomerular hemodynamics, Ang II also has direct effects on proximal tubule transport mediated by Ang II receptors on both the apical and basolateral membranes (Douglas, 1987).

ANG protein and mRNA are present in the proximal tubule and are up-regulated by sodium depletion (Ingelfinger, 1990; 1986). Angiotensin Converting Enzyme has been localized in the apical membrane and to a lesser extent the basolateral membrane of the proximal tubule (Marchetti, 1987). Both renin activity and mRNA were low in the cultured proximal tubule cells, approximately 2% of that measured in isolated JG cells. However, when rats are given Captopril, renin mRNA is clearly detectable, predominantly in the S_2 proximal tubule (Moe, 1993-b).

The existence of all components of the RAS in the proximal tubule suggests that this segment may be an important source of locally generated Ang II. Theoretically, renin could be secreted from the apical or basolateral membrane along with ANG and Ang I would then be generated extracellularly. Alternatively, renin can cleave ANG and generate Ang I intracellularly. Since ACE is present on the plasma membrane, one would expect the intracellularly generated Ang I to be transported extracellularly to the converting enzyme. Ang II generated from Ang I can then act on the same proximal tubule cell in an autocrine mode or be delivered downstream to act on another proximal tubule cell in a paracrine fashion (Moe, 1993-a).

The renal proximal tubule absorbs 50% of the NaCl and 80% of the $NaHCO_3$ filtered at the glomerulus (Alpern, 1990). Hormones such as Ang II, norepinephrine, and dopamine regulate transepithelial transport by modifying apical and basolateral Na transporters via second messenger systems. The presence of a local proximal tubule RAS presents the possibility for novel mechanisms of regulation of these transporters.

In conclusion, a complete RAS is present in the mammalian proximal tubule that is potentially autocrine and paracrine in nature. This RAS may be important in the regulation of proximal tubule NaCl and $NaHCO_3$ transport. It may play a pathogenic role in certain patients with essential hypertension and in the secondary hypertension.

X. ANG GENE REGULATION

Under normal conditions, ANG concentrations circulating in the plasma are rate limiting for the maximum velocity of Ang I formation. In the liver, the major site of circulating ANG synthesis, ANG expression is under exquisite hormonal control. Inflammation activates ANG gene transcription as a result of the macrophage-derived cytokines interleukin-1 and tumor necrosis factor-α (TNF-α) (Brasier, 1996). Activation of the RAS, through production of Ang II, results in feedback stimulation of ANG synthesis (the "positive feedback loop") (Eggena, 1993).

X.1. THE 5'-Flanking Sequence

The 5'-flanking region of the ANG gene is important for tissue- and cell type-specific expression of the gene *in vitro* and *in vivo*. Tamura et al (1992) disclosed that the promoter region of the mouse ANG gene is able to direct transcription in HepG2 cells. Clouston's group (1989) showed that the 750-bp promoter element from the immediate 5'-flanking region is capable of directing most, but not all, tissue-specific and hormonal regulation of the ANG minigene in transgenic mice. Altogether, the 5'-flanking sequences play a major role in the regulation of the ANG gene expression.

Tamura's group (1994) further reported that, the proximal promoter region of the mouse ANG gene from -96 to +22 is able to confer HepG2-specific transcriptional activity and identified two cis-acting elements that contributed to this specificity, ANG gene-activating element 2 (AGE2) (-96/-52) and AGE3 (-6/+22). Electrophoretic mobility shift assay identified two nuclear factors, ANG gene-activating factor 2 (AGF2) and AGF3. AGF2 bound to AGE2 in HepG2-specific manner, whereas AGF3 interacted with AGE3 in all cell lines examined. The heterologous thymidine kinase promoter assay showed that AGE2 and AGE3 synergistically conferred HepG2-specific ANG gene expression and suggested that the synergistic interplay between AGF2 and AGF3 is important for the ANG promoter activation.

During the hormonal induction of the differentiation of 3T3 adipoblasts into adipocytes, ANG gene transcription and corresponding mRNA levels are induced from undetectable to high levels and remain elevated for as long as the adipocytes are maintained in culture (Ron, 1990). The same group demonstrated a differentiation-specific element located at -982 to -996 in the promoter of the ANG gene that has the remarkable property of being responsible for the sustained irreversible expression of the ANG gene (1993). The cloning of 3T3 adipocyte cDNA has been reported that encodes a 150 Kd protein, designated Differentiation Specific Element Binding Protein that exhibits sequence-specific binding to the Differentiation-specific Element. Differentiation-specific Element Binding Protein may be both a transcription factor and a DNA-replication factor (McGehee, 1995).

X.2. Mechanisms of ANG Gene Transcription by Glucocorticoids

Inducible control of gene expression can be affected by changes in: transcription rates, mRNA stability and processing, in selected situations and translational initiation rates. In the liver, which is the major site for plasma protein biosynthesis and which lacks the ability to store presynthesized proteins, translation and secretion of proteins through the constitutive pathway seem to be the primary actions. In mammalian hepatocytes changes in transcriptional initiation rates have been observed to be a common and important mechanism in controlling gene expression (Tewksbury, 1990).

ANG gene transcription is responsive to diverse hormonal mediators. These agents include circulating steroid hormones: glucocorticoids, estrogens (Kett, 1993), and thyroid hormones (Hong-Brown, 1992). Among them, a glucocorticoid excess and estrogen administration are associated with elevated ANG levels as well as Ang II-dependent hypertension.

Glucocorticoids are the most potent pharmacological activators of ANG gene expression. Administration of natural or potent synthetic glucocorticoids to humans increases circulating ANG (Krakoff, 1973); in experimental animals, glucocorticoids administration induces accumulation of ANG mRNA transcripts in the liver (Kalinyak, 1987). Conversely, adrenalectomized animals have suppressed levels of circulating ANG (Clauser, 1985).

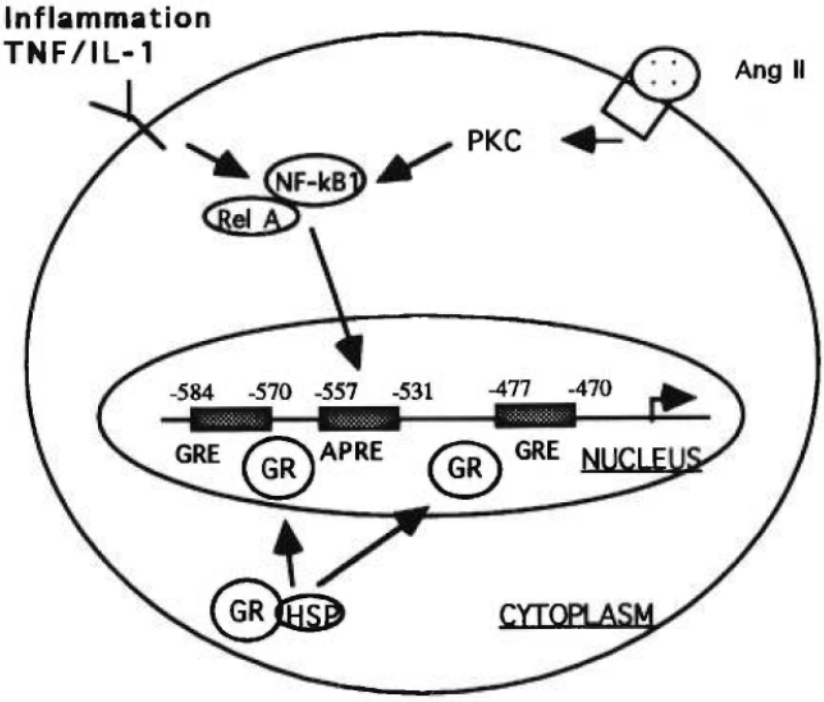
Adrenal-derived glucocorticoids produce the translocation of the glucocorticoid receptors (GR) into the nucleus. The mechanisms of action of glucocorticoids are: GR is a ligand-inducible transcription factor that forms a complex with cytoplasmic heat-shock proteins. Upon binding to glucocorticoid, this complex dissociates and GR enters the nucleus. The activated GR is now competent to bind to high affinity binding sites within the genome and thereby modulates gene expression (Pratt, 1992).

Studies with the rodent ANG gene revealed that, the first 700 base pairs upstream of the transcription starting site are sufficient for tissue-specific and hormonally regulated expression (Brasier, 1989). Nuclear run-on assays and gene-transfer studies have shown that glucocorticoid exert their effects at the transcriptional level (Brasier, 1986). Moreover, the fact that the glucocorticoid induction of ANG gene expression is dose dependent, saturable, and antagonized by the antiglucocorticoid RU486, these studies indicate that this transcriptional event is mediated by the GR (Brasier, 1996).

Within the first 700 base pairs of the rodent ANG gene, there are two glucocorticoid-responsive element (GRE) sites (Fig. 11). GRE I, a near-palindromic GR containing the sequence 5'-AGAACATTTTGTTTC-3' (-584 to -570) is essential for glucocorticoid induction. GRE II, a hexameric "half-site" sequence located between -472 and -477, is insufficient for glucocorticoid induction in the absence of a functional GRE I. However, it is required for maximal glucocorticoid inducibility of the ANG gene promoter. Both GRE

I and GRE II bind to recombinant GR in *in vitro* DNA binding assays. These data suggest that GREs in the ANG gene promoter are hierarchical and synergistic (Brasier, 1996).

Figure 11. Mechanisms of ANG Gene Transcription.



(Modified form Brasier & Li, Hypertension. 1996;27pt2:473. TNF: tumor necrosis factor; IL-1: interleukin-1; NF-κB1: necrosis factor-κB1; PKC: protein kinase C; Ang II: angiotensin II; GRE: glucocorticoid response element; GR: glucocorticoid receptor; APRE: acute-phase response element; HSP: heat-shock protein.)

Studies with the rat ANG gene by our group (Chan, 1992) have also demonstrated that glucocorticoid stimulates gene expression within the first 700 base pairs upstream of the transcription site (-688/+18). Moreover, previous studies (Chan, 1990) showed that there are two putative GREs located on N-478 to N-472 and on N-585 to N-579. Further studies are underway in our laboratory.

X.3. Mechanisms of ANG Gene Activation by Inflammation

The hepatic acute-phase response (APR) is another well-characterized physiological activator of ANG expression. This activation is a species-conserved stereotypic response of the mammalian liver to the initiation of inflammation.

In the APR, local injury or inflammation results in cytokine elaboration; these hormones induce a switch in hepatic gene transcription to producing proteins involved in macrophage opsonization and wound repair (Birch, 1986). The APR, initiated experimentally by bacterial lipopolysaccharide (LPS), is a potent inducer of hepatic ANG expression (Kageyama, 1985). In the LPS induced APR, the macrophage-derived cytokines TNF-α and interleukin-1 are the key activators of hepatic ANG expression (Brasier, 1996).

Studies of the ANG genes have shown that, the crucial DNA control element of the ANG 5'-flanking sequences, located between -557 and -531 in the rat gene, is absolutely required for cytokine induction of ANG promoter activity (Fig. 11). This region was termed acute-phase response element (APRE) (Brasier, 1989). The APRE functions as a cytokine-inducible enhancer. It is a binding site for the potent necrosis factor (NF-κB) transcription factor complex (including NF-κB1 and nuclear factor-κBp65 subunit (Rel A)) (Baeuerle, 1991). Upon binding of the transcription complex and APRE, the gene transcription is then stimulated.

X.4. Transcriptional Mechanisms of the RAS Positive Feedback Loop

Intravascular Ang II formation controls the activity of the RAS through negative feedback regulation of renin secretion and positive feedback regulation of ANG synthesis (Eggena, 1993). In the hepatocyte, Ang II stimulates ANG gene expression in part through enhanced transcription (Eggena, 1993). The RAS "positive feedback loop" is important to ensure that sufficient ANG is in supply to respond to any future hypotensive challenges. It may also play a role in the malignant phase of essential or reno-

vascular hypertension, in which enhanced ANG synthesis initially sustains the elevated blood pressure (Peach, 1977).

Upon binding to the Ang II type 1 receptor, Ang II stimulates the formation of DAG with consequent PKC activation, mobilization of intracellular calcium, and inhibition of hormone-stimulated AC activity (Griendling, 1986).

Continuously perfused primary hepatocytes have been used to demonstrate that Ang II induces the accumulation of the ANG mRNA and the secretion of the protein (Shade 1973). In the same Manner and gel mobility shift assay, Brasier's group (1996) has found a four-fold increase of APRE binding activity by Ang II in a dose-dependent fashion. Moreover, the increase in APRE DNA binding activity is rapid and observed as early as 1 hour after stimulation. Competition analysis showed that Ang II-activated APRE binding is sequence specific for the NF-κB protein family (Fig. 11). Their conclusion is that cytokines and the vasoactive Ang II peptide produce ANG transcription through the same cytoplasmically inducible transcription factor -- the NF-κB1/Rel-A complex.

XI. INTERACTION OF THE RAS AND SYMPATHETIC NERVOUS SYSTEMS

The RAS system is mainly involved in the regulation of arterial blood pressure and fluid balance. One of the main stimuli for the secretion of renin present in the renal juxtamedullary cells, but also in certain other tissues, is provided by the sympathetic nervous system via the action of NE on β_1-ARs (Kawasaki, 1984). There is good evidence in animal experiments that Ang II facilitates sympathetic neurotransmission by several mechanisms, including an increase in the rate of NE synthesis, an increase in neuronal release of NE in response to nerve stimulation, inhibition of neuronal

reuptake, and increased vascular smooth muscle reactivity to NE (Starke, 1977). All of which seem to involve distinct, but perhaps heterogeneous, Ang II receptors. Acting within the central nervous system, angiotensin augments sympathetic nerve outflow directly, but probably also by inhibiting the reflex decrease in sympathetic nerve activity following an increase in arterial pressure (Bickerton, 1961; Reid, 1984). Ang II also stimulates adrenomedullary and ganglionic transmission as well as enhances the release of sympathetic transmitter by a presynaptic action (Peach, 1971; Starke, 1977). In addition, there is some evidence that angiotensin can inhibit NE reuptake and augment its biosynthesis and responses mediated via both extrasynaptic α_2- and intrasynaptic α_1-ARs (Khairallah, 1971).

Brain Ang II plays a key role in blood pressure control in part by interacting with CAT and by stimulation of sympathetic pathways. Ang II causes both acute and chronic stimulation of NE uptake in neuronal cultures of Wistar Kyoto rat brain. The acute stimulation appears to be a postranscriptional event and does not involve PKC or NE transporter system, whereas the chronic stimulation of NE uptake involves PKC, c-fos, and the NE transporter system. Both effects are via the AT_1 receptor subtype (Lu, 1996).

In rabbits, intravertebral infusions of Ang II produce a dose-dependent shift of the midrange of the baroreflex curve toward higher pressures. Pretreatment with Prazosin (α_1-AR antagonist) via the vertebral artery markedly reduces the shift in the baroreflex curve induced by Ang II. This experiment proved that Ang II modulates arterial baroreflex function via a central α_1-AR mechanism (Nishida, 1995).

Faria's group (1992) examined the angiotensin-induced potentiation of noradrenergic transmission in the isolated mesenteric arteries of one-kidney, one clip (1K1C) hypertensive rats, in which the induced hypertension is considered to be independent of the circulating RAS (Ferrario, 1984). Faria's group found that the angiotensin converting

enzyme (ACE) activity measured in plasma does not change during the development of hypertension, whereas the activity measured in the aortic tissue is significantly augmented 28 days after the renal artery is clipped. Although the pressor responses to nerve stimulation are basically unaltered, a significant increase in the sensitivity to NE develops during hypertension. The 1K1C preparations presented an increased sensitivity to the facilitatory effect of Ang II on the response to periarterial nerve stimulation. The facilitatory effect of Ang II on both nerve stimulation and exogenous NE was blocked by Saralasin, AT_1 receptor antagonist.

Studies with human cortical kidney slices also showed that Ang II enhances a stimulation-induced outflow of NE in a concentration-dependent manner (Rump, 1995). Ichihara's group (1995) showed that the NE-induced renin secretion rate is amplified in the presence of Ang II in JG cells harvested from Sprague-Dawley rats. The NE-induced intracellular calcium response is also potentiated by Ang II. Prazosin blocks the NE threshold renin secretion rate responses and abolishes the agonist-related enhancements. It suggests that the response of NE is mediated via the α_1-ARs.

The above studies indicate that there is an interaction between RAS and the SNS. However, whether the renal SNS is involved in the regulation of ANG gene expression in the kidney via its specific receptors is unknown.

HYPOTHESIS

There is a functional interaction between the catecholamines (i.e. activation of the renal sympathetic nervous system) and the activation of the renin-angiotensin system in the renal proximal tubules.

The adrenergic receptors α and ß are both involved in mediating the stimulatory effect of catecholamines. There might be an interaction between the two receptors on the expression of the ANG gene and this interaction is probably mediated by the "cross-talk" of PKA and PKC signal transduction pathways via the cAMP-responsive element (CRE) in the 5'-flanking region of the rat angiotensinogen (ANG) gene.

OBJECTIVES

1. To study whether α-adrenergic and dopaminergic receptors have any effect on ANG gene expression in the opossum proximal tubular (OK) cells;

2. To study the effect of NE (via both α- and ß-ARs) on the expression of the ANG gene in OK cells;

3. To study which signal transduction pathways (PKC or PKA) is involved in the effect of catecholamines on the expression of the ANG gene in OK cells;

4. To study the molecular mechanisms of the effect of the adrenergic receptors on the expression of the ANG gene in OK cells.

MATERIALS

I. Cells and Culture

I.1. OK Cells

The opossum kidney proximal tubular cell line was obtained from the American Type Tissue Culture Collection (ATCC). It was derived from the kidneys of an adult female American opossum (Koyama, 1978). This cell line has been characterized with respect to morphology, chromosome constitution, tissue-culture requirements, and attainable mitotic arrest. The cells are morphologically epithelial-like.

OK cells have renal epithelial properties such as the polarized distribution of plasma membrane proteins, the expression of an apical microvilli, and the presence of renal transport systems that are characteristic of the proximal tubule (Caverzasio, 1985), i.e. parathyroid hormone (PTH), forskolin, and prostaglandin E1 increase cAMP formation in OK cells. PTH inhibits sodium-dependent phosphate transport in OK cells. Furthermore, these cells have been shown to possess most of the adrenergic receptors. Therefore, this cell line is used as the model for proximal tubule cells by various investigators (Caverzasio, 1985; Cole, 1989; Murphy, 1988-a, -b) and has been chosen as a prototypical model for our studies.

I.2. Culture Dishes

The 6-, 24-, and 96-well plates and 100 X 20 mm petri dishes were purchased from GIBCO Bethesda Research Laboratories (BRL) (Quebec, Canada).

I.3. Disposable Sterile Pipettes

Pipettes (5- and 10-ml) were purchased from Costar Corporation (Cambridge, MA, USA).

I.4. Cell Culture Media and Supplements

All culture media and supplements were purchased from GIBCO BRL (Quebec, Canada):

I.4.1. Dulbecco's Modified Eagle's Medium (D-MEM) (CAT.# 12800-082) with high glucose, L-glutamine, 110 mg/L sodium pyruvate, without sodium bicarbonate;

I.4.2. Fetal Bovine Serum (FBS) (CAT.#1-140-048), heat-inactivated;

I.4.3. Dulbecco's phosphate Buffered Saline (PBS) (CAT.#14200-026), 10X, without calcium chloride or magnesium chloride;

I.4.4. Trypsin-EDTA, 1X (CAT.#25300-021).

II. Drugs and Hormones

		F.W.	CAT.#
(±)-Atenolol	(β_1-receptor antagonist)	266.34	A-139
8-Bromo-cAMP	(cAMP analog)	430.9	B-130
6-Chloro-APB, HBr (SKF 82958)	(D_1-receptor full agonist)	410.74	C-130
Epinephrine bitartrate	(adrenergic agonist)	333.29	E-104
Dopamine	(dopaminergic receptor agonist)	189.6	D-019
Forskolin	(adenylate cyclase activator)	410.5	F-105
ICI-118,511	(β_2-receptor antagonist)	313.9	I-127
ß-Iodoclonidine HCl	(α_2-receptor agonist)	392.46	I-144
(±)-Isoproterenol HCl	(ß-receptor agonist)	397.38	I-104
Ketanserin tartrate	(Selective 5-HT2/5-HT1c serotonin receptor blocker)	545.5	S-006
(±)-Norepinephrine	(ß, α_1-receptor agonist)	219.67	N-112
Phorbol 12-myristate 13-acetate (PMA or TPA)	(Phorbol ester activator of protein kinase C)	616.84	P-145

PPHT HCl ((±)-(N-0434))	(D_2-receptor agonist)	345.9	P-105
Prazosin hydrochloride	(Peripheral α_1-receptor antagonist)	419.9	P-115
R(+)-SCH-23390 HCl	(D_1-receptor antagonist)	324.1	D-054
R(-)-Phenylephrine HCl	(α_1-receptor agonist)	203.67	P-133
Rp-cAMPs triethylamine salt	(inhibitor of cAMP-		
	dependent Protein kinase I & II)	446.46	A-165
Spiperone HCl	(D_2-receptor antagonist)	431.94	D-050
Staurosporine	(PKC inhibitor)	466.54	S-137
U73122	(Phospholipase C and A_2 inhibitor)	464.65	U-107
Yohimbine HCl	(α_2-receptor antagonist)	390.91	Y-100

The above chemicals were purchased from Research Biochemicals International (RBI, Nattick, MA, USA).

III. Reagents

From Sigma Chemical Corporation (St. Louis, MO, USA)

	F.W.	CAT.#
Hepes	238.3	P-133
Streptomycin Sulfate (729u/mg)	1457.38	860-1860
Penicillin "G"	1650 u/mg	860-1830
From Boehringer Mannheim Gmbh (German)		
Ampicillin	371.4	835-269
From Dupont, NEN (Boston, MA, USA)		
^{125}I-Na	5 mCi/50 µl	NEZ-033A
^{35}S-dATP	1 mCi/50 µl	NEG-734H
γ-^{32}P-dATP	250 µCi/25 µl	NEG-502A
α-^{32}P-dCTP	250 µCi/25 µl	NEG-513H
^{14}C-Chloramphenicol	50 µCi/500 µl	NEC-408

From Bethesda Research Laboratories (GIBCO BRL, Burlington, Canada)

Geneticin (G418 sulfate)	860-1811

From Bio-Rad Laboratories (Richmond, CA, USA)

AG 1X8 Ion Exchange Resin	140-1441

All other reagents (e.g. Agarose) were of molecular biology grade and purchased either from Sigma Chemicals, Bethesda Research Laboratories (GIBCO BRL), Boehringer-Mannheim, Pharmacia Inc, or Promega-Fisher Inc.

IV. Bacteria and Culture

IV.1. Bacteria

	Company	CAT.#
HB101	Promega	L2011
TB-1	New England BioLabs	800
DH 5α competent cells	GIBCO BRL	18265-017

IV.2. Bacterial Culture

Tryptone	DIFCO Laboratories	0123-07-5
Yeast-extract	DIFCO Laboratories	0127-17-9

IV.3. Culture Media

IV.3.1. LB media: 1% Tryptone, 0.5% Yeast-extract, 1% NaCl, autoclave;

IV.3.2. Rich LB media: 1% Trptone, 0.5% Yeast-extract, 0.5% NaCl, 0.2% glucose;

IV.3.3. Agar plates: 1% Tryptone, 0.5% Yeast-extract, 1% NaCl, 1.5% agar, autoclave.

(For ampicillin plates: after autoclavation, cool down until 45-50°C, add ampicillin to a final concentration of 25 μg/ml and plate the medium into dishes.)

V. Restriction and Modifying Enzymes

All enzymes were purchased from GIBCO BRL (Burlington, Ontario, Canada), Boehringer-Mannheim (Doval, Quebec, Canada), Pharmacia Inc. (Baie d'Urfé, Quebec, Canada), or Sigma Biochemicals (St-Louis, MO, USA).

VI. Expression Vectors and Oligonucleotides

VI.1. pGEM-3 (Promega-Biotech, Madison, WI, USA)

It is a mammalian expression vector containing multiple polylinker sites (Hind III, Sph I, etc.) with Sp6 and T_7 promoter on the 5' and 3' ends, respectively.

VI.2. pOGH & pTKGH (Nichols Institute of Diagnostics, La Jolla, CA, USA)

These plasmids contain the entire genomic sequence of the human growth hormone (hGH) gene with or without thymidine kinase (TK) enhancer/promoter sequence fused to the 5'-end of the hGH gene;

VI.3. pRSV-Neo

This plasmid was a gift from Dr. Teresa Wang (Dept. of Pathology, Stanford University, Stanford, CA, USA). It contains the coding sequence for Neomycin (Neo) with Rouse Sarcoma Virus (RSV) enhancer/promoter sequence fused to the 5'-end of the Neomycin gene;

VI.4. pRC/RSV (Invetrogen Corporation, San Diego, CA, USA)

It is a mammalian expression vector, contains the RSV/LTR promoter/enhancer plus a bovine growth hormone poly-adenylation signal for polyadenylation of transcribed mRNAs;

VI.5. pMAL-c (New England BioLabs, New England)

This vector allows the cloned gene inserted downstream from the *malE* gene, which encodes maltose-binding protein (MBP), and results in the expression of an MBP fusion protein.

VI.6. pcDNA I (Invetrogen Corporation, San Diego, CA, USA)

It is a mammalian expression vector and contains the CMV fused with a polylinker site.

VI.7. Oligonucleotides

		-806 -779
ANG-CRE	5'AGC TTA AGA GAT TAC T**TG ACG TAC** TGG ATG CAA A3'	
(containing rat ANG N-806/N-779)	5'CTA GAT TGC ATC CA**G TAC GTC A**AG TAA TCT CTT A3'	
ANG-CREM1	5'AGC TTA AGA GAT TAC T**TG ACI TAC** TGG ATG CAA A3'	
	5'CTA GAT TGC ATC CA**G TA**A **GTC A**AG TAA TCT CTT A3'	
ANG-CREM2	5'AGC TTA AGA GAT TAC T**TG A**AT **TAC** TGG ATG CAA A3'	
	5'CTA GAT TGC ATC CA**G TA**A T**TC A**AG TAA TCT CTT A3'	
ANG-CREM3	5'AGC TTA AGA GAT TAC TTAT AT **TAC** TGG ATG CAA A3'	
	5'CTA GAT TGC ATC CA**G TA**A TAT **A**AG TAA TCT CTT A3'	

rat CREB cDNA primers

5' end	5'TCG AAT TCA TGA CCA TGG ACT CTG GA3'
3' end with 6-histidine	5'GAA GAA TTC TTA GTG ATG GTG ATG GTG ATG ATC TGA CTT GTG GCA GTA AAG GTC3'

The oligonucleotides were synthesized by Bio Synthesis Inc., Lewisville, TX, USA.

VII. The Kits

VII.1. Radioimmunoassay kit for Human Growth Hormone (RIA-hGH)

The RIA-hGH kit was a gift from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases (NIADDA), NIH, USA.

It contains human growth hormone antigen (NIDDK-hGH-I-3 (AFP-11019B)) for iodination, a hormone standard (2.0 mg/bottle), and human growth hormone antiserum (rabbit) (NIDDK-anti-hGH-2) (1.0 ml of 1:50 dilution).

VII.2. ^{T7}Sequencing kit (Pharmacia Inc., CAT. #27-1682-01), contains:

"A", "C", "G" & "T" Mixes-short and -Long	Annealing Buffer
FPLC*pure* T_7 DNA Polymerse, Cloned	Labeling Mix-dATP
Enzyme Dilution Buffer	Labeling Mix-dCTP
Universal Primer	Stop Solution
Control Template	Instruction booklet

VII.3. Polymerase Chain Reaction (PCR) kit (Perkin Elmer Cetus, CT, USA), contains:

Taq DNA Polymerase	dNTP stock solution, 10 mM
PCR reaction buffer, 10X	

VII.4. TA Cloning kit (Invitrogen Corporation, San Diego, CA, USA), contains:

pCR2.1 vector	T_4 DNA ligase
10X ligation buffer	Sterile water

VII.5. Geneclean II kit (BIO 101 Inc., La Jolla, CA, USA), contains:

NaI, 6 M sodium iodide	Glassmilk	TBE modifier

NEW concentrate (concentrated solution of NaCl, Tris & EDTA for washing)

VIII. Buffer Systems

VIII.1. Cells

VIII.1.1. RIA buffer: 0.05 M Phosphate buffer (pH7.4), 0.01 M EDTA (pH 8.0), 0.2 ml% Triton X-100, 0.5% Bovine Serum Albumin (BSA);

VIII.1.2. Calcium phosphate transfection buffer:

VIII.1.2.1. 2 M $CaCl_2$

VIII.1.2.2. 2X Hepes Buffered with Sodium Phosphate (HBSP): 1.5 mM Na_2HPO_4, 10 mM KCl, 280 mM NaCl, 12 mM Glucose, 50 mM Hepes; pH 7.4;

VIII.2. DNA

VIII.2.1. 10X Ligation Buffer: 200 mM Tris·Cl, 50 mM $MgCl_2$, 50 mM Dithiothreitol, 500 µg/ml BSA;

VIII.2.2. Transformation

VIII.2.2.1. FSB: 10 mM KAc, 100 mM KCl, 45 mM $MnCl_2{\cdot}4H_2O$, 10 mM $CaCl_2{\cdot}2H_2O$, 3 mM $HACoCl_3$, 10% Redistilled glycerol;

VIII.2.2.2. SOB: 2% Tryptone, 0.5% Yeast-extract, 10 mM NaCl, 2.5 mM KCl, autoclave; SOC: to SOB, add 10 mM $MgCl_2/MgSO_4$ and 20 mM glucose;

VIII.2.3. Annealing Buffer: 0.1 M NaCl, 10 mM Tris·Cl (pH 7.8), 1.0 mM EDTA;

VIII.2.4. Hybridization buffers

VIII.2.4.1. 20X SSPE: 3 M NaCl, 0.2 M NaH_2PO_4, 0.02 M EDTA;

VIII.2.4.2. 20X SSC: 3 M NaCl, 0.3 M $Na_3Citrate{\cdot}2H_2O$;

VIII.2.4.3. Hybridization buffer: 5X SSPE, 5X Denhardt, 100 µg/ml denatured Salmon Sperm DNA, 1% SDS;

VIII.2.5. DNA Sequencing Buffer

VIII.2.5.1. 40% Acrylamide: 10 g N'N-Methylenebisacrylamide, 190 g Acrylamide in 500 ml total volume with dH_2O, filtered through a 0.45 µM Millipore filter;

VIII.2.5.2. 20X TBE: 1.8 M Tris·base, 1.8 M boric acid, 0.04 M EDTA;

VIII.2.5.3. Sequencing Gel -- 8% Acrylamide Gel:

8% Acrylamide (from 40% Acrylamide), 8 M urea, 1X TBE, filtered through a 0.45 µM Millipore filter, when using, add 1% Temed, 0.01% Ammonium Persulfate;

VIII.2.6. Mini-Agarose Gel

VIII.2.6.1. 50X TAE: 2 M Tris·base, 5.7% acetic acid, 50 mM EDTA (pH 8.0);

VIII.2.6.2. Mini-Agarose gel: 0.8% Low EEO Agarose in 1X TAE;

Running buffer: 1X TAE;

VIII.2.7. DNA Preparation

VIII.2.7.1. Solution I: 50 mM glucose, 25 mM Tris·Cl (pH 8.0), 10 mM EDTA (pH 8.0);

VIII.2.7.2. Solution II: 0.2 N NaOH, 1% SDS;

VIII.2.7.3. Solution III: 5 M potassium acetate 60 ml, glacial acetic acid 11.5 ml, add dH_2O to 100 ml.

VIII.2.7.4. TE buffer: 0.01 M Tris·HCl, 1 mM EDTA

VIII.3. Proteins

VIII.3.1. SDS-PAGE electrophoresis

VIII.3.1.1. 2X SDS-PAGE sample buffer: 0.125 mM Tris·base, 4% SDS, 10% glycerol, 0.02% Bromophenol blue, 4% ß-mercaptoethanol;

VIII.3.1.2. 10X SDS-PAGE running buffer: 0.25 M Tris·base, 1.9 M glycine, 1% SDS;

VIII.3.1.3. Transfer buffer: 0.1 M Tris·base, 0.77 M glycine, 20% methanol;

VIII.3.1.4. 30% Acrylamide: 1 g N'N-Methylenebisacrylamide, 29 g Acrylamide in

100 ml total volume with dH_2O;

VIII.3.2. Gel-Mobility Shift Sample Buffer: 0.3 units of poly (dI-dC) in 20 mM Hepes, pH 7.6, 1 mM EDTA, 50 mM KCl, 2 mM spermidine, 1 mM DDT, 0.5 mM PMSF and 10% glycerol;

VIII.3.3. Binding Buffer for South-Western Blot: 10 mM Hepes, pH 7.0, 10 mM MgCl, 50 mM NaCl, 0.25 mM EDTA and 2.5% glycerol;

VIII.3.4. Protein Purification

VIII.3.4.1. Maltose Affinity Column

Washing Buffer: 10 mM Na-phosphate/0.5 M NaCl/1 mM Azide/10 mM ß-ME/ 1 mM EGTA;

Elution Buffer: 10 mM Na-phosphate/0.5 mM NaCl/1 mM azide/10 mM ß-ME/ 1 mM EGTA/10 mM maltose.

VIII.3.4.2. HiTrap Protein-G Affinity Column

Coupling Buffer: 0.1 M $NaHCO_3$/O.5 M NaCl;

Washing Buffer: 20 mM Na-phosphate, pH 7.0;

Elution Buffer: 0.1 M Glycine-HCl, pH 2.7.

VIII.3.4.3. CNBr Sepharose 4B Affinity Column

Washing Buffer: 0.1M Acetate/0.5 M NaCl, pH 4.0; 0.1 M Tris-HCl/0.5 M NaCl, pH 8;

Equilibration Buffer: 0.1 M Tris-HCl pH 8.0;

Binding Buffer: 50 mM Na-phosphate/0.5 M NaCl, pH 6.3;

Elution Buffer: 50 mM Glycine-HCl/0.15 M NaCl, pH 2.5;

Neutralization Buffer: 1 M Tris-HCl, pH 9.0.

IX. Protein Purifications

	Company	Cat.#
Amylose Resin	New England BioLabs	800-21
HiTrap Protein G	Pharmacia Biotech	17-0404-03
CNBr-activated Sepharose 4B	Pharmacia Biotech	17-0430-01
Centricon-30 concentrators	Amicon	4208

X. Equipment

DNA thermal cycler (Perkin Elmer Cetus)

Thermostatic Circulator (2219 Multitemp II; LKB, Bromma)

DU-6 Spectrophotometer (Beckman)

Incubator Shaker (Series 25; New Brunswick Scientific Co.)

Refrigerated Superspeed Centrifuge (Sorvall RC-5B; Du Pont Instruments)

Microplate Reader (Model 3350; BIO-RAD)

METHODS

I. Cells

I.1. Cell Culture

The passages of the cells used for the present studies are:

OK 27 and OK 13 cells, 15 - 25;

OK 960, OK 280, and OK 53, 15 - 20;

OK 95, OK 95/M1, OK 95/M2, and OK 95/M3, 15 - 20;

OK 96, OK 96/M1, OK 96/M2, and OK 96/M3, 15 - 20;

The cells were initially grown in 100 X 20 mm plastic petri dishes in Dulbecco's Modified Eagle's Medium (DMEM), pH 7.45, supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 mg/ml streptomycin. The cells were grown in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. For transfection, cells were trypsinized (0.05% trypsin and EDTA) and plated at 2.5 X 10^5 cells/plate in 6-well plates.

I.2. Depleted Fetal Bovine Serum (dFBS)

The dFBS was prepared by incubation of the FBS with 1% activated charcoal and 1% AG 1X8 ion exchange resin for 16 hours or more at room temperature with gentle rotation. The serum was then filtered with 0.22 μm bottle top filter and stored at -20°C before use. This procedure removes endogenous steroid and thyroid hormones from the FBS (Samuels, 1979).

I.3. Selection of Stable Transformants

Geneticin (G418) is an aminoglycoside related to Gentamicin and is used as a selective agent in molecular genetic experiments. It is toxic to both prokaryotic and eukaryotic cells. The resistance gene (neomycin, neo) can be introduced and expressed in eukaryotic cells. Efficient expression is achieved when the neo DNA sequence is linked to eukaryotic DNA sequences that permit transcription and processing of the Neo co-

ding sequence into mRNA (Jimenez, 1980).

The G418 had no effect on OK cells transfected with pRSV-neo (which contains neo gene) at 500 mg/ml in the media. The stable transformants which were able to grow in the presence of G418 (500 mg/ml) and secrete high levels of IR-hGH into the medium were further subcloned using the method of limiting dilution.

Cells which have passed through at least three repetitions of limiting dilution and which still secreted high levels of IR-hGH after three months in the presence of G418 were considered to be a stable clone.

We have obtained independent clones which have the plasmids integrated into their genomes and were used for present studies:

pOGH (ANG N-1498/+18) + pRSV-neo	OK 27
pOGH (ANG N-960/+18) + pRSV-neo	OK 960
pOGH (ANG N-280/+18) + pRSV-neo	OK 280
pOGH (ANG N-53/+18) + pRSV-neo	OK 53
pOGH (ANG/CRE/-53/+18) + pRSV-neo	OK 95
pOGH (ANG/CREM1/-53/+18) + pRSV-neo	OK 95/M1
pOGH (ANG/CREM2/-53/+18) + pRSV-neo	OK 95/M2
pOGH (ANG/CREM3/-53/+18) + pRSV-neo	OK 95/M3
pOGH (ANG/CRE/-53/+18) + pRSV/CREB	OK 96
pOGH (ANG/CREM1/-53/+18) + pRSV/CREB	OK 96/M1
pOGH (ANG/CREM2/-53/+18) + pRSV/CREB	OK 96/M2
pOGH (ANG/CREM3/-53/+18) + pRSV/CREB	OK 96/M3
pTKGH + pRSV-neo	OK 13

I.4. Effect of Different Hormones and Drugs on the Expression of ANG Fusion Genes and pTKGH in OK Cells

OK permanent cell lines were plated at a density of 2 X 10^5 cells/plate of 6-well plate and incubated overnight in DMEM medium containing 10% FBS. Then, the cell growth was arrested by the incubation in serum-free DMEM for 24 hours.

Subsequently, the cells were incubated for up to 24 hours in the DMEM medium containing 1% dFBS and various concentrations of different reagents. At the end of the incubation period, the media were collected and kept at -20°C until assay.

II. Assays

II.1. Protein Assay

The samples were mixed with BIO-RAD protein dye, and were read by a BIO-RAD microplate reader at 595 nm with a protein standard (various concentrations) of bovine serum albumin (BSA) mixed with the dye. The assay was performed in duplicate.

II.2. Radioimmunoassay

II.2.1. Iodination of Human Growth Hormone (hGH)

A Sephadex G-100 column (medium, 20 X 1.5 cm) was equilibrated with 0.025 M Tris·HCl, pre-run with 2.5% BSA (1 ml) and washed with 0.025 M Tris·HCl buffer for 3 bed volumes.

The reaction was performed by adding 10 μg of hGH, 10 μl of 0.5 M phosphate buffer (pH 7.0), 10 μl (1 mCi) Na-^{125}I, 10 μg of lactoperoxidase (1μg/μl) and 10 μl of 0.001% H_2O_2 (25 μl of 30% H_2O_2 in 375 ml dH_2O). The reaction mixture was incubated at room temperature (RT) for 5 minutes. Then, another 10 μl of 0.001% H_2O_2 was added and incubated at RT for 5 minutes. Finally, the reaction mixture was applied onto the pre-equilibrated Sephadex G-100 column.

The eluates were collected in aliquots. The radioactivity in each fraction was counted in a LKB gamma counter. The fractions containing the first radioactive peak were collected and stored at -20°C for further testing and use.

II.2.2. Radioimmunoassay (RIA)

The procedure for RIA was performed according to the method of Chan et al (Chan, 1978). Briefly, 100 µl of RIA buffer, 100 µl of sample or standard hGH and 100 µl of hGH antibody (rabbit, 1:400,000 dilution) were added to the test tubes and incubated at 4°C for overnight. The next day, 100 µl of radioactive hGH (~20,000 cpm) wer added and further incubated for 24 hours. On the third day, 50 µl of goat-anti-rabbit antibody (1:10) and 50 µl of normal rabbit serum (1:100) were added and incubated for overnight at 4°C. On the fourth day, the tubes were centrifuged at 3500 X g for 30 minutes at 4°C. The supernatant was aspirated and the pellet was counted in the LKB gamma counter.

All the samples were prepared in triplicate. The lower limit of sensitivity of the assay was 0.1 ng/ml. The linearity of the standard curve was from 0.2 to 10 ng/ml. The inter- and intra-assay coefficients of variation were 10% (n=10) and 12% (n=10), respectively. The total specific binding was about 15%.

III. Construction of the Plasmids

III.1. Fusion genes pOGH (ANG N-1498/+18), pOGH (ANG N-960/+18), pOGH (ANG N-688/+18), pOGH (ANG N-280/+18), pOGH (ANG N-53/+18)

These plasmids were constructed by Dr. John SD Chan (1990). Briefly, a DNA fragment (28 bp) (XhoII-XhoII, N-53 to N+75) was removed from the pGEM-ANG I and inserted into pGEM-3 via the polylinker site, BamHI. This plasmid was termed pGEM (ANG N-53/+75). The orientation and the sequence of the DNA insert were determined

by DNA sequencing using both T_7 and SP_6 primers.

Deletion at the 3'-border of the pGEM (ANG N-53/+75) was created by using restriction site, EcoRI, at the polylinker region of pGEM-3 and then sequentially removing the nucleotides by digestion with endonuclease BAL-31. Following repair with DNA polymerase I large fragment and digestion with HindIII, the DNA fragment was isolated from low melting point Agarose and inserted into pOGH via polylinker sites (HindIII/HincII).

The plasmid containing the angiotensinogen (N-53/+18) insert was isolated and designated as pOGH (ANG N-53/+18). This angiotensinogen-growth hormone (ANG-GH) fusion gene has a 3'-boundary of n+18) relative to the transcriptional start site.

The pOGH (ANG N-1498/+18) fusion gene was constructed by isolating the DNA fragment (HindIII-XhoI, N-1498 to N-35) from pGEM-ANG I and inserting it into the pOGH (ANG N-53/+18) which had been previously digested with restriction enzymes HindIII-XhoI to release DNA fragments (N-53 to N-36).

The pOGH (ANG N-960/+18) fusion gene was constructed by isolating the DNA fragment (XbaI-XhoI, N-960 to N-36) from pOGH (ANG N-1498/+18) and inserting it into the pOGH (ANG N-53/+18) which had been previously digested with restriction enzymes HindIII-XhoI to release DNA fragments (N-53 to N-36).

The pOGH (ANG N-688/+18) was constructed by isolating the DNA fragment (BamHI-BamHI, N-688 to N+18) from pOGH (ANG N-1498/+18) and re-inserting it into pOGH via the polylinker site, BamHI.

The pOGH (ANG N-280/+18) fusion gene was constructed by isolating the DNA fragment (Nci-1-XhoI, N-280 to N-36) from pOGH (ANG N-1498/+18) and inserting it into

the pOGH (ANG N-53/+18) which had been previously digested with restriction enzymes HindIII-XhoI to release DNA fragments (N-53 to N-36).

After ligation, the plasmids were transferred into bacteria HB101. The positive clones were selected by Southern blotting with the labeled oligonucleotides. The plasmid DNA was isolated by the procedures of mini-preparation. The sequences for all fusion genes were confirmed by dideoxy sequencing.

III.2. pRSV/CREB and pMALc-CREB

These plasmids were constructed by Dr. J-F Qian (a former post-doctoral fellow in our lab) (1997). Briefly, total cellular RNA was prepared from adult male rat liver (Wistar-Kyoto) by guanidium isothiocyanate/cesium chloride gradient as previously described (Chan, 1992). 20 μg of total RNA was used to synthesize the double-strand cDNA for CREB by the method of reverse transcription and amplification by polymerase-chain reaction (PCR) (Thermal Cycler) according to the manual supplied by the supplier (Perkin Elmer Cetus Inc.).

The nucleotide sequences for the two oligonucleotide primers corresponding to the first 18 nucleotides (5'ATG ACC ATG GAC TCT GGA3') and the last 18 nucleotides (5'TTA ATC TGA CTT GTG GCA3') in the opening reading frame of the rat CREB (Gonzalez, 1989-b), respectively, were used in PCR. 1 kb CREB cDNA fragment was amplified and then subcloned in the plasmid pBluescript (SK^-) (Stratagene Inc.), tentatively designated as pBluescript-CREB. The sequence for the CREB cDNA was confirmed by dideoxy sequencing with T_3 and T_7 primers.

After digesting the plasmid pBluescript-CREB with EcoRI to release the CREB cDNA, the cDNA was isolated by Geneclean. The cDNA was then religated into plasmid pMALc or pRC/RSV which were previously digested with EcoRI. The sequences for the

CREB cDNA was confirmed by dideoxy sequencing with Mal-E or RSV primers, respectively.

III.3. pOGH (ANG/CRE/-53/+18), pOGH (ANG/CREM1/-53/+18), pOGH (ANG/CREM2/-53/+18), and pOGH (ANG/CREM3/-53/+18)

The plasmid pOGH (ANG N-53/+18) was digested with restriction enzymes HindIII and XbaI on the polylinker site, and ran in a 0.8% mini-Agarose gel, then the linearized plasmid was isolated by Geneclean (BIO 101) following the instructions provided.

The oligonucleotides ANG-CRE, ANG-CREM1, ANG-CREM2, ANG-CREM3 were as follows:

	-806 -779
ANG-CRE	5'AGC TTA AGA GAT TAC **TTG ACG TAC** TGG ATG CAA A3'
(containing rat ANG N-806/N-779)	5'CTA GAT TGC ATC CA**G TAC GTC A**AG TAA TCT CTT A3'
ANG-CREM1	5'AGC TTA AGA GAT TAC **TTG ACT TAC** TGG ATG CAA A3'
	5'CTA GAT TGC ATC CA**G TAA GTC A**AG TAA TCT CTT A3'
ANG-CREM2	5'AGC TTA AGA GAT TAC **TTG A**AT **TAC** TGG ATG CAA A3'
	5'CTA GAT TGC ATC CA**G TAA TTC A**AG TAA TCT CTT A3'
ANG-CREM3	5'AGC TTA AGA GAT TAC TTAT AT **TAC** TGG ATG CAA A3'
	5'CTA GAT TGC ATC CA**G TAA** TAT **A**AG TAA TCT CTT A3'

They were annealed and religated back to the plasmid pOGH (ANG N-53/+18) individually. The DNAs were transferred into bacteria HB101 and a Southern blot was done with γ-^{32}P-dATP kinased single strand oligonucleotides, respectively. The positive clones were selected and the plasmid DNAs were prepared by mini-preparation. The sequences were confirmed by dideoxy sequencing with primer SP_6.

III.4. pRSV/CREB(6 his)

The plasmid pMALc-CREB was used as a template for PCR. The primer MalE (5'GGT CGT CAG ACT GTC GAT GAA GCC3') and the last 18 nucleotides in the opening reading frame of the rat CREB (Gonzalez, 1989-b) plus six histidine (5'GAA GAA TTC TTA GTG ATG GTG ATG GTG ATG ATC TGA CTT GTG GCA GTA AAG GTC3'), were used in PCR. 1 kb CREB cDNA fragment was amplified.

The PCR product was digested with EcoRI and then ran in a 0.8% Agarose gel. The DNA was isolated by the method of Geneclean and then religated into the plasmid pcDNA I, which was previously digested with EcoRI and dephosphorylated. The ligated DNA was transformed into bacteria DH5α competent cells. The positive clones were selected and the plasmid DNAs were prepared. The sequence of the plasmids was confirmed by dideoxy sequencing with primer T_7. The plasmid was designated as pcDNA I/CREB (6 his).

The plasmid pcDNA I/CREB (6 his) was digested with HindIII/XbaI to release the CREB cDNA and separated on a 0.8% Agarose gel. The DNA was isolated by the method of Geneclean and religated into the plasmid pRC/RSV which was previously digested with HindIII/XbaI. The ligated DNA was transferred into bacteria DH5α competent cells. The positive clones were selected and the plasmid DNAs were prepared by mini-preparations. The sequence of the plasmids was confirmed by dideoxy sequencing with primer RSV. The plasmid was designated as pRSV/CREB (6 his).

IV. General Molecular Biology Techniques

IV.1. Dephosphorylation

The enzyme alkaline phosphatase (CIP, calf intestine alkaline phosphatase) was used to catalyze the removal of 5'-phosphate residues from DNA and RNA, ribo- and deoxy-ribonucleo-tide triphosphates.

Briefly, DNA (10 - 20 μg) was incubated with 5 μl of CIP (5 u/100μl) and CIP buffer at 37°C for 1 hour. Then, the reaction solution was heated at 65°C for 5 minutes. Another 5 μl of CIP (5 u/100μl) was added and incubated at 37°C for 1 hour. The solution was heated at 65°C for 15 minutes, followed by extraction with phenol/chloroform (twice) and then alcohol precipitation.

IV.2. Annealing of DNA

Equal amounts of the complementary oligonucleotides or single strand DNA were mixed in annealing buffer and incubated at 45°C for 30 minutes. Then the reaction mixture were slowly cooled down to RT. After ethanol precipitation and lyophilization, the DNA was rehydrated and the concentration was measured at $O.D._{260\ nm}$.

IV.3. Ligation

Various concentrations of the DNA insert were prepared and added to vector DNA (usually the concentrations of the insert DNA were about 2 times of the vector DNA in molarity). The DNAs were heated at 45°C for 5 min, then placed on ice. 1 μl of 10X ligation buffer, 1 μl 5 mM ATP, 2 u T_4 DNA ligase, and dH_2O until a final volume 10 μl were added and incubated at 16°C in a circulating water bath for overnight.

IV.4. Preparation of Competent Cells (HB101 or TB1)

A single colony of bacteria were grown overnight. Then the culture was diluted at 1:100 with SOC. The bacteria was further grown until $O.D._{650}$ was 0.3 ~ 0.4. The bac-

teria were then centrifuged down and resuspended with FSB (1/3 volume), i.e. 16 ml per 50 ml culture and incubated on ice for 15 minutes. The bacteria were spun down and resuspended again with FSB (1/12.5 volume) i.e. 4 ml. 140 µl of DMSO were added and swirled, and incubated on ice for 5 min. Another 140 µl of DMSO was added and incubated on ice for 10 min. The bacteria were aliquoted (200 µl/vial) and stored at -80°C.

IV.5. Transformation

The ligated DNA was added to the competent cells in a tube and incubated on ice for 30 min. Then the cells were heat shocked at 42°C for 90" and placed on ice for 2 minutes. Up to 800 µl of SOC were added to each tube and cultured with shaking at 37°C for 1 hour. The bacteria culture was spread onto agar/ampicillin plates and incubated at 37°C for overnight.

IV.6. Kinasing

10 pmol of DNA, 1 µl of 0.1 M DTT, 2 µl 10X 5'-protruding kinasing buffer, 1 u of T_4 kinase, 12 µl of dH_2O and 30 µCi of γ-^{32}P-dATP were added to an Eppendorf vial and incubated at 37°C for 1 hour. The reaction was stopped with 1 µl of 0.5 M EDTA and the reaction solution was applied onto a pre-equilibrated (with dH_2O) Sephadex G-50 column. The eluted fractions were collected and the fractions in the first peak were stored at -20°C.

IV.7. Southern Blotting

For Agarose gels: After transfer, the nitrocellulose (NC) paper was removed from the gel and rinsed with 2X SSC. The filter was dried at 80°C for 2 hours.

For agar plates: The filter was placed on top of the agar, then slowly peeled off. Then the filter was denatured in 0.5 M NaOH/NaCl for 10 min, and renatured in 0.5 M Tris/3

M NaCl for 30 min. The filter was rinsed with 2X SSC and dried at 80°C for 2 hours.

Blotting: The filter was prehybridized at 42°C for 1 hour with 5X SSPE, 5X Denhardt, 1% SDS and 100 μg/ml of denatured salmon sperm DNA. It was then replenished with the same buffer containing the probe (~10^6 cpm/10 ml) and hybridized for overnight at 42°C. The filter was washed with 2X SSC/0.1% SDS at RT for 20 min, and then washed with 2X SSC/0.1% SDS at 55°C for 20 min. The filter was exposed for autoradiography at -70°C for overnight.

IV.8. Mini-Preparation of Plasmid DNA

A single bacteria colony was chosen and grown in 10 ml of media for overnight. 1.5 ml of the bacteria culture was taken and spun down. 100 μl Solution I were added and vortexed, 200 μl of Solution II were added and the tube was inverted and placed on ice for 5 min. 150 μl of Solution III were added and the tube was inverted and placed on ice for 5 min. Then the bacteria were spun down and the supernatant was extracted twice with phenol/chloroform and precipitated with 95% ethanol, after being washed with 75% ethanol, the DNA was dried in a speed-vacuum and reconstituted with TE buffer. The DNA plasmid was further digested with RNAse A, RNAse T_1 and proteinase K. The digested solution was extracted with phenol/chloroform and the DNA was precipitated with 7.5 M NH_4Ac and 95% ethanol. After being washed with 75% ethanol and vacuum dried, the DNA was ready for sequencing.

IV.9. DNA Sequencing

The DNA (3-4 μg) was denatured with 2 μl 2 M NaOH for 10 minutes and precipitated with 3 M NaAc and 95% ethanol for 30 minutes. After being washed with 75% ethanol and vacuum dried, the DNA pellet was dissolved in 10 μl of dH_2O, and 2 μl of annealing buffer and 2 μl of primer were added. The mixture was incubated at 37°C for 20 min and slowly cooled down to RT. 3 μl of dATP labeling mix, 1 μl of ^{35}S-dATP and 5 u

of T_7 DNA polymerase were added to the DNA mixture and placed at RT for 5 min. At the same time, an aliquot of 2.5 µl of the G, A, T, C mix were added into different Eppendorf vials. Then, 4.5 µl of the labeled DNA sample was added to each mix and incubated at 37°C for 5 min. The reaction was stopped by adding 5 µl of stop solution to each tube. The samples were heated at 90°C for 2 min before being applied to the sequencing gel. The gel was dried at 80°C for 2 hours after running and exposed for autoradiography at RT for overnight.

IV.10. Maxi-Preparation of Plasmid DNA

500 ml of bacteria culture were grown for overnight at 37°C with shaking. The bacteria were spun down (4K, 4°C, 20 min) and resuspended in 15 ml of Solution I with vortexing. 30 ml of Solution II were added and mixed by swirling and placed on ice for 10 min. 22.5 ml of Solution III were added and mixed and placed on ice for 15 min. After centrifugation (7K, 4°C, 30 min), the supernatant was filtered through gauze and precipitated with 95% ethanol at RT for 10 min. The DNA was centrifuged (8K, 4°C, 30min) and redissolved in TE buffer. RNA and proteins were precipitated by adding 7.5 M NH_4Ac, and placing on ice for 30 min. After centrifugation (10K, 4°C, 10 min), the DNA was precipitated with 95% ethanol at -80°C for 1 hour. The solution was centrifuged (12K, 4°C, 20 min) and the pellet was dried with speed vacuum. Then the DNA was redissolved with TE buffer and digested with RNAse A, RNAse T_1 and proteinase K, respectively. The reaction solution was extracted twice with phenol/ chloroform and precipitated with 95% ethanol and 7.5 M NH_4Ac. The pellet was washed with 75% ethanol and vacuum dried and finally resuspended in TE buffer. The concentration of the DNA plasmid was measured ($O.D._{260/280}$).

IV.11. Transfection of Plasmid DNA

The plasmids were transfected into OK cells utilizing calcium phosphate-mediated endocytosis (Chen, 1987).

The media of the cells were replenished with fresh DMEM containing 10% FBS and incubated for 2 hours before transfection. The calcium phosphate precipitate was prepared by adding 2M $CaCl_2$ (186 µl), DNA (15-60 µg) and dH_2O (1314 µl - x µl of DNA) into a 15 ml centrifuge tube. Then 1500 µl of 2X HBSP were added slowly into the reaction mixture by inserting the pipette near the bottom of the tube. The reaction solution was left at RT for 5 minutes and then applied to a 6-well plate, 1 ml/well.

The cells were incubated for 24 hours and then the medium was replaced with fresh DMEM containing 10% FBS. The next day, the media were collected for RIA, or the cells were rinsed with DMEM and various drugs or hormones dissolved in DMEM were added plus 1% dFBS. At day 4, the cell media were collected and kept at -20°C until assay.

IV.12. Purification of Fusion Protein pMAL-CREB

The bacteria were cultured in rich LB medium with agitation at 37°C until the $O.D._{600}$ reached ~ 0.5. IPTG was then added to give a final concentration of 2.5 mM and the incubation was continued for another 4 hours. The cells were harvested by centrifugation (4K, 4°C, 10 min), the pellet was resuspended in NCB + proteinase inhibitors and stored at -20°C overnight. The next day, the freeze-thaw (to RT) cycle was repeated twice and the cells were sonicated. After spinning down the cells (9K, 4°C, 20 min), the supernatant was applied to an amylose resin column. The column was washed with NCB and eluted with NCB + 20 mM Maltose. The fractions of the first peak (tested with protein assay) were kept and concentrated with Centricon-30.

IV.13. Purification of Rabbit IgG

1 ml of HiTrap Protein G affinity column was washed with at least three column volumes of washing buffer to wash out the ethanol preservative and was further

equilibrated with 5X column volumes of washing buffer. 2 ml of dialyzed rabbit antiserum (overnight in washing buffer, 4°C) was applied each time onto the column. The effluent was re-applied for 5 cycles. The column was then washed with 7X column volumes of washing buffer. The IgG was eluted with 5X column volumes of elution buffer. The elutes were collected in 1 ml aliquots at a flow rate of 2 ml/min and immediately neutralized (4:1 in neutralization buffer). The purified IgG fractions were finally concentrated to a small volume and were then analyzed on SDS-PAGE.

IV.14. Purification of Protein pRSV/CREB

IV.14.1. Coupling of IgG to Sepharose 4B

20 mg of purified IgG (dialyzed against coupling buffer for overnight at 4°C) was mixed with 1.5 ml of the sepharose gel and rotate overnight at 4°C. The next day, the excess ligand was washed away with 5X gel volumes of coupling buffer. The gel was then equilibrated and allowed to stand for 2 hrs. The gel was then washed with alternating pH of washing buffer for 3 cycles and packed to the column.

IV.14.2. Purification of Protein pRSV/CREB

The column was washed with 5X binding buffer. 5 ml of the cell extract was then applied each time for 5 cycles. The column was then washed for 7X of its volume. The protein was eluted with 5X volume of the column. The elutes were collected in 1 ml aliquots and neutralized immediately (4:1 in neutralization buffer). The purified protein was concentrated to a small volume and was analyzed with Western blot.

IV.15. Western Blotting

The filter was blocked with PBS containing 5% milk protein for 4 hours at 4°C and then replenished with PBS containing 5% milk protein and the first antibody and incubated at 4°C with shaking for more than 2 hours or overnight. After being washed 3X with 1X PBS, the filter was incubated with TBS containing 5% milk protein and the second an-

tibody (ARP-conjugated goat anti-rabbit immunoglobulin, 1:1000 dilution) and incubated at 4°C with shaking for 1 hour or overnight. Finally, the filter was washed 3X with TBS, and developed (If the second antibody was horseradish peroxidase conjugated, the developing solution was the fresh mixture of 30 mg of 4-chloronaphthol in 10 ml of methanol and 30 µl of 30% H_2O_2 in 40 ml TBS).

IV.16. Gel Mobility Shift Assay

The DNA fragments, ANG N-806 to N-779 was 5'-end labeled with γ-^{32}P-dATP using T_4 polynucleotide kinase. OK cell extract (10 µg) or bovine serum albumin (10 µg) in the presence of 0.3 units of poly (dI-dC) in 20 mM Hepes, pH 7.6, 1 mM EDTA, 50 mM KCl, 2 mM spermidine, 1 mM DDT, 0.5 mM PMSF and 10% glycerol were incubated at RT for 30 min. The 5'-labeled probe (0.1 pmole) was added and further incubated at RT for 30 min. After chilling on ice, the mixture was run on a 8% non-denaturing polyacrylamide gel and exposed for autoradiography.

In competition assays, a 100- to 200-fold excess or higher of unlabeled DNA fragments were added to the reaction mixture and incubated at RT for 30 min prior the incubation with the labeled probe.

IV.17. South-Western Blotting

OK cell nuclear proteins (50-200 mg) were resolved on a 8 to 15% gradient polyacrylamide gel containing sodium dedocyl sulfate (PAGE-SDS) and then electrotransferred onto a nitrocellulose membrane (0.45 µM). The membrane was incubated with 10% non-fat milk proteins in a binding buffer containing 10 mM Hepes, pH 7.0, 10 mM $MgCl_2$, 50 mM NaCl, 0.25 mM EDTA and 2.5% glycerol for one hour at 4°C. The membrane was then washed at least twice with the binding buffer containing 0.25% non-fat milk proteins. Subsequently, the membrane was hybridized with γ-^{32}P-labeled double stranded oligonucleotides (approximately 1.0 to 2.0 pmole, 10^6 cpm/ml) in binding

buffer containing 0.25% non-fat milk proteins and 300 mg/ml of non-denatured herring sperm DNA at 4°C for overnight. The membrane was washed, air-dried and exposed for autoradiography.

In competition assays, a 50 to 100-fold excess of unlabeled DNA fragments was incubated with the membrane overnight before blotting with the radioactive ANG N-806/-779.

V. Rabbit Immunization

The rabbit was immunized with 30 μg of the fusion protein (i.e. the maltose binding protein fused with CREB in small aliquots of 0.1 M NH_4HCO_3) mixed with 1 ml of 0.9% NaCl and 1 ml of Freund's complete adjuvant (1:1). The suspension was administered subcutaneously once every two weeks for 3 months and then once a month.

*N.B., the incomplete adjuvent was used with the same solution after the first injection.

VI. Statistical Analysis

The experiments were performed at least two to three times in triplicate, the data shown was from the result of one experiment.

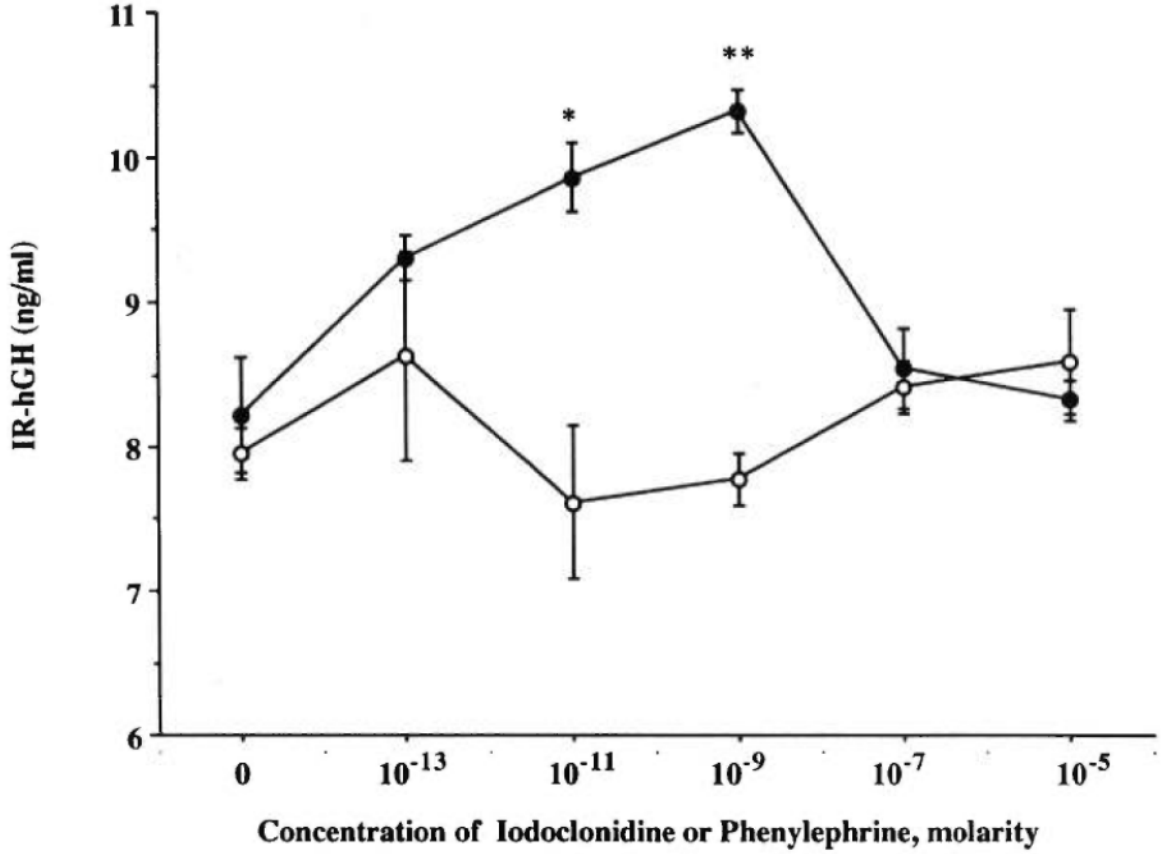
The data were analyzed by Student's "t" test unpaired or Annova analysis. A probability level of $p \leq 0.05$ was regarded as significant.

RESULTS

RESULTS-1 Alpha-Adrenoceptors and Angiotensinogen Gene Expression in OK Cells

(Wang TT, LaChance S, Delalandre A, Carrière S & Chan JSD. Kidney Int 1995;48:139-145)

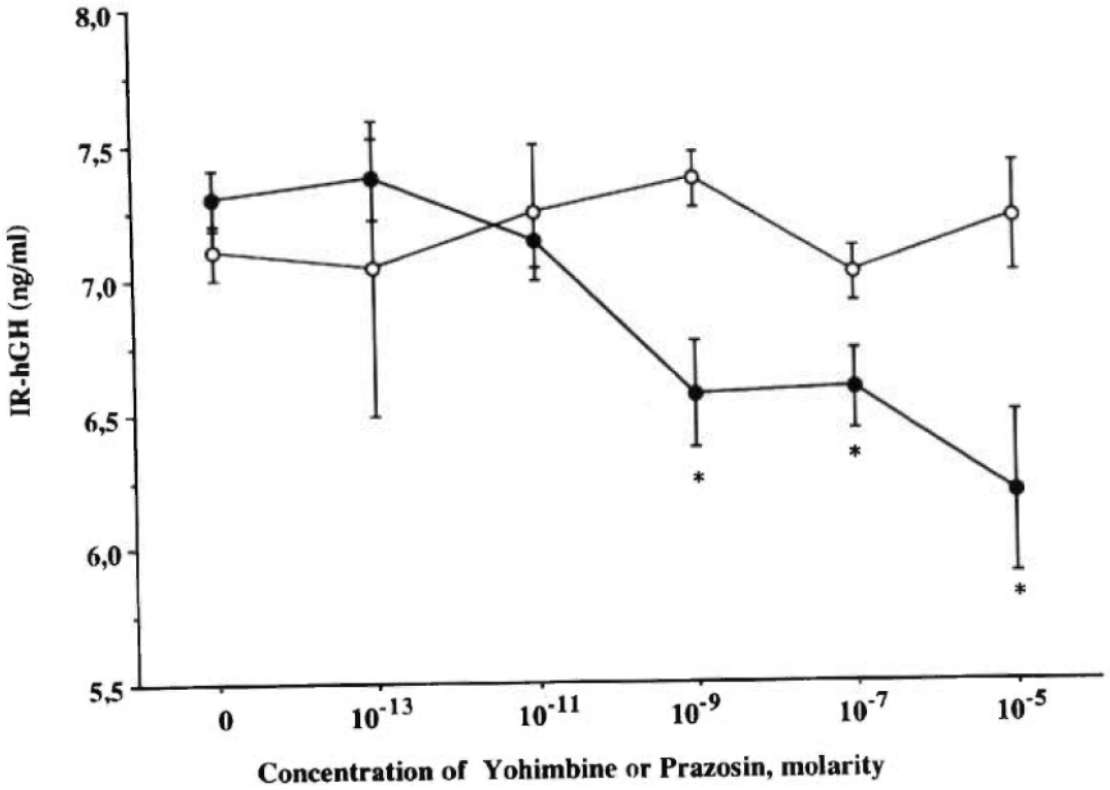
FIG.1. EFFECT OF IODOCLONIDINE (α_2-ADRENOCEPTOR AGONIST) OR PHENYLEPHRINE (α_1-ADRENOCEPTOR AGONIST) ON THE EXPRESSION OF pOGH (ANG N-1498/+18) IN OK 27 CELLS



OK 27 cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, various concentrations of iodoclonidine (10^{-13} to 10^{-5} M) or phenylephrine (10^{-13} to 10^{-5} M) were added to the culture medium containing 1% dFBS and incubated for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). The concentration of IR-hGH in the absence of iodoclonidine or phenylephrine is considered as the control level. Each point represents the mean±SD of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). The experiment has been done 6 times in triplicates.

A dose-dependent relationship between iodoclonidine (●) and the stimulation of the expression of pOGH (ANG N-1498/+18) was observed for iodoclonidine at 10^{-11} to 10^{-9} M. The maximal stimulation was found with 10^{-9} M iodoclonidine. At concentrations greater than 10^{-9} M, the stimulatory effect of iodoclonidine was minimal or inhibited. Phenylephrine (O) had no effect on the gene expression.

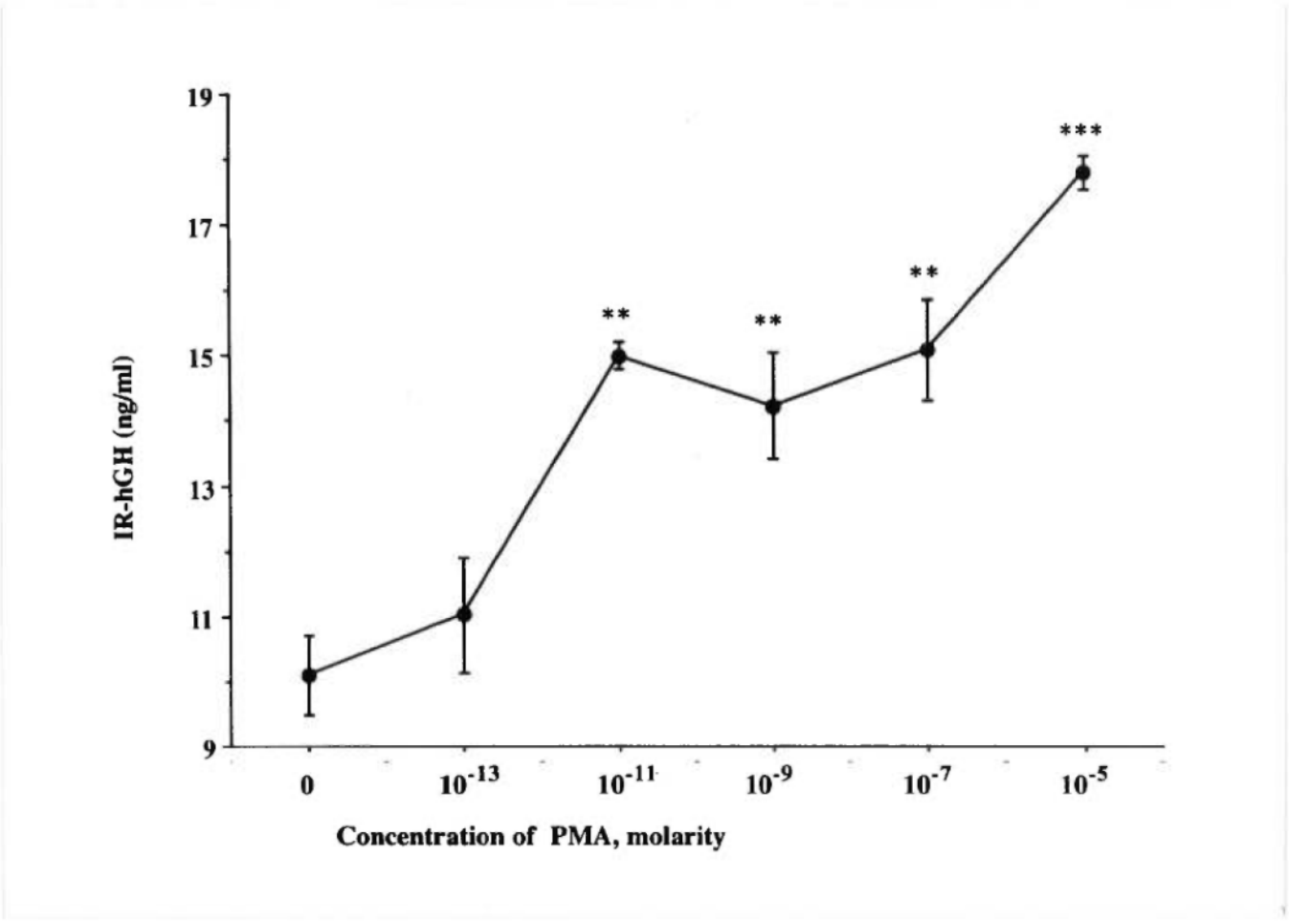
FIG.2. EFFECT OF YOHIMBINE (α_2-ADRENOCEPTOR ANTAGONIST) OR PRAZOSIN (α_1-ADRENOCEPTOR ANTAGONIST) ON THE EXPRESSION OF pOGH (ANG N-1498/+18) IN OK 27 CELLS STIMULATED BY IODOCLONIDINE



Cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, various concentrations of yohimbine (10^{-13} to 10^{-5} M) or prazosin (10^{-13} to 10^{-5} M) were added to the culture medium and co-incubated with iodoclonidine (10^{-9} M) for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). The concentration of IR-hGH in the absence of yohimbine or prazosin is considered as the control level. Each point represents the mean±SD of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). The experiment has been done 7 times in triplicates.

The addition of yohimbine (●) inhibited the stimulatory effect of iodoclonidine (10^{-9} M) in a dose-dependent manner. The effective doses were found with 10^{-9} to 10^{-5} M yohimbine whereas prazosin (O) had no effect on the stimulation of iodoclonindine on the expression of pOGH (ANG N-1498/+18). These studies suggested that the stimulatory effect of α-adrenoceptors on the expression of pOGH (ANG N-1498/+18) in OK 27 cells is mediated via the α_2-adrenoceptors.

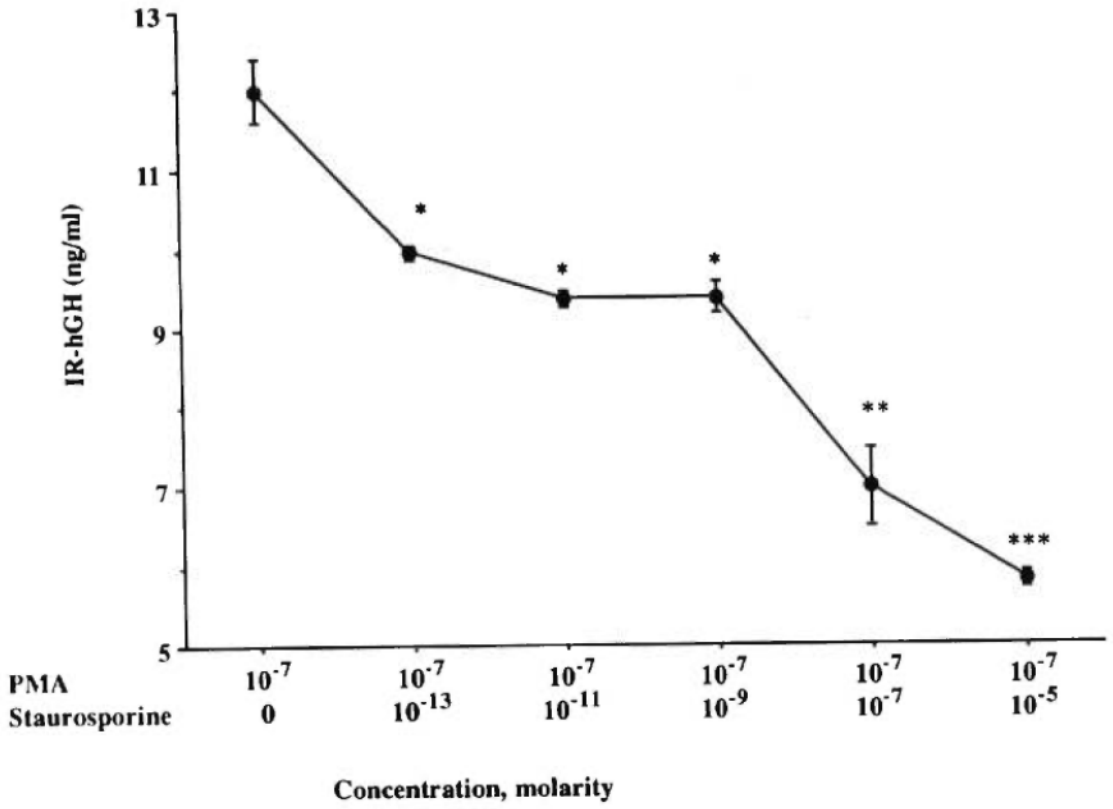
FIG.3. EFFECT OF PHORBOL 12-MYRISTATE 13-ACETATE (PMA) (PKC ACTIVATOR) ON THE EXPRESSION OF pOGH (ANG N-1498/+18) IN OK 27 CELLS



Cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, various concentrations of PMA (10^{-13} to 10^{-5} M) were added to the culture medium containing 1% dFBS and incubated for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). The concentration of IR-hGH in the absence of PMA is considered as the control level. Each point represents the mean±SD of at least three dishes (*p≤0.05, **p≤0.01 and ***p≤0.005). The experiment has been done 3 times in triplicates.

A dose-dependent relationship between PMA and the stimulation of expression of pOGH (ANG N-1498/+18) was observed for PMA at 10^{-11} to 10^{-5} M. The maximal stimulation was found with 10^{-5} M PMA.

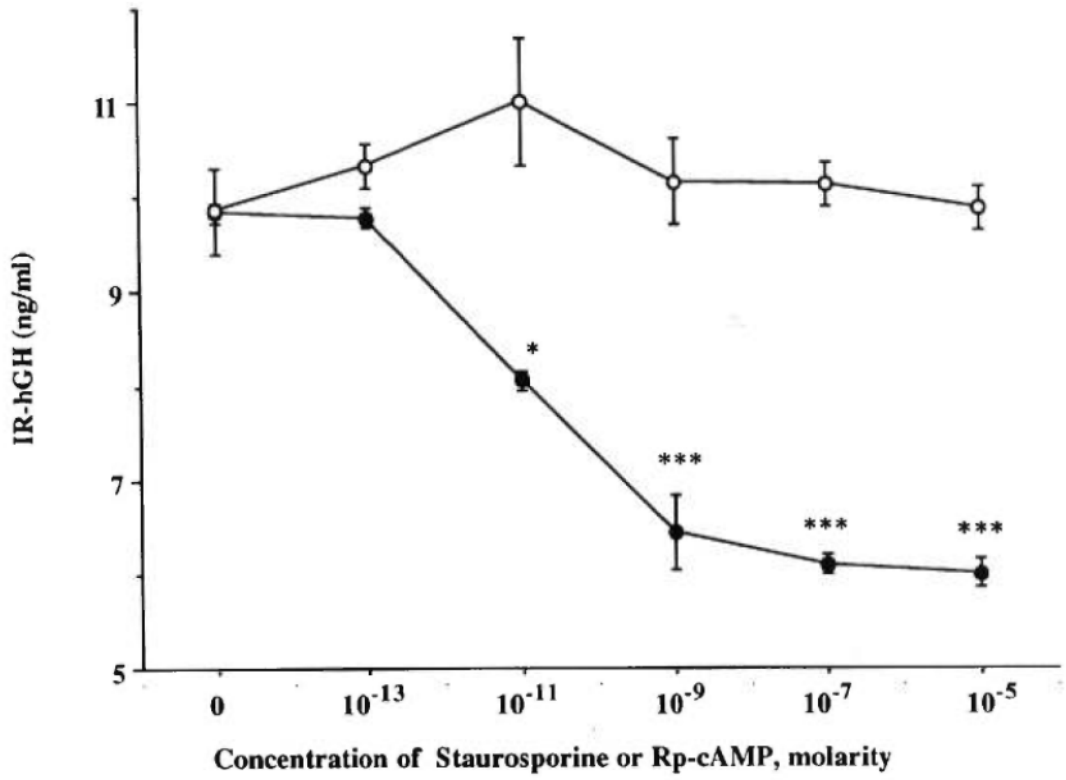
FIG.4. INHIBITORY EFFECT OF STAUROSPORINE (INHIBITOR OF PROTEIN KINASE C) ON THE EXPRESSION OF pOGH (ANG N-1498/+18) IN OK 27 CELLS STIMULATED BY PMA



Cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, various concentrations of staurosporine (10^{-13} to 10^{-5} M) were added to the culture medium containing 1% dFBS and co-incubated with PMA (10^{-7} M) for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). The concentration of IR-hGH in the absence of PMA or staurosporine is considered as the control level. Each point represents the mean±SD of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). The experiment has been done 4 times in triplicates.

The addition of staurosporine inhibited the stimulatory effect of PMA (10^{-7} M) in a dose-dependent manner. The effective doses were found with 10^{-13} to 10^{-5} M staurosporine. These studies suggested that the regulation of the expression of pOGH (ANG N-1498/+18) in OK 27 cells is mediated via the PKC pathway.

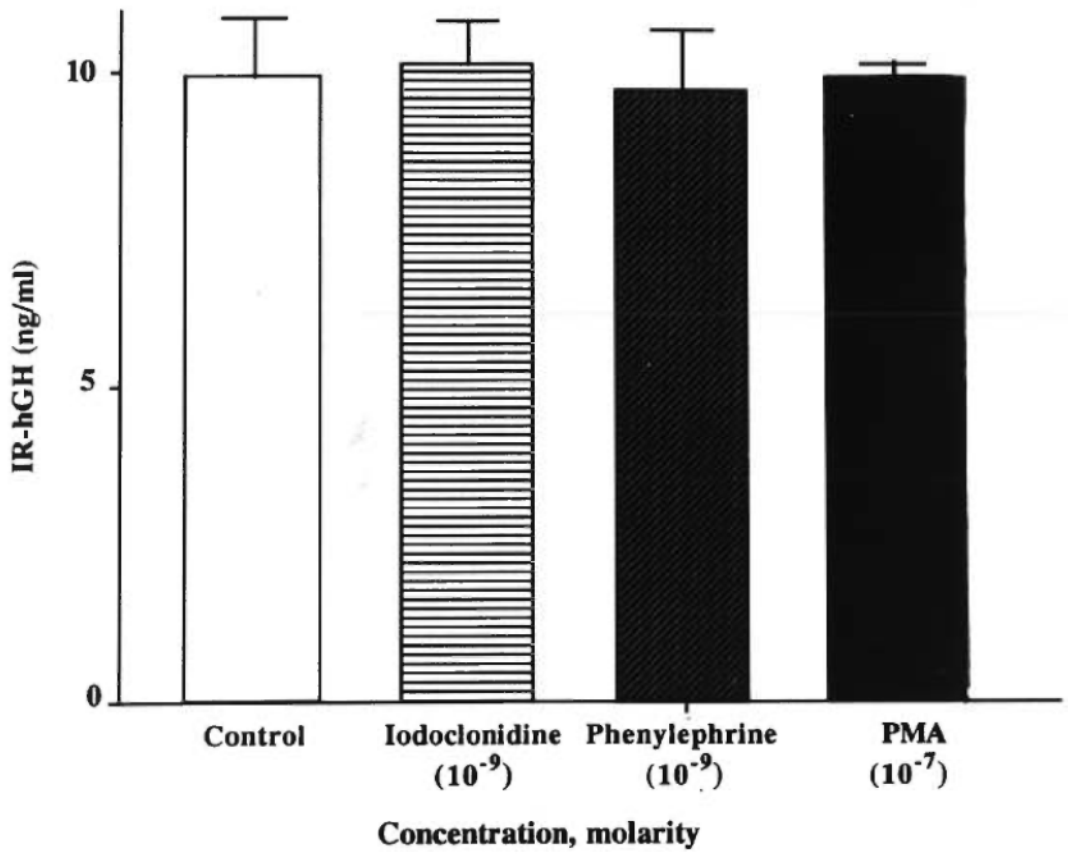
FIG.5. INHIBITORY EFFECT OF STAUROSPORINE OR Rp-cAMP (INHIBITOR OF cAMP-DEPENDENT PROTEIN KINASE I AND II) ON THE EXPRESSION OF pOGH (ANG N-1498/+18) IN OK 27 CELLS STIMULATED BY IODOCLONIDINE



Cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, various concentrations of staurosporine (10^{-13} to 10^{-5} M) or Rp-cAMP (10^{-13} to 10^{-5} M) were added to the culture medium containing 1% dFBS and co-incubated with iodoclonidine (10^{-9} M) for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). The concentration of IR-hGH in the absence of iodoclonidine staurosporine or Rp-cAMP is considered as the control level. Each point represents the mean±SD of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). The experiment has been done 4 times.

The addition of staurosporine (●) inhibited the stimulatory effect of iodoclonidine (10^{-9} M) in a dose-dependent manner. The effective doses were found with 10^{-11} to 10^{-5} M staurosporine whereas Rp-cAMP (O) had no inhibitory effect on the stimulation of iodoclonidine on the expression of pOGH (ANG N-1498/+18). These studies suggested that the stimulatory effect of α_2-adrenoceptors on the expression of pOGH (ANG N-1498/+18) in OK 27 cells is probably mediated via the PKC pathway.

FIG.6. EFFECT OF IODOCLONIDINE, PHENYLEPHRINE AND PMA ON THE EXPRESSION OF pTKGH IN OK 13 CELLS



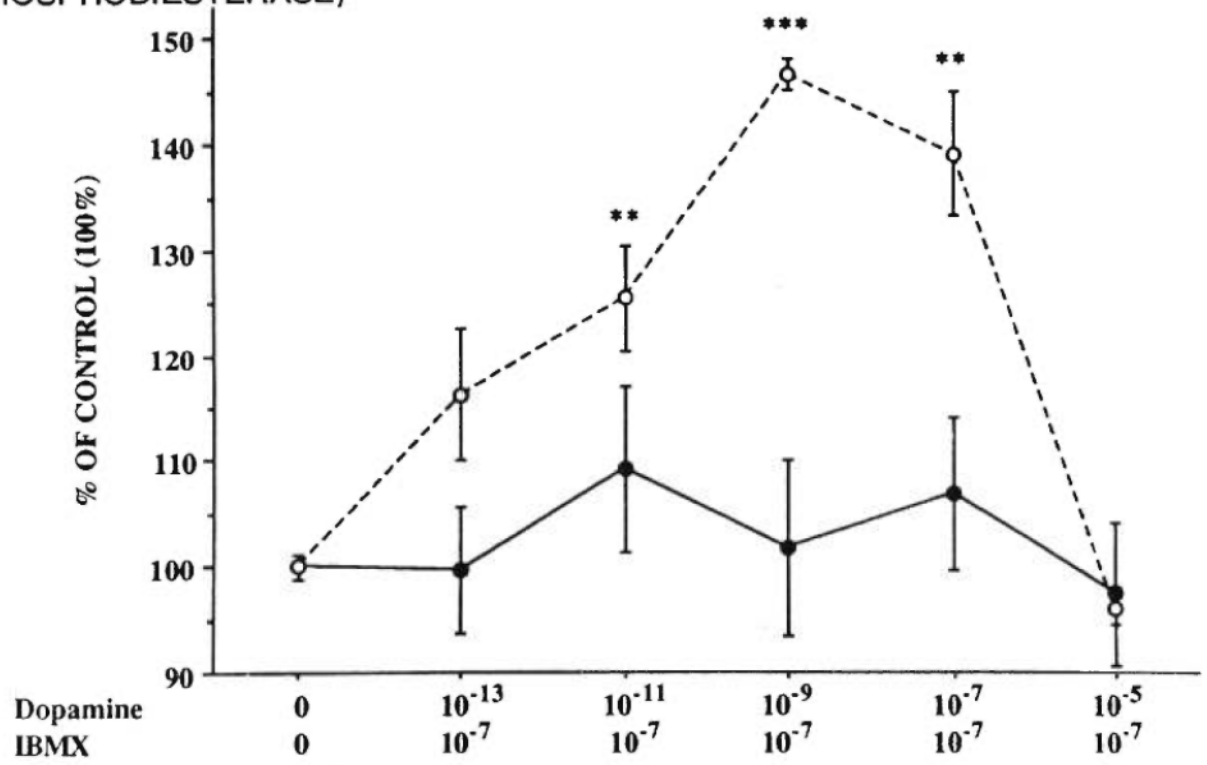
OK 13 cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, iodoclonidine (10^{-9} M) or phenylephrine (10^{-9} M) or PMA (10^{-7} M) was added to the culture medium containing 1% dFBS and incubated for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). The concentration of IR-hGH in the absence of iodoclonidine or phenylephrine or PMA is considered as the control level. Each point represents the mean±SD of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). The experiment has been done 4 times.

Neither iodoclonidine, phenylephrine nor PMA could stimulate the expression of the pTKGH in OK 13 cells compared to the the control (absence of iodoclonidine, phenylephrine or PMA). These studies suggested that the stimulatory effect of α_2-adrenoceptors on the expression of pOGH (ANG N-1498/+18) in OK 27 cells is gene-specific and mediated via the 5'-flanking sequences of the rat ANG gene and not mediated via the DNA sequence of the hGH reporter gene.

RESULTS-2 Dopaminergic Receptors and Angiotensinogen Gene Expression in Opossum Kidney Cells

(Wang TT, LaChance S, Delalandre A, Carrière S & Chan JSD. Am J Physiol 1996;271:R519-R527)

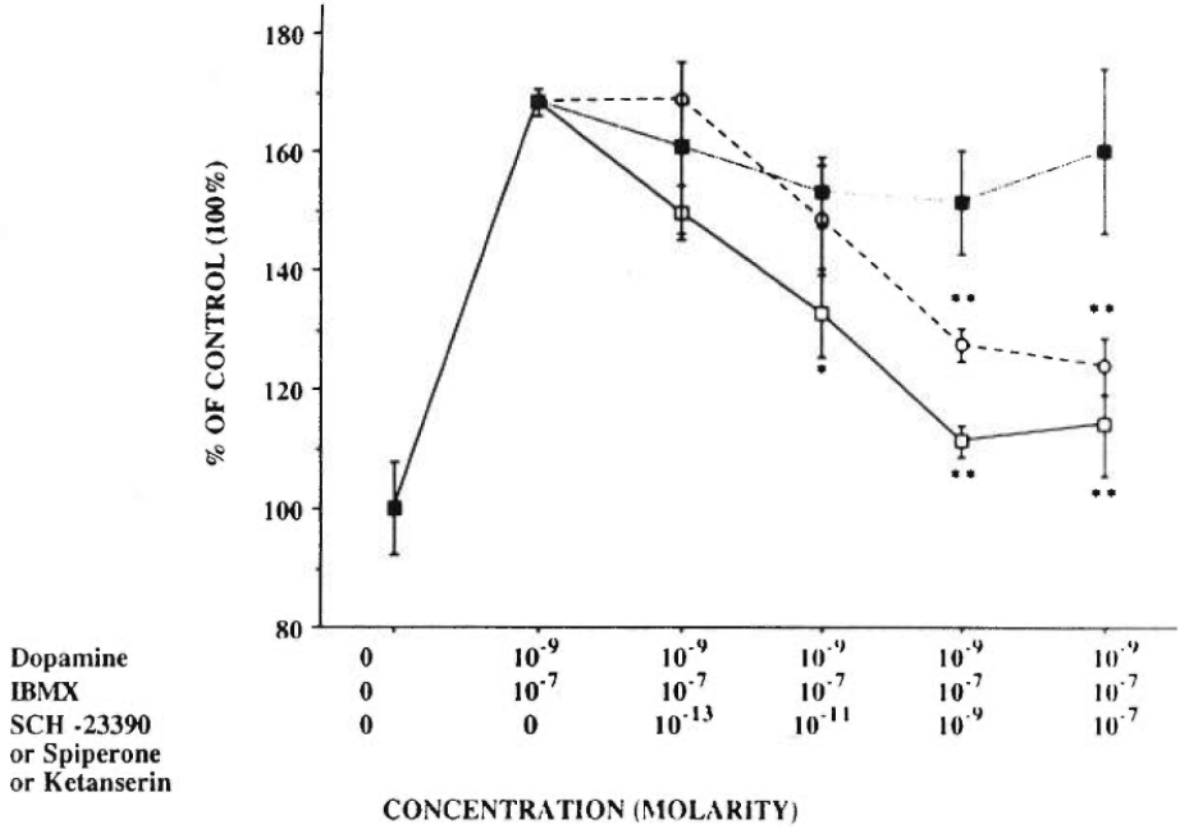
FIG.7. EFFECT OF DOPAMINE (DOPAMINERGIC RECEPTOR AGONIST) ON THE EXPRESSION OF pOGH (ANG N-1498/+18) IN OK 27 CELLS IN THE PRESENCE OR ABSENCE OF 3-ISOBUTYL-1-METHYLXANTHINE (IBMX) (INHIBITOR OF PHOSPHODIESTERASE)



OK 27 cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, various concentrations of dopamine (10^{-13} to 10^{-5} M) were added to the culture medium containing 1% dFBS with or without 0.1 µM IBMX and incubated for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). Levels of IR-hGH in the medium (with or without IBMX) in the absence of dopamine are expressed as 100% (control). Each point represents the mean±SD of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). The experiment has been done 4 times in triplicates, similar results were obtained from 3 other experiments. The basal levels of IR-hGH in the controls were 5.4±0.06 ng/ml. In subsequent experiments, levels of IR-hGH in the controls ranged from 4 to 10 ng/ml.

A dose-dependent relationship between dopamine and the stimulation of expression of pOGH (ANG N-1498/+18) was observed for dopamine at 10^{-11} to 10^{-7} M in the presence of IBMX (O). The maximal stimulation of expression of the pOGH (ANG N-1498/+18) was found with 10^{-9} M dopamine whereas dopamine had no stimulatory effect on the expression of pOGH (ANG N-1498/+18) in OK 27 cells in the absence of IBMX (●). These studies suggested that the expression of pOGH (ANG N-1498/+18) in OK 27 cells is stimulated by low concentrations of dopamine (10^{-13} to 10^{-7} M) and required the presence of IBMX to inhibit endogenous phosphodiesterase activity.

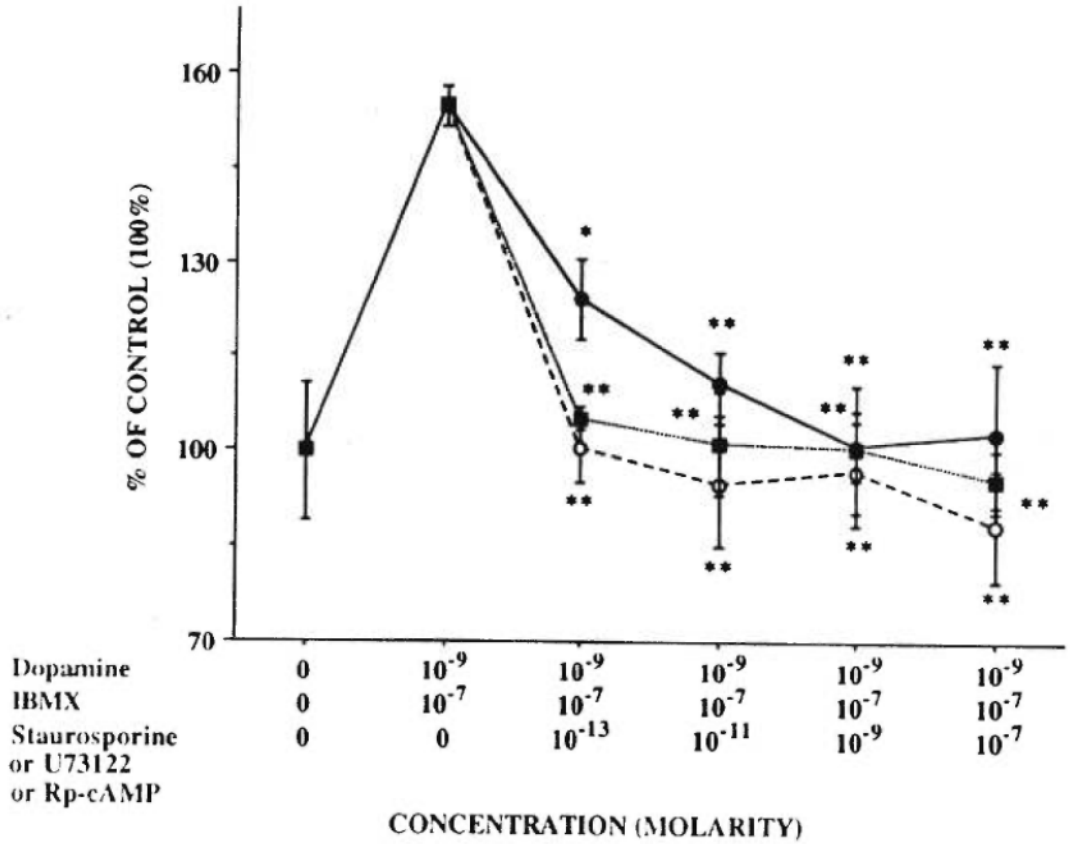
FIG.8. INHIBITORY EFFECT OF SCH-23390 (D_1-DOPAMINERGIC RECEPTOR ANTAGONIST) OR SPIPERONE (D_2-DOPAMINERGIC RECEPTOR ANTAGONIST) OR KETANSERIN (SEROTONERGIC RECEPTOR ANTAGONIST) ON THE EXPRESSION OF pOGH (ANG N-1498/+18) IN OK 27 CELLS STIMULATED BY DOPAMINE



Cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, various concentrations of SCH-23390 (10^{-13} to 10^{-5} M) or spiperone (10^{-13} to 10^{-5} M) or ketanserin (10^{-13} to 10^{-5} M) were added to the culture medium containing 1% dFBS and co-incubated with dopamine (10^{-9} M) and IBMX (10^{-7} M) for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). Levels of IR-hGH in the medium in the absence of dopamine and IBMX or the antagonists are expressed as 100% (control). Each point represents the mean±SD of at least three dishes (*p≤0.05, **p≤0.01 and ***p≤0.005). The experiment has been done 5 times in triplicates.

The addition of SCH-23390 (❑) or spiperone (O) inhibited the stimulatory effect of dopamine (10^{-9} M) in a dose-dependent manner. The effective doses were found with 10^{-11} to 10^{-7} M SCH-23390 or 10^{-9} to 10^{-7} M spiperone, respectively. Ketanserin (■) had no inhibitory effect on the stimulation of dopamine on the expression of pOGH (ANG N-1498/+18). These studies suggested that the stimulatory effect of dopamine on the expression of pOGH (ANG N-1498/+18) in OK 27 cells is mediated via DA_1 and DA_2 receptors, but not via serotonergic receptors.

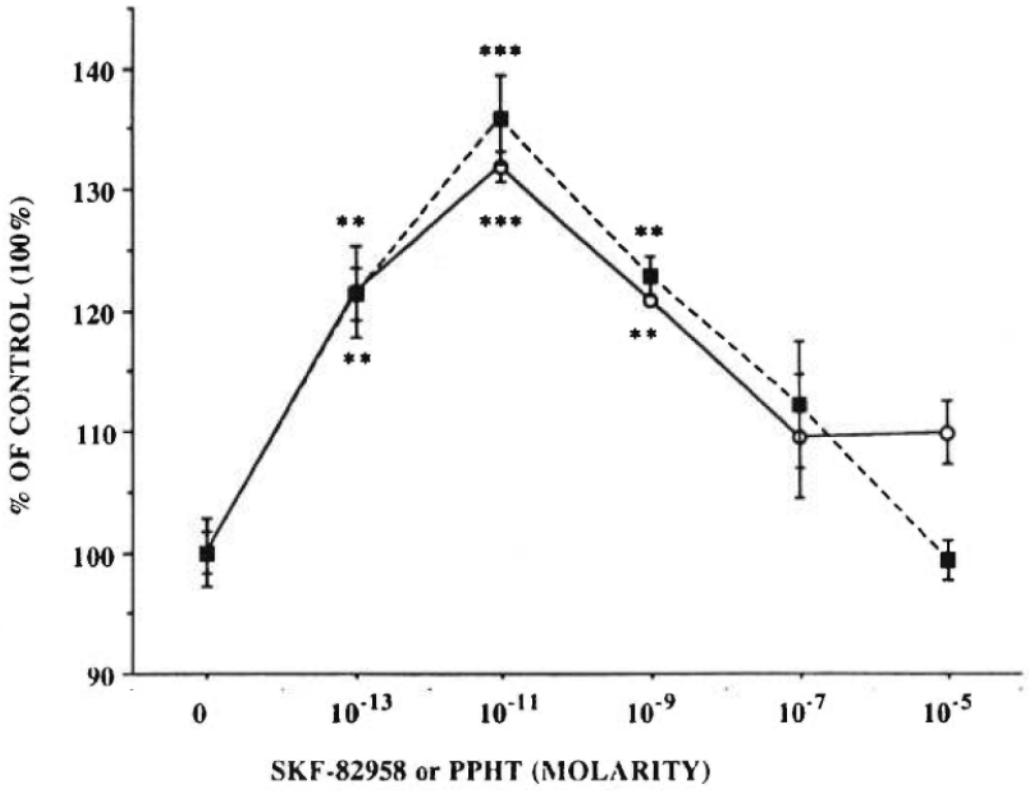
FIG.9. INHIBITORY EFFECT OF Rp-CAMP, STAUROSPORINE OR U73122 (INHIBITOR OF PHOSPHOLIPASE C) ON THE EXPRESSION OF pOGH (ANG N-1498/+18) IN OK 27 CELLS STIMULATED BY DOPAMINE



Cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, various concentrations of Rp-cAMP (10^{-13} to 10^{-5} M) or staurosporine (10^{-13} to 10^{-5} M) or U73122 (10^{-13} to 10^{-5} M) were added to the culture medium containing 1% dFBS and co-incubated with dopamine (10^{-9} M) and IBMX (10^{-7} M) for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). Levels of IR-hGH in the medium in the absence of dopamine and IBMX or Rp-cAMP or staurosporine or U73122 are expressed as 100% (control). Each point represents the mean±SD of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). The experiment has been done 5 times in triplicates.

The addition of staurosporine (○) or U73122 (■) inhibits the stimulatory effect of dopamine (10^{-9} M) in the presence of IBMX (10^{-7} M) with the maximal effect at 10^{-13} M. The addition of Rp-cAMP (●) inhibits the stimulatory effect of dopamine (10^{-9} M) in the presence of IBMX (10^{-7} M) in a dose-dependent manner. The effective doses were found with 10^{-13} to 10^{-7} M of the inhibitors. These studies suggested that the stimulatory effect of dopamine on the expression of pOGH (ANG N-1498/+18) in OK 27 cells is probably mediated via both PKA and PKC pathways.

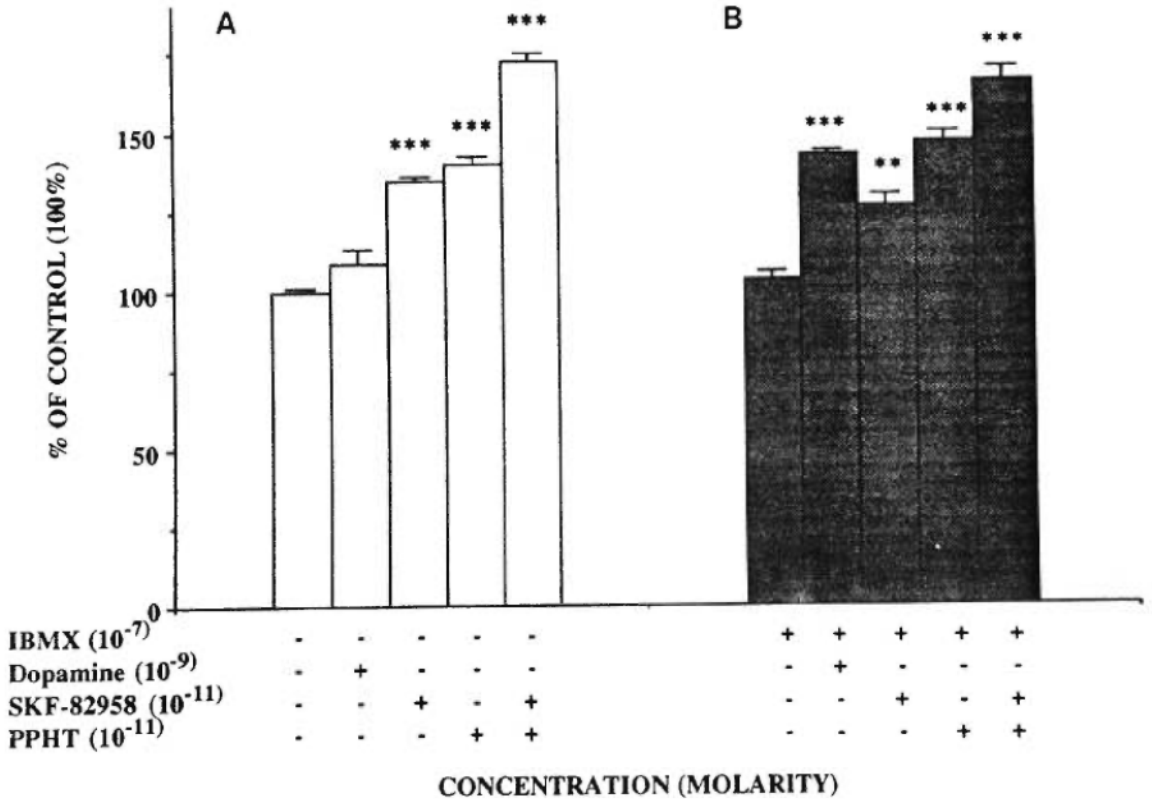
FIG.10. EFFECT OF SKF-82958 (D_1-DOPAMINERGIC RECEPTOR AGONIST) OR PPHT (D_2-DOPAMINERGIC RECEPTOR AGONIST) ON THE EXPRESSION OF pOGH (ANG N-1498/+18) IN OK 27 CELLS



Cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, various concentrations of SKF-82958 (10^{-13} to 10^{-5} M) or PPHT (10^{-13} to 10^{-5} M) were added to the culture medium containing 1% dFBS and incubated for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). Levels of IR-hGH in the medium in the absence of SKF-82958 or PPHT are expressed as 100% (control). Each point represents the mean±SD of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). The experiment has been done 4 times in triplicates.

A dose-dependent relationship between SKF-82958 (O) or PPHT (■) and the stimulation of expression of pOGH (ANG N-1498/+18) was observed at10^{-13} to 10^{-9} M. The maximal stimulation was found with 10^{-11} M SKF-82958 or PPHT, respectively. At concentrations greater than 10^{-9} M, the stimulatory effect was minimal or inhibited. These studies suggested that the stimulatory effect of dopamine on the expression of pOGH (ANG N-1498/+18) in OK 27 cells is probably mediated via both DA_1 and DA_2 receptors.

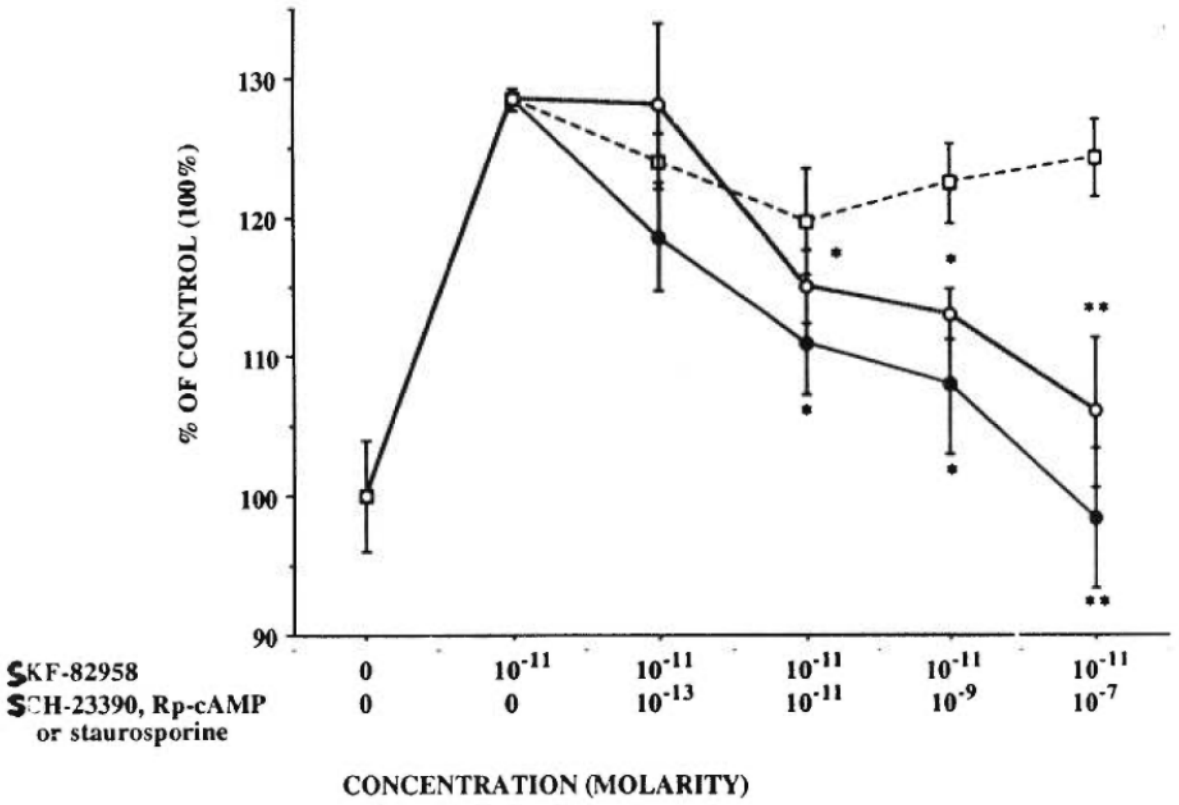
FIG.11 EFFECT OF DOPAMINE, SKF-82958, PPHT OR A COMBINATION OF BOTH SKF-82958 AND PPHT ON THE EXPRESSION OF pOGH (ANG N-1498/+18) IN OK 27 CELLS IN THE PRESENCE OR ABSENCE OF IBMX



Cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, dopamine (10^{-9} M) or SKF-82958 (10^{-11} M) or PPHT (10^{-11}M) or a combination of both SKF-82958 (10^{-11} M) and PPHT (10^{-11}M) was added to the culture medium containing 1% dFBS with or without IBMX (10^{-7} M) and incubated for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). Levels of IR-hGH in the medium in the absence of dopamine or SKF-82958 or PPHT with or without IBMX are expressed as 100% (control). Each point represents the mean±SD of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). The experiment has been done 7 times in triplicates.

The presence or absence of IBMX did not change the levels of the stimulatory effect of SKF-82958 (10^{-11} M) or PPHT (10^{-11} M). The combination of SKF-82958 (10^{-11} M) and PPHT (10^{-11} M) had an additive effect on the gene expression, but the levels did not change with or without IBMX. These studies suggested that there is probably an additive effect of both DA_1 and DA_2 receptors on the expression of pOGH (ANG N-1498/+18) in OK 27 cells.

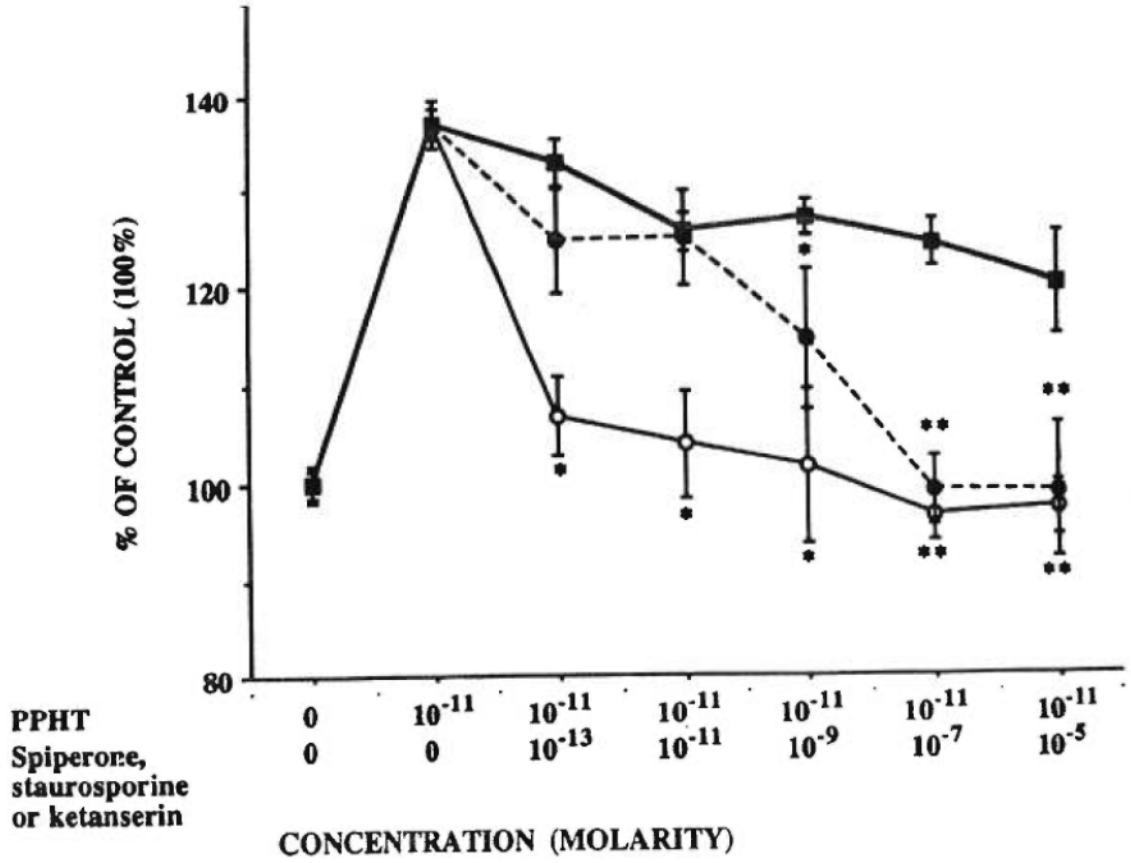
FIG.12. EFFECT OF SCH-23390 OR Rp-cAMP OR STAUROSPORINE ON THE EXPRESSION OF pOGH (ANG N-1498/+18) IN OK 27 CELLS STIMULATED BY SKF-82958



Cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, various concentrations of SCH-23390 (10^{-13} to 10^{-5} M) or Rp-cAMP (10^{-13} to 10^{-5} M) or staurosporine (10^{-13} to 10^{-5} M) were added to the culture medium containing 1% dFBS and co-incubated with SKF-82958 (10^{-11} M) for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). Levels of IR-hGH in the medium in the absence of SKF-82958 or SCH-23390 or Rp-cAMP or staurosporine are expressed as 100% (control). Each point represents the mean±SD of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). The experiment has been done 7 times in triplicates.

The addition of SCH-23390 (●) or Rp-cAMP (○) inhibited the stimulatory effect of SKF-82958 (10^{-9} M) in a dose-dependent manner. The effective doses were found with 10^{-11} to 10^{-7} M of SCH-23390 or Rp-cAMP, respectively. Staurosporine (□) had no inhibitory effect on the stimulation of SKF-82958 on the expression of pOGH (ANG N-1498/+18). These studies suggested that the stimulatory effect of DA_1 receptor on the rat ANG gene expression is mediated via the PKA but not the PKC pathway.

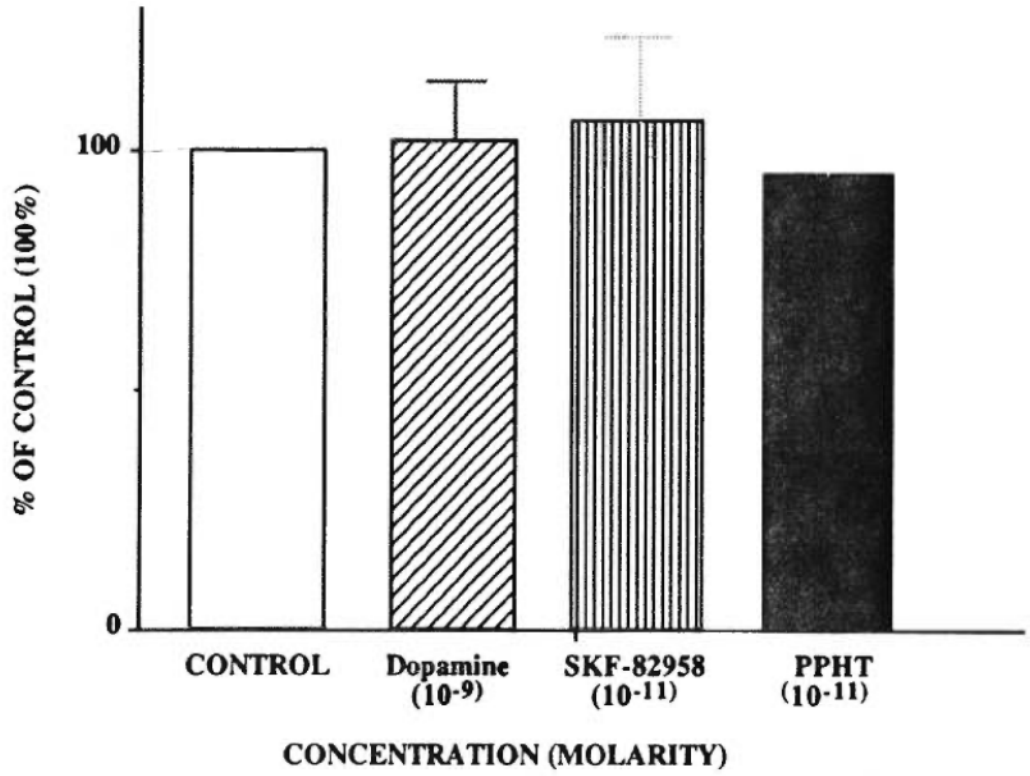
FIG.13. EFFECT OF SPIPERONE OR STAUROSPORINE OR KETANSERINE ON THE EXPRESSION OF pOGH (ANG N-1498/+18) IN OK 27 CELLS STIMULATED BY PPHT



Cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, various concentrations of spiperone (10^{-13} to 10^{-5} M) or ketanserine (10^{-13} to 10^{-5} M) or staurosporine (10^{-13} to 10^{-5} M) were added to the culture medium containing 1% dFBS and co-incubated with PPHT (10^{-11} M) for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). Levels of IR-hGH in the medium in the absence of PPHT or spiperone or sturosporine or ketanserine are expressed as 100% (control). Each point represents the mean±SD of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). The experiment has been done 5 times in triplicates.

The addition of spiperone (O) or staurosporine (●) inhibited the stimulatory effect of PPHT (10^{-11} M) in a dose-dependent manner. The effective doses were found with 10^{-13} to 10^{-5} M of spiperone or 10^{-11} to 10^{-5} M staurosporine, respectively. Ketanserine (■) had no inhibitory effect on the stimulation of PPHT on the expression of pOGH (ANG N-1498/+18). These studies suggested that the stimulatory effect of DA_2 receptor on the rat ANG gene expression is mediated via the PKC pathway.

FIG.14. EFFECT OF DOPAMINE, SKF-82958 AND PPHT ON THE EXPRESSION OF pTKGH IN OK 13 CELLS



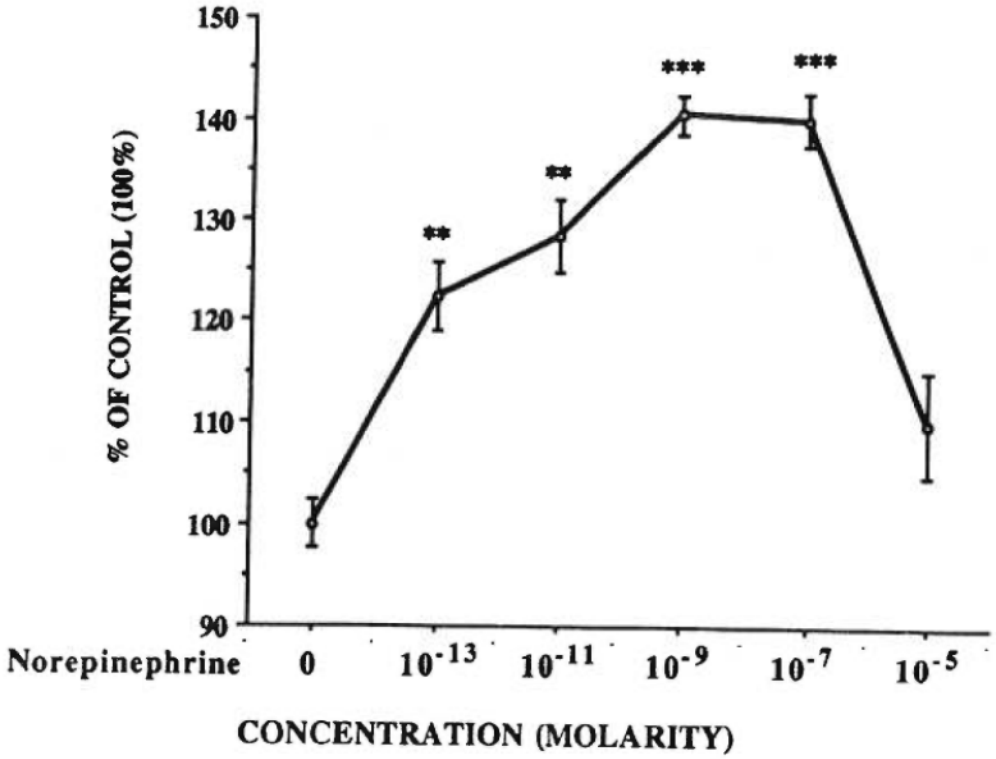
OK 13 cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, dopamine (10^{-9} M) and IBMX (10^{-7} M) or SKF-82958 (10^{-11} M) or PPHT (10^{-11} M) was added to the culture medium containing 1% dFBS and incubated for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). The concentration of IR-hGH in the absence of dopamine, SKF-82958 or PPHT is considered as the control level (100%). Each point represents the mean±SD of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). The experiment has been done 3 times.

Neither dopamine, SKF-82958 nor PPHT could stimulate the expression of the pTKGH in OK 13 cells compared to the the control (absence of dopamine, SKF-82958 or PPHT). These studies suggested that the stimulatory effect of dopaminergic receptors on the expression of pOGH (ANG N-1498/+18) in OK 27 cells is gene-specific and is mediated via the 5'-flanking sequences of the rat ANG gene and not mediated via the DNA sequence of the hGH reporter gene.

RESULT-3 The Molecular Mechanism(s) of Norepinephrine on the Expression of Angiotensinogen Gene in Opossum Kidney Cells

(Wang TT, Wu XH, Zhang SL & Chan JSD. Kidney Int 1998, under rivision)

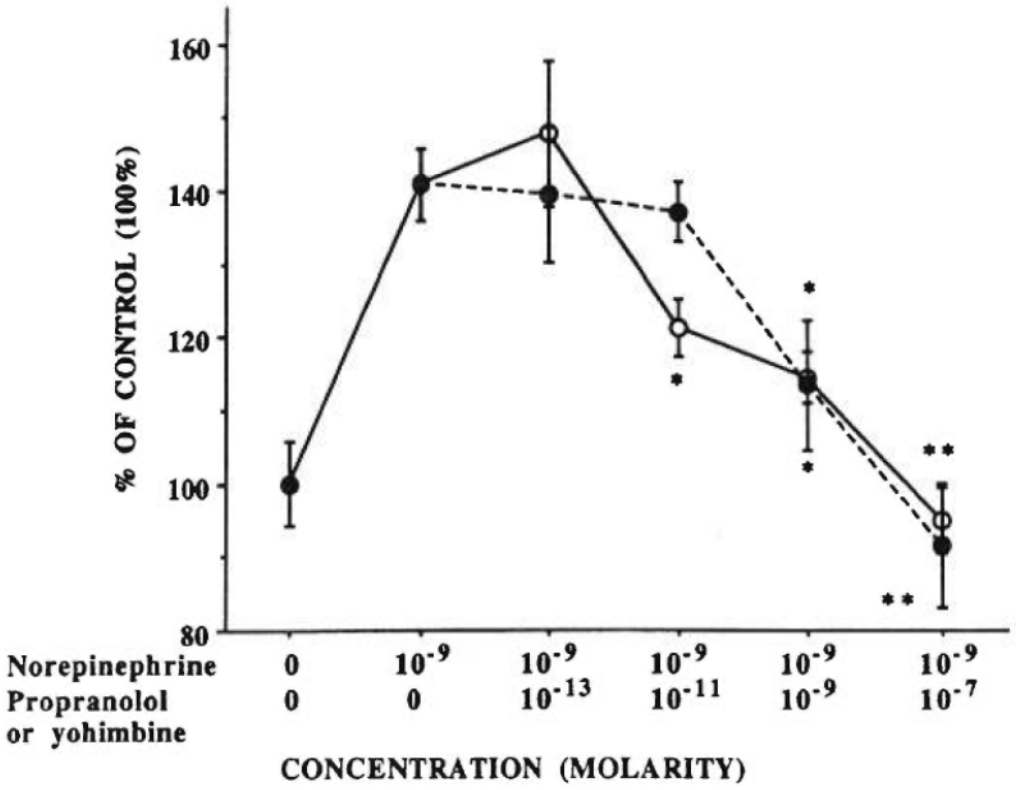
FIG.15. EFFECT OF NOREPINEPHRINE ON THE EXPRESSION OF pOGH (ANG N-1498/+18) IN OK 27 CELLS



OK 27 cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, various concentrations of norepinephrine (10^{-13} to 10^{-5} M) were added to the culture medium containing 1% dFBS and incubated for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). Levels of IR-hGH in the medium in the absence of norepinephrine are expressed as 100% (control). Each point represents the mean±SD of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). The basal levels of IR-hGH in the controls were 5.2±0.05 ng/ml. In subsequent experiments, levels of IR-hGH in the controls ranged from 4 to 10 ng/ml. The experiment has been done 3 times in triplicates.

In the presence of various concentrations of norepinephrine, a dose-dependent relationship was observed at 10^{-13} to 10^{-7} M. The maximal stimulation of expression of the pOGH (ANG N-1498/+18) was found with 10^{-9} M norepinephrine. These studies suggested that norepinephrine stimulates the rat ANG gene expression in OK 27 cells.

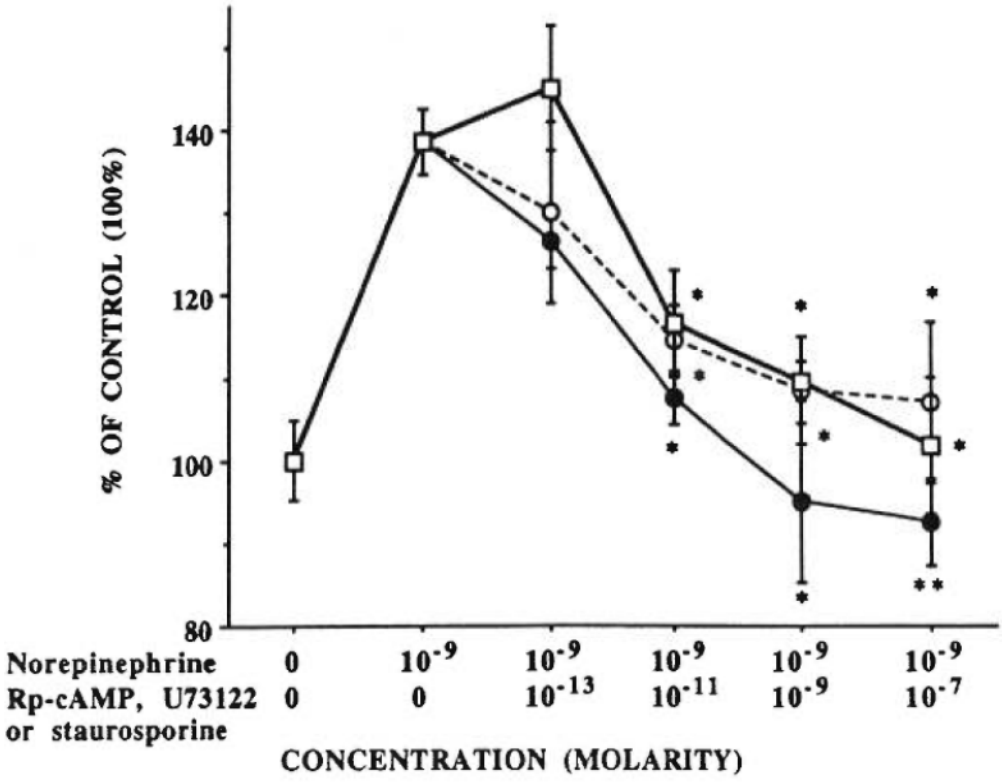
FIG.16. EFFECT OF YOHIMBINE OR PROPRANOLOL (ß-ADRENERGIC RECEPTOR ANTAGONIST) ON THE EXPRESSION OF pOGH (ANG N-1498/+18) IN OK 27 CELLS STIMULATED BY NOREPINEPHRINE



OK 27 cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, various concentrations of yohimbine (10^{-13} to 10^{-5} M) or propranolol (10^{-13} to 10^{-5} M) were added to the culture medium containing 1% dFBS and co-incubated with norepinephrine (10^{-9} M) for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). Levels of IR-hGH in the medium in the absence of norepinephrine or yohimbine or propranolol are expressed as 100% (control). Each point represents the mean±SD of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). The experiment has been done 4 times in triplicates.

In the presence of various concentrations of yohimbine (O) or propranolol (●), the stimulatory effect of norepinephrine (10^{-9} M) was inhibited in a dose-dependent manner. The effective doses were found with 10^{-11} to 10^{-7} M of propranolol, or 10^{-9} to 10^{-7} M yohimbine, respectively. These studies indicated that the stimulatory effect of norepinephrine on the expression of pOGH (A N-1498/+18) in OK 27 cells is mediated via both α_2- and ß-adrenoceptors.

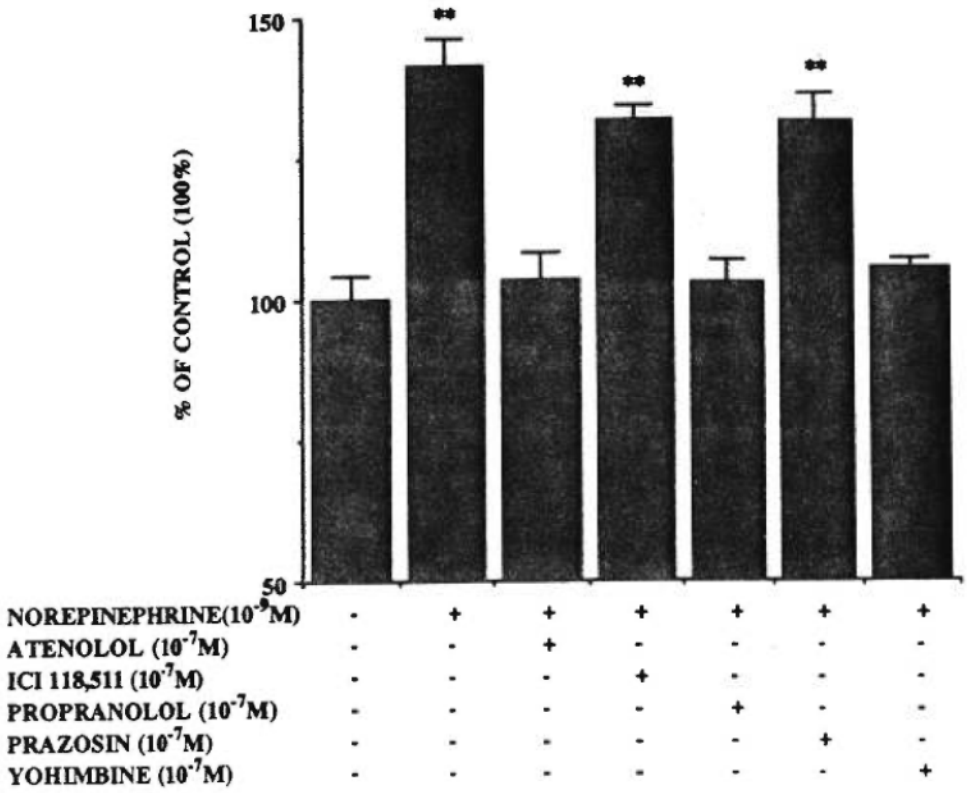
FIG.17. INHIBITORY EFFECT OF STAUROSPORINE OR Rp-cAMP OR U73122 ON THE EXPRESSION OF pOGH (ANG N-1498/+18) IN OK 27 CELLS STIMULATED BY NOREPINEPHRINE



OK 27 cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, various concentrations of staurosporine (10^{-13} to 10^{-5} M) or Rp-cAMP (10^{-13} to 10^{-5} M) or U73122 (10^{-13} to 10^{-5} M) were added to the culture medium containing 1% dFBS and co-incubated with norepinephrine (10^{-9} M) for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). Levels of IR-hGH in the medium in the absence of norepinephrine or staurosporine or Rp-cAMP or U73122 are expressed as 100% (control). Each point represents the mean±SD of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). The experiment has been done 5 times in triplicates.

In the presence of various concentrations of staurosporine (●) or Rp-cAMP (❑) or U73122 (○), the stimulatory effect of norepinephrine (10^{-9} M) was inhibited in a dose-dependent manner. The effective doses were found with 10^{-11} to 10^{-7} M staurosporine or Rp-cAMP or U73122. These studies indicated that the stimulatory effect of norepinephrine on the expression of pOGH (A N-1498/+18) in OK 27 cells is mediated via both PKA and PKC signal transduction pathways.

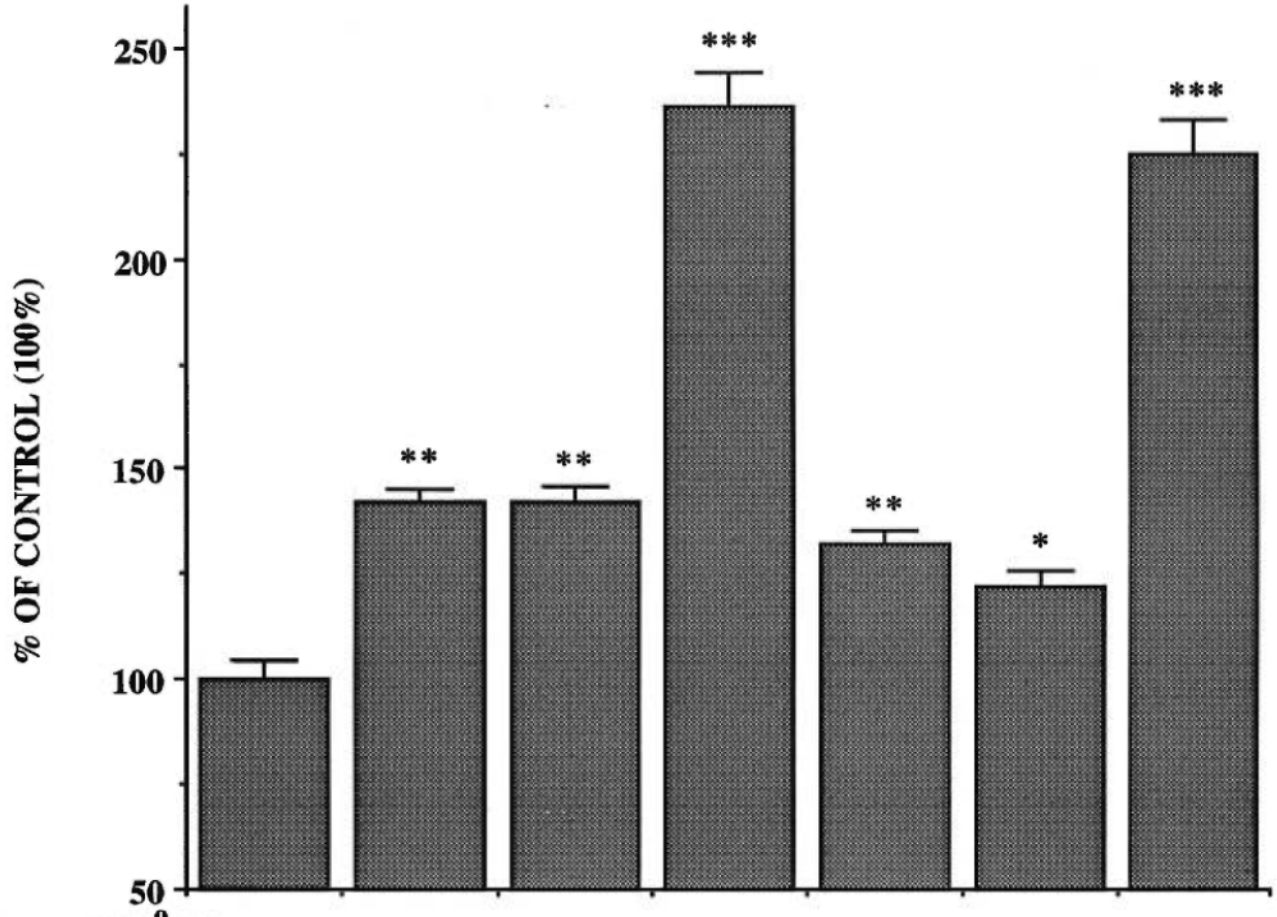
FIG. 18. THE INHIBITORY EFFECT OF VARIOUS ADRENOCEPTOR ANTAGONISTS ON THE EXPRESSION OF THE pOGH (ANG N-1498/+18) IN OK 27 CELLS



OK 27 cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, various concentrations of the antagonists (10^{-13} to 10^{-5} M) were added to the culture medium containing 1% dFBS and co-incubated with norepinephrine (10^{-9} M) for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). Levels of IR-hGH in the medium in the absence of norepinephrine or any of the antagonists are expressed as 100% (control). Each point represents the mean±SD of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). The experiment has been done 7 times in triplicates.

The stimulatory effect of norepinephrine (10^{-9} M) was inhibited by atenolol (β_1-adrenergic receptor antagonist) (10^{-7} M), propranolol (10^{-7} M), or yohimbine (10^{-7} M), but not by ICI 118,511 (β_2-adrenergic receptor antagonist) (10^{-7} M), or prazosin (10^{-7} M). These studies indicated that the stimulatory effect of norepinephrine on the expression of pOGH (ANG N-1498/+18) in OK 27 cells is mediated via both α_2- and β_1-adrenoceptors.

FIG.19. EFFECT OF IODOCLONIDINE AND ISOPROTERENOL (ß-ADRENOCEPTOR AGONIST) OR PMA AND 8-Br-cAMP (cAMP ANALOG) ON THE EXPRESSION OF pOGH (ANG N-1498/+18) IN OK 27 CELLS

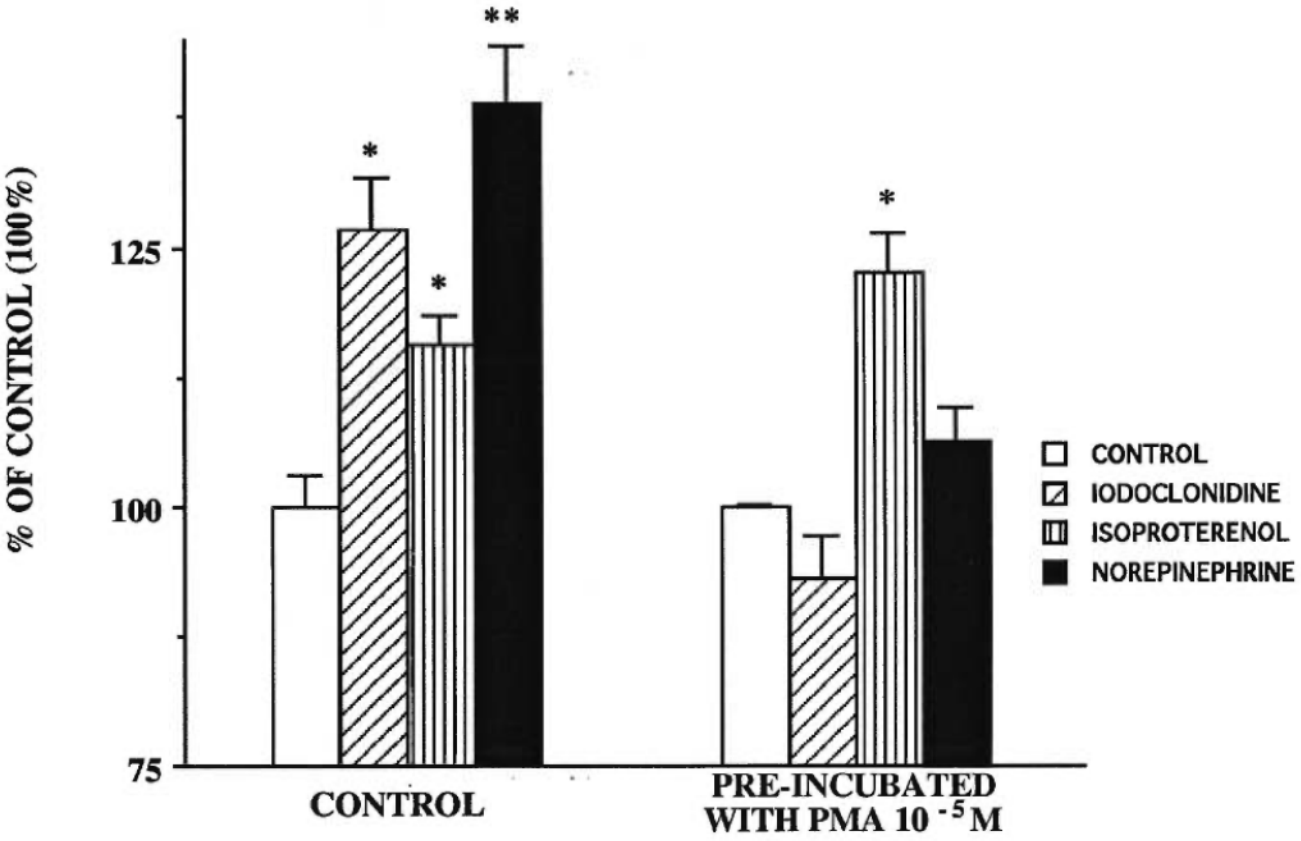


›oproterenol(10^{-9}M)	-	+	-	+	-	-	-
›doclonidine (10^{-9}M)	-	-	+	+	-	-	-
·Br-cAMP (10^{-3}M)	-	-	-	-	+	-	+
MA (10^{-11}M)	-	-	-	-	-	+	+

Cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, isoproterenol (10^{-9} M) or iodoclonidine (10^{-9} M) or 8-Br-cAMP (10^{-3} M) or PMA (10^{-11} M) were added to the culture medium containing 1% dFBS and incubated for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). Levels of IR-hGH in the medium in the absence of isoproterenol or iodoclonidine or 8-Br-cAMP or PMA are expressed as 100% (control). Each point represents the mean±SD of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). The experiment has been done 3 times in triplicates.

These studies suggested that the combination of isoproterenol (10^{-9} M) and iodoclonidine (10^{-9} M) or 8-Br-cAMP (10^{-3} M) and PMA (10^{-11} M) had a synergistic effect on the ANG gene expression in OK 27 cells.

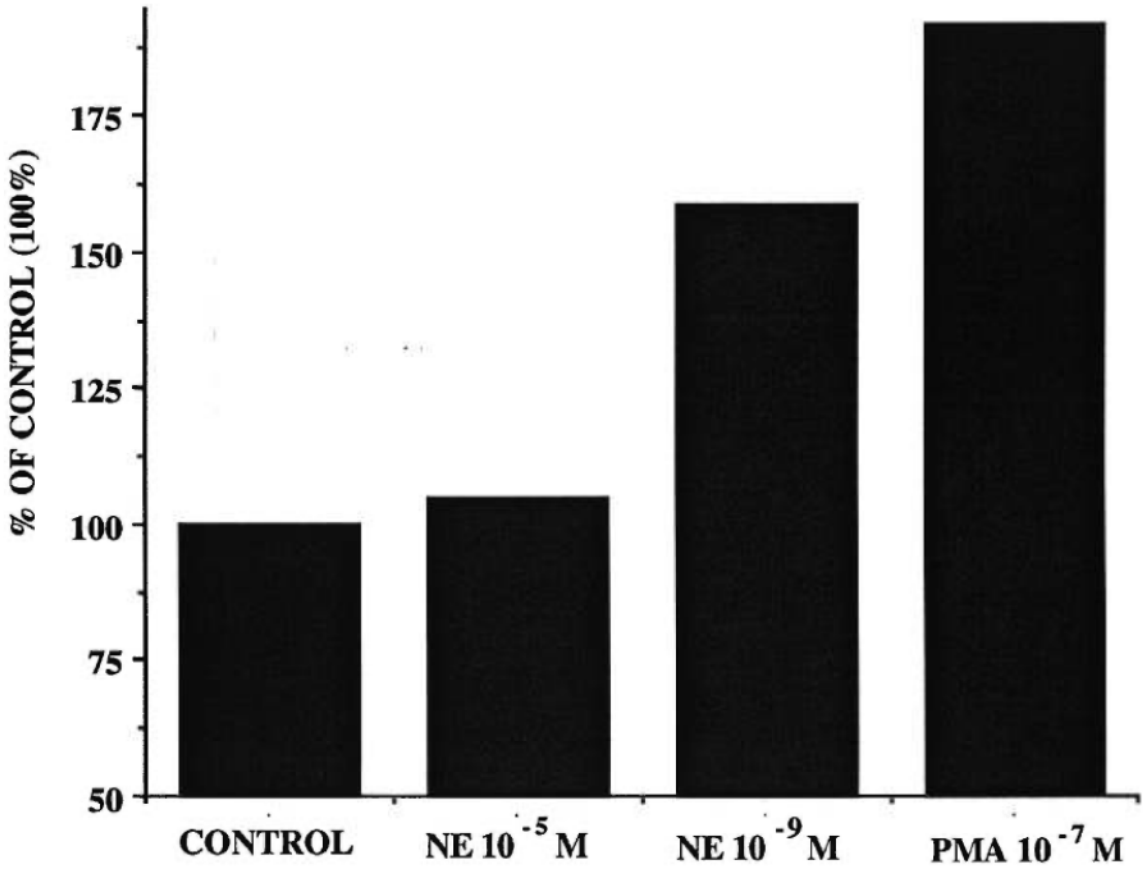
FIG.20. EFFECT OF ADDITION OF IODOCLONIDINE, ISOPROTERENOL OR NOREPINEPHRINE ON THE EXPRESSION OF pOGH (ANG N-1498/+18) IN OK 27 CELLS WHEN THE CELLS WERE PRE-INCUBATED IN THE ABSENCE/PRESENCE OF A HIGH LEVEL OF PMA



Cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, the cells were incubated in the absence or presence of PMA (10^{-5} M) with the culture medium containing 1% dFBS for 24 hours. Then, isoproterenol (10^{-9} M) or iodoclonidine (10^{-9} M) or norepinephrine (10^{-9} M) was added to the culture medium containing 1% dFBS and incubated for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). Levels of IR-hGH in the medium in the absence of isoproterenol or iodoclonidine or norepinephrine are expressed as 100% (control). Each point represents the mean±SD of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). The experiment has been done once in triplicates.

The figure showed that pre-incubation of PMA (10^{-5} M) abolished the stimulatory effect of iodoclonidine or norepinephrine, but not that of isoproterenol. These studies indicated that the stimulatory effect of norepine-phrine and iodoclonidine is mediated via the PKC pathway.

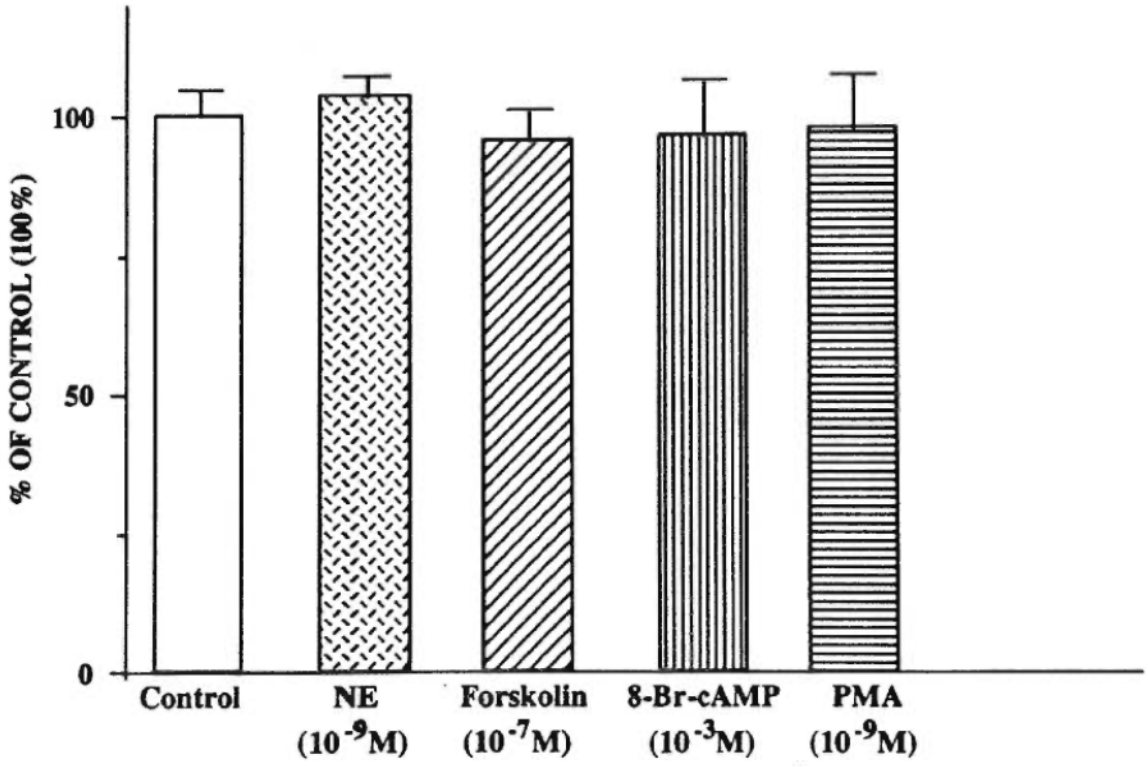
FIG.21. EFFECT OF NOREPINEPHRINE ON THE RATIO OF MEMBRANE/ CYTOSOLIC PKC ACTIVITY IN OK 27 CELLS



Cells were plated at a density of 2 X 10^6 cells in 150X100 mm petri-dishes for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, norepinephrine (10^{-5} M or 10^{-9} M) or PMA (10^{-7} M) was added to the culture medium containing 1% dFBS and incubated for 30 minutes. Cells were harvested after the incubation and assayed for PKC activity. The ratio of membrane/cytosolic PKC activity in the absence of norepinephrine or PMA are expressed as 100% (control). The experiment has been done 3 times in duplicates.

The figure showed that norepinephrine (10^{-9} M) or PMA (10^{-7} M) stimulated the cellular PKC activity. These studies indicated that norepinephrine (10^{-9} M) or PMA (10^{-7} M) stimulated the translocation of PKC from cytosol to membrane in OK 27 cells.

FIG.22. EFFECT OF NOREPINEPHRINE, FORSKOLIN (ADENYLYL CYCLASE ACTIVATOR), 8-Br-cAMP OR PMA ON THE EXPRESSION OF pTKGH IN OK 13 CELLS



OK 13 cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, norepinephrine (10^{-9} M) or forskolin (10^{-11} M) or 8-Br-cAMP (10^{-3} M) or PMA (10^{-11} M) was added to the culture medium containing 1% dFBS and incubated for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). The concentration of IR-hGH in the absence of norepinephrine or forskolin or 8-Br-cAMP or PMA is considered as the control level (100%). Each point represents the mean±SD of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). The experiment has been done 3 times.

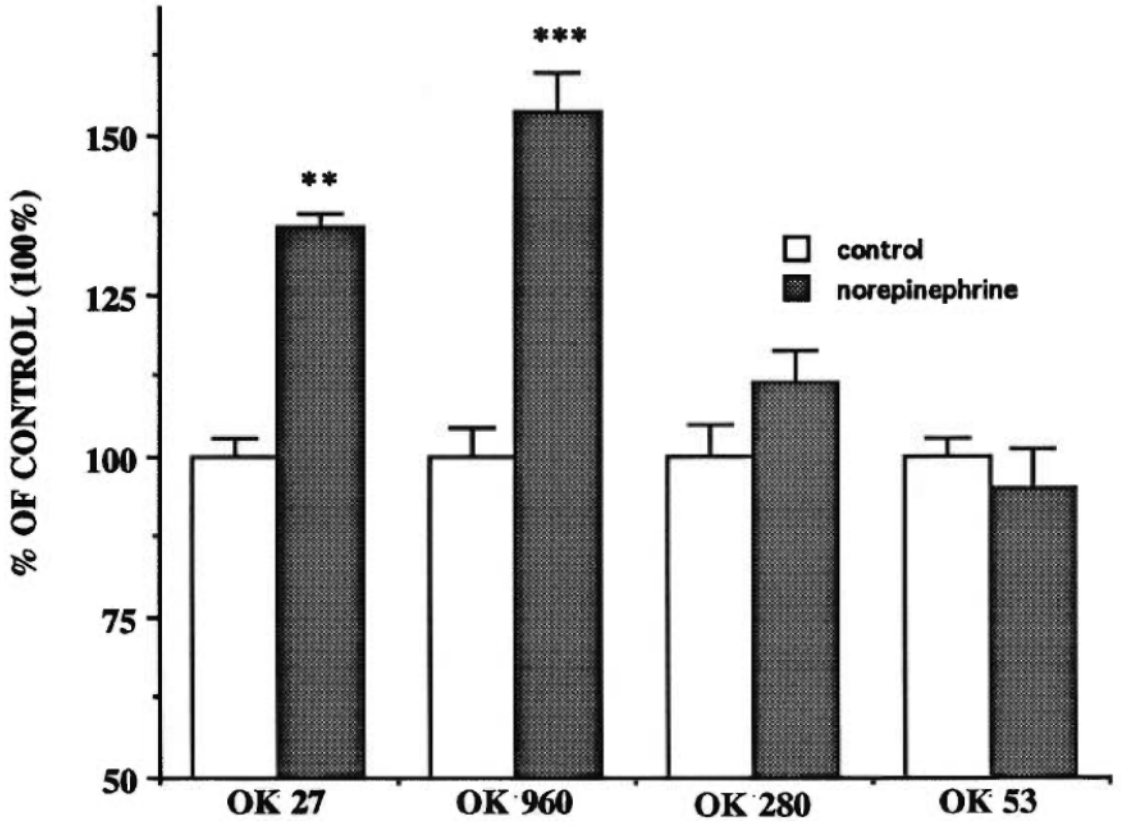
Neither norepinephrine, forskolin, 8-Br-cAMP or PMA could stimulate the expression of the pTKGH in OK 13 cells compared to the the control (absence of norepinephrine, forskolin, 8-Br-cAMP or PMA). These studies suggested that the stimulatory effect of adrenergic receptors on the expression of pOGH (ANG N-1498/+18) in OK 27 cells is gene-specific and is mediated via the 5'-flanking sequences of the rat ANG gene and not mediated via the DNA sequence of the hGH reporter gene.

RESULTS-4 Molecular mechanisms of the Action of CREB on the Expression of the Rat ANG Gene in OK Cells

(Qian JF, Wang TT, Wu XH, Wu J, Ge C, Lachance S, Carrière S & Chan JSD: Angiotensinogen gene expression is stimulated by the cyclic AMP-responsive element binding protein (CREB) in opossum kidney cells. J Am Soc Nephrol 1997;8:1072)

(Wang TT, Wu XH, Zhang SL & Chan JSD: Molecular mechanism(s) of action of isoproterenol on the expression of the angiotensinogen gene in opossum kidney proximal tubular cells. In preparation)

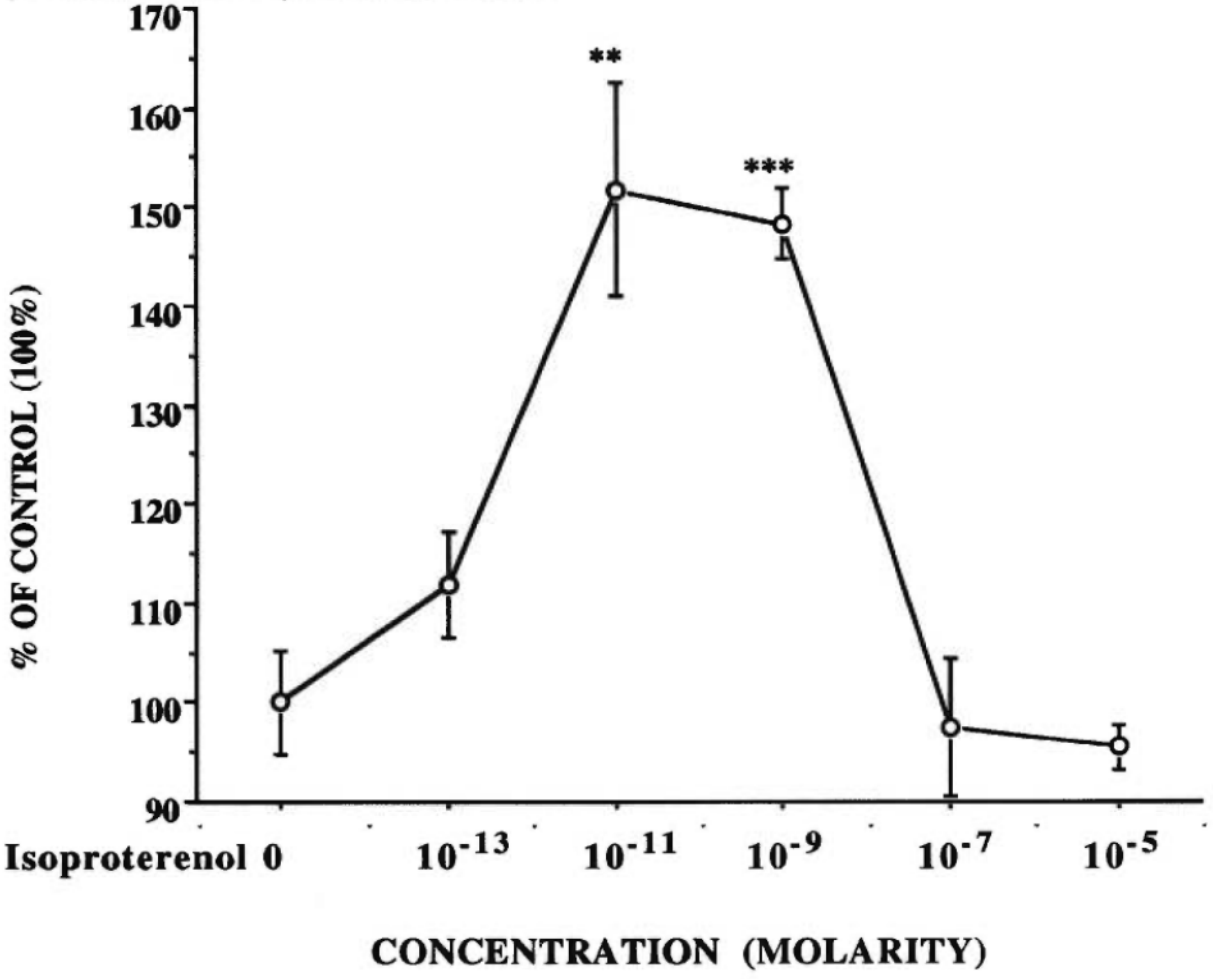
FIG. 23. EFFECT OF NOREPINEPHRINE ON THE EXPRESSION OF DIFFERENT ANG-GH FUSION GENES IN OK CELLS



Cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, norepinephrine (10^{-9} M) was added to the culture medium containing 1% dFBS and incubated for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). The concentration of IR-hGH in the absence of norepinephrine is considered as the control level (100%). Each point represents the mean±SD of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). The basal levels of IR-hGH in the controls were 4.5±0.03 ng/ml. In subsequent experiments, levels of IR-hGH in the controls ranged from 4 to 10 ng/ml. The experiment has been done 4 times in triplicates.

The addition of norepinephrine (10^{-9} M) stimulated the expression of pOGH (ANG N-1498/+18) and pOGH (ANG N-960/+18) in OK 27 and OK 96 cells. Norepinephrine had no effect on the expression of pOGH (ANG N-280/+18) or pOGH (ANG N-53/+18) in OK 280 or OK 53 cells, respectively. These studies suggested that there might be a specific DNA sequence between -960 to -280 in the 5'-flanking sequence of the rat ANG gene which responded to the stimulation of norepinephrine.

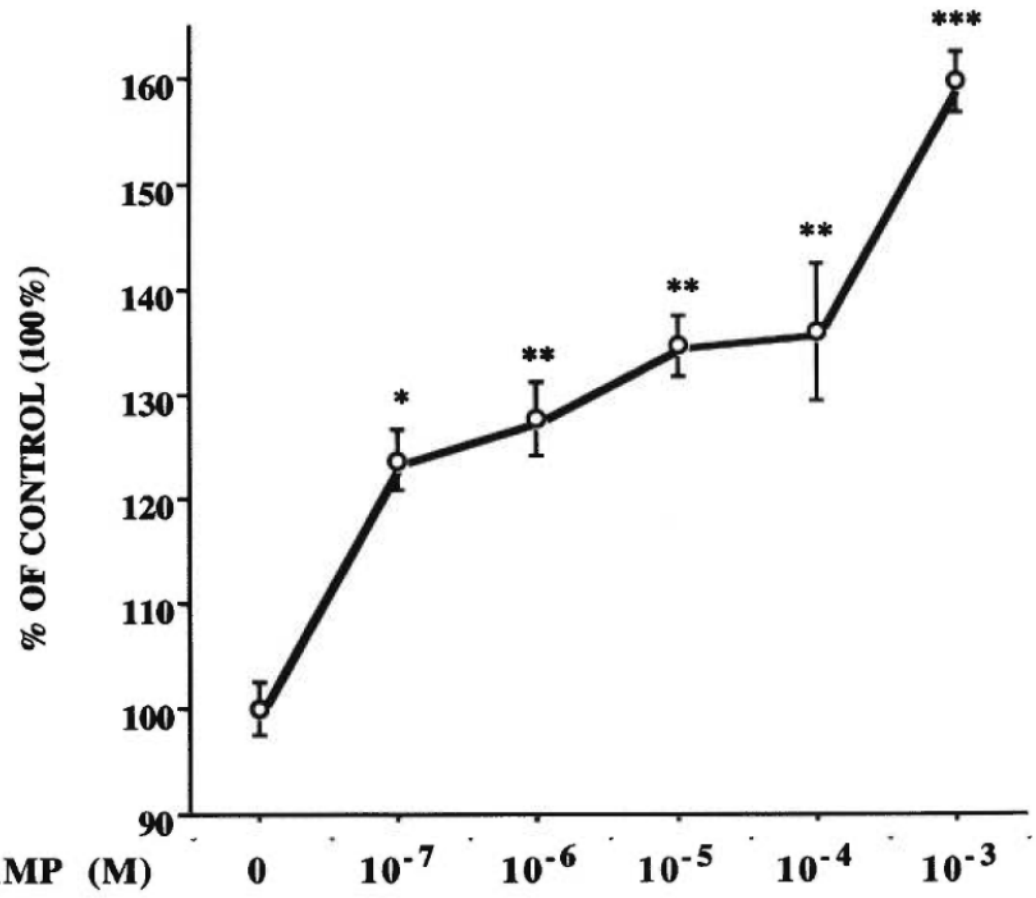
FIG.24. EFFECT OF ISOPROTERENOL ON THE EXPRESSION OF pOGH (ANG/CRE/-53/+18) IN OK 95 CELLS



OK 95 cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, various concentrations of isoproterenol (10^{-13} to10^{-5} M) were added to the culture medium containing 1% dFBS and incubated for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). The concentration of IR-hGH in the absence of isoproterenol is considered as the control level (100%). Each point represents the mean±SD of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). The experiment has been done 4 times in triplicates.

A dose-dependent relationship was observed at 10^{-11} to 10^{-9} M of isoproterenol. The maximal stimulation of expression of the pOGH (ANG/CRE/-53/+18) was found with 10^{-9} M isoproterenol.

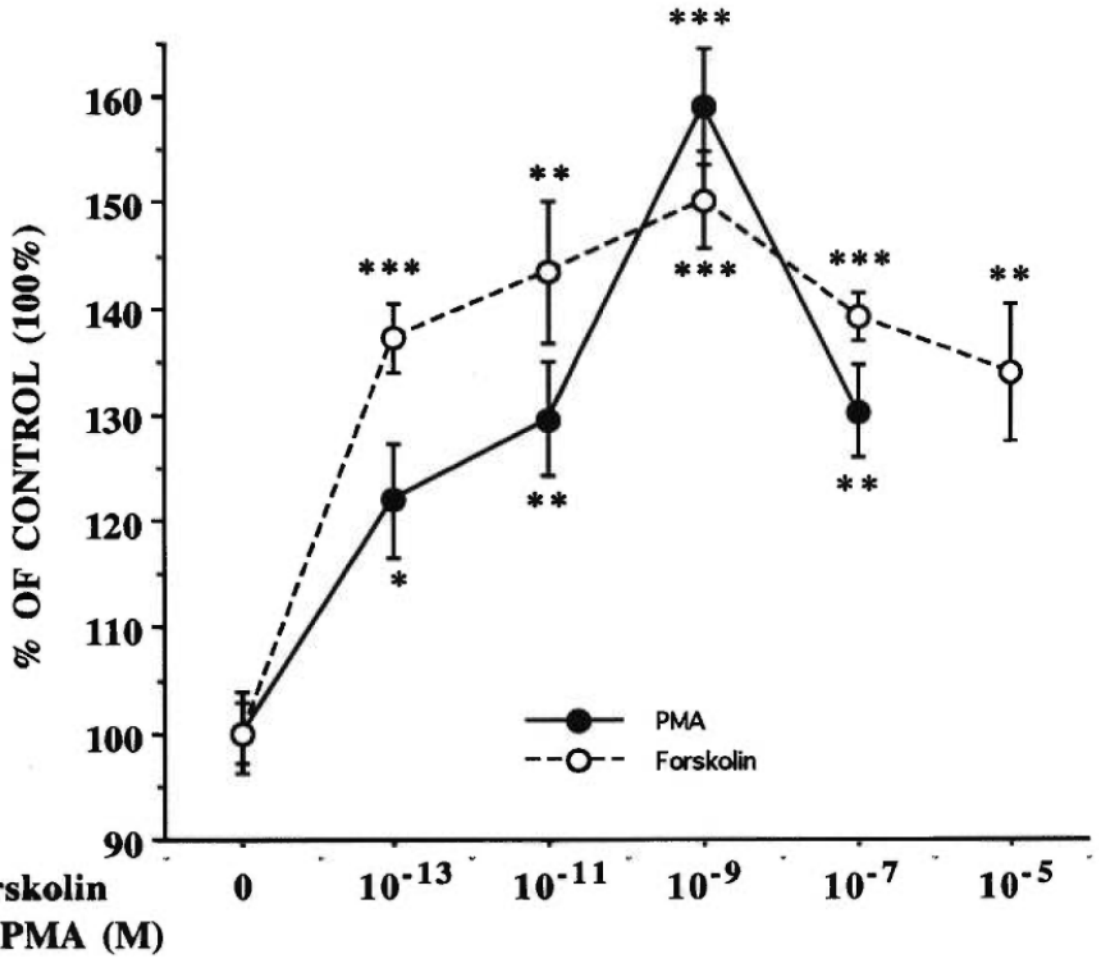
FIG.25. EFFECT OF 8-Br-cAMP ON THE EXPRESSION OF pOGH (ANG/CRE/-53/+18) IN OK 95 CELLS



OK 95 cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, various concentrations of 8-Br-cAMP (10^{-7} to10^{-3} M) were added to the culture medium containing 1% dFBS and incubated for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). The concentration of IR-hGH in the absence of 8-Br-cAMP is considered as the control level (100%). Each point represents the mean±SD of at least three dishes (*p≤0.05, **p≤0.01 and ***p≤0.005). The experiment has been done 3 times in triplicates.

A dose-dependent relationship between 8-Br-cAMP and the stimulation of expression of pOGH (ANG/CRE/-53/+18) was observed for 8-Br-cAMP at 10^{-7} to 10^{-3} M. The maximal stimulation was found with 10^{-3} M 8-Br-cAMP. These studies suggested that PKA pathway is involved in the regulation of the ANG gene expression.

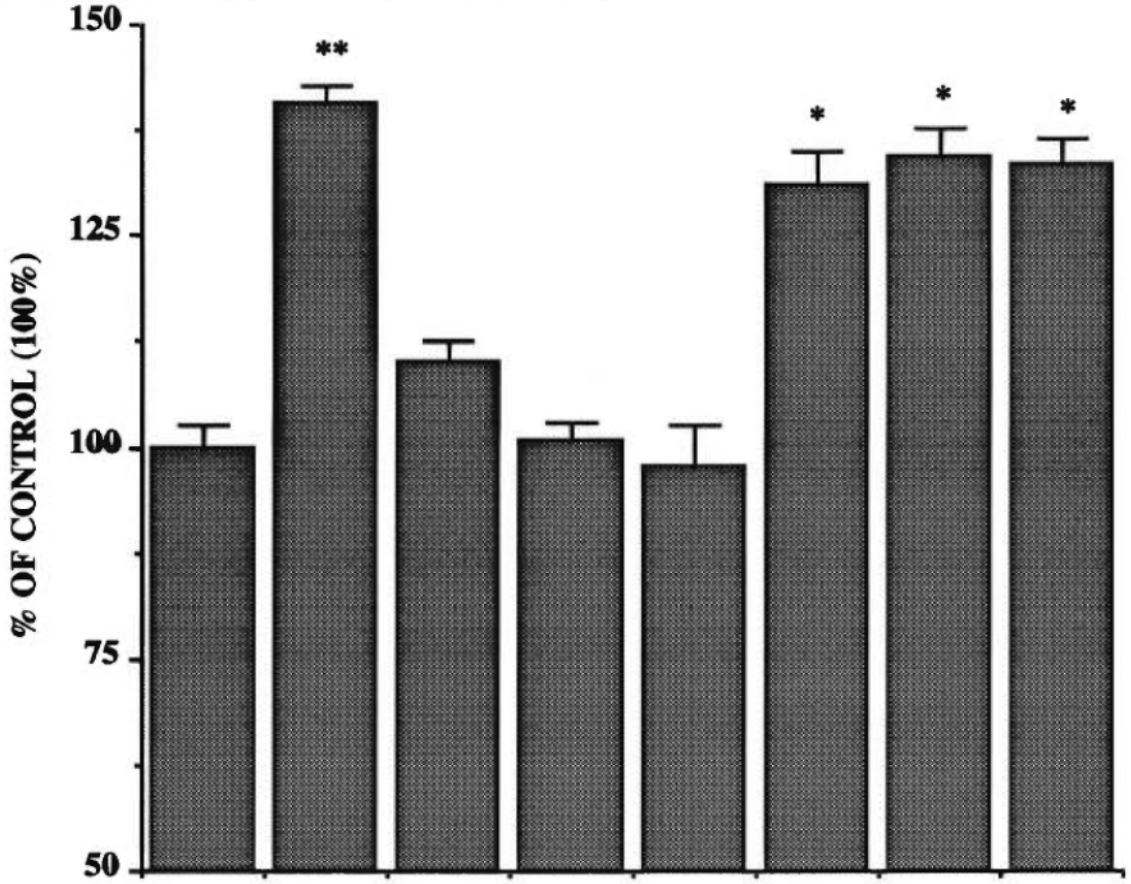
FIG.26. EFFECT OF PMA OR FORSKOLIN ON THE EXPRESSION OF pOGH (ANG/CRE/-53/+18) IN OK 95 CELLS



OK 95 cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, various concentrations of PMA (10^{-13} to10^{-5} M) or forskolin (10^{-13} to 10^{-5} M) were added to the culture medium containing 1% dFBS and incubated for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). The concentration of IR-hGH in the absence of PMA or forskolin is considered as the control level (100%). Each point represents the mean±SD of at least three dishes (*p≤0.05, **p≤0.01 and ***p≤0.005). The experiment has been done 5 times in triplicates.

A dose-dependent relationship between PMA or forskolin and the stimulation of expression of pOGH (ANG/CRE/-53/+18) was observed at 10^{-11} to 10^{-5} M. The maximal stimulation was found with 10^{-9} M PMA or forskolin, respectively. These studies suggested that PKA and PKC signaling pathways are probably both involved in the regulation of the ANG gene expression.

FIG.27. EFFECT OF ADRENERGIC RECEPTOR ANTAGONISTS OR Rp-cAMP OR STAUROSPORINE OR U73122 ON THE EXPRESSION OF pOGH (ANG/CRE/-53/+18) IN OK 95 CELLS STIMULATED BY ISOPROTERENOL

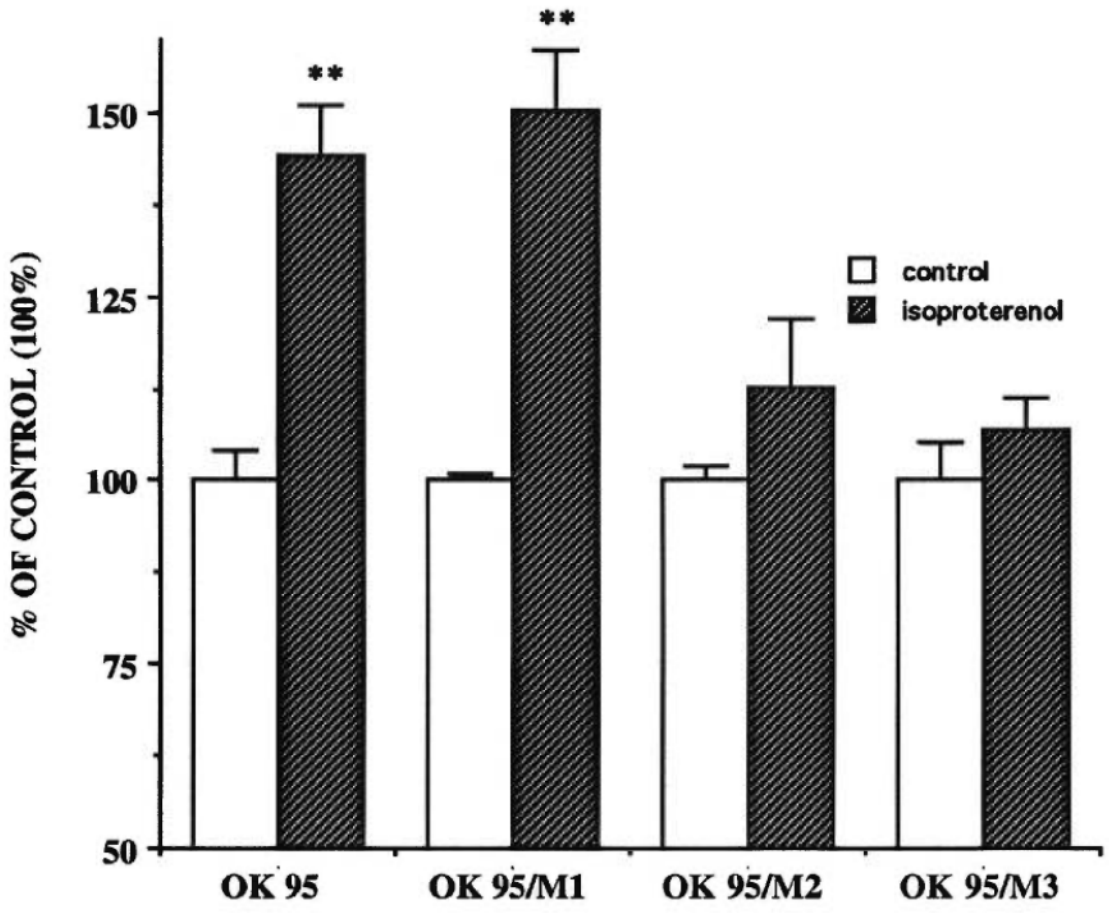


Isoproterenol(10^{-9} M)	-	+	+	+	+	+	+	+
Propranolol (10^{-7} M)	-	-	+	-	-	-	-	-
Atenolol (10^{-7} M)	-	-	-	+	-	-	-	-
Rp-cAMP (10^{-7} M)	-	-	-	-	+	-	-	-
ICI 118,511 (10^{-7} M)	-	-	-	-	-	+	-	-
Staurosporine(10^{-7} M)	-	-	-	-	-	-	+	-
U73122 (10^{-7} M)	-	-	-	-	-	-	-	+

OK 95 cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, various atagonists or inhibitors (10^{-7} M) was added to the culture medium containing 1% dFBS and co-incubated with isoproterenol (10^{-9} M) for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). The concentration of IR-hGH in the absence of isoproterenol or antagonists or inhibitors is considered as the control level (100%). Each point represents the mean±SD of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). The experiment has been done 7 times in triplicates.

The addition of atenolol (10^{-7} M), propranolol (10^{-7} M), or Rp-cAMP (10^{-7} M), but not ICI 118,511 (10^{-7} M), staurosporine (10^{-7} M) or U73122 (10^{-7} M) inhibited the stimulatory effect of isoproterenol (10^{-9} M) on the expression of pOGH (ANG/CRE/-53/+18) in OK 95 cells. These studies suggested that the effect of isoproterenol is mediated via β_1-AR and PKA pathway.

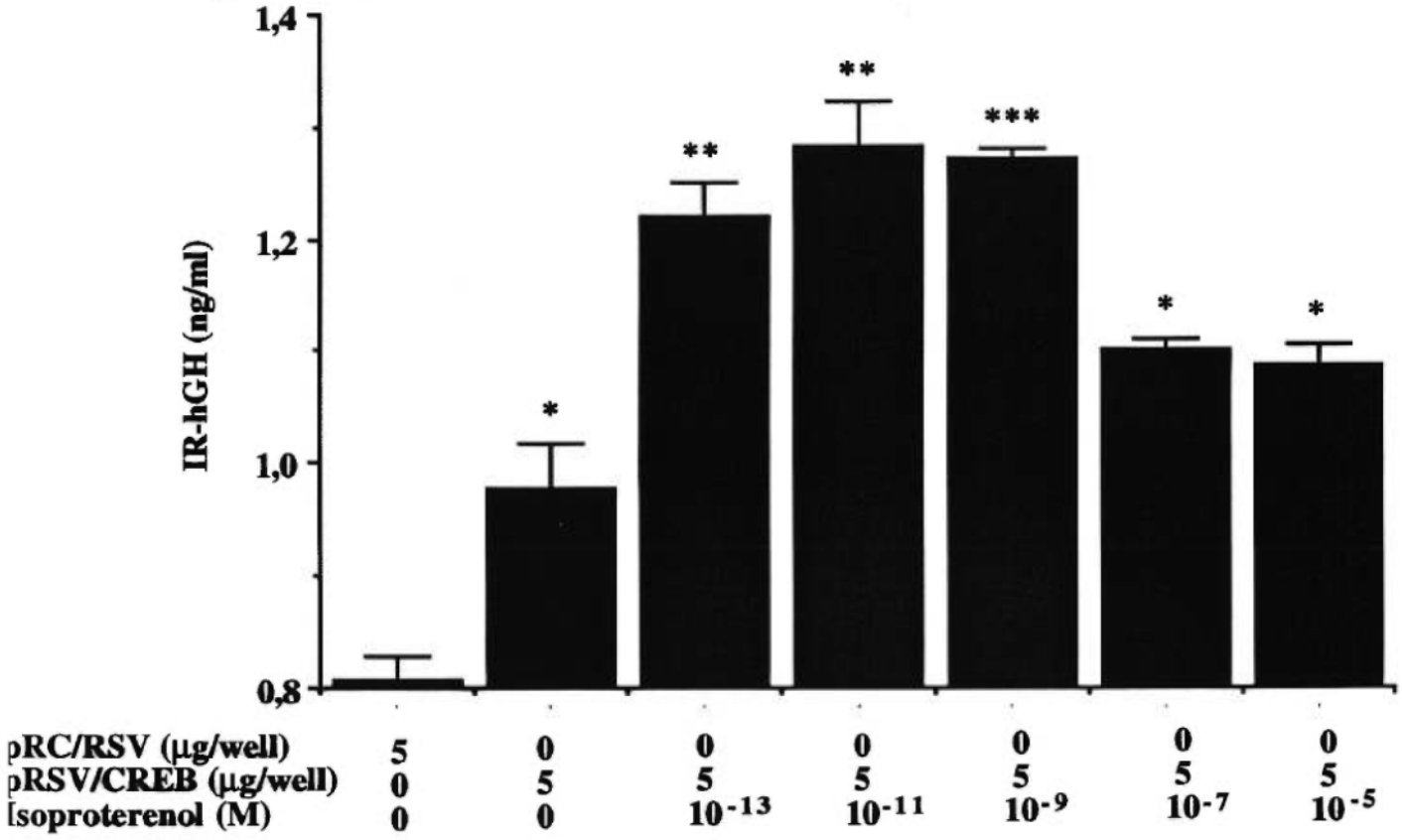
FIG.28. EFFECT OF ISOPROTERENOL ON THE EXPRESSION OF pOGH (ANG/CRE/-53/+18) OR DIFFERENT MUTANTS IN OK CELLS



OK 95 or OK 95/mutant cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, isoproterenol (10^{-9} M) was added to the culture medium containing 1% dFBS and incubated for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). The concentration of IR-hGH in the absence of isoproterenol is considered as the control level (100%). Each point represents the mean±SD of at least three dishes (*p≤0.05, **p≤0.01 and ***p≤0.005). The experiment has been done 5 times in triplicates.

The addition of isoproterenol (10^{-9} M) stimulated the expression of pOGH (ANG/CRE/-53/+18) or pOGH (ANG/CREM1/-53/+18), respectively, whereas isoproterenol had no effect on OK 95/M2 or OK 95/M3. These studies suggested that the stimulatory effect of isoproterenol is mediated via the ANG-CRE sequence of the 5'-flanking region of the rat ANG gene.

FIG.29. EFFECT OF pRSV/CREB ON THE EXPRESSION OF pOGH (ANG N-1498/+18) IN OK 27 CELLS

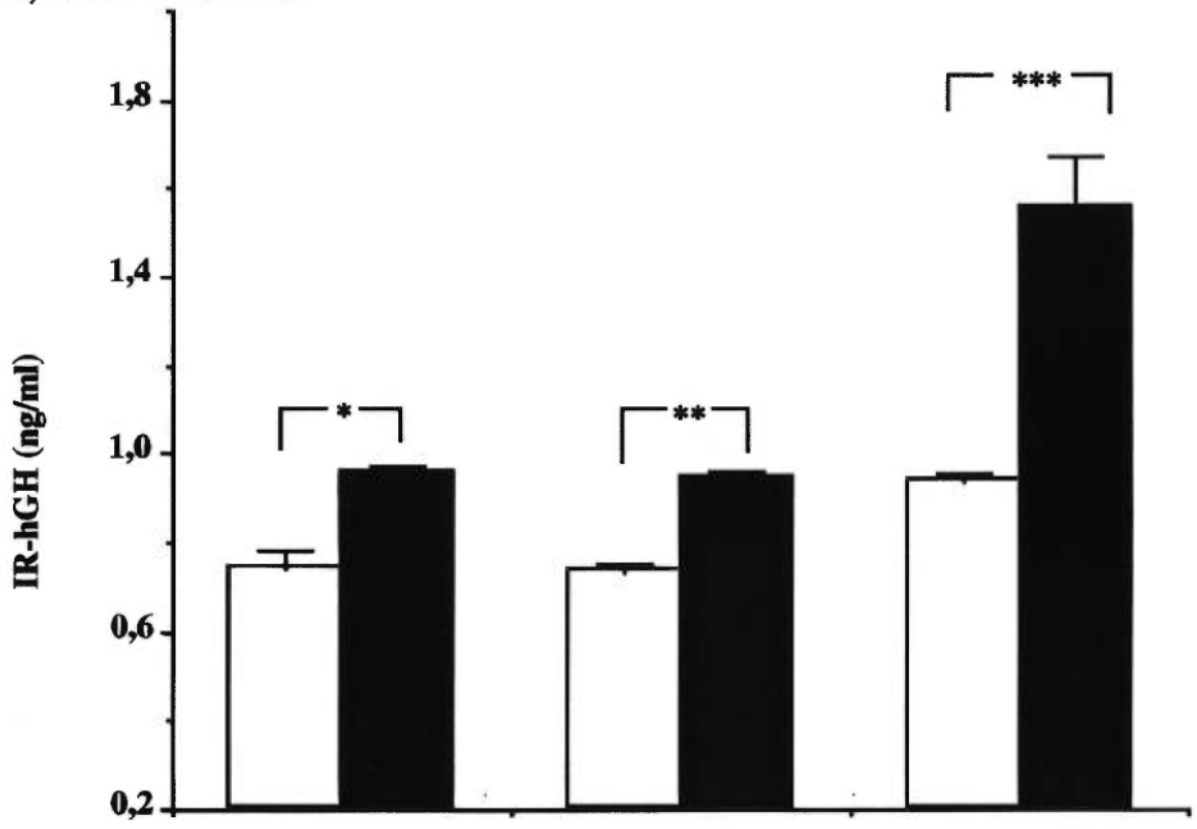


OK 27 cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. Then, 5 µg of pRSV/CREB or pRC/RSV per well were transiently transfected into the cells. After an overnight incubation, the media were replaced with media containing 1% dFBS and various concentratins (10^{-13} to 10^{-5} M) of isoproterenol and incubated for 24 hours. Media were collected and kept at -20°C until assayed. The effect of isoprterenol is compared with the control cells (transfected with 5 µg of pRSV/CREB but without the addition of isoproterenol). Each point represents the mean±SD of at least three determinations. Similar results were obtained in four independent experiments. The DNA transfection efficiency for this experiment was approximately 25% compared with pRSVCAT (*p≤0.05, **p≤0.01 and ***p≤0.005).

The expression of pRSV/CREB stimulated the ANG fusion gene expression. Various concentrations of isoproterenol further stimulated the expression of pOGH (ANG N-1498/+18) in a dose-dependent manner. These studies suggested that the stimulatory effect of isoproterenol is mediated via PKA-CREB pathway.

(Qian, 1997)

FIG.30. EFFECT OF pRSV/CREB ON THE EXPRESSION OF pOGH (ANG N-1498/+18) IN OK 27 CELLS

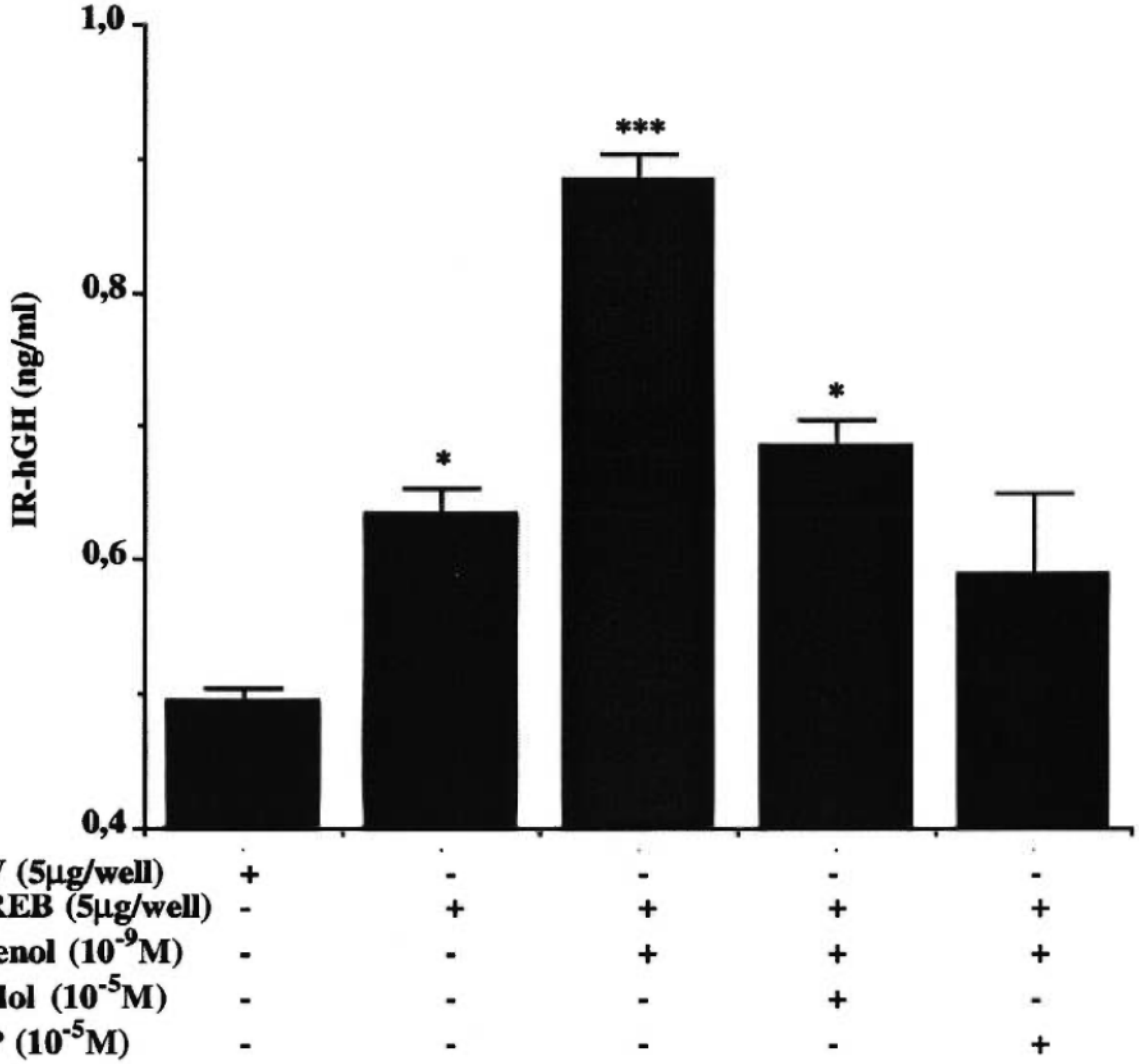


OK 27 cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. Then, 5 µg of pRSV/CREB or pGEM-3 per well were transiently transfected into the cells. After an overnight incubation, the media were replaced with media containing 1% dFBS with or without isoproterenol (10^{-9} M) and incubated for 24 hours. Media were collected and kept at -20°C until assayed. The effect of isoproterenol is compared with the control cells (transfected with 5 µg of pGEM-3 or 5 µg of pRSV/CREB but without the addition of isoproterenol). Each point represents the mean±SD of at least three determinations. Similar results were obtained in four independent experiments. The DNA transfection efficiency for this experiment was approximately 30% compared with pRSVCAT (*p≤0.05, **p≤0.01 and ***p≤0.005).

The addition of pRSV/CREB stimulated the basal expression of the fusion gene. The addition of isoproterenol further enhanced the effect of pRSV/CREB on the expression of pOGH (ANG N-1498/+18). There was no stimulatory effect observed when transfected with pGEM-3. These studies suggested that the effect of CREB on the regulation of the expression of the ANG gene is specific.

(Qian, 1997)

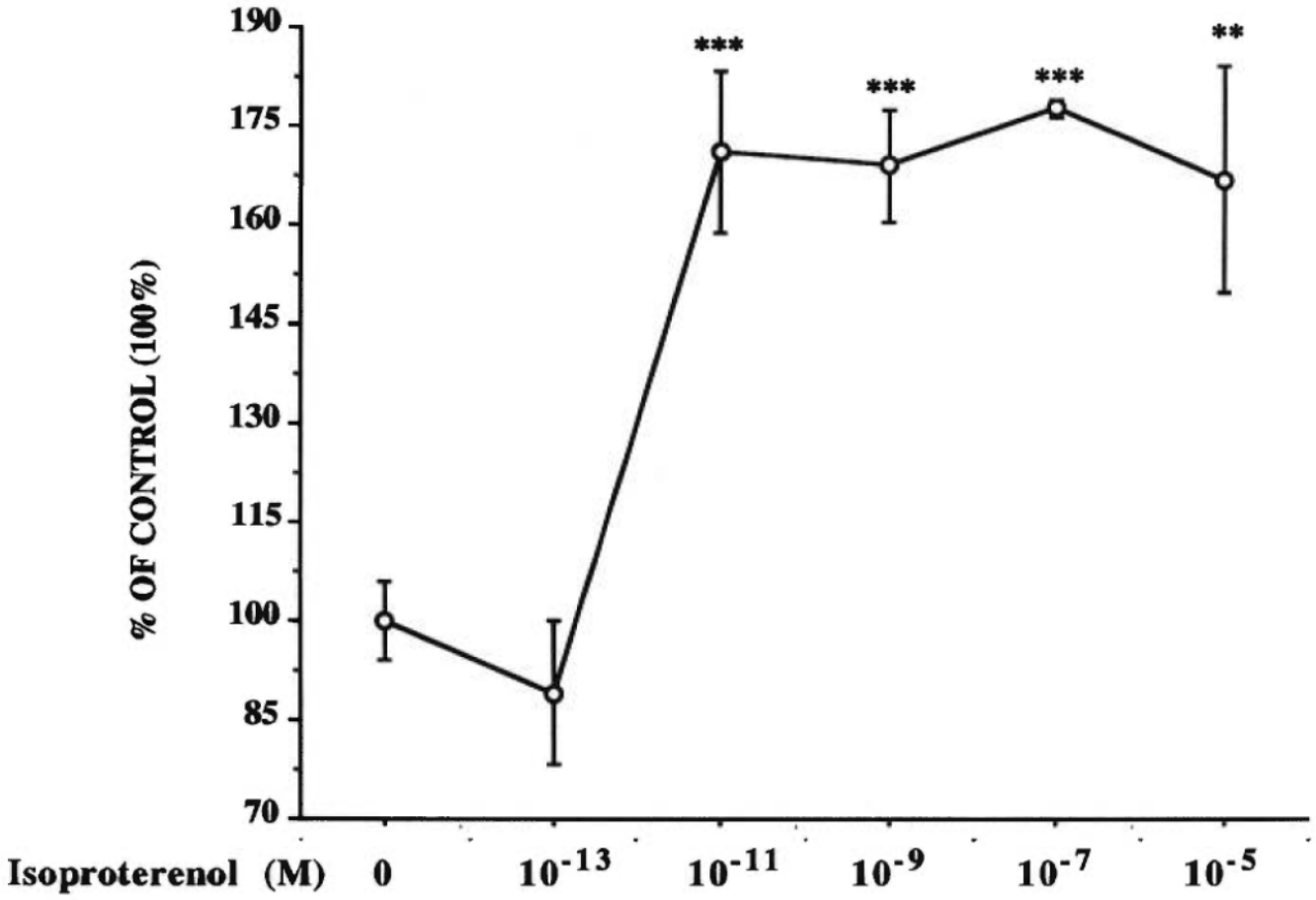
FIG.31. EFFECT OF PROPRANOLOL OR Rp-cAMP ON THE EXPRESSION OF pOGH (ANG N-1498/+18) IN OK 27 CELLS TRANSFECTED WITH pRSV/CREB AND STIMULATED BY ISOPROTERENOL



OK 27 cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. Then, 5 µg of pRSV/CREB or pRC/RSV per well were transiently transfected into the cells. After an overnight incubation, the media were replaced with media containing 1% dFBS in the presence of isoproterenol (10^{-9} M) with or without propranolol (10^{-5} M) or Rp-cAMP (10^{-5} M) and incubated for 24 hours. Media were collected and kept at -20°C until assayed. The concentration of IR-hGH in medium of cells transfected with 5 µg of pRC/RSV is considered as the control levels. Each point represents the mean±SD of at least three determinations. Similar results were obtained in four independent experiments. The DNA transfection efficiency for this experiment was approximately 55% compared with pRSVCAT (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$).

The addition of propranolol (10^{-5} M) or Rp-cAMP (10^{-5} M) inhibited the stimulatory effect of isoproterenol (10^{-9} M) in the presence of pRSV/CREB (5 µg/well) on the expression of pOGH (ANG N-1498/+18). These studies suggested that the stimulatory effect of isoproterenol is mediated via ß-AR and PKA-CREB pathway.
(Qian, 1997)

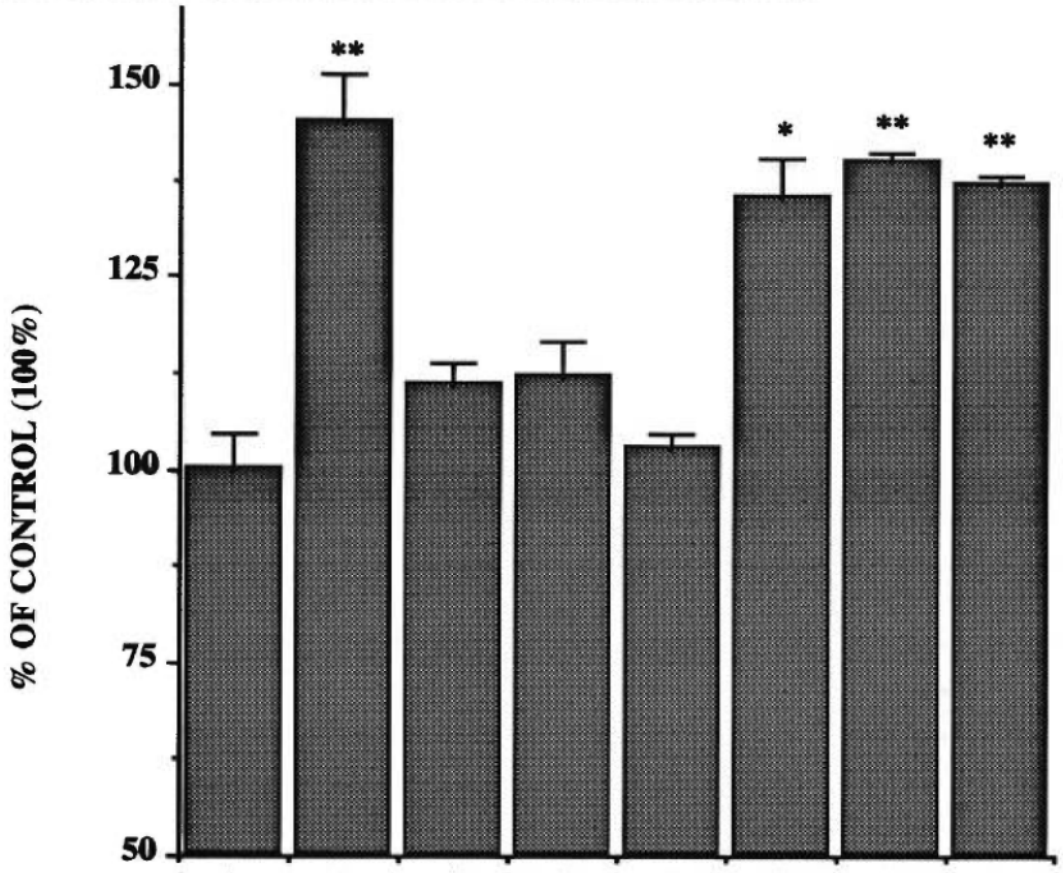
FIG.32. EFFECT OF ISOPROTERENOL ON THE EXPRESSION OF pOGH (ANG/CRE/-53/+18)/pRSV/CREB IN OK 96 CELLS



OK 96 cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, various concentrations of isoproterenol (10^{-13} to10^{-5} M) were added to the culture medium containing 1% dFBS and incubated for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). The concentration of IR-hGH in the absence of isoproterenol is considered as the control level (100%). Each point represents the mean±SD of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). The experiment has been done 4 times in triplicates.

A dose-dependent relationship was observed at 10^{-11} to 10^{-5} M of isoproterenol. The expression of pOGH (ANG/CER/-53/+18)+pRSV/CREB in OK 96 cells was stimulated by 10^{-13} to 10^{-5} M isoproterenol. These studies suggested that the stimulatory effect of isoproterenol is mediated via the interaction of the ANG-CRE and CREB.

FIG.33. EFFECT OF ADRENERGIC RECEPTOR ANTAGONISTS OR Rp-cAMP OR STAUROSPORINE OR U73122 ON THE EXPRESSION OF pOGH (ANG/CRE/-53/+18) /pRSV/CREB IN OK 96 CELLS STIMULATED BY ISOPROTERENOL

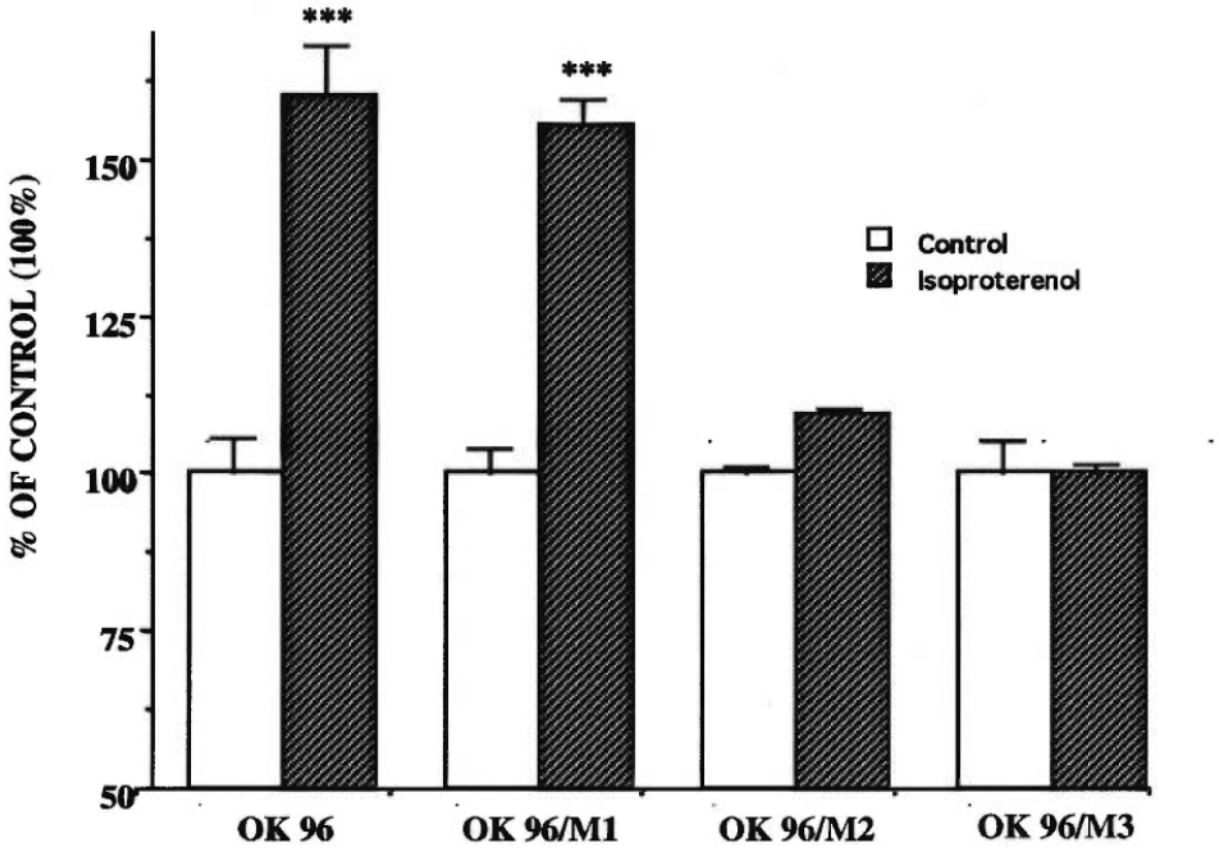


Isoproterenol(10^{-9} M)	-	+	+	+	+	+	+	+
Propranolol (10^{-7} M)	-	-	+	-	-	-	-	-
Atenolol (10^{-7} M)	-	-	-	+	-	-	-	-
Rp-cAMP (10^{-7} M)	-	-	-	-	+	-	-	-
ICI 118,511 (10^{-7} M)	-	-	-	-	-	+	-	-
Staurosporine(10^{-7} M)	-	-	-	-	-	-	+	-
U73122 (10^{-7} M)	-	-	-	-	-	-	-	+

OK 96 cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, various atagonists or inhibitors (10^{-7} M) was added to the culture medium containing 1% dFBS and co-incubated with isoproterenol (10^{-9} M) for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). The concentration of IR-hGH in the absence of isoproterenol or antagonists or inhibitors is considered as the control level (100%). Each point represents the mean±SD of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). The experiment has been done 7 times in triplicates.

The addition of atenolol (10^{-7} M), propranolol (10^{-7} M), or Rp-cAMP (10^{-7} M), but not ICI 118,511 (10^{-7} M), staurosporine (10^{-7} M) or U73122 (10^{-7} M) inhibited the stimulatory effect of isoproterenol (10^{-9} M) on the expression of pOGH (ANG/CRE/-53/+18) in OK 96 cells. These studies suggested that the effect of isoproterenol is mediated via β_1-AR and PKA pathway.

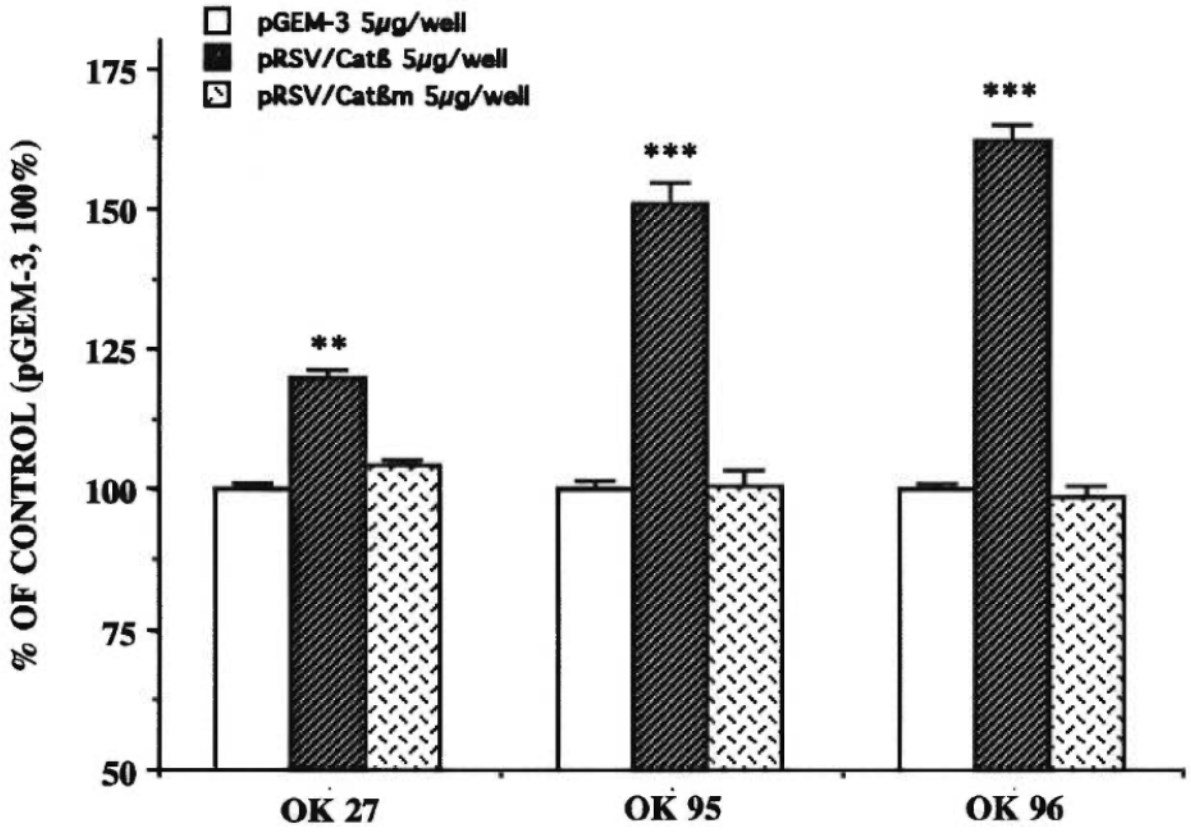
FIG.34. EFFECT OF ISOPROTERENOL ON THE EXPRESSION OF pOGH (ANG/CRE/-53/+18)/pRSV/CREB OR DIFFERENT MUTANTS IN OK CELLS



OK 96 or OK 96/mutant cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, isoproterenol (10^{-9} M) was added to the culture medium containing 1% dFBS and incubated for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). The concentration of IR-hGH in the absence of isoproterenol is considered as the control level (100%). Each point represents the mean±SD of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). The experiment has been done 5 times in triplicates.

The addition of isoproterenol (10^{-9} M) stimulated the expression of pOGH (ANG/CRE/-53/+18)/ pRSV/CREB or pOGH (ANG/CREM1/-53/+18)/ pRSV/CREB, respectively, whereas isoproterenol had no effect on OK 96/M2 or OK 96/M3. These studies suggested that the stimulatory effect of isoproterenol is mediated via the ANG-CRE sequence of the 5'-flanking region of the rat ANG gene.

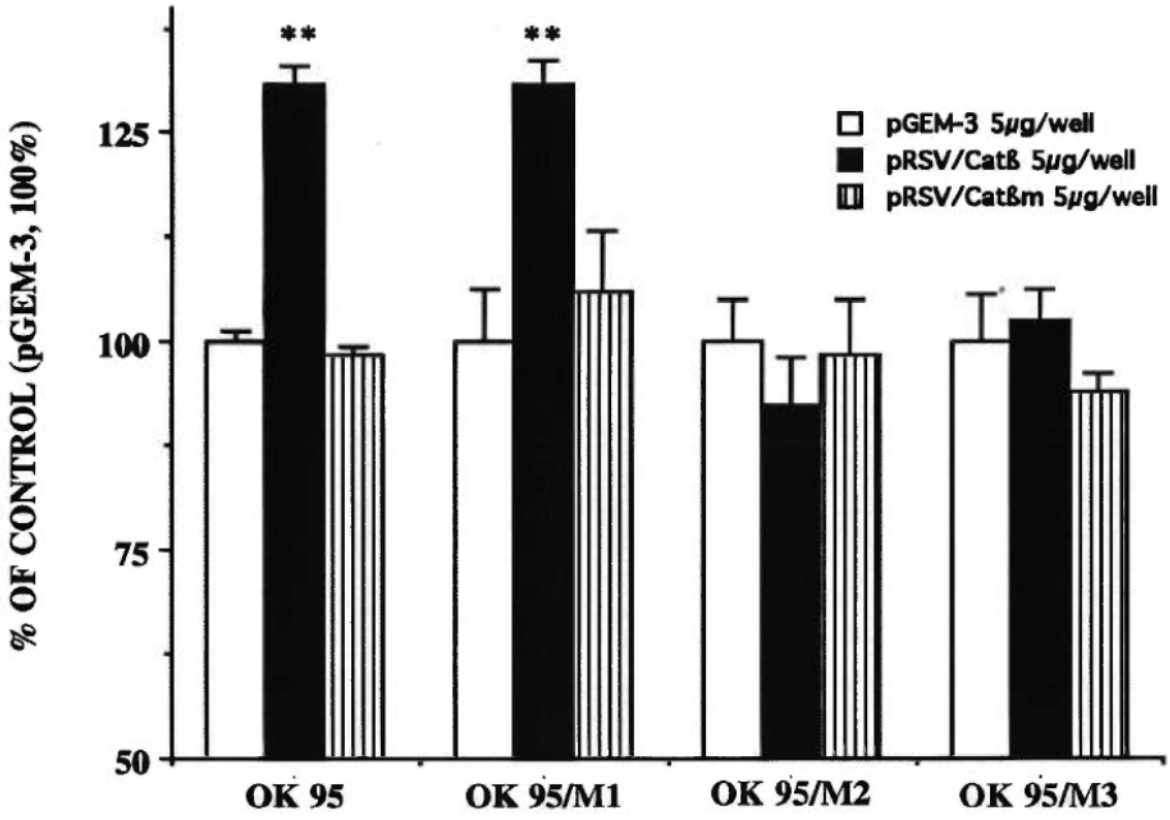
FIG.35. EFFECT OF PKA CATALYTIC SUBUNIT ON THE EXPRESSION OF RAT ANG FUSION GENES IN OK CELLS



OK 27, OK 95 or OK 96 cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. Then, 5 μg of pGEM-3 or pRSV/Catß or pRSV/Catßm per well were transiently transfected into the cells. After an overnight incubation, the media were replaced with media containing 10% dFBS and incubated for 24 hours. Media were collected and kept at -20°C until assayed. The effect of PKA catalytic subuint (pRSV/Catß) or the mutant of PKA catalytic subuint (pRSV/Catßm) is compared with the control cells (transfected with 5 μg of pGEM-3) (100%). Each point represents the mean±SD of at least three determinations. Similar results were obtained in four independent experiments. The DNA transfection efficiency for this experiment was approximately 30% compared with pRSVCAT (*p≤0.05, **p≤0.01 and ***p≤0.005).

In the presence of the PKA catalytic subunit, the expression of different ANG fusion genes pOGH (A N-1498/+18), pOGH (ANG/CRE/-53/+18) or pOGH (ANG/CRE/-53/+18)/pRSV/CREB in OK 27, OK 95 or OK 96 cells were stimulated, respectively. The maximal effect was found in OK 96 cells. These studies suggested that PKA is involved on the regulation of the expression of the ANG gene is specific.

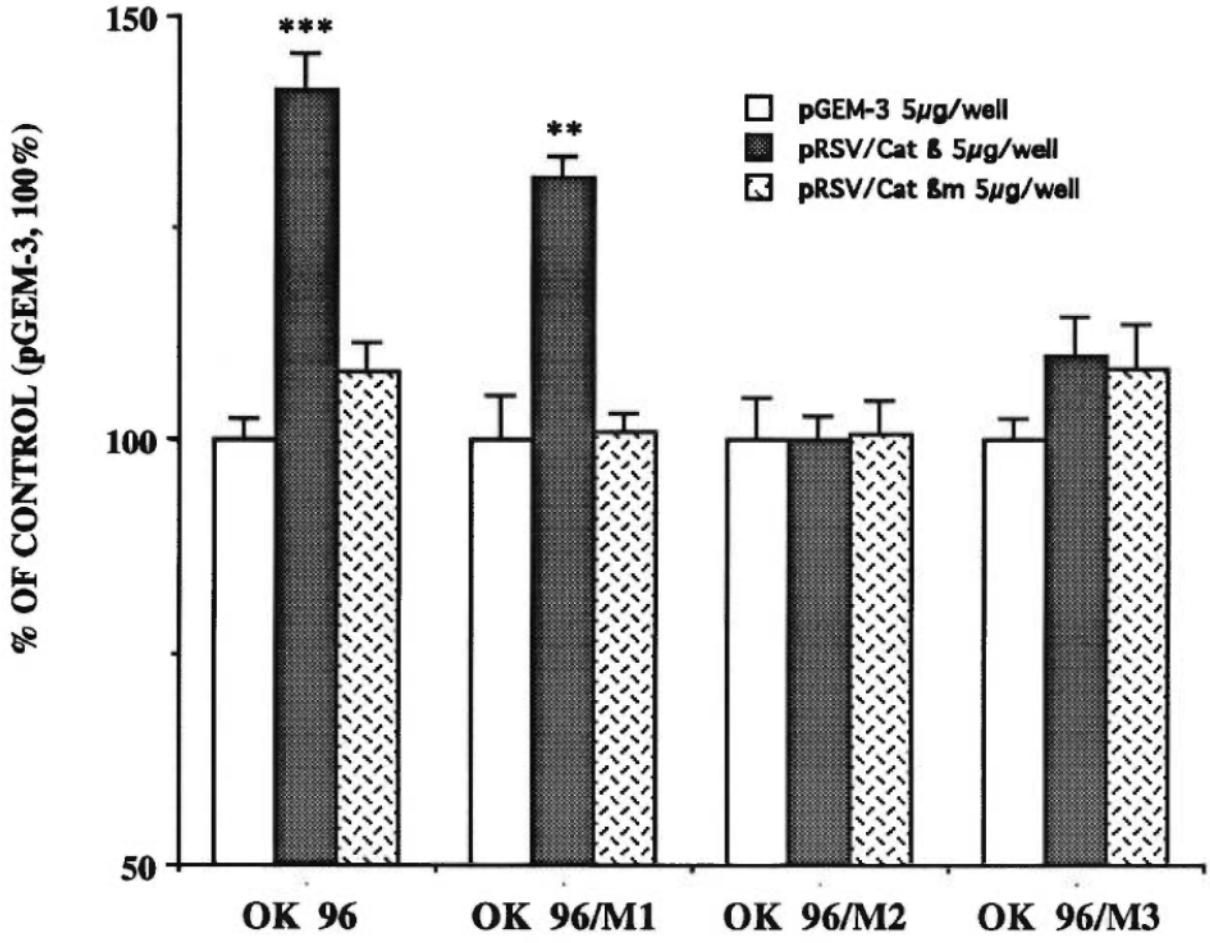
FIG.36. EFFECT OF PKA CATALYTIC SUBUNIT ON THE EXPRESSION OF pOGH (ANG/CRE/-53/+18) OR DIFFERENT MUTANTS IN OK CELLS



OK 95 or OK 95/mutant cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. Then, 5 µg of pGEM-3 or pRSV/Catß or pRSV/Catßm per well were transiently transfected into the cells. After an overnight incubation, the media were replaced with media containing 10% dFBS and incubated for 24 hours. Media were collected and kept at -20°C until assayed. The effect of PKA catalytic subuint (pRSV/Catß) or the mutant of PKA catalytic subuint (pRSV/Catßm) is compared with the control cells (transfected with 5 µg of pGEM-3) (100%). Each point represents the mean±SD of at least three determinations. Similar results were obtained in 3 independent experiments. The DNA transfection efficiency for this experiment was approximately 35% compared with pRSVCAT (*p≤0.05, **p≤0.01 and ***p≤0.005).

In the presence of the PKA catalytic subunit, the expression of ANG-CRE or ANG-CREM1 fusion genes in OK 95 or OK 95M1 cells were stimulated, respectively. These studies suggested that the effect of PKA is mediated via the ANG-CRE on the 5'-flanking sequence of the rat ANG gene.

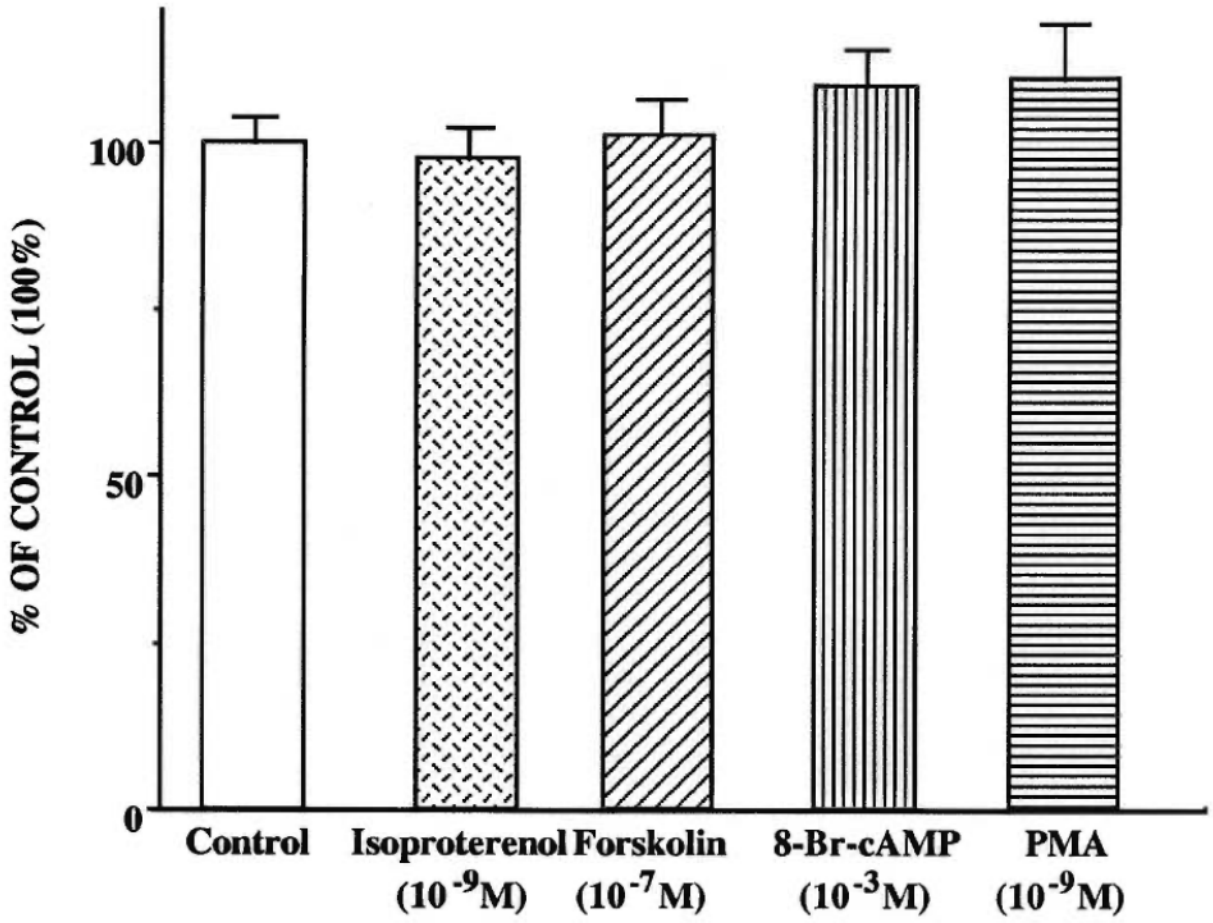
FIG.37. EFFECT OF PKA CATALYTIC SUBUNIT ON THE EXPRESSION OF pOGH (ANG/CRE/-53/+18)+pRSV/CREB OR DIFFERENT MUTANTS+pRSV/CREB IN OK CELLS



OK 96 or OK 96/mutant cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. Then, 5 μg of pGEM-3 or pRSV/Catß or pRSV/Catßm per well were transiently transfected into the cells. After an overnight incubation, the media were replaced with media containing 10% dFBS and incubated for 24 hours. Media were collected and kept at -20°C until assayed. The effect of PKA catalytic subuint (pRSV/Catß) or the mutant of PKA catalytic subuint (pRSV/Catßm) is compared with the control cells (transfected with 5 μg of pGEM-3) (100%). Each point represents the mean±SD of at least three determinations. Similar results were obtained in 3 independent experiments. The DNA transfection efficiency for this experiment was approximately 25% compared with pRSVCAT (*p≤0.05, **p≤0.01 and ***p≤0.005).

In the presence of the PKA catalytic subunit, the expression of pOGH (ANG/CRE/-53/+18) or pOGH (ANG/CREM1/-53/+18) in OK 96 or OK 96M1 cells were stimulated, respectively. These studies suggested that the effect of PKA and CREB is mediated via the ANG-CRE on the 5'-flanking sequence of the rat ANG gene.

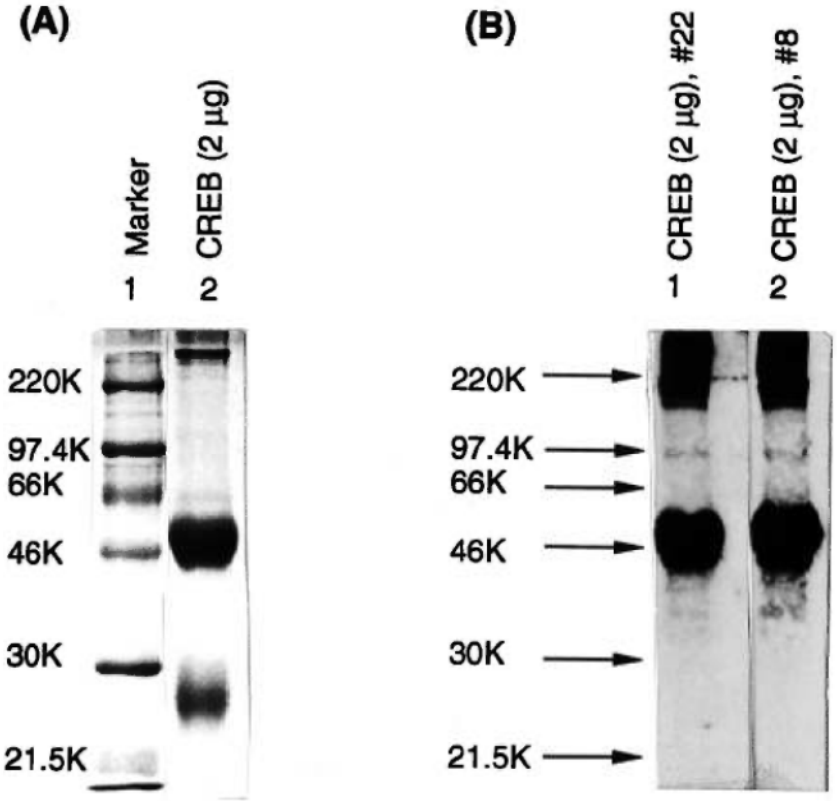
FIG.38. EFFECT OF ISOPROTERENOL, FORSKOLIN, 8-Br-cAMP OR PMA ON THE EXPRESSION OF pOGH (ANG N-53/+18) IN OK 53 CELLS



OK 53 cells were plated at a density of 2 X 10^5 cells/well in six-well plates for up to 24 hours and incubated overnight in DMEM containing 10% FBS. The growth of the cells was arrested by the incubation in serum-free DMEM for 24 hours. Subsequently, isoproterenol (10^{-9} M) or forskolin (10^{-7} M) or 8-Br-cAMP (10^{-3} M) or PMA (10^{-9} M) was added to the culture medium containing 1% dFBS and incubated for 24 hours. Media were harvested after the incubation and assayed for immunoreactive human growth hormone (IR-hGH). The concentration of IR-hGH in the absence of isoproterenol or forskolin or 8-Br-cAMP or PMA is considered as the control level. Each point represents the mean±SD of at least three dishes (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$). The experiment has been done 3 times.

Neither isoproterenol, forskolin, 8-Br-cAMP or PMA could stimulate the expression of the pOGH (ANG N-53/+18) in OK 53 cells compared to the the control (absence of isoproterenol, forskolin, 8-Br-cAMP or PMA). These studies suggested that the stimulatory effect of ß-ARs or PKA or PKC on the expression of pOGH (ANG/CRE/-53/+18) in OK 95 or OK 96 cells is gene-specific and is mediated via the ANG-CRE sequence of the rat ANG gene.

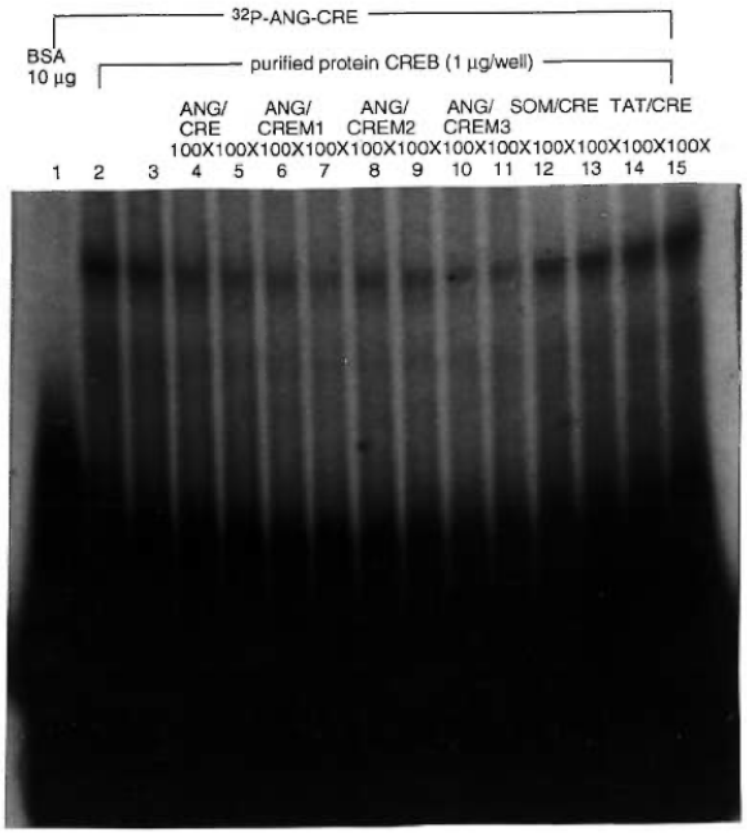
FIG.39. SDS-PAGE AND WESTERN BLOT ANALYSIS OF PURIFIED RECOMBINANT 43 kDa-CREB



The COS-7 cells with the pRSV/CREB stably integrated into the genome were grown confluent in 20 plates (25 X 150 mm). The cells were harvested by trypsin-EDTA. The cells were frozen and thawed and sonicated in hypotonic buffer (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT and 10 µg of trypsin inhibitor). The recombinant CREB was purified by passing twice sequentially through the IgG-affinity columns.

The 43 kDa-CREB was purified by two consecutive IgG-affinity columns. It is evident that at least two major molecule species with apparent molecular weights of approximately 43-46 kDa and 25-27 kDa (A) were observed by 10% polyacrylamide gel electrophoresis containing sodium dodecyl sulfate (SDS-PAGE). Western blot analysis (B), however, shows that the rabbit polyclonal antiserum (Rb #8) against the amino acid residues 135-150 of the 43 kDa-CREB or rabbit polyclonal antiserum (Rb #22) against the recombinant 43 kDa-CREB interacted with the purified 43-46 kDa CREB but did not interact with the 25-27 kDa species. An additional immunoreative band with an apparant molecular weight of 220 kDa was also observed in (B). These studies indicated that the rabbit polyclonal antibodies (#8, #22) interact with the rat 43 kDa-CREB.

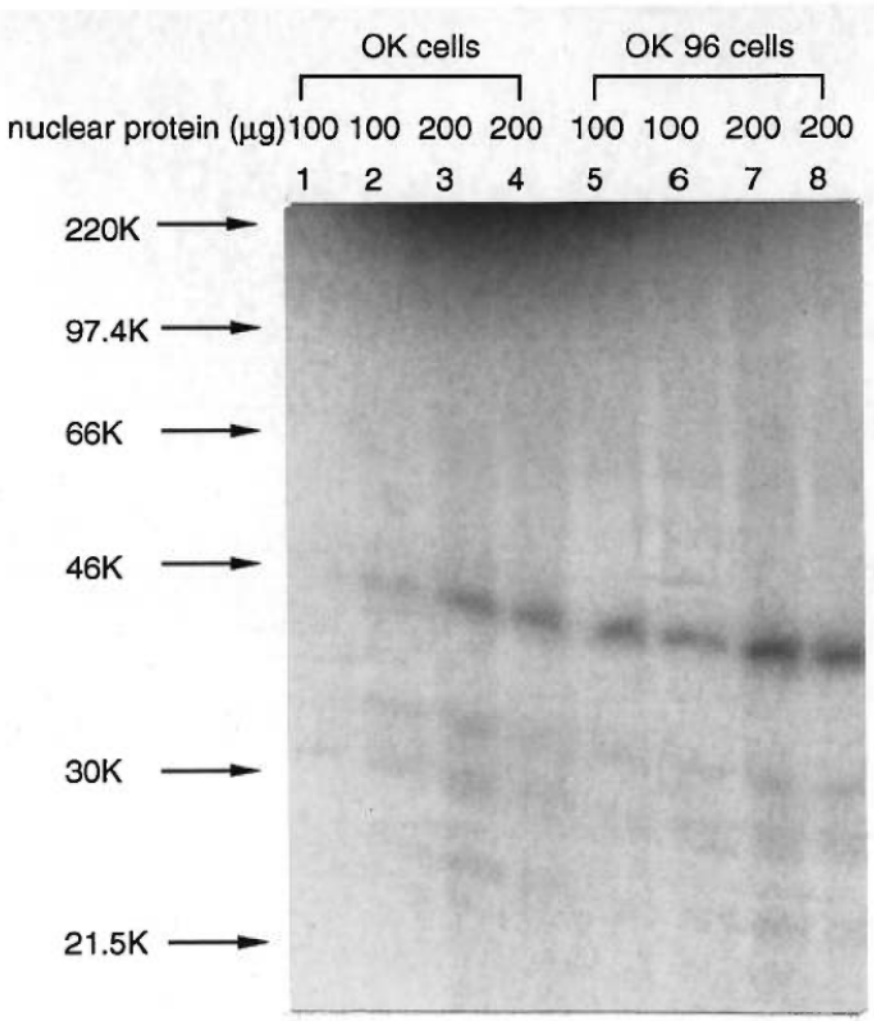
FIG.40. GEL MOBILITY SHIFT ASSAY OF ^{32}P-ANG-CRE WITH THE PURIFIED CREB PROTEIN



The DNA fragments, ANG-CRE (ANG N-806 to N-779) was 5'-end labeled with γ-^{32}P-dATP using T_4 polynucleotide kinase. A 100-fold excess of unlabeled DNA fragments (ANG-CRE, lanes 4,5; ANG-CREM1, lanes 6,7; ANG-CREM2, lanes 8,9; ANG-CREM3, lanes 10,11; SOM-CRE, lanes 12,13; TAT-CRE, lanes 14,15) and the purified recombinant 43 kDa-CREB (1.0 µg/lane, lanes 2 - 15) or bovine serum albumin (BSA, 10 µg, lane 1) in the presence of 0.5 units of poly (dI-dC) in 20 mM tris-glycine, pH 7.6 and 1 mM EDTA were incubated for 30 minutes at room temperature. Then the 5'-labeled probe (0.1 pmole) was added and further incubated for 30 minutes at room temperature. After chilling on ice, the mixture was run on a 8% non-denaturing polyacrylamide gel and exposed for autoradiography.

The purified recombinant CREB bind to the labeled ANG-CRE (lanes 2,3). The mutants of ANG-CRE (i.e. M1, M2, M3 on lanes 6 - 11) were less effective in displacing the binding of the labeled ANG-CRE with the purified recombinant CREB as compared to the unlabeled ANG-CRE (lanes 4,5). The SOM-CRE and the TAT-CRE were more effective than the ANG-CRE in interacting with the 43 kDa-CREB. These studies indicated that the binding of CREB with the ANG-CRE is weaker than the binding of CREB with the SOM-CRE and with the TAT-CRE and that the intact CRE-motif is important for the binding with the CREB.

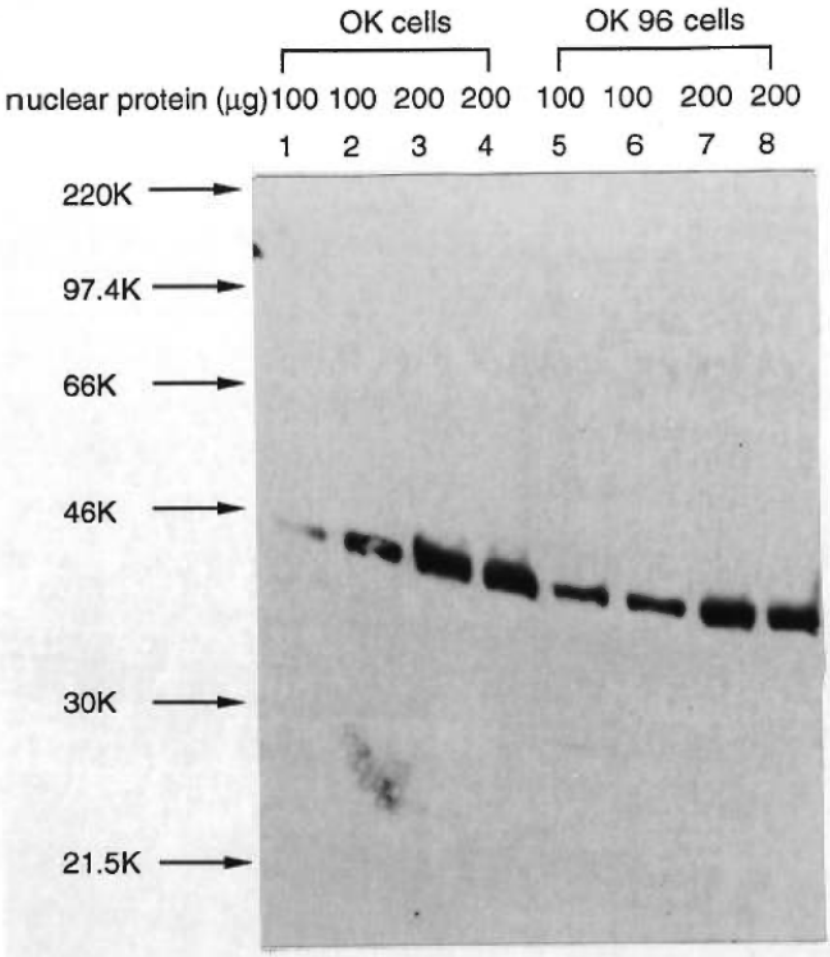
FIG.41. SOUTHWESTERN BLOT OF 43 kDa-CREB FROM THE OK AND OK 96 CELLULAR NUCLEAR PROTEIN(S)



OK (lanes 1 - 4) and OK 96 (lanes 5 - 8) cell nuclear proteins (100-200 µg) were resolved on a 8 to 15% gradient polyacrylamide gel containing sodium dedocyl sulfate (PAGE-SDS) and then electrotransferred onto a nitrocellulose membrane (0.45 µM). The membrane was incubated with 10% non-fat milk proteins in a binding buffer containing 10 mM Hepes, pH 7.0, 10 mM $MgCl_2$, 50 mM NaCl, 0.25 mM EDTA and 2.5% glycerol for one hour at 4°C. The membrane was then washed at least twice with the binding buffer containing 0.25% non-fat milk proteins. Subsequently, the membrane was hybridized with γ-^{32}P-dATP labeled ANG-CRE (5'-end) (approximately 1.0 to 2.0 pmole, 10^6 cpm/ml) in binding buffer containing 0.25% non-fat milk proteins and 300 mg/ml of non-denatured herring sperm DNA at 4°C for overnight. The membrane was washed with the binding buffer, air-dried and exposed for autoradiography.

Both OK or OK 96 nuclear proteins interact with ANG-CRE, the binding is at ~43 kDa. The interaction is dose dependent. OK 96 nuclear proteins have a stronger interaction with ANG-CRE (lanes 5 - 8) than OK cell nuclear proteins (lanes 1 - 4) with the same amount of proteins. These studies indicated that the binding of OK and OK 96 cell nuclear proteins to ANG-CRE is at 43 kDa and OK 96 cells probably contain more of 43 kDa protein.

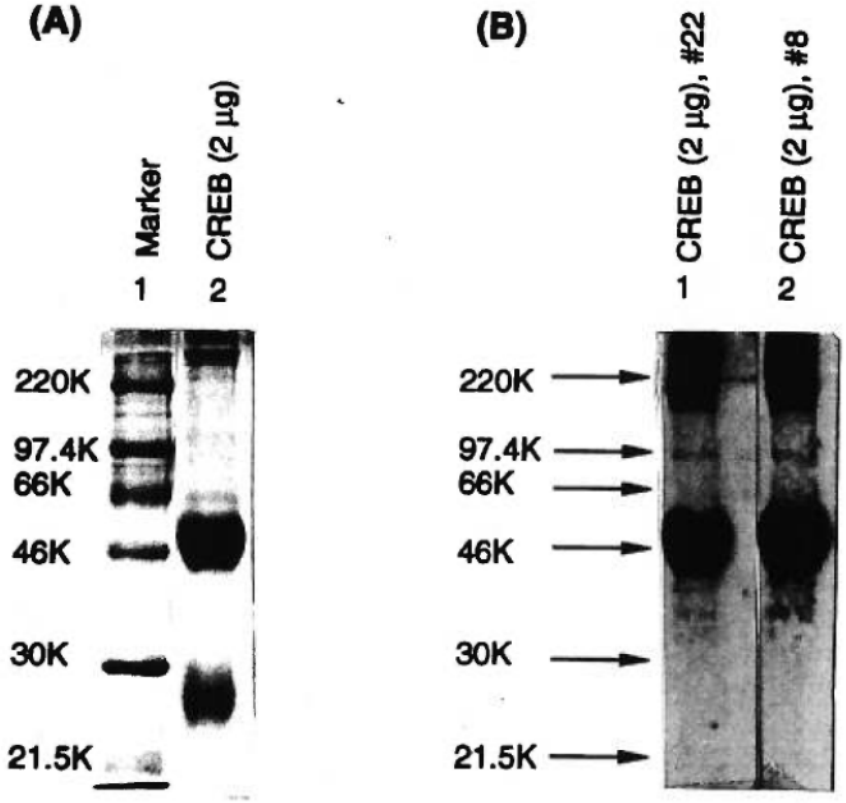
FIG.42. WESTERN BLOT ANALYSIS OF 43 kDa-CREB FROM THE OK AND OK 96 CELLULAR NUCLEAR PROTEIN(S)



The filter from the South-Western blot was blocked with PBS/5% skim milk for 4 hours at 4°C and then replenished with the same solution containing rabbit polyclonal antiserum (Rb #8) against the amino acid residues 135-150 of the 43 kDa-CREB and incubated at 4°C with shaking for more than 2 hours. After being washed 3X with 1X PBS, the filter was soaked with TBS/5% skim milk containing the horseradish peroxidase conjugated goat anti-rabbit and incubated at 4°C with shaking for 1 hour. Finally, the filter was washed 3X with TBS, and developed with the fresh mixture of 30 mg 4-chloronaphthol in 10 ml methanol and 30 µl 30% H_2O_2 in 40 ml TBS.

Both OK and OK 96 nuclear protein interact with anti-CREB antibody (Rb. #8) at ~43 kDa (lanes 1 - 8). The interaction is dose dependent. These studies indicated that both OK and OK 96 cells contain the 43 kDa-CREB protein.

FIG.39. SDS-PAGE AND WESTERN BLOT ANALYSIS OF PURIFIED RECOMBINANT 43 kDa-CREB



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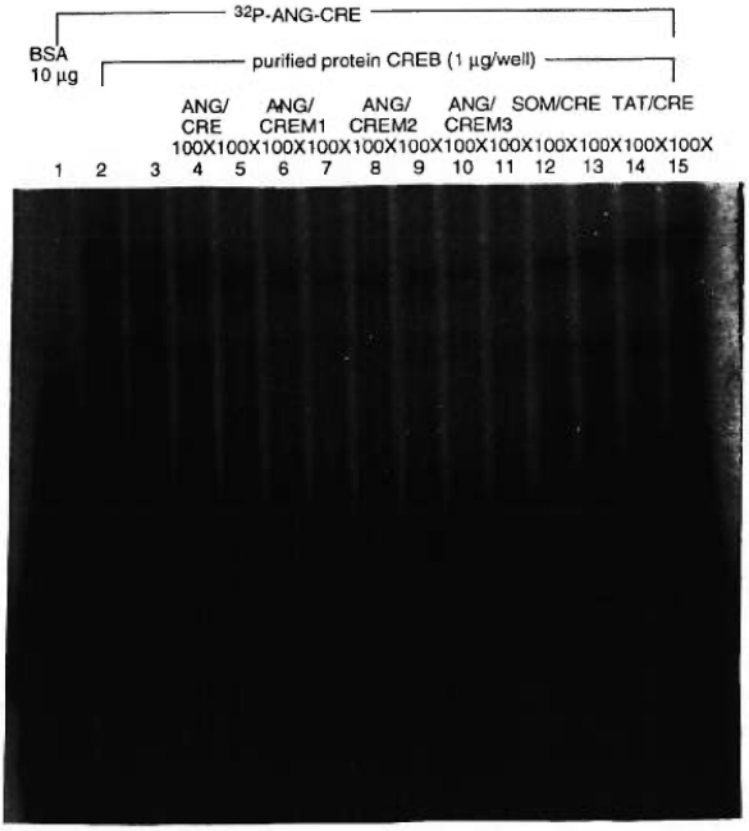

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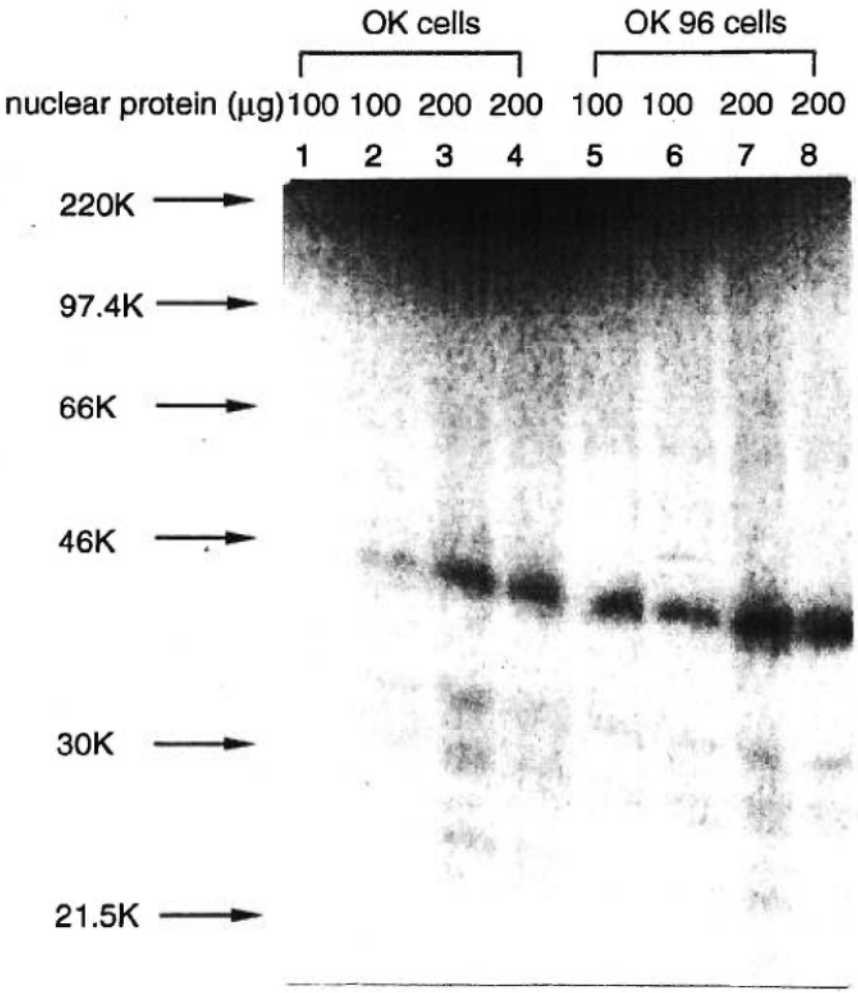

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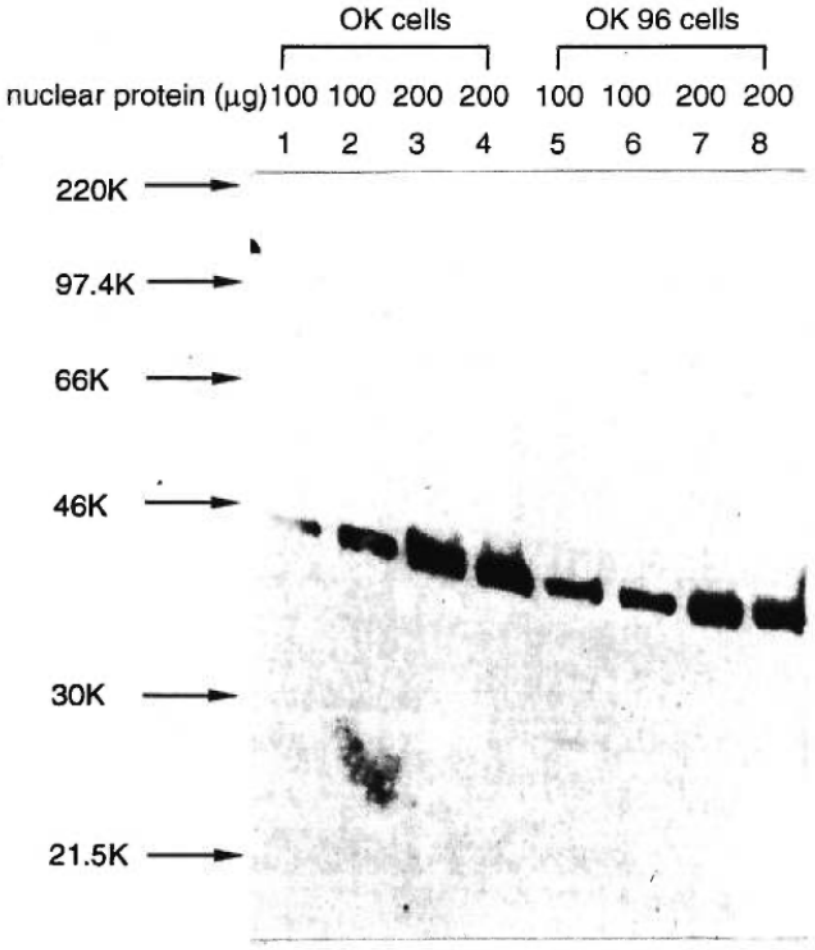

The filter from the South-Western blot was blocked with PBS/5% skim milk for 4 hours at 4°C and then replenished with the same solution containing rabbit polyclonal antiserum (Rb #8) against the amino acid residues 135-150 of the 43 kDa-CREB and incubated at 4°C with shaking for more than 2 hours. After being washed 3X with 1X PBS, the filter was soaked with TBS/5% skim milk containing the horseradish peroxidase conjugated goat anti-rabbit and incubated at 4°C with shaking for 1 hour. Finally, the filter was washed 3X with TBS, and developed with the fresh mixture of 30 mg 4-chloronaphthol in 10 ml methanol and 30 µl 30% H_2O_2 in 40 ml TBS.

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DISCUSSION

I. EFFECT OF α-ARS AND DOPAMINERGIC RECEPTORS ON ANG GENE EXPRESSION IN OK CELLS

I.1. α-Adrenergic Receptors in the Proximal Tubular Cells

I.1.1. Distribution

α-ARs mediate sympathetically controlled renal vascular resistance and may contribute to vasoconstrictor hyper-responsiveness. In the rat kidney, α_2-ARs predominate numerically in a ratio of approximately 2:1 (α_2:α_1) (Drew, 1979). This ratio is 2.9 in SHR rats whereas both renal α_1 and α_2-AR density is higher in SHR than in WKY rats at all ages (Sanchez, 1986). α_1-ARs have been shown to mediate adrenergic-induced renovasoconstriction (Schmitz, 1981). These evidence may suggest an important developmental role of α receptors in hypertension.

α_2-ARs, known to be coupled in an inhibitory fashion to renal AC, are largely restricted to the renal cortex where they are believed to be closely associated with the proximal tubular segment of the nephron (Stephenson, 1985; Young, 1980) in rats.

The opossum proximal tubular (OK) cell is a continuous cell line derived from the kidney of an American opossum. The cells retain several properties of proximal tubular epithelia as mentioned in [Materials]. Morphologically, OK cells appear as polarized epithelial cells with apical microvilli, tight junctions and develop discreet sites of solute and water accumulation on the basolateral growth surface (Murphy, 1988). It appears that the α_2-ARs in OK cells is a unique subtype than other α_2-ARs. Murphy's group first identified the existence of α_2-AR which is negatively coupled to the production of cAMP in OK cells. The pharmacological profile fits the characteristics of the α_{2B} subtype more closely than the α_{2A} subtype. They tentatively termed it α_{2C}-AR. With binding studies, Gleason and Hieble (1992) showed that a human retinoblastoma cell line (Y79) also expresses the alpha 2C-adrenoceptor subtype. In

1997, Schaak's group, using radioligand binding studies and RT-PCR demonstrated that the hepatoma cell line HepG2 and the neuroblastoma cell line SK-N-MC possess α_2-AR of the α2C subtype, and the receptor was coupled to pertussis toxin-sensitive G-proteins (G_i). The α_2-AR agonist was able to decrease forskolin-stimulated cAMP production.

Our studies showed that α_2-AR, but not α_1-AR has a regulatory role on the ANG gene expression (Fig. 1), which further confirmed the presence of α_2-AR in OK cells.

I.1.2. Effects of α_2-AR on ANG Gene Expression in OK Cells

We proposed that stimualtion of α_1- or α_2-ARs might have effects on the expression of ANG gene. Our studies showed that the α_2-AR agonist, iodoclonidine, stimulates the expression of the pOGH (ANG N-1498/+18) in OK 27 cells (Fig. 1), and this effect can be blocked by α_2-AR antagonist, yohimbine (Fig. 2). These results indicated that α_2-AR has a positive effect on the ANG gene expression.

We did not observe any significant stimulation of the pOGH (ANG N-1498/+18) by phenylephrine (α_1-AR agonist) in OK 27 cells (Fig. 1). Prazosin (α_1-AR antagonist) at concentrations as high as 10^{-5} M does not inhibit the stimulatory effect of iodoclonidine (Fig. 2).This might be explained by the fact that there is a low or undetectable amount of α_1-AR in OK cells, as demonstrated by the binding studies of Murphy (1988).

These results support the previous studies by other groups that OK cells express α_2-AR but not α_1-AR. These results also indicate that the activation of renal nerves may stimulate the expression of the renal ANG gene via α_2-ARs *in vivo*.

I.1.3. Desensitization or Downregulation

Adrenergic receptors are subject to desensitization (Kurose, 1994). Studies on the mechanisms of α_1-AR vascular desensitization (Kiuchi, 1992) revealed that NE (0.1 μg/kg/min in dog) or chronic α_1-AR stimulation induced decrease to pressor and vasoconstrictor responses. Furthermore, α_1-AR density from vessels, as determined by binding studies was decreased. The α_{2A}-AR undergoes rapid agonst-promoted desensitization which is due to phosphorylation of the receptor. One kinase that has been shown to phosphorylate α_{2A}-AR is the βARK, which is a member of the family of G protein-coupled receptor kinases (Jewell-Motz, 1996). However, long term agonist-induced desensitization of α_{2A}-AR was found to be due in part to a decrease in the amount of cellular G_i, but not by a decrease in receptor expression (Liggett, 1992).

Our studies showed that higher concentrations of iodoclonidine (Fig.1) (greater than 10^{-9} M) have lower stimulatory effects on the expression of the pOGH (ANG N-1498/+18) in OK cells. One possible explanation may be that the exposure of OK cells to high levels of α_2-AR agonists may desensitize their own adrenoceptors.

Another explanation could be that prolong incubation with the agonist may induce downregulation of the receptors. Barturen's group (1992) observed that sustained stimulation of α_{2A}-AR by endogenous NE would increase the α_{2A}-AR desappearance rate (degradation), leading to a reduction in receptor number. Jones' group (1990) also observed that preincubation with an α_2-AR agonist resulted in a downregulation of the agonist's inhibition of parathyroid hormone-stimulated cAMP production in OK cells.

However, we have not investigated whether the mechanisms of the minimal effect of the α_{2A}-AR agonist is due to desensitization or downregulation. Further studies on receptor binding and phosphorylation are warranted.

I.2. Dopaminergic Receptors in the Proximal Tubular Cells

I.2.1. Distribution

Dopamine receptors of DA_1 and DA_2 subtypes are located at various regions within the kidney, which include the renal vasculature, the JG cells, and the renal tubules (Willems 1985; Lokhandwala, 1988). The DA_1 receptor in the kidney is associated with renal vasodilation and an increase in electrolyte excretion. The DA_1-related vasodilation and inhibition of electrolyte transport is mediated by cAMP. The role of renal DA_2 receptors remains to be clarified. Although DA_1 and DA_2 receptors may act in concert to decrease transport in the renal proximal convoluted tubule, the overall function of DA_2 receptors may be actually the opposite of those noted for DA_1 receptors (Jose, 1992).

In the kidney, DA_1 receptors are localized at both the luminal and basolateral membranes at the level of the proximal tubules. Activation of these DA_1 receptors by dopamine and by selective DA_1 receptor agonists results in natriuresis and diuresis. The cellular signaling mechanisms responsible for this response appear to be DA_1 receptor-induced activation of adenylate cyclase and PLC, which via the generation of various intracellular messenger systems cause inhibition of Na^+-H^+ antiport (luminal) and Na^+-K^+-ATPase (basolateral), respectively. Both of these events consequently inhibit sodium reabsorption leading to natriuresis and diuresis (Lokhandwala, 1991). Felder's group (1984) showed that the addition of dopamine increases AC via DA_1 receptors in the proximal convoluted tubule of the rabbit. Nash et al (1993) demonstrated that DA_1 receptor mRNA is expressed in OK cells.

In the dog, Jose et al (1986) demonstrated the existence of DA_2 receptors which is not linked to the AC system. Further studies with RT-PCR demonstrated the presence of the D_2-like receptor mRNA in the glomeruli, proximal tubules, outer medulla, inner medulla, and renal microvessels of the rat kidney (Gao, 1994).

Renal proximal tubular cells have been shown to express aromatic L-amino acid decarboxylase (L-AAAD), which converts L-dopa into dopamine and 5 hydroxytryptophan [(OH)Try] into 5-hydroxytryptamine (5-HT, serotonin). In OK cells, 5-HT synthesis and (OH)Try concentration increase with incubation time and is abolished by benserazide, an L-AAAD inhibitor, suggesting local formation of dopamine (Hafdi, 1996). Further studies showed that L-dopa stimulates cAMP generation and inhibits Na-dependent phosphate (Pi) uptake in OK cells. The effects of L-dopa can be mimicked by SKF 38393, a DA_1 receptor agonist, and are potentiated by S-sulpiride, a DA_2 receptor antagonist. These results support the involvement of DA_1 and DA_2 receptors, positively and negatively coupled into AC respectively, in modulation of renal phosphate transport (Perrichot, 1995).

I.2.2. Effects of Dopaminergic Recepters on ANG Gene Expression in OK Cells

We proposed that stimualtion of DA_1- or DA_2-dopaminergic recepters might have effects on the expression of ANG gene. Our studies showed that, in the presence of IBMX, dopamine stimulates the expression of pOGH (ANG N-1498/+18) in OK 27 cells at low concentrations (10^{-11} to 10^{-7} M) (Fig. 7). Addition of the D_1-dopaminergic receptor antagonist, SCH-23390, or D_2-dopaminergic receptor antagonist, spiperone, inhibit the stimulatory effect of dopamine (Fig. 8). These data suggested that the effect of dopamine is mediated via both D_1 and D_2 receptors in OK cells.

The addition of D_1-dopaminergic receptor agonist, SKF-82958, or D_2-dopaminergic receptor agonist, PPHT, also stimulate the expression of pOGH (ANG N-1498/+18) in OK 27 cells (Fig. 10), which further supports the presence of both D_1 and D_2 receptors in OK cells.

The D_2-receptor agonist, PPHT also has stimulatory effect on serotonergic receptors. However, when we added Ketanserine, which is the antagonist of serotonergic

receptors, it did not block the effect of PPHT (Fig. 13). These data indicated that the stimulatory effect of PPHT on ANG fusion gene expression is specific.

Our studies support the existence of functional DA_1 and DA_2 receptors in OK cells, however, both receptors have a positive effect on the regulation of the expression of the ANG gene.

I.2.3. Desensitization and Downregulation

Ng's group (1994) showed that exposure of cells expression D_1 receptor to dopamine for 15 min resulted in a reduction in the maximal dopamine stimulated AC activity, which was accompanied by an increased phosphorylation of the receptor and a rapid redistribution of surface D_1 receptor as detected by in situ immunofluorescence. These are the evidence that human D_1 receptor undergoes agonist-dependent desensitization and phosphorylation. Further studies revealed that the agonist-dependent desensitization of D_1 receptor was substantially increased in cells overexpressing various G protein-coupled receptor kinases (types 2,3 and 5). And the G protein-coupled receptor kinases-phosphorylated D_1 receptors display a differential reduction of functional coupling to AC. Dopamine also induces a pharmacologically specific inhibition of forskolin-stimulated AC activity via D_2 receptors. This effect involves a functional uncoupling of the D_2 receptor as well as a loss of its ligand binding activity (Barton, 1991).

In our studies, high concentrations (greater than 10^{-9} M) of SKF-82958 or PPHT has a minimal effect on the expression of pOGH (ANG N-1498/+18) in OK 27 cells (Fig. 10). The possible explanation may be that the higher concentrations of the dopaminergic receptor agonists rapidly desensitize or downregulate its own receptors. Bates et al (1991, 1993) observed that dopamine pretreatment which increases the levels of intracellular cAMP, induces rapid downregulation of D_1-receptor in OK cells. However,

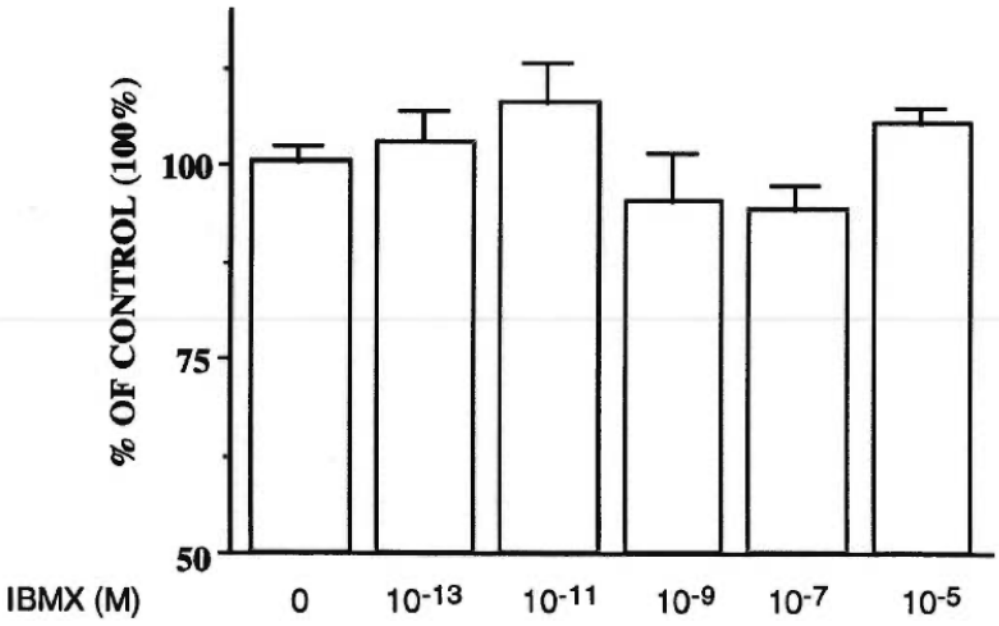
the increase of cAMP is not necessary for dopamine-induced desensitization.

Until now, we do not have the evidence to demonstrate that OK cells undergo desensitization or downregulation after the treatment of dopamine or dopaminergic receptor agonists. More studies are underway in our lab to differentiate the mechanisms of the minimal effect of dopamine and dopaminergic receptor agonists.

I.2.4. Effect of IBMX

IBMX is an inhibitor of phosphodiesterase. We demonstrated that dopamine has a minimal effect on the expression of pOGH (ANG N-1498/+18) in OK 27 cells in the absence of IBMX (Fig. 7). One possible explanation may be that the intracellular cAMP (stimulated by dopamine) is rapidly degraded by the endogenous phosphodiesterase. This possibility is also supported by Cheng et al (1990), who showed that dopamine is much more effective in the stimulation of intracellular cAMP in OK cells in the presence of IBMX.

On the other hand, addition of IBMX (10^{-13} to 10^{-5} M) alone does not affect the basal expression of pOGH (ANG N-1498/+18) in OK 27 cells (unpublished data, Fig. below).



This result suggestes that the inhibition of the endogenous phosphodiesterase alone by IBMX is not sufficient to affect the basal expression of the ANG gene expression in OK 27 cells. However, in the absence of IBMX, SKF-82958 or PPHT alone could stimulate the ANG fusion gene expression (Fig.11). These data suggest that the lack of response by dopamine could not be explained solely on the rapid degradation of cAMP. It is possible that other factors, such as binding affinity and specificity of the ligand to its receptor, may also play an important role.

I.2.5. Synergistic Effect of DA_1 and DA_2 Receptors

Functional D_1-D_2 receptor linkage (synergy) had been shown in studies of dopamine-stimulated modulation of Na^+-K^+-ATPase and arachidonate release (Bertorello, 1990; Piomelli, 1991). These studies used cell populations known to produce both D_1 and D_2 receptors, suggesting an intracellular rather than an intercellular interaction. Dopamine receptor interactions have also been demonstrated *in vitro* with the use of radioligand binding techniques, where D_1- and D_2-like receptors displayed pharmacological coupling. Antagonist occupation of the D_1 receptors can modulate the ability of D_2 receptor agonists to bind to the D_2 receptor (Seeman, 1989). Whether this D_1-D_2 receptor link interacts with or involves the receptor number is unknown.

Under normal conditions, D_1 and D_2 receptors interact synergistically in the striatum, and immediate early gene induction by dopaminergic drugs is not observed after stimulation of either receptor subtype alone. The combined administration of low doses of D_1 and D_2 agonists significantly enhances striosomal c-fos mRNA and Fos-LI expression, an effect blocked by pretreament with a combination of D_1 and D_2 antagonists (LaHoste, 1993).

D_1 and D_2 receptors can act synergistically to produce many electrophysiological and behavioral responses (Bertorello, 1990; Piomelli, 1991). This synergism can occur at

the level of single neurons, as well as signaling pathway activated by dopamine. In CHO cells transfected with D_2 receptor cDNA, D_2 agonists potently enhance arachidonic acid release initiated by increasing intracellular Ca^{2+}. When D_1 and D_2 receptors are coexpressed, activation of both subtypes results in a marked synergistic potentiation of arachidonic acid release.

Dopamine inhibits NaKATPase activity in rat renal proximal convoluted tubules segments (Aperia, 1987). However, there is no inhibition of NaKATPase activity observed when incubate proximal convoluted tubule segments with either a highly specific DA_1 or DA_2 agonist (Bertorello, 1988). Bertorello's study showed that the presence of both the DA_1 and DA_2 agonistic effects is needed to activate the dopamine receptors that cause inhibition of NaKATPase activity. It is likely that the dopamine effect is mediated by an inhibitory DA_2 receptor than a DA_1 receptor, since activated DA_1 receptors will activate AC and increase cAMP production, and cAMP stimulate NaKATPase.

In our studies, the combination of both SKF-82958 and PPHT is more effective than either dopamine or SKF-82958 or PPHT alone (Fig. 11), suggesting an additive effect of SKF-82958 and PPHT. This effect can be due to the synergism between the two receptors, although the mechanism is not yet known. One explanation is that there could be a "cross-talk" between SKF-82958 and PPHT. That is, PPHT may enhance the effect of SKF-82958 by inducing the phosphorylation of AC.

In summary, our studies showed that low levels of dopamine plus IBMX, SKF-82958, or PPHT stimulate the expression of pOGH (ANG N-1498/+18) in OK 27 cells. The stimulatory effect can be blocked by the receptor antagonists, respectively. Furthermore, there is an additive effect of DA_1 and DA_2 receptors in the regulation of the ANG gene expression in OK 27 cells.

I.3. OK 13 Cells

The OK 13 cell is a cell line into which has been stably integrated a fusion gene: pTKGH containing the promoter/enhancer DNA sequence of the viral thymidine kinase gene fused with a human growth hormone gene as a reporter. The expression of the pTKGH in OK 13 cells is driven by the promoter/enhancer DNA sequence of the TK gene. Therefore, we used OK 13 cells as a control cell line to examine the effect of AR or dopaminergic receptor agonists.

We do not observe any significant stimulation of expression of the pTKGH by neither iodoclonidine, phenylephrine, nor PMA in OK 13 cells (Fig. 6). These studies demonstrated that the promoter/enhancer DNA sequence of the TK gene is not responsive to the addition of iodoclonidine, phenylephrine, or PMA. The effect of iodoclonidine, phenylephrine, and PMA in OK 27 cells is mediated specifically via the 5'-flanking region of the rat ANG gene but not mediated via the DNA sequence of the hGH reporter gene.

Similarly, neither dopamine, SKF-82958, nor PPHT have any effect on the expression of pTKGH in OK 13 cells (Fig. 14). These studies demonstrated that the promoter/enhancer DNA sequence of TK gene is not responsive to addition of dopamine, SKF-82958 or PPHT. The effect of dopamine, SKF-82958 and PPHT in OK 27 cells is mediated specifically via the 5'-flanking region of the rat ANG gene but not mediated via the DNA sequence of the hGH reporter gene.

II. EFFECT OF NOREPINEPHRINE ON THE EXPRESSION OF ANG GENE IN OK CELLS

II.1. Biological Effects of Norepinephrine

All of the effects of increased renal sympathetic nerve activity (RSNA) have the potential of contributing to the initiation, development, and maintenance of hypertension (DiBona, 1991). In which, stimulation of renin secretion rate would lead to increases in both intrarenal and circulating Ang II concentrations whose effects are well known to participate in hypertension.

Studies have shown that increases in RSNA produce sustained hypertension. Chronic renal sympathetic nerve stimulation produces sustained hypertension in dogs (Kottke, 1945). In rabbits, renal denervation decreases plasma renin activity and cumulative renal sodium retention, as well as prevents the development of hypertension (Weinstock, 1996). When NE has been infused into the renal artery of rats and dogs to obtain a chronic renal sympathetic nerve stimulation, sustained hypertension resulted (Katholi, 1977), which is accompanied by an increase in plasma angiotensin (Reinhart, 1995). Individuals with essential hypertension have been characterized by increased renal sympathetic vascular tone. Low-level elevation of intrarenal adrenergic neurotransmitter (NE) produces sustained arterial hypertension (Plato, 1996).

Besides hypertension, studies also indicate that functional abnormalities of the SNS contribute to the genesis and maintenance of hypertension associated chronic renal failure. In hypertensive chronic renal failure, the plasma NE of the patients is significantly higher than the patients with essential hypertension (Cottone, 1995), suggesting that NE plays a role in hypertensive nephropathy.

The above studies suggested that increased RSNA, or NE release and RAS play important roles in hypertension and nephropathy, and there might be some

interactions between the two systems.

II.2. Effect of Norepinephrine on the Expression of ANG Gene in OK Cells

We hypothesized that NE may modulate the expression of the ANG gene in OK cells via either/both ß- or α_2-ARs. Our studies showed that addition of NE directly stimulates the expression of pOGH (ANG N-1498/+18) in OK 27 cells in a dose-dependent manner (Fig. 15). The stimulatory effect of NE can be inhibited by the presence of propranolol or yohimbine or atenolol (a $ß_1$-AR antagonist) (Fig. 16, 18). These results demonstrate that the effect of NE may be mediated via either the $ß_1$- or α_2-ARs. These results also confirmed our previous studies that the activation of the ß- or α_2-ARs alone stimulates the expression of pOGH (ANG N-1498/+18) in OK 27 cells (Wang, 1994; Discussion I.1.2.).

We observed that higher concentrations of NE (greater than 10^{-7} M) has no stimulatory effects on the expression of pOGH (ANG N-1498/+18) in OK 27 cells. One possible explantion may be that the exposure of OK cells to high levels of NE may desensitize its own ß- or α_2-adrenoceptors (as discussed in Introduction III.1.2 and Discussion I.1.3).

II.3. Synergistic Effect of ß- and α-ARs

Studies (Morris, 1991) have shown that epinephrine, which acts at both α_1- and ß-ARs, induced a short term increase in the abundance of α_1-AR mRNA. The ß-AR agonist attenuated the effect of epinephrine but did not affect the decrease in α_1-AR-specific ligand binding. This observation indicated that crosss-regulation between α_1- and ß-AR occurs at the level of mRNA. Moreover, Birnbaum's group (1995) demonstrated that in oocytes co-injected with α_2- and ß-AR mRNAs, isobolographic analysis (on currents carried by cAMP-regulated chloride channels) revealed an additive interaction between α_2- and ß-ARs.

Our studies demonstrated that the stimulatory effect of the addition of a combination of both isoproterenol and iodoclonidine on the expression of pOGH (ANG N-1498/+18) in OK 27 cells is significantly higher than the addition of isoproterenol and iodoclonidine alone (Fig. 19), suggesting a synergistic effect of β_1- and α_2-adrenoceptors. One explanation could be that the cross-reaction occurs at the α_2-AR mRNA level as discussed above. Other possibility is that since the effect of β_1- and α_2-adrenoceptors are mediated via the protein kinase A and protein kinase C in OK 27 cells (will be discussed later), respectively, we speculated that there might be a "cross-talk" between the β_1- and α_2-adrenoceptors or between the activation of PKA and PKC on the expression of pOGH (ANG N-1498/+18) in OK 27 cells.

The molecular mechanism(s) for the synergistic effect of the β_1- and α_2-adrenoceptors or the PKA and PKC signal transduction pathways is not yet known, more experiments are required to clarify these observations.

II.4. OK 53 Cells

The expression of the pOGH (ANG N-53/+18) in OK 53 cells is driven by the minimal promoter (i.e. ANG N-53 to +18 contains the putative "CCAT" and "TATA" boxes on nucleotides -50 and -30 upstream of the transcriptional site, respectively (Chan, 1990)) of the rat ANG gene. Our results showed that neither NE, forskolin, 8-Br-cAMP nor PMA has any effect on the expression of the pOGH (ANG N-53/+18) in OK 53 cells (Fig. 20). These studies demonstrate that the minimal promoter of the rat ANG gene is not sufficient to respond to the addition of NE, forskolin, 8-Br-cAMP or PMA.

In summary, our studies demonstrate that NE stimulates the expression of pOGH (ANG N-1498/+18) in OK 27 cells. The stimulatory effect of NE was blocked by the presence of β_1- and α_2-adrenoceptor antagonists. Furthermore, we demonstrate that the effect of

a combination of both isoproterenol and iodoclonidine on the expression of pOGH (ANG N-1498/+18) in OK 27 cells is significantly higher than the addition of isoproterenol and iodoclonidine alone. Our studies raise the possibility that the expression of the ANG gene in the renal proximal tubule is stimulated by NE via either the ß- and α_2-adrenoceptor alone or via the interaction ("cross-talk") of both $ß_1$- and α_2-adrenoceptors *in vivo*. The local formation of renal Ang II might then modulate the sodium and fluid reabsorption by the proximal tubular cells. Hence, local intrarenal RAS plays a significant role in the modulation of sodium reabsorption.

III. SIGNAL TRANSDUCTION PATHWAYS INVOLVED IN THE EFFECT OF CATECHOLAMINES ON THE EXPRESSION OF THE ANG GENE IN OK CELLS

III.1. Signal Transduction Pathways Mediated by α-Adrenergic Receptors

The major coupling system of the α_1-AR is activation of PLC via a pertussis toxin-insensitive G-protein. α_1-AR stimulation can also increase intracellular cAMP by a mechanism that does not involve direct activation of AC (Cotecchia, 1991).

α_{2A} receptors stimulation in brain, kidney and adipose tissue results in the inhibition of AC (Limbird, 1986). By utilizing transient and stable expression of recombinant cDNAs encoding the α_2-AR subtypes, Cotecchia et al (1991) showed that the primary effect of α_{2B} receptors is to decrease intracellular cAMP concentrations, the same as α_{2A}. In addition, both α_2-ARs have a modest effect to stimulate directly phosphotidylinositol (PI) metabolism. The stimulation of PI metabolism is independent of cAMP levels. The two effects are both mediated by pertussis toxin-sensitive G-proteins.

We observed that staurosporine (a potent inhibitor of PKC) inhibits the expression of the pOGH (ANG N-1498/+18) stimulated by iodoclonidine in OK 27 cells in a dose-

dependent manner (Fig. 5). We also observed that the addition of PMA (phorbol 12-myristate 13-acetate) stimulates the expression of pOGH (ANG N-1498/+18) in OK 27 cells in a dose-dependent manner and the stimulatory effect can be blocked by staurosporine (Fig.3, 4). These data support the hypothesis that the effect of α_2-AR on the expression of ANG gene is via the PKC pathway and not via the PKA pathway, since Rp-cAMP (an inhibitor of cAMP-dependent protein kinase A) does not inhibit the stimulatory effect of iodoclonidine (Fig. 5).

Since staurosporine is an protein kinase inhibitor with some selectivity to PKC, we applied another potent PKC inhibitor, H7. Our data showed that H7 has the same effect as staurosporine on the regualtion of ANG fusion genes (data not shown).

U73122 is an inhibitor of PLC and PLA_2, it is conceivable that the addition of U73122 might prevent the hydrolysis of PIP2 and would subsequently inhibit the activation of PKC in OK 27 cells. This possibility is supported by the studies of Martin et al (1994) who showed that the addition of U73122 abolishes the increase in particulate PKC activity stimulated by parathyroid hormone in OK cells.

Our data also showed that the stimulatory effect of NE (10^{-9} M) and iodoclonidine (10^{-9} M) was abolished after a 24 hour pre-incubation of OK 27 cells with high concentration of PMA (10^{-5} M) (Fig. 20). These results are in agreement that the exposure to PMA will downregulate the PKC activity (Hug, 1993). Moreover, our preliminary studies showed that NE at 10^{-9} M increased the cellular level of PKC activity in OK 27 cells but not NE at 10^{-5} M (Fig. 21). All these studies support the notion that the stimulatory effect of NE is mediated, at least in part, via the PKC signal tranduction pathway.

In conclusion, the addition of iodoclonidine and NE directly stimulates the expression of the ANG-GH fusion gene in OK 27 cells via α_2-AR and PKC signal transduction

pathway.

III.2. Signal Transduction Pathways Mediated by Dopaminergic Receptors

In rat renal cortical membranes, DA_1 receptor agonists have been shown to stimulate cAMP production and the release of inositol phosphates (Felder, 1984, 1989). Dopexamine hydrochloride (DPX), a dopamine DA_1 and beta 2-adrenergic receptor agonist, causes concentration related increases in cAMP levels in rat kidney membrane particles. The effect can be completely abolished by a combined presence of D_1 receptor antagonist, SCH 23390 and ß-adrenoceptor antagonist, propranolol suggesting that both binding sites of DPX are linked to AC (Vyas, 1991-a). It has also been shown that DA_1 receptor activation leads to stimulation of PLC activity, most likely at the basolateral membrane of the rat proximal convoluted tubule (Vyas, 1991-b). It is also reported that a defect in DA_1 receptor AC coupling in the proximal convoluted tubule in the SHR may contribute to the diminished natriuretic response to DA_1 receptor agonists, and a similar defect is also present in DA_1 receptor-coupled PLC pathway (Chen, 1992) .

The presence of DA_2 receptors negatively coupled to cAMP generation in the kidney cortex has also been characterized (Ricci, 1991). However, D_2 receptors in different cell lines are likely to have different couplings of signal transduction pathways. The studies of Vallar's group showed that when both pituitary cell line GH_4C_1 cells and mouse fibroblast Ltk^- cells are transfected with D_2 receptor cDNA, the D_2 receptor induces a rapid stimulation of inositol (1,4,5)-trisphosphate followed by an immediate increase of $[Ca^{2+}]_i$ due to both Ca^{2+} mobilization form internal stores and influx from the extracellular medium in Ltk^- fibroblasts. Whereas in GH_4C_1 cells, the transfected receptor fails to affect phosphoinositide hydrolysis and induces a decrease of $[Ca^{2+}]_i$. In both cell lines, the D_2 receptor response is mediated by G-proteins sensitive to pertussis toxin. This study suggested that in certain cells D_2 receptors are efficiently

coupled to the stimulation of phosphoinositide hydrolysis (Vallar, 1990).

In OK cells, Cheng et al (1990) showed that dopamine stimulates the cAMP production in a dose dependent manner and markedly higher levels are observed in the presence of dopamine plus a phosphodiesterase inhibitor (3-isobutyl-1-metylxanthine, IBMX). They also found that a DA_1-receptor agonist can mimic the effect of dopamine on cAMP production, whereas a DA_2-receptor agonist can not. The stimulatory effects of dopamine and DA_1 receptor agonist are abolished by the specific DA_1 receptor antagonist but not DA_2 receptor antagonist. These results suggest the existence of DA_1-receptors linked to AC in OK cells.

In our studies, addition of Rp-cAMP or staurosporine completely inhibits the stimulatory effect of dopamine (Fig. 9). This result implies that the stimulatory effect of dopamine on the expression of pOGH (ANG N-1498/+18) is probably via both PKC and cAMP-PKA pathways in OK 27 cells. The involvement of the PKC pathways is further supported by the observation that U73122 (PLC inhibitor) inhibits the stimulatory effect of dopamine (Fig. 9). It is conceivable that the addition of U73122 might prevent the hydrolysis of phosphatidyl inositol 4,5-bisphosphate and subsequently inhibits the activation of PKC in OK 27 cells. This possibility is supported by the studies of Martin et al (1994), who showed that the addition of U73122 abolishes the increase in particulate PKC activity stimulated by PTH in OK cells.

The effect of SKF-82958 is blocked by the presence of SCH-23390 and Rp-cAMP but not by staurosporine (Fig. 12), suggesting that the effect of the D_1-receptor agonist on the expression of pOGH (ANG N-1498/+18) in OK 27 cells is mediated via the cAMP-PKA pathway.

Spiperone or staurosporine inhibits the effect of PPHT in a dose-dependent manner

(Fig. 13), suggesting that the PKC pathway is involved in mediating the effect of PPHT on the expression of pOGH (ANG N-1498/+18) in OK 27 cells. These studies further support that PKC may be involved in the expression of the ANG gene in OK cells.

The above studies suggest that in OK cells, both DA_1 and DA_2 receptors are involved in the regulation of the expression of the ANG gene via PKA and PKC pathway respectively.

Our studies raise the possibility that the expression of the ANG gene in renal proximal tubules may be modulated by dopaminergic receptors *in vivo*. However, the physiological significance remains to be a paradox since the effect of dopamine on increase of renal blood flow and sodium excretion is via the activation DA_1 receptor and inhibition of DA_2 receptor. In our result, both receptors are all positively regulated the expression of the ANG-GH fusion gene in OK 27 cells. At present, the physiological significance of the stimulation of expression of the ANG gene by dopamine is not known, further studies are warrant.

IV. MOLECULAR MECHANISMS OF THE EFFECT OF THE ADRENERGIC RECEPTORS ON THE EXPRESSION OF THE ANG GENE IN OK CELLS

IV.1. The effect of 8-Br-cAMP or/and PMA on the expression of the ANG gene

Our previous studies demonstrated that the stimulatory effect of ß-AR agonist isoproterenol on the ANG fusion gene expression is mediated via the PKA-cAMP pathway (Wang, 1994). The cAMP analog 8-Br-cAMP can stimulate the expression of the gene in OK 27 cells. This implies the possibility that cAMP induces the expression and phosphorylation of cAMP responsive element binding protein(s) (i.e. CREB or CREB-like proteins). The phosphorylated CREB or CREB-like protein(s) then interact(s) with ANG-CRE, and trigger the gene transcription.

Our present studies also showed that PMA directly stimulates the ANG-GH fusion gene expression and the stimulatory effect can be blocked by PKC inhibitor, staurosporine (Fig. 3, 4). Since PMA is a potent stimulator of PKC, it is suggested that the PKC pathway is also involved in the expression of the ANG gene in OK cells. This raised the possibility that the route of iodoclonidine, in exerting its effect, is mediated via the PKC pathway. Indeed, our results showed that staurosporine, but not Rp-cAMP, inhibits the expression of the pOGH (ANG N-1498/+18) stimulated by iodoclonidine in OK 27 cells (Fig. 5). These data also support the hypothesis that the effect of α_2-AR on the expression of the ANG gene is via the PKC but not the PKA pathway.

The addition of low concentrations of staurosporine or Rp-cAMP or U73122 completely block the stimulatory effect of dopamine (Fig. 9). These studies suggested that the stimulatory effect of dopamine is via both PKC and PKA pathway on the expression on ANG-GH fusion gene in OK cells. Since U73122 is an inhibitor of PLC and PLA_2, it is conceivable that the addition of U73122 might prevent the hydrolysis of phosphatidyl inositol 4,5-bisphosphate and subsequently inhibits the activation of PKC in OK 27 cells. This possibility is supported by the studies of Martin et al (1994) who showed that the addition of U73122 abolishes the increase in particulate PKC activity stimulated by PTH in OK cells.

Further studies showed that the effect of SKF-82958 is blocked by the presence of Rp-cAMP but not by staurosporine (Fig. 12), suggesting that the effect of the D_1 receptor on the expression of the ANG fusion gene is mediated via the cAMP-dependent PKA pathway. The effect of PPHT can be inhibited by staurosporine (Fig. 13), suggesting that the effect of the D_2 receptor on the expression of the ANG fusion gene is mediated via the PKC pathway. Indeed, the combination of both SKF-82958 and PPHT is more effective than SKF-82958 or PPHT alone in OK 27 cells (Fig. 11), suggesting an additive effect of SKF-82958 and PPHT.

It is interesting that both β_1- and α_2-AR stimulate the expression of the ANG fusion gene in OK 27 cells. The addition of both isoproterenol and iodoclonidine is significantly enhanced the expression of the ANG-GH fusion gene as compared to isoproterenol or iodoclonidine alone (Fig. 19). Since α_2-AR is known to uncouple the AC system in OK cells (Murphy, 1988), it is possible that the α_2-AR can modulate the effect of ß-ARs on the expression of ANG gene or vise versa.

The above results raised a possibility that there might be an interaction between the PKA and PKC pathway in the regulation of the ANG gene expression. Our hypothesis is confirmed by the result that PMA and 8-Br-cAMP have a synergistic effect on the stimulation of the ANG-GH gene expression in OK 27 cells (Fig. 19). Thus, these results suggested strongly that there might be a "cross-talk" between the ß- and α_2-AR or between the activation of PKA and PKC on the expression of pOGH (ANG N-1498/+18) in OK 27 cells.

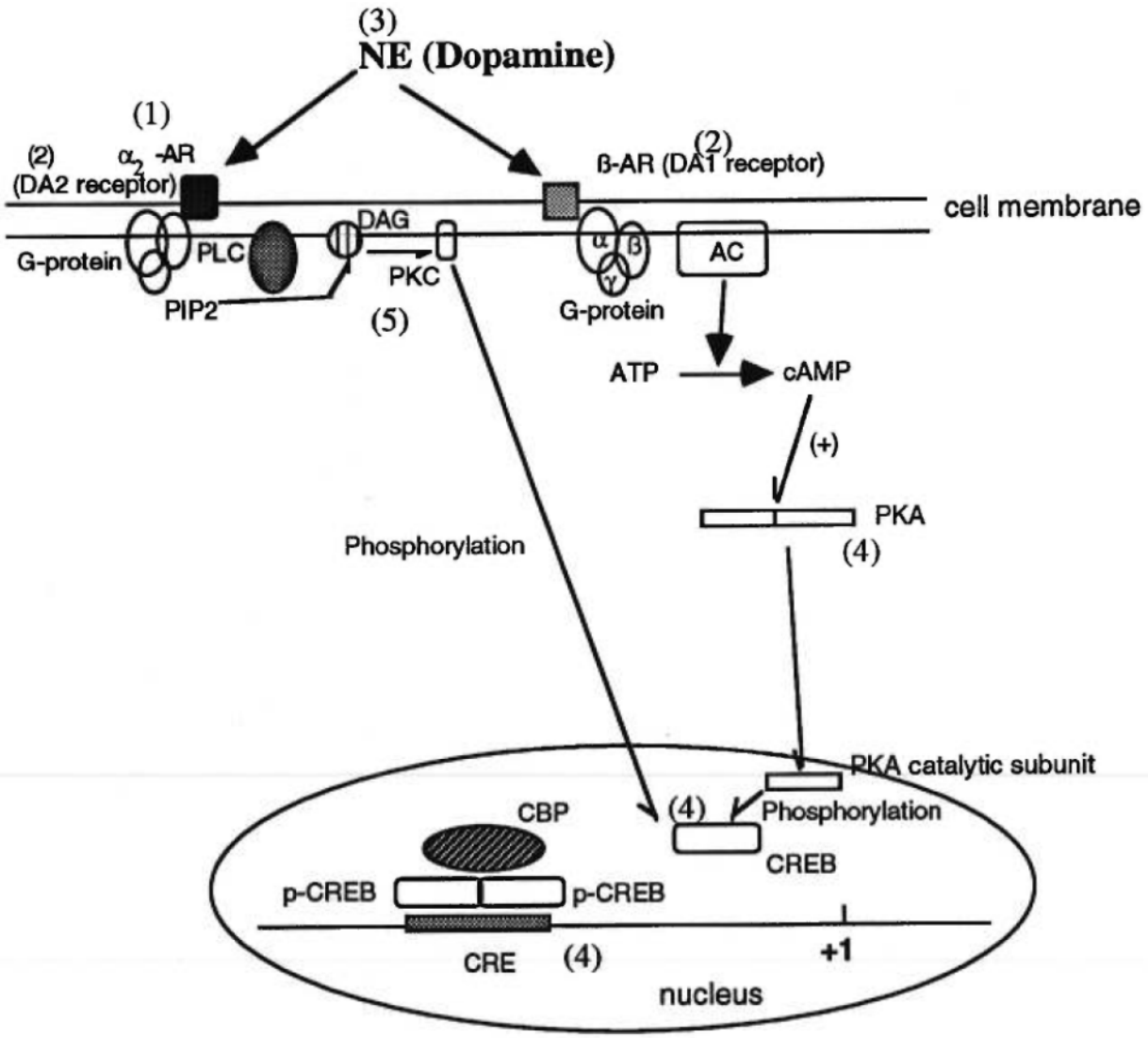
IV.2. The cyclic AMP responsive element (CRE)

The molecular mechanism(s) for the synergistic effect of PKA and PKC in the expression of the ANG gene is unknown. There might be several possibilities. First, the activation of PKC might induce the expression of early-immediate protooncogenes, that is, c-*Fos* and *c-Jun* genes. The products of these protooncogenes form an heterodimer complex (AP-1 complex) and then bind to the putative AP-1 DNA sequence. Subsequently, the AP-1 complex enhances the expression of the ANG gene. Indeed, our previous studies (Chan, 1990) showed that the DNA sequence (N-758 to N-752, TGACTAC) in the 5'-flanking regulatory region of the rat ANG is similar to the consensus TRE (TGACTCA) (Rauscher, 1988), with the exception that the last two nucleotides are in reverse order. Second, PKC (Bell, 1984) may stimulate directly AC and increase cAMP synthesis, or the activation of PKC phosphorylates the CREB or CREB-like nuclear protein(s) , since CREB contains the site of phosphorylation by PKC

(Gonzalez, 1989-b). Third, the increased intracellular Ca^{2+} activates CaM kinases IV. The activated CaM kinase IV could then phosphorylate CREB (Enslen, 1995).

Our hypothesis was that the catecholamines stimulate the expression of ANG gene via both PKA and PKC (via direct stimulation of AC and increase cAMP synthesis) pathways, resulting in phosphorylation of CREB or CREB-like proteins, which then interact with the CRE in the 5'-flanking region of the rat ANG gene, and trigger the gene transcription (see the graph below).

Model of the Molecular Mechanism(s) of the Effect of PKA and PKC Pathways on the Expression of the rat ANG Gene in OK Cells



In this model, we proposed that since NE interacts with both the α- and ß-ARs in the renal proximal tubules (DiBona, 1982, 1985; Hanson, 1995; Insel, 1985), it might modulate the expression of the ANG gene in OK cells via both the α_2- and $ß_1$-ARs. We obtained several other permanent cell lines with different segments of the ANG 5'-flanking sequence fused with growth hormone reporter gene, which are OK 960 (pOGH (A N-960/+18)), OK 280 (pOGH (A N-280/+18)), and OK 30 (pOGH (A N-53/+18)). Our data showed that NE at 10^{-9} M can stimulate the expression of pOGH (A N-960/+18) and pOGH (A N-1498/+18) in OK 960 and OK 27 cells, respectively, but not pOGH (A N-280/+18) and pOGH (A N-53/+18) in OK 280 and OK 53 cells, respectively (Fig. 21). These results demonstrate that the stimulatory effect of NE occurs between N-960 to N-280 of the rat ANG 5'-flanking sequence.

Studies by Fukamizu et al (1990) reported that a putative CRE is located in the 5'-flanking region (ANG N-839 to N-833) of the human ANG gene. Our previous studies Chan, 1990) on the DNA structure of the 5'-flanking sequence of the rat ANG gene showed that the DNA sequence of nucleotides N-806 to N-779 (5'TGACGTAC3') is almost identical to the CRE (5'TGACGTCA3') of the somatostatin gene (Montminy, 1986), vasoactive intestinal peptide gene (Tsukada, 1987), α-chorionic gonadotropin gene (Silver, 1987), and phosphoenolpyruvate carboxykinase gene (Short, 1986) except that the last two nucleotides are in reverse order. The sequence flanking the CRE of the rat ANG gene, however, is not homologous (less than 40%) with the above genes. We raised the question of whether the sequence ANG N-806 to N-779 could be a putative CRE and we tested this possibility.

Previous studies in our lab observed that the addition of forskolin or 8-Br-cAMP stimulates the expression of pTKCAT (ANG N-814/-689) and pTKCAT (ANG N-814/-761) in OK cells (Ming, 1995).

We obtained another permanent cell line, OK 95, which contains the ANG putative CRE (N-806/-779) (5'AGC TTA AGA GAT TAC **TTG ACG TAC** TGG ATG CAA A3') plus the basal promoter of the ANG gene (N-53/+18) fused with growth hormone reporter gene (pOGH (ANG/CRE/-53/+18)) integrated into the genome. We tested the effect of 8-Br-cAMP or PMA (Fig. 27, 28). We found that, 8-Br-cAMP stimulates the expression of ANG-CRE in OK 95 cells. We also found that forskolin (Fig. 28), which is an AC activator, as well as PMA, stimulate the expression of ANG-CRE in OK 95 cells. Furthermore, the effect of isoproterenol on the expression of the pOGH (ANG/CRE/-53/+18) can be blocked by Rp-cAMP in OK 95 cells (Fig. 25), suggesting that the stimulatory effect of ß-AR agonist is via the PKA signal transduction pathway which interact with the ANG-CRE.

To further confirm ANG N-806 to N-779 is the ANG-CRE, we have synthesized several mutants, which are mutant 1 (5'AGC TTA AGA GAT TAC **TTG ACT TAC** TGG ATG CAA A3'), mutant 2 (5'AGC TTA AGA GAT TAC **TTG A**AT **TAC** TGG ATG CAA A3') and mutant 3 (5'AGC TTA AGA GAT TAC TTAT AT **TAC** TGG ATG CAA A3'). These mutants were inserted upstream of the rat ANG basal promoter, ANG N-53/+18. Permanent cell lines OK 95/M1, OK 95/M2 and OK 95/M3 with different mutants plus ANG gene basic promoter fused with growth hormone reporter gene (i.e. pOGH (ANG/CREM1/-53/+18), pOGH (ANG/CREM2/-53/+18), pOGH (ANG/CREM3/-53/+18)) were obtained. We investigated the effect of isoproterenol on the expression of ANG-CRE mutant genes. The result (Fig. 26) showed that isoproterenol stimulates the gene expression of mutant 1 but not mutant 2 and 3, suggesting that ANG N-806 to N-779 is the ANG-CRE and the mutations of more than two nucleotides abolished the responsiveness to catecholamines.

IV.3. The cyclic AMP Responsive Element Binding Protein (CREB)

Studies have shown that the molecular mechanism of the action of cAMP is probably mediated via the phosphorylation of a 43K nuclear protein which binds selectively to the CRE. When added to nuclear extracts, purified CREB specifically stimulated transcription of CRE-containing genes like somatostatin, suggesting that CREB was a transcription factor as well as a DNA-binding protein (Montminy, 1987). In addition, the transcriptional efficacy of CREB is regulated by PKA phosphorylation (Yamamoto, 1988).

Studies have also shown that CREB can be phosphorylated at serine 133 by cAMP-dependent PKA (Gonzalez, 1989-a), or by PKC (Yamamoto, 1988) and phosphorylation increases the binding affinity of 43-kDa-CREB to CRE (Nichols, 1992) and subsequently enhances the gene expression.

The rat 43-kDa CREB was cloned from the adult male rat (Wistar-Kyoto) liver in our lab Qian, 1997). The expression plasmid containing the cDNA for 43-kDa-CREB, pRSV/CREB was constructed. Transient gene transfection of pRSV/CREB directly stimulates the expression of pOGH (ANG N-1498/+18) in OK 27 cells (Fig. 31). The addition of isoproterenol further enhances the stimulatory effect of pRSV/CREB (Fig. 31), as compared to those transfected with pGEM-3 (Fig. 32). Moreover, the stimulatory effect of isoproterenol via pRSV/CREB can be blocked by propranolol or Rp-cAMP (Fig. 33). These studies, as well as our previous studies (Wang, 1994), demonstrated that isoproterenol stimulates the synthesis of intracellular cAMP. The elevated intracellular cAMP then activates the cAMP-dependent PKA and phosphorylates the nuclear protein CREB.

To study if the phosphorylated CREB could interact with ANG-CRE, we integrated both pRSV/CREB and pOGH (ANG/CRE/-53/+18) or pOGH (ANG/CREM1/-53/+18), or

pOGH (ANG/CREM2/-53/+18), or pOGH (ANG/CREM3/-53/+18) into the OK cells, and obtained the permanent cell lines, OK 96, OK 96/M1, OK 96/M2 and OK 96/M3, respectively. We found that, isoproterenol stimulates the expression of pOGH (ANG/CRE/-53/+18) and pOGH (ANG/CREM1/-53/+18) in OK 96 and OK 96/M1 cells but not OK 96/M2 or OK 96/M3, respectively (Fig. 29, 34). The stimulatory effect of isoproterenol can be blocked by atenolol (β_1-AR antagonist), propranolol and Rp-cAMP but not by ICI 118,511 (β_2-AR antagonist), staurosporine or U73122 (Fig. 30). These results suggest that the CREB binds to the CRE of the rat ANG gene and enhances the gene expression.

Studies have shown that the CREB could be phosphorylated directly by the catalytic subunit of the PKA. (Yamamoto, 1988) To confirm if the transcriptional efficacy of CREB is regulated by PKA phosphorylation, we transiently transfected the expression plasmid of PKA catalytic subunit (Maurer, 1989), pRSV/Catß into OK 27, OK 95 or OK 96 cell lines. The results showed that the PKA catalytic subunit, but not the mutant (pRSV/Catßm), directly stimulates the ANG gene expression in the three cell lines, the maximal effect is found in OK 96 cell lines (Fig. 35). Furthermore, the plasmid pRSV/Catß has a similar effect on the expression of the ANG-CRE mutant-1 genes in OK 95/M1 and OK 96/M1 (Fig. 36, 37), respectively. The pRSV/Catß has no effect on OK 95/M2, OK 95/M3, OK 96/M2 or OK 96/M3. These results indicated that the effect of ß-AR and cAMP-dependent PKA are involved in the expression of the ANG gene and is mediated via the phosphorylation of the CREB and the CRE of the ANG gene.

Rabbit polyclonal antibodies (Rb. #22) against the bacterially-expressed recombinat 43 kDa-CREB were used to establish the affinity column and then the IgG-affinity column was used to purify the 43 kDa-CREB expresed from COS-7 cells. It is apparent that the 43 kDa-CREB preparation was not pure (Fig. 39A). At most, it was probably 70-75% pure. On the other hand, the lower molecular species (25-27 kDa) did not interact

with the rabbit polyclonal antibodies (Rb. #8) against the amino acid residues 135-150 of the 43 kDa-CREB nor interact with the polyclonal antibodies Rb. #22 (Fig. 39B). At present we do not know the molecular structure of this lower molecular weight species. It is possible that this lower molecular species might be contaminated protein that bound non-specifically in the IgG-affinity column.

An additional immunoreactive high molecular species (220 kDa) was observed in Western blot analysis (Fig. 39B). We speculate that this high molecular weight species might be the aggregated form of the 43 kDa-CREB. More experiments are needed to define the lower and higher molecular weight species.

To investigate whether the CREB is able to interact with the ANG-CRE, we performed gel mobility shift assay. Our studies showed that the purified CREB interacted with the 5'-end labeled ANG-CRE (^{32}P-ANG-CRE). The binding of ^{32}P-ANG-CRE to the CREB is displaced by the presence of unlabeled ANG-CRE, ANG-CREM1, SOM-CRE and TAT-CRE but not by the presence of ANG-CREM2 and ANG-CREM3 (Fig. 40). These studies demonstrate that the intact CRE-motif of the ANG-CRE is essential for the binding to the CREB.

Our Southwestern blot experiments showed that ^{32}P-ANG-CRE binds to CREB proteins with an apparent molecular weight of 43 kDa (Fig. 41). This experiment suggested that ANG N-806/-779 interacts with a putative 43kDa-CREB. Further analysis of the Southwestern blot revealed that the polyclonal antibodies Rb. #22 of the 43 kDa-CREB interacts with the 43 kDa molecular species (Fig. 42). These data demontrated that the ANG-CRE interacts with the 43 kDa-CREB.

The reason(s) for the relatively weak displacement by the unlabeled ANG-CRE with the recombinant CREB as compared to the unlabeled SOM-CRE and TAT-CRE (Fig. 40) is

not clear. One possible explanation may be due to the asymmetrical palindrome of the ANG-CRE motif (TGACGTAC), since studies of Benbrook and Jones (1994) demonstrated that the mutations of consensus CRE-motif (TGACGTCA) greatly diminished the binding of the CREB to the mutants of CRE-motif.as compared to SOM-CRE.

These results demonstrate that the ANG-CRE interacts with CREB protein, OK and OK 96 cellular nuclear proteins, whereas the 43 kDa molecular species is immunologically similar to the 43- kDa-CREB as reported by Gonzalez et al (1989-b).

In summary, the effect of NE (or both α and ß-ARs), via renal nerves, is probably via the phosphorylation of nuclear protein CREB. The activated CREB then bind to ANG-CRE to trigger the gene expression.

Based on our studies, we have confirmed that (numbers refer to the mechanism graph on Page 164):

1. The α_2-AR has a direct stimulatory effect on ANG gene expression in OK cells, the signal transduction pathway is probably mediated via PKC;

2. The dopaminergic receptors (DA_1 and DA_2) have direct stimulatory effect on ANG gene expression in OK cells, the signal transduction pathways are probably mediated via both PKA and PKC;

3. NE has a stimulatory effect on ANG gene expression in OK cells via both α_2- and β_1-ARs and the signal transduction pathways are probably mediated via both PKA and PKC, respectively;

4. The molecular mechanisms of the effect of catecholamines on the expression of the ANG gene in OK cells is: activation of PKA, then the activated PKA phosphorylates CREB, the phosphorylated CREB binds to ANG-CRE thereafter and trigger the gene transcription;

5. NE stimulates the translocation of PKC in OK cells.

We have not yet confirmed that:

1. Whether NE or isoproterenol will phosphorylate CREB;

2. The molecular mechanisms of the synergistic/additive effect of both PKA and PKC on the expression of the ANG gene in OK cells.

V. SIGNIFICANCE OF THE PRESENT STUDIES

Studies have shown that the intrarenal Ang II formation responses to different experimental and clinical conditions and, the high intrarenal conversion of Ang I to II, the higher concentration of Ang II in the luminal liquid than that in the blood. Furthermore, Ang II formed in the postglomerular capillary and/or interstitial environment can reduce glomerular and proximal tubular function. These results all support the idea that the effects of Ang II on the renal hemodynamics may be mainly due to the effects of the locally formed peptide (Rosivall, 1994).

In vivo studies showed that in the early stage of SHR, the renin mRNA level in denervated kidney decreases significantly, whereas in the Wistar rats the renal renin mRNA level does not change at any age and is not affected by renal denervation. The renal ANG mRNA level gradually increases with age in both rat strains and is not affected by denervation, but much higher levels are attained in the Wistar rats than in SHR, suggesting that renal ANG gene expression is depressed in SHR. These results suggest that the renal sympathetic nerves elevates renal renin gene expression in the prehypertensive stage, although their influence decreases as hypertension developed (Nakamura, 1995b).

Our present studies also demonstrate a functional relationship between the catecholamine(s) and the activation of expression of the rat ANG gene in OK cells, the results support the *in vivo* study by Nakamura and Johns (1996). However, since OK cells are from opossum and the ANG gene we studied is the rat gene, there might be difference on the regulation of the human ANG gene in human cells regarding the effect of catecholamines. Up till now, we have not found other evidence on the regulation of human ANG gene by catecholamine *in vivo* or *in vitro*. Indeed, we are going to investigate the regulation of human ANG gene with human proximal tubular cells (*in vitro* study).

Our studies raise the possibility that the molecular mechanism(s) of the effect of renal nerve on the expression of the renal ANG gene *in vivo* is probably mediated via the PKA or PKC, or via the metabolism of both PKA and PKC on the phosphorylation of the nuclear protein CREB. The phosphorylated CREB than interacts with the ANG-CRE in the 5'-flanking region of the ANG gene and subsequently enhances the expression of the ANG gene in the proximal tubules. The local formation of renal Ang II might then modulate the sodium and fluid reabsorption by the renal proximal tubular cells. Hence, local intrarenal RAS might play a significant role in the modulation of sodium reabsorption.

In summary, all these studies showed that the SNS plays a regulatory role on the regulation of the ANG gene expression in the kidney. The renal RAS plays an important role in the development of hypertension and nephropathy.

CONCLUSIONS

1. α_2-AR agonist, iodoclonidine, stimulates the expression of the pOGH (ANG N-1498/+18) in OK 27 cells, and this effect can be blocked by α_2-AR antagonist, yohimbine;

2. Staurosporine (PKC inhibitor) inhibits the expression of the pOGH (ANG N-1498/+18) stimulated by iodoclonidine in OK 27 cells in a dose-dependent manner;

3. In the presence of IBMX, dopamine stimulates the expression of pOGH (ANG N-1498/+18) in OK 27 cells and the addition of SCH-23390 (D_1-dopaminergic receptor antagonist), or spiperone (D_2-dopaminergic receptor antagonist), inhibits the stimulatory effect of dopamine;

4. The addition of Rp-cAMP (AC inhibitor) or staurosporine completely inhibits the stimulatory effect of dopamine;

5. The addition of SKF-82958 (D_1-dopaminergic receptor agonist), or PPHT (D_2-dopaminergic receptor agonist), also stimulates the expression of pOGH (ANG N-1498/+18) in OK 27 cells and the stimulatory effect can be blocked by the respective antagonist;

6. The stimulatory effect of SKF-82958 is blocked by the presence of SCH-23390 and Rp-cAMP, whereas staurosporine inhibits the effect of PPHT;

7. The combination of both SKF-82958 and PPHT is more effective than either dopamine or SKF-82958 or PPHT alone, suggesting an additive effect;

8. The addition of NE directly stimulates the expression of pOGH (ANG N-1498/+18) in OK 27 cells and this effect can be inhibited by the presence of propranolol (ß-AR antagonist) or yohimbine or atenolol ($ß_1$-AR antagonist);

9. The stimulatory effect of the addition of a combination of both isoproterenol (ß-AR agonist) and iodoclonidine on the expression of pOGH (ANG N-1498/+18) in OK 27 cells is significantly higher than the addition of isoproterenol and iodoclonidine alone, suggesting a synergistic effect;

10. NE can also stimulate the expression of pOGH (A N-960/+18) in OK 960 cells but not the expression of pOGH (A N-280/+18) and pOGH (A N-53/+18) in OK 280 and OK 30 cells, respectively;

11. Isoproterenol, 8-Br-cAMP, forskolin or PMA stimulates the expression of ANG-CRE (pOGH (ANG N-806/-779/-53/+18) in OK 95 cells and the effect of isoproterenol can be blocked by Rp-cAMP in OK 95 cells;

12. Transient gene transfection of pRSV/CREB directly stimulates the expression of pOGH (ANG N-1498/+18) in OK 27 cells and the addition of isoproterenol further enhances the stimulatory effect of pRSV/CREB;

13. Isoproterenol stimulates the expression of pOGH (ANG/CRE/-53/+18) in OK 96 cells; the stimulatory effect of isoproterenol can be blocked by atenolol (β_1-AR antagonist), propranolol and Rp-cAMP;

14. The PKA catalytic subunit directly stimulates the ANG gene expression in OK 27, OK 95 or OK 96 cell lines;

15. OK cell nuclear extract interacted with the ANG-CRE;

16. The ANG-CRE binds to two OK cellular nuclear proteins with an apparent molecular weight of 56 and 43 kDa and antibodies against the CREB interact with the 43 kDa molecular species.

Our present studies demonstrate a functional relationship between the catecholamine(s) and the activation of expression of the rat ANG gene in OK cells. The molecular mechanism(s) of the effect of renal nerve on the expression of the renal ANG gene *in vivo* is probably mediated via the PKA or PKC, or via the metabolism of both PKA and PKC on the phosphorylation of the nuclear protein CREB. The phosphorylated CREB than interacts with the ANG-CRE in the 5'-flanking region of the ANG gene and subsequently enhances the expression of the ANG gene in the proximal tubules. The local formation of renal Ang II might then modulate the sodium and fluid reabsorption by the renal proximal tubular cells. Hence, local intrarenal RAS might play a significant role in the modulation of sodium reabsorption.

FUTURE WORK

1. Phosphorylation of CREB

In order to confirm if the 43-KDa CREB is phosphorylated by PKA or PKC, we will apply forskolin or PMA to OK 27, OK 95 and OK 96 cells, then take the cellular extract for Western blotting with polyclonal antibodies against phosphorylated CREB;

2. PKC isoforms

Since we confirmed that PKC is involved in the regulation of ANG gene expression, we will differentiate the isoforms of PKC in OK 27 cells;

3. Molecular mechanism(s) of the additive or synergistic effect of PKA and PKC

a). Transiently transfect the expression vectors of a- and ß-AR cDNA into OK 27 cells to investigate whether the effect is at the receptor level;

b). Transiently transfect the expression vectors of c-fos/c-jun cDNA and pRSV/CREB into OK 27 cells to investigate whether the activity of AP-1 and CREB is enhanced mutually;

c). Mobility shift analysis and South-Western blotting to investigate whether both AP-1 and CREB interact with ANG-CRE to induce the synergistic effect.

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REFERENCES

Aghajanian GK, Vandermaelen CP. α_2-Adrenoceptor-mediated hyperpolarization of locus coeruleus neurons: Intracellular studies in vivo. Science 1982;215:1394;

Alblas J, van Corven EJ, Hordijk PL, Milligan G & Moolenaar WH. G_i-mediated activation of the $p21^{ras}$, mitogen-activated protein kinase pathway by α_2-adrenergic receptors expressed in fibroblasts. J Biol Chem 1993;268:22235;

Alessandrini A, Crews CM & Erikson RL. Phorbol ester stimulates a protein-tyrosine/threonine kinase that phosphorylates and activates the *Erk*-1 gene product. Proc Natl Acad Sci USA 1992;89:8200;

Alpern RJ. Cellular mechanisms of proximal tubule acidification. Physiol Rev 1990;70:1;

Andrews PM. Investigations of cytoplasmic contractile and cytoskeletal elements in the kidney glomerulus. Kidney Int 1981;20:549;

Angel P, Imagawa M, Chiu R, Stein B, Imbra RJ, Rahmsdorf HJ, Jonat C, Herrlich P & Karin M. Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulated transacting factor. Cell 1987;49:729;

Aperia A & Bertorello A. Dopamine is an intrarenal natriuretic hormone. Kidney Int 1987;31:258;

Ashendel CL. The phorbol ester receptor: a phospholipid-regulated protein kinase. Biochi Biophy Acta 1985;822:219;

Ausiello DA, Kreisberg JI, Roc C & Karnovsky MJ. Contraction of rat glomerular cells of apparent mesangial origin after stimulation with angiotensin II and arginine vasopressin. J Clin Inv 1980;65:754;

Avruch J, Zhang XF & Kyriakis JM. Raf meets Ras: completing the framework of a signal transduction pathway. Trends Biochem Sci 1994;19:279;

Baeuerle PA. The iducible transcription activator NF-kappa B: regulation by distinct protein subunits. Biochim Biophys Acta 1991;1072:63;

Baichwal VR & Tjian R. Control of c-Jun activity by interaction of a cell-specific inhibitor with regulatory domain δ : Differences between v- and c-Jun. Cell 1990;63:815;

Bakalyar HA & Reed RR. Identification of a specialized adenylyl cyclase that may mediate odorant detection. Science 1990;250:1403;

Ballermann, Skorecki KL & Brenner BM. Reduced glomerular angiotensin II receptor density in early untreated diabetes mellitus in the rat. Am J Physiol 1985;247:F110;

Barajas L, Powers K & Wang P. Innervation of the renal cortical tubules: a quantitative study. Am J Physiol 1984;247:F50;

Barajas L, Salido EC & Smolens P. Pathology of the juxtaglomerular apparatus including Bartter's syndrome. in Tisher CC & Brenner BM (eds.). Renal pathology. With clinical and functional correlations. J.B. Lippincott, Philadelphia 1989:877;

Barajas L. Innervation of the renal cortex. Federa Proc 1978;37:1192;

Barraclough MA, Jones NF & Marsden CD. Effect of angiotensin on renal function in the rat. Am J Physiol 1967;212:1153;

Barton AC, Black LE & Sibley DR. Agonist-induced desensitization of D2 dopamine receptors in human Y-79 retinoblastoma cells. Mol Pharmacol 1991;39:650;

Barturen F & Garcia-Sevilla JA. Long term treatment with desipramine increases the turnover of alpha 2-adrenoceptors in the rat brain. Mol Pharmacol 1992;42:846;

Bates MD, Olsen CL, Becker BN, Albers FJ, Middleton JP, Mulheron JG, Catherine-Jin SL, Conti M & Raymond JR. Elevation of cAMP is required for down-regulation, but not agonist-induced desensitization of endogenous dopamine D_1 receptors in opossum kidney cells. J Biol Chem 1993;268:14757;

Bates MM, Caron G & Raymond JR. Desensitization of DA_1 dopamine receptors coupled to adenylyl cyclase in opossum kidney cells. Am J Physiol 1991;260:F937;

Baumbach L & Skott O. Renin release from different parts of rat afferent arterioles in vitro. Am J Physiol 1986;251:F12;

Beach RE, Schwab SJ, Brazy PC & Dennis VW. Norepinephrine increases Na^+-K^+-ATPase and solute transport in rabbit proximal tubules. Am J Physiol 1987;252:F215;

Bell JD & Brunton LL. Multiple effects of phorbol esters on hormone-sensitive adenylate cyclase activity in S49 lymphoma cells. Am J Physiol 1987;252:6Pt1:E783;

Bell JD, Buxton ILO & Brunton LL. Enhancement of adenylate cyclase activity in S49 lymphoma cells by phorbol esters: putative effect of C kinase on α_s-GTP-catalytic subunit interaction. J Biol Chem 1985;256:2625;

Bello-Reuss E, Colindres RE, Pastoriza-Munoz E, Gottschalk CW & Mueller RA. Effects of acute unilateral renal denervation in the rat. J Clin Invest 1975;56:208;

Benbrook DM & Jones NC. Different binding specificities and transactivation of variant CRE's by CREB complexes. Nucleic Acids Res 1994;8:1463;

Benbrook DM & Jones NC. Heterodimer formation between CREB and JUN proteins. Oncogene 1990;5:295;

Bencsath P, Szalay L, Demeczky L & Takacs L. Effects of chlorothiazide and furosemide on sodium and water excretion after unilateral splanchnicotomy in the dog. Nephron 1971;8:329;

Berg JM. Potential metal-binding domains in nucleic acid binding proteins. Science 1986;232:485;

Berger SL, Pina B, Silverman N, Marcus GA, Agapite J, Regier JL, Triezenberg SL & Guarente L. Genetic isolation of ADA2: a potential transcriptional adapter required for function of certain acidic activation domains. Cell 1992;70:251;

Berkowitz LA & Gilman MZ. Two distinct forms of active transcription factor CREB (cAMP-response element binding protein). Proc Natl Acad Sci USA 1990;87:5258;

Berridge MJ & Irvine RF. Inositol triphosphate, a novel second messenger in cellular signal transduction. Nature 1984;312:315;

Berridge MJ. Inositol trisphosphate and diacylglycerol: two interacting second messengers. Ann Rev Biochem 1987;56:159;

Berthelsen S & Pettinger WA. A functional basis for the classification of alpha adrenergic receptors. Life Sci 1977;21:595;

Bertorello A & Aperia A. Both DA_1 and DA_2 receptor agonists are necessary to inhibit NaKATPase activity in proximal tubules from rat kidney. Act Physiol Scand 1988;132:441;

Bertorello AM, Hopfield JE, Aperia A. Inhibition by dopamine receptor interactions. Nature 1990;347:386;

Bickerton RK & Buckley JP. Evidence for a central mechanism in angiotensin induced hypertension. Proc Soc Exp Biol Med 1961; 106:832;

Billah MM & Canthes JC. The regulation and cellular functions of phosphatidylcholine hydrolysis. Biochem J 1990;269:281;

Birch H & Schreiber G. Transcriptional regulation of plasma protein synthesis during inflammation. J Biol Chem 1986;261:8077;

Birnbaum AK, Wotta DR, Law PY & Wilcox GL. Functional expression of adrenergic and opioid receptors in Xenopus oocytes: interaction between alpha 2- and beta 2-adrenergic receptors. Brain Res Mol Res 1995;1:72;

Birnbaumer L. G proteins in signal transduction. Annu Rev Phar Tox 1990;30:675;

Boyle WJ, Smeal T, Defize LHK, Angel P, Woodget JR, Karin M & Hunter T. Activation of protein kinase C decreases phosphorylation of c-Jun at sites that negatively regulate its DNA-binding activity. Cell 1991;64:573;

Brasier AR & Li J. Mechanisms of inducible control of angiotensinogen gene transcription. Hypertension 1996;27pt2:465;

Brasier AR, Philippe J, Campbell DJ & Habener JF. Novel expression of the angiotensinogen gene in a rat pancreatic islet cell line: transcriptional regulation by glucocorticoids. J Biol Chem 1986;261:16148;

Brasier AR, Tate JE, Ron D & Habener JF. Multiple cis-acting DNA regulatory elements mediate hepatic angiotensinogen gene expression. Mol Endocrenol 1989;6:1022;

Brindle P, Linke S & Montminy M. Protein-kinase-A-dependent activator in transcription factor CREB reveals new role for CREM repressors. Nature (London) 1993;364:821;

Brooks VL. Chronic infusion of angiotensin II resets baroreflex control of heart rate by an arterial pressure-independent mechanism. Hypertension Dallas 1995;26:420;

Bruna RD, Kurtz A, Corvol P & Pinet F. Renin mRNA quantification using polymerase chain in cultured juxtaglomerular cells. Circ Res 1993;73:639;

Bührle C, Hackenthal E & Helmchen U. The hydronephrotic kidney of the mouse as a tool for intravital microscopy and in vitro electrophysiological studies of renin-containing cells. Lab Invest 1986;54:462;

Bunzow JR, Van Tol HHM, Grandy DK, Alber P, Salon J, Christie M, Machida CA, Neve KA & Civelli O. Cloning and expression of a rat D2 dopamine receptor cDNA. Nature (Lond) 1988;336:783;

Burnham CE, Hawelu-Johnson CL, Frank BM & Lynch KR. Molecular cloning of rat renin cDNA and its gene. Proc Natl Acad Sci USA 1987;84:5605;

Bylund DB. Heterogeneity of alpha-2 adrenergic receptors. Pharma Biochem Behav 1985;22:835;

Caldicott WJH, Taub KJ, Margulies SS & Hollenberg NK. Angiotensin receptors in glomeruli differ from those in renal arterioles. Kidney Int 1981;19:687;

Cambier JC, Newell MK, Justement LB, McGuire JC, Leach KL & Chen ZZ. Ia binding ligands and cAMP stimulate nuclear translocation of PKC in B lymphocytes. Nature 1987;327:629;

Campbell DJ & Habener JF. Cellular localization of angiotensinogen gene expression in brown adipose tissue and mesentery: quantification of messenger ribonucleic acid abundance using hybridization in situ. Endocrinology 1987;121:1616; -a

Campbell DJ. Tissue renin-angiotensin system: sites of angiotensin formation. J Card Phar 1987;10: suppl7:S1; -b

Campbell WB, Graham RM & Jackson EK. Role of renal prostaglandins in sympathetically mediated renin release in the rat. J Clin Inves 1979;64:448;

Carlson GM, Bechtel PJ & Graves DJ. Chemical and regulatory properties of phosphorylase kinase and cyclic AMP-dependent protein kinase. Adv Enzymol 1979;50:41;

Carty DJ, Padrell E, Codina J, Birnbaumer L, Hildebrandt JD & Iyengar RJ. Distinct guanine nucleotide binding and release properties of the three Gi proteins. J Biol Chem 1990;265:6268;

Cassis LA, Lynch KR & Peach MJ. Localization of angiotensinogen mRNA in rat aorta. Circ Res 1988;62:1259;

Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkawa U & Nishizuka Y. Direct activation of calcium-activated phospholipid-dependent protein kinase by tumor promoting phorbol esters. J Biol Chem 1982;257:7847;

Catt KJ. Angiotensin II receptors. (in) The Renin Angiotensin System. (ed) Robertson JIS & Nichols MG. Gower Medical Publishing, London 1993:12.1;

Cerione RA, Staniszewski C, Benovic JL, Lefkowitz RJ & Caron MG. Specificity of the functional interactions of the ß-adrenergic receptor and rhodopsin with guanine nucleotide regulatory proteins reconstituted in phospholipid vesicles. J Biol Chem 1985;260:1493;

Chan JSD, Chan AHH, Jiang Q, Nie ZR, Lachance S & Carrière S. Molecular cloning and expression of the rat angiotensinogen gene. Pediat Nephrol 1990;4:429;

Chan JSD, Ming M, Nie ZR, Sikstrom R, Lachance S & Carrière S: Hormonal regulation of expression of the angiotensinogen gene in cultured opossum kidney proximal tubular cells. Am Soc Nephrol 1992;2:1516;

Chan JSD, Robertson HA & Friesen HG. Maternal and fetal concentration of ovine placental lactogen measured by radioimmunoassay. Endocrinology 1978;102:1606;

Chan YL. Adrenergic control of bicarbonate absorption in the proximal convoluted tubule of the rat kidney. Pfluegers Arch 1980;388:159; -a

Chan YL. The role of norepinephrine in the regulation of fluid absorption in the rat proximal tubule. J Pharma Exp Ther 1980;215:65; -b

Chen CJ, Vyas SJ, Eichberg J & Lokhandwala MF. Diminished phospholipase C activation by dopamine in spontaneously hypertensive rats. Hypertension 1992;19:102;

Cheng L, Precht P, Frank D & Liang CT. Dopamine stimulation of cAMP production in cultured opossum kidney cells. Am J Physiol 1990;258:F877;

Chiu AT, Roscoe WA & McCall DE. Angiotensin II-1 receptors mediated both vasoconstrictor and hypertrophic responses in rat aortic smooth muscle cells. Receptor 1991;1:133;

Chrivia JC, Kwok RPS, Lamb N, Haglwara M, Montmigy MR & Goodman RH. Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature 1993;365:855;

Chruscinski, AJ, Link RE, Daunt DA, Barsh GS & Kobilka BK. Cloning and expression of the mouse homolog of the human α_2-C2 adrenergic receptor. Biochem Biophys Res Comm 1992;186:1280;

Clark RB. Desensitization of hormonal stimuli coupled to regulation of cyclic AMP levels. Advances-Cyclic Nucleotides and Protein Phosphorylation Res 1986;20:151;

Clauser E, Bouhnik J, Jaramillo HN, Auzan C, Corvol P & Menard J. Angiotensinogen production and consumption in the adrenalectomized rat. Endocrinology 1985;116:247;

Clemens MJ, Trayner I & Menaya J. The role of protein kinase C isoenzymes in the regulation of cell proliferation and differentiation. J Cell Sci 1992;103:881;

Clouston WM, Lyon IG & Richards RI. Tissue-specific and hormonal regulation of angiotensinogen minigenes in transgenic mice. EMBO J 1989;8:3337;

Cooke JP, Rimele TJ, Flavahan NA & Vanhoutte PM. Nimodipine and inhibition of alpha adrenergic activation of the isolated canine saphenous vein. J Pharma Exp Ther 1985;234:598;

Corbin JD, Thomas MK, Wolfe L, Shabb JB, Woodford TA & Francis SH. New insights into cGMP action. In: Advances in Second Messenger and Phophoprotein Research: The Biology and Medicine of Signal Transduction. 1990;24:411; Nishizuka Y, Endo M & Tanaka C (eds) Raven Press, New York;

Cotecchia S, Kobilka BK, Daniel KW, Nolan RD, Lapetina EG, Caron MG, Lefkowitz RJ & Rega JW. Multiple second messenger pathways of α-adrenergic receptor subtypes expressed in eukaryotic cells. J Biol Chem 1991;265:63;

Cotecchia S, Schwinn DA, Randall RR, Lefkowitz RJ, Caron MG & Kobilka BK. Molecular cloning and expression of the cDNA for the hamster α_1-adrenergic receptor. Proc Natl Acad Sci USA 1988;85:7159;

Cotecchia S. Molecular cloning and expression of cDNA for a novel α-adrenergic receptor subtype. J Biol Chem 1990;265:8183;

Cottone S, Panepinto N, Vdala A, Zagarrigo C, Galione P, Volpe V & Cerasola G. Sympathetic overeactivity and 24-hour blood pressure pattern in hypertensives with chronic renal failure. Renal Failure 1995;17:751;

Coussens L, Parker PJ & Rhee L. Multiple, distinct forms of bovine and human protein kinase C suggest diversity in cellular signaling pathways. Science 1986;233:859;

Dal Toso R, Sommer B, Ewert M, Herb A, Pritchett DB, Bach A, Shivers BD & Seeburg PH. The dopamine D2 receptor: two molecular forms generated by alternative splicing. EMBO J 1989;8:4025;

Darbon JM, Oury F, Clamens S & Bayard F. TPA induces subcellular translocation and subsequent down-regulation of both phorbol ester binding and protein kinase C activity. Biochem Biophys Res Comm 1987;146:537;

Dasarathy Y & Fanburg BL. Involvement of second messenger systems in stimulation of angiotensin converting enzyme of bovine endothelial cells. J Cell Physiol 1991; 148:327;

de Gasparo M, Whitebread S & Mele M. Biochemical characterization of two angiotensin II receptor subtypes in the rat. J Cardiovas Pharm 1990;suppl 4:S31;

de Gasparo M, Whitebread S, Kalenga MK, de Mertogh R, Crevoisier P & Thomas K. Down regulation of the angiotesin II receptor subtype AT2 in human myometrium during pregnancy. Regul Pet 1994;53:39;

Dearry A, Gingrich JA, Falardeau P, Fremeau RT Jr, Bates MD & Caron MG. Molecular cloning and expression of the gene for a human D1 dopamine receptor. Nature (Lond) 1990;347:72;

Dekker LV & Parker PJ. Protein kinase C - a question of specificity. Trends Biochem Sci 1994;19:73;

Delmas V, Laoide BM, Masquilier D, de Groot RP, Foulkes NS & Sassone-Corsi P. Alternative usage of initiation codons in mRNA encoding the cAMP-responsive-element modulator (CREM) generates regulators with opposite functions. Proc Natl Acad Sci USA 1992;89:4226;

Deutsch PJ, Hoeffler JP, Jameson L & Haberner JF. Cyclic AMP and phorbol ester-stimulated transcription mediated by similar DNA elements that bind distinct proteins. Proc Natl Acad Sci USA 1988;85:7922;

DiBona GF & Kopp UC. Neural control of renal function. Physiol Rev 1997;77:75;

DiBona GF. Neural control of renal function: role of renal alpha adrenoceptors. J Cardiovasc Pharmacol 1985;7suppl 8:S12;

DiBona GF. Renal neural activity in hepatorenal syndrome. Kidney Int 1984;25:841;

DiBona GF. Role of renal nerves in hypertension. Semin Nephrol 1991;11:503;

DiBona GF. The function of renal nerves. Rev Physiol Biochem Pharmacol 1982;94:75;

Dilts RP Jr, Helton TE & McGinty JF. Selective induction of Fos and FRA immunoreactivity within the mesolimbic and mesostriatal dopamine terminal field. Synapse 1993;13:251;

Divecha N, Banfic H & Irvine RF. The polyphosphoinositide cycle exists in the nuclei of Weiss 3T3 cells under the control of a receptor (for IGF-1) in the plasma membrane, and stimulation of the cycle increases nuclear diacylglycerol and apparently induces translocation of protein kinase C to the nucleus. EMBO J 1991;10:3207;

Dohlman H, Caron MG & Lefkowitz RF. A family of receptors coupled to guanine nucleotide regulatory proteins. Biochemistry 1987;26:2657;

Dohlman HG, Thomer J, Caron MC Lefkowitz RJ. Model systems for the study of seven-transmembrane-segment receptors. Annu Rev Biochem 1991;60:653;

Douglas JF. Estrogen effects on angiotensin receptors are modulated by pituitary in female rats. Am J Physiol 1987;252:F57;

Drew GM & Whiting SB. Evidence for two distinct types of postsynaptic alpha-adrenoceptor in vascular smooth muscle *in vivo*. Br J Pharma 1979;67:207;

Dwarki VJ, Montminy M & Verma IM. Both the basic region and the 'leucine zipper' domain of the cyclic AMP response element binding (CREB) protein are essential for transcriptional activation. EMBO J 1990;9:225;

Dzau VJ. Renal and circulatory mechanisms in congestive heart failure. Kidney Int 1987;31:1402; -b

Edwards RM. Effects of prostaglandins on vasoconstrictor action in isolated renal arterioles. Am J Physiol 1985;248:F779;

Egan TM, Henderson G, North RA & Williams JT. Noradrenaline-mediated synaptic inhibition in rat locus coeruleus neurons. J Physiol1983;345:477;

Eggena P, Zhu JH, Clegg K & Barrett JD. Nuclear angiotensin receptors induce transcription of renin and angiotensinogen mRNA. Hypertension 1993;22:496;

Ehmke H, Persson P, Fischer S, Hackenthal E & kirchheim H. Resetting of pressure-dependent renin release by intrarenal alpha1-adrenoceptors in conscious dog. Pfluegers Arch 1989;413:261;

Einhorn LC, Gregerson KA & Oxford GS. D2 dopamine receptor activation of potassium channels in identified rat lactotrophs: whole-cell and single-channel recording. J Neurosci 1991;11:3237;

Ellison DH, Velazquez H & Wright FS. Mechanisms of sodium, potassium and chloride transport by the renal distal tubule. Miner Electrolyte Metab 1987;6:422;

Enslen H, Tokumitsu H & Soderling TR. Phosphorylation of CREB by CaM-kinase IV activated by CaM-kinase IV kinase. Bioche Biophy Res Comm 1995;207:1038;

Esler M, Jennings G, Lambert G, Meredith I, Horne M & Eisenhofer G. Overflow of catecholamine neurotransmitters to the circulation: source, fate and function. Physiol Rev 1990;70:963;

Fabris B, Jackson B, Kohzuki M, Perich R & Johnston CI. Increased cardiac angiotensin-converting enzyme in rats with chronic heart failure. Clin Exp Pharm Physiol 1990;17:309;

Faraj AH, Lindop GBM & Morley AR. Three-dimensional reconstruction of human juxtaglomerular apparatus (JGA). APMIS 1992;100:29;

Faria FAC & Salgado MCO. Facilitation of noradrenergic transmission by angiotensin in hypertensive rats. hypertension 1992;19:II30;

Farquhar MG. The glomerular basement membrane: A selective macromolecular filter. In Hay ED (ed.). Cell biology of extracellular matrix. Plenum Press, New York, 1981:335;

Feinstein PG, Schrader KA, Bakalyar HA, Tang WJ, Kuprinski J, Gilman AG & Reed RR. Molecular cloning and characterization of a Ca2+/calmodulin-insensitive adenylyl cyclase from rat brain. Proc Natl Acad Sci USA 1991;88:10173;

Felder RA, Blecher M, Eisner GM & Jose PA. Cortical, tubular and glomerular dopamine receptors in the rat kidney. Am J Physiol 1984;246:F557;

Ferrario CM & Carretero OA. Hemodynamics of experimental renal hypertension. in de Jong W (ed): Handbook of hypertension, Volume 4: Experimental and genetic models of hypertension. Amsterdam, Elsevier Science Publishers, 1984:54;

Ferreri K, Gill G & Montaminy M. The cAMP-regulated transcription factor CREB interacts with a component of the TFIID complex. Proc Natl Acad Sci USA 1994;91:1210;

Fields AP, Pincus SM, Kraft AS & May WS. Interleukin-3 and bryostatin mediate rapid nuclear envelope protein phosphorylation in growth factor-dependent FDC-P1 hematopoietic cells. J Biol Chem 1989;264:21896;

Fields AP, Tyler G, Kraft AS & May WS. Role of nuclear protein kinase C in the mitogenic response to platelet-derived growth factor. J Cell Sci 1990;96:107;

Fink JS, Verhave M, Walton K, Mandel G & Goodman RH. Cyclic AMP- and phorbol ester-induced transcriptional activation are mediated by the same enhancer element in the human vasoactive intestinal peptide gene. J Biol Chem 1991;266:3882;

Fischer EH. Cellular regulation by protein phosphorylation. Bull L'Institut Pasteur 1983;81:7;

Foidart J, Sraer J, DeLarue F, Mahieu P & Ardaillou R. Evidence for mesangial glomerular receptors for angiotensin II linked to mesangial cell contractility. FEBS I 1980;121:333;

Folkow B, DiBona GF, Hjemdahl P, Thoren P & Wallin G. Measurements of plasma norepinephrine concentrations in human primary hypertension: a word of caution concerning their applicability for assessing neurogenic contributions. Hypertension Dallas 1983;5:399;

Forray C, Bard JA, Wetzel JM, Chui G, Shapiro E, Tang R, Lepor H, Hartig PR, Weinshank RL, Branchek TA & Gluchowski C. The α_1-adrenergic receptor that mediates smooth muscle contraction in human prostate has the pharmacological properties of the cloned human α_{1C} subtype. Mol Pharma 1994;45:703;

Foulkes NS & Sassone-Corsi P. More is better: activators and repressors from the same gene. Cell 1992;68:411; -a

Foulkes NS, Borrelli E & Sassone-Corsi P. CREM gene: use of alternative DNA binding domains generates multiple antagonists of cAMP-induced transcription. Cell 1991;64:739; -a

Foulkes NS, Laoide BM, Schlotter F & Sassone-Corsi P. Transcriptional antagonist CREM down-regulates c-fos cAMP-induced expression. Proc Natl Acad Sci USA 1991;88:5448; -b

Foulkes NS, Mellstrom B, Benusiglio E & Sassone-Corsi P. Developmental switch of CREM function during spermatogenesis: from antagonist to transcriptional activator. Nature 1992;355:80; -b

Fray JCS. Stretch receptor model for renin release with evidence from perfused rat kidney. Am J Physiol 1976;231:936;

Friedland J, Setton C & Silverstein E. Angiotensin converting enzyme induction by steroids in rabbit alveolar macrophages in culture. Science 1977;197:64;

Fujiwara Y, Kitamura E, Ueda N, Fukunaga M, Orita Y & Kamada T. Mechanism of action of angiotensin II on isolated rat glomeruli. Kidney Int 1989:36:985;

Fukamizu A, Takahashi S, Seo MS, Tada M, Tanemoto K, Mehana S & Murakami K. Structure and expression of the human angiotensinogen gene. J Biol Chem 1990;265:7576;

Ganten D, Kreutz R, Bader M & Wagner J. Progress in molecular medicine. From experimental genetics to the treatment of hypertensive patients. Chin Med J 1993;106:375;

Gao B & Gilman AG. Cloning and expression of a widely distributed (type IV) adenylyl cyclase. Proc Natl Acad Sci USA 1991;88:10178;

Gao B & Kunos G. Transcription of the rat α_{1B}-adrenergic receptor gene in liver is controlled by three promoters. J Biol Chem 1994;269:15762;

Gellersen B, Kempf R & Telgmann R. Human endometrial stromal cells express novel isoforms of the transcriptional modulator CREM and up-regulate ICER in the course of decidulization. Mol Endo 1997;11:97;

Gerfen CR, Engber TM, Susel Z, Chase TN, Mahan LC, Monsma FJ Jr & Sibley DR. D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. Science 1990;250:1429;

Gibbons GH, Pratt RE & Dzau VJ. Vascular smooth muscle cell hypertrophy vs hyperplasia. Autocrine transforming growth factor-b1 expression determines growth response to angiotensin II. J Clin Invest 1992;90:456;

Giros B, Sokoloff P, Martres MP, Riou JF, Emorine LJ & Schwartz JC. Alternative splicing directs the expression of two D2 dopamine receptor isoforms. Nature (Lond) 1989;342:923;

Gleason MM & Hieble JP. The alpha 2-adrenoceptors of the human retinoblastoma cell line (Y79) may represent an additional example of the alpha 2C-adrenoceptor. Brit J Pharm 1992;107:222;

Gonzalez GA & Montminy MR. Cyclic AMP stimulates somatostatin gene transcription by phsphorylation of CREB at serine 133. Cell 1989;59:675; -a

Gonzalez GA, Yamamoto KK & Fischer WH. A cluster of phsphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. Nature 1989;337:749; -b

Goodman RH. Regulation of neuropeptide gene expression. A Rev Neurosci 1990;13:111;

Grandy DK, Zhang Y, Bouvier C, Zhou QY, Johnson RA, Allen L, Buck K, Bunzow JR, Salon J & Civelli O. Multiple human D5 dopamine receptor genes: a functional receptor and two pseudogenes. Proc Natl Acad Sci USA 1991;88:9175;

Griendling KK, Rittenhouse SE, Brock TA, Ekstein LS, Gimbrone MA Jr & Alexander RW. Sustained diacylglycerol formation from inositol phospholipids in angiotensi II-stimulated vascular smooth muscle cells. J Biol Chem 1986;261:5901;

Guyer CA, Horstman DA, Wilson AL, Clark JD, Cragoe EJ Jr & Limbird LE. Cloning, sequencing, and expression of the gene encoding the porcine α_2-adrenergic receptor. J Biol Chem 1990;265:17307;

Hafdi Z, Couette S, Cornoy E, Prie D, Amiel C & Friedlander C. Locally formed 5-Hydroxytryptamine stimulates phosphate transport in cultured opossum kidney cells and in rat kidney. Biochem J 1996;320:615;

Hai TW, Liu F, Coukos WJ & Green MR. Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. Genes Dev 1989;3:2083;

Haller H, Quass P, Lindschau C, Luft FC & Distler A. Platelet-derived growth factor and angiotensin II induce different spatial distribution of protein kinase C-α and -ß in vascular smooth muscle cells. Hypertension 1994;23:848;

Hanley MR. Molecular and cell biology of angiotensin receptors. J Car Phar 1991;18suppl 2:S7;

Hardman JA, Hort YJ, Catanzaro DF, Tellan JT, Baxter JD, Morris BJ & Shine J. Primary structure of the human renin gene. DNA 1984;3:457;

Harris PJ, Young JA. Dose-dependent stimulation and inhibition of proximal tubule sodium reabsorption by angiotensin II in the rat kidney. Pflugers Arch 1977;367:295;

Healy DP, Munzel PA & Insel PA. Localization of $ß_1$- and $ß_2$-adrenergic receptors in rat kidney by autoradiography. Circ Res 1985;57:278;

Hepler JR & Gilman AG. G proteins. Trends Biche Sci 1992;17:383;

Hervé D, Lévi-Strauss M, Marey-Sempler I, Verney C, Tassin JP, Glowinski J & Girdult JA. G_{olf} and G_s in rat basal ganglia: possible involvement of G_{olf} in the coupling of dopamine D1 receptor with adenylyl cyclase. J Neurosci 1993;13:2237;

Heyduk T, Lee JC, Ebright YW, Blatter EE, Zhou Y & Ebright RH. CAP interacts with RNA polymerase in solution in the absence of promoter DNA. Nature 1993;364:548;

Hocevar BA, Morrow DM, Tykocinski ML & Fields AP. Protein kinase C isotypes in human erythroleukemia cell proliferation and differentiation. J Cell Sci 1992;101:671;

Hoeffler JP, Meyer TE, Yun Y, Jameson JL & Habener JF. Cyclic AMP-responsive DNA-binding protein: Structure based on a cloned placental cDNA. Science 1988;242:1430;

Hollenberg NK, Adams DF, Solomon H, Chenitz WR, Burger BM, Abrams HL & Merill JP. Renal vascular tone in essential and secondary hypertension. Medicine 1975;54:29;

Holmer S, Rinne B, Eckardt KU & Kurtz A. Role of renal nerves for the expression of renin in adult rat kidney. Am J Physiol 1994;266:F738;

Hong-Brown LQ & Deschepper CF. Effects of thyroid hormones on angiotensinogen gene expression in rat liver, brain, and cultured cells. Endocrinology 1992;130:1231;

Horn JP & McAffee DA. Alpha-adrenergic inhibition of calcium-dependent potentials in rat sympathetic neurons. J Physiol 1980;301:191;

Hsueh W & Baxter JD. Human prorenin. Hypertension 1991;17:469;

Hubert C, Hoot AM, Cormol P & Soubrier F. Structure of the angiotensin I-converting enzyme gene. Two alternate promoters correspond to evolutionary steps of a duplicated gene. J Biol Chem 1991;266:15377;

Hug H & Sane TF. Protein kinase C isozymes: divergence in signal transduction? Biochem J 1993;291:329;

Humphreys MH. Mechanisms and management of nephrotic edema. Kidney Int 1994;45:266;

Ichihara A, Suzuki H, Murakami M, Naitoh M, Matsumoto A & Saruta T. Interactions between angiotensin II and norepinephrine on renin release by juxtaglomerular cell. Euro J Endocri 1995;133:569;

Ikemoto F, Song G & Tominaga M. Angiotensin converting enzyme predominates in the inner cortex and medulla of the rat kidney. Biochem Biophys Res Comm 1987;144:915;

Ingelfinger JR, Pratt RE & Ellison KE. Sodium regulation of angiotensinogen mRNA expression in rat kidney cortex and medulla. J Clin Invest 1986;78:1311;

Ingelfinger JR, Zau WM & Fon EA. In situ hybridization evidence for angiotensinogen mRNA in the rat proximal tubule. J Clin Invest 1990;85:417;

Insel PA. Adrenergic receptors. Evolving concepts on structure and function. Am J Hypert 1989;2:112S;

Ishikawa Y, Katsushika S, Chen L, Halnon NJ, Kawabe J & Homcy CJ. Isolation and characterization of a novel cardiac adenylylcyclase cDNA. J Biol Chem 1992;267:13553;

Iwao H, Fukui K, Kim S, Nakayama K, Ohkubo S, Nakanishi S & Abe Y. Sodium balance effects on renin angiotensinogen and atrial natriuretic peptide mRNA levels. Am J Physiol 1988;255:E129;

Iyengar R. Molecular and functional diversity of mammalian Gs-stimulated adenylyl cyclases. FASEB J 1993;7:768;-b

Iyengar R. Multiple families of Gs-regulated adenylyl cyclases. Adv in Sec Mes Phos Res 1993;28:27-a

Jackson TR & Hanley MR. Tumor promoter 12-O-tetradecanoylphobol 13-acetate inhibits mas/angiotensin receptor-stimulated inosito phosphate production and intracellular Ca^{2+} elevation in the 401L-C3 neuronal cell line FEB 1989;251:27;

Jacobowitz O & Iyengar R. Phorbol ester-induced stimulation and phosphorylation of adenylyl cyclase 2. Proc Natl Acad Sci USA 1994;91:10630;

Jacobowitz O, Chen J, Premont RT & Iyengar R. Stimulation of specific types of Gs-stimulated adenylyl cyclases by phorbol ester treatment. J Biol Chem 1993;268:3829;

Jacobs WR & Chan YL. Evidence for the presence of functional beta-adrenoceptor along the proximal tubule of the rat kidney. Biochem Biophys Res Commun 1986;141:334;

Jewell-Motz EA & Liggett SB. G protein-coupled receptor kinase specificity for phosphorylation and desensitization of alpha2-adrenergic receptor subtypes. J Biol Chem 1996;271:18082;

Jiang HP, Wu D & Simon MI. Activation of phospholipase C beta 4 by heterotrimeric GTP-binding proteins. J Biol Chem 1994;269:7593;

Johnson MD, Freese JW & Schmitt DE. Effects of an ß-adrenoceptor agonist, prenalterol, on renal function and renin secretion rate in anesthetized dogs. J Cardiov Pharm 1984;6:627;

Jones N. Transcriptional regulation by dimerization: two sides to an incestuous relationship. Cell 1990;61:9;

Jose PA, Felder RA, Robillard JE, Felder CC & Eisner GM. Dopamine-2 receptor in the canine kidney (abstract) Kidney Int 1986;29:385;

Jose PA, Raymond JR, BAtes MD, Aperia A, Felder RA & Carey RM.The renal dopamine receptors. J Am Soc Nephrol 1992;8:1265;

Kageyama R, Ohkubo H & Nakanishi S. Induction of rat liver angiotensinogen mRNA following acute inflammation. Biochem Biophys Res Comm 1985;129:826;

Kalinyak JE & Perlman AJ. Tissue-specific regulation of angiotensinogen mRNA accumulation by dexamethasone. J Biol Chem 1987;262:460;

Kaprinski BA, Morle GD, Huggenvik J, Uhler MD & Leiden JM. Molecular cloning of human CREB-2: an ATF/CREB transcription factor than can negatively regulate transcription from the cAMP response element. Proc Natl Acad Sci USA 1992;89:4820;

Karin M & Smeal T. Control of transcription factors by signal transduction pathways: the beginning of the end. Trends Biochem Sci 1992;17:418;

Katholi RE, Carey RM, Ayers CR, Vaughan ED, Yancey MR & Morton CL. Production of sustained hypertension by chronic intrarenal norepinephrine infusion in conscious dogs. Circ Res 1977;40 suppl I:118;

Katsushika S, Chen L, Kawabe JI, Nilakantan R, Halnon NJ, Homcy CJ & Ishikawa Y. Cloning and characterization of a sixth adenylyl cyclase isoform: types V and VI constitute a subgroup within the mammalian adenylyl cyclase family. Proc Natl Acad Sci USA 1992;89:8774;

Katz AI, Doucet A & Morel F. Na-K-ATPase activity along the rabbit, rat, and mouse nephron. Am J Physiol 1979;237:F114;

Kawasaki H, Cline WH Jr, & Su C. Involvement of the vascular renin-angiotensin system in beta adrenergic receptor-mediated facilitation of vascular neurotransmission in spontaneously hypertensive rats. J Pharma Exp Ther 1984;231:23;

Kebabian JW & Calne DB. Multiple receptors for dopamine. Nature 1979;277:93;

Keeton TK & Campbell WB. The pharmacologic alteration of renin release. Pharmacol Rev 1980;32:81;

Kett C, Hellmann W, Hackenthal E & Ganten D. Modulation of tissue angiotensinogen gene expression by glucocorticoids, estrogens, and androgens in SHR and WKY rats. Clin Exp Hypertens 1993;15:683;

Khairallah PA, Davila D, Papanicolaou N, Gende NM & Meyer P. Effects of angiotensin infusion on catecholamine uptake and reactivity in blood vessels. Cir Res 1971;28:96;

Kifor I & Dzau VJ. Endothelial renin-angiotensin pathway: Evidence for intracellular synthesis and secretion of angiotensins. Circ Res 1987;60:422;

Kinne R, Schmitz JE & Kinne-Saffran E. The localization of the Na^+-K^+-ATPase in the cells of rat kidney cortex: A study on isolated plasma membrane. Pflügers Arch 1971;329:191;

Kiuchi K, Vatner DE, Uemura N, Bigaud M, Hasebe N, Hempel DM, Graham RM & Vatner SF. Mechanisms of alpha 1-adrenergic vascular desensitization in conscious dogs. Circ Res 1992;71:1185;

Knepper MA. Urea transport in isolated thick ascending limbs and collecting ducts from rats. Am J Physiol 1983;245:F634;

Kobilka BK, Matsui H, Kobilka TS, Yang-Feng TL, Francke U, Caron MG, Lefkowitz RJ & Regan JW. Cloning, sequencing, and expression of the gene coding for the human platelet α_2-adrenergic receptor. Science 1987;238:650;

Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, Marmé D & Rapp UR. Protein kinase Cα activates RAF-1 by direct phosphorylation. Nature 1993;364:249;

Kottke FJ, Kubicek WG & Visscher MB. The production of arterial hypertension by chronic renal artery nerve stimulation. Am J Physiol 1945;145:38;

Kraft AS, Anderson WB, Cooper L & Sando JJ. Decrease in cytosolic calcium/phospholipid-dependent protein kinase activity following phorbol ester treatment of EL4 thymoma cells. J Biol Chem 1983;257:13196;

Krakoff LR. Measurement of plasma-renin substrate by radioimmunoassay of angiotensin I: concentration in syndromes associated with steroid excess. J Clin Endoc Metab 1973;37:110;

Krug E. Beitmann HP & Tashjian AH Jr. Evidence for increased synthesis as well as increased degradation of protein kinase C after treatment of human osteosarcoma cells with phorbol ester. J Biol Chem 1987;262:11852;

Krulewitz AK & Fanburg BL. Stimulation of bovine endothelial cell angiotensin converting enzyme activity by cAMP related agents. J Cell Physiol 1986;129:147;

Krupinski J, Coussn F, Bakalyar HA, Tank WJ, Feinstein PG, Orth K, Slaughter C, Reed RR & Gilman AG. Adenylyl cyclase amino acid sequence: Possible channel- or transporter-like structure. Science 1989;244:1558;

Kudo K, Kondo Y, Abe K, Igarashi Y, Tada K & Yoshinaga K. Evidence for presence of functional ß-adrenoceptor in rabbit S_2 proximal straight tubules. Am Physiol Soc 1991;261:F393;

Kurose H & Lefkowitz RJ. Differential desensitization and phosphorylation of three cloned and transfected α_2-adrenergic receptor subtypes. J Biol Chem 1994;269:10093;

Kwok RPS, Lundblad JR, Chrivia JC, Richards JP, Bächinger HP, Brennan RG, Roberts SGE, Green MR & Goodman RH. Nuclear protein CBP is a coactivator for the transcription factor CREB. Nature 1994;370:223;

La Grange RG, Sloop CH & Schmid HE. Selective stimulation of renal nerves in the anesthetized dog. Circ Res 1973;33:704;

LaHoste GJ, Yu J & Marshall JF. Striatal Fos expression is indicative of dopamine D1/D2 synergism and receptor supersensitivity. Proc Natl Acad Sci USA 1993;90:7451;

Lamers WH, Hanson RW & Meisner HM. cAMP stimulates transcription of the gene for cytosolic phosphoenolpyruvate carboxykinase in rat liver nuclei. Proc Natl Acad Sci USA 1982;79:5137;

Landshulz WH, Johnson PF & McKnight SL. The leucine zipper: A hypothetical structure common to a new class of DNA binding proteins. Science 1988;240:1759;

Lanier SM, Downing S, Duzic E & Homcy CJ. Isolation of rat genomic clones encoding subtypes of the α_2-adrenergic receptor. J Biol Chem 1991;266:10470;

Laoide BM, Foulkes NS, Schlotter F & Sassone-Corsi P. The functional versatility of CREM is determined by its modular structure. EMBO J 1993;12:1179;

Leach KL, Ruff VA, Jarpe MB, Adams LD, Fabbro D & Raben DM. α-thrombin stimulates nuclear diglyceride levels and differential nuclear localization of protein kinase C isozymes in IIC9 cells. J Biol Chem 1992;267:21816;

Lefkowitz RJ, Cotecchia S, Kjelsberg MA, Pitcher J, Koch WJ, Inglese J & Caron MG. Adrenergic receptors: recent insights into their mechanism of activation and desensitization. Adv Second Messen Phosphop Res 1993;28:1;

Lenormand P, Sardet C, Pagès G, L'Allemain G, Brunet A & Pouysségur J. Growth factors induce nuclear translocation of MAP kinases ($p42^{mapk}$ and $p44^{mapk}$) but not of their activator MAP kinase kinase ($p45^{mapkk}$) in fibroblasts. J Cell Biol 1993;122:1079;

Leprêtre N, Mironneau J & Morel JL. Both α_{1A}- and α_{2A}-adrenoceptor subtypes stimulate voltage-operated L-type calcium channels in rat portal vein myocytes. J Biol Chem 1994;269:29546;

Liggett SB, Ostrowski J, Chesnut LC, Kurose H, Raymond JR, Caron MG & Lefkowitz RJ. Sites in the third intracellular loop of the alpha 2A-adrenergic receptor confer short term agonis-promoted desensitzation. Evidence for a receptor kinase-mediated mechanism. J Biol Chem 1992;267:4740;

Limbird LE & Sweatt JD. (in) The receptors. (ed) Conn M. Academic Press, Orlando, FL. 1986;Vol II:281;

Lindpainter K, Jin M, Nidermaier N, Wilhelm MJ & Ganten D. Cardiac angiotensinogen and its local activation in the isolated perfused beating heart. Circ Res 1990;67:564;

Liu FY, Cogan MG. Angiotensin II stimulation of hydrogen ion secretion in the rat early proximal tubule: Modes of action, mechanisms, and kinetics. J Clin Invest 1988;2:601;

Liu M & Simon MI. Regulation by cAMP-dependent protein kinase of a G-protein-mediated phospholipse C. Nature 1996;382:83;

Lokhandwala MF & Amenta F. Anatomical distribution and function of dopamine receptors in the kidney. FASEB J 1991;15:3023;

Lokhandwala MF. Cadiovascular and renal effects of dopamine receptor agonists. ISI Atlas of Science: Pharmacology 1988;2:261;

Lomasney JW, Cotecchia S, Lorenz, W, Leung WY, Schwinn DA, Yang-Feng TL, Brownstein M, Lefkowitz RJ & Caron MG. Molecular cloning and expression of the cDNA for the α_{1A}-adrenergic receptor. J Biol Chem 1991;266:6365;

Lomasney JW, Lorenz W, Allen LF, King K, Regan JW, Yang-Feng TL, Caron MG & Lefkowitz RJ. Expansion of the α_2-adrenergic receptor family: cloning and characterization of a human α_2-adrenergic receptor subtype, the gene for which is located on chromosome 2. Proc Natl Acad Sci USA 1990;87:5094;

Lote CJ.Principles of renal physiology. Chapman & Hall, London. 1994;

Lu D, Yu K, Paddy MR, Rowland NE & Raizada MK. Regulation of norepinephrine transport system by angiotensin II in neuronal cultures of normotensive and spontaneously hypertensive rat brains. Endocrinology 1996;137:763;

Lundblad JR, Kowk RPS, Laurance ME, Harter ML & Goodman RH. Adenoviral E1A-associated protein p300 as a functional homologue of the transcriptional co-activator CBP. Nature 1995;374:85;

Luttrell LM, Ostrowski J, Cotecchia S, Kendall H & Lefkowitz RJ. Antagonism of catecholamine expression of cytoplasmic domains of the receptors. Science 1993;259:1453;

MacNulty EE, McClue SJ, Carr IC, Jess T, Wakelam MJO & Milligan G. α_2-C10 adrenergic receptors expressed in Rat 1 fibroblasts can regulate both adenylylcyclase and phospholipase D-mediated hydrolysis of phosphatidylcholine by interacting with pertussis toxin-sensitive guanine nucleotide-binding proteins. J Biol Chem 1992;267:2149;

Madri JA, Roll FJ, Furthmayr H & Foidart JM. Ultrastructural localization of fibronectin and laminin in the basement membranes of the murine kidney. J Cell Biol 1980;86:682;

Mahan LC, Burch RM, Monsma FJ & Sibley DR. Expression of striatal D1 dopamine receptors coupled to inositol phosphate production and Ca^{2+} mobilization in Xenopus oocytes. Proc Natl Acad Sci USA 1990;87:2196;

Mahan LC, McKernan RM & Insel PA. Metabolism of alpha- and beta-adrenergic receptors in vitro and in vivo. Ann Rev Pharma Tox 1987;27:215;

Mann JF, Rascher W, Schomig A, Buu T, Kuchel O, Boucher R & Genest J. Contribution of the sympathetic nervous system to the centrally-induced pressor action of angiotensin II in rats. Clin Exp Pharmacol Physiol 1982;2:193;

Marchetti J, Rouseau S, Alhenc-Gelas F. Angiotensin I converting enzyme and kinin-hydrolyzing enzyme along the rabbit nephron. Kidney Int 1987;31:744;

Martin KC, McConkey CL, Jacob AK, Gonzalez EA, Khan M & Baldassar JJ. Effect of U73122, an inhibitor of phospholipase C on actions of parathyroid hormone in opossum kidney cells. Am J Physiol 1994:266:F254;

Martin KJ, McConkey CL, Jacob AK, Gonzalez EA, Khan M & Baldassare JJ. Effect of U-73122, an inhibitor of phospholipase C on actions of parathyroid hormone in opossum kidney cells. Am J Physiol 1994;266:F254;

Maurer RA. Both isoforms of the cAMP-dependent protein kinase catalytic subunit can activate transcription of the prolactin gene. J Biol Chem 1989;264:6870;

McCaa RE, Guton AC & Young DB. Role of angiotensin II in the regulation of aldosterone biosynthesis. (in) Johnson JA & Anderson RR (eds). The Renin-Angiotensin System. New York, Plenum Publishing Corp. 1980:227;

McCaa RE. Role of the renin-angiotensin system in the regulation of aldosterone biosynthesis and arterial pressure during sodium deficiency. Circ Res 1977;40(supp):157;

McGehee RE Jr & Habener JF. Differentiation-specific element binding protein (DSEB) binds to a defined

element in the promoter of the angiotensinogen gene required for the irreversible induction of gene expression during differentiation of 3T3-L1 adipoblasts to adipocytes. Mol Endo 1995;9:487;

McGehee RE, Ron D, Brasier AR & Habener JF. Differentiation-specific element: a cis-acting developmental switch required for the sustained transcriptional expression of the angiotensinogen gene during hormonal-induced differentiation of 3T3-L1 fibroblasts to adipocytes. Mol Endo 1993;7:551;

Meador-Woodruff JH, Mansour A, Healy DJ, Kuehn R, Zhou QY, Bunzow JR, Akil H, Civelli O, & Watson SJ. Comparison of the distributions of D1 and D2 dopamine receptor mRNAs in rat brain. Neuropsychopharmacology 1991;5:231;

Meister B, Holgert H, Aperia A & Hökfelt T. Dopamine D1 receptor mRNA in rat kidney: localization by in situ hybridization. Acta Physiol Scand 1991;4:447;

Menard J, Clauser E, Bounik J & Corvol P. Angiotensinogen: biochemistry. (in) The Renin Angiotensin System. (ed) Robertson JIS & Nichols MG. Gower Medical Publishing, London 1993:8.1;

Meyer TE & Habener JF. Cyclic adenosine 3',5'-monophosphate response element binding protein (CREB) and related transcription-activating deoxyribonucleic acid-binding proteins. Endo Rev 1993, 14:269;

Michell RH, Kirk CJ, Jones LM, Downes C & Creba JA. The stimulation of inositol lipid metabolism that accompanies calcium mobilization in stimulated cells: defined characteristics and unanswered questions. Phyl Trans Roy Soc Lond [Biol] 1981;296:123;

Ming M, Wang TT, Lanchance S, Delalandre A, Carriere S & Chan JSD. Expression of the angiotensinogen gene by 8-bromo-cAMP and dexamethasone in opossum kidney proximal tubular cells. Am J Physiol 1995;268:R105;

Minowa MT, Minowa T, Monsma FJ Jr, Sibley DR & Mouradian MM. Characterization of the 5' flanking region of the human D_{1A} dopamine receptor gene. Proc Natl Acad Sci USA 1992;89:3045;

Moe OW, Alpern RJ, Henrich WL. The renal proximal tubule renin-angiotensin system. Sem Nephro 1993;13:552; -a

Moe OW, Ujiie K, Star RA. Renin expression in renal proximal tubule. J Clin Invest 1993;91:774; -b

Moffat DB. The fine structure of the blood vessels of the renal medulla with particular reference to control of the medullary circulation. J Ultrastruct Res 1967;216:693;

Molina CA, Foulkes NS, Lalli E & Sassone-Corsi P. Inducibility and negative autoregulation of CREM: an alternative promoter directs the expression of ICER, an early response repressor. Cell 1993;75:875;

Montminy MR & Bilezikjian LM. Binding of nuclear protein to the cyclic-AMP response element of the somatostatin gene. Nature 1987;328:175;

Montminy MR, Gonzalez GA & Yamamoto KK. Characteristics of the cAMP response Unit. Metabolism 1990;39suppl2:6;

Montminy MR, Low MJ, Tapia-Arancibia L, Reichlin S, Mandel G & Goodman RH. Cyclic AMP regulates somatostatin mRNA accumulation in primary diencephalic cultures and in transfected fibroblast cells. J Neurosci 1986;6:1171; -a

Montminy MR, Sevarino KA, Wagner JA, Mandel G & Goodman RH. Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. Proc Natl Acad Sci USA 1986;83:6682; -b

Morimoto BH & Koshland DE Jr. Conditional activation of cAMP signal transduction by protein kinase C. J Biol Chem 1994;269:4065;

Morris BJ, Catanzaro DF, Hardman J, Mesterovic N, Tellam J, Hort Y, Bennetts BH & Shine J. Structure of human renin and expression of the renin gene. Clin Exp Pharmacol Physiol 1984;11:369;

Morris GM, Hadcock JR & Malbon CC. Cross-regulation between G-protein-coupled receptors. Activation of beta 2-adrenergic receptors increases alpha 1-adrenergic receptor mRNA levels. J Biol Chem 1991;266:2233;

Morrow AL & Creese I. Characterization of α_1-adrenergic receptor subtypes in rat brain: a reevaluation of [^{3}H]WB4104 and [^{3}H] prazosin binding. Mol Pharma 1986;29:321;

Mujais SK, Kauffman S & Katz AI. Angiotensin II binding sites in individual segments of the rat nephron. J Clin Invest 1986;77:315;

Muller U, Roberts MP, Engel DA, Doerfler W & Shenk T. Induction of transcription factor AP-1 by adenovirus E1A protein and cAMP. Genes & Dev 1989;3:1991;

Mullins JJ, Burt DW, Windass JD, MTurk P, George H & Brammar WJ. Molecular cloning of two distinct renin genes from the DBA/2 mouse. EMBO J 1982;1:1461;

Mullins JJ, Peters J & Ganten D: Fulminate hypertension in transgenic rats harbouring the mouse Ren-2 gene. Nature 1990;344:541;

Murphy TJ & Bylund DB. Characterization of alpha-2 adrenergic receptors in the OK cell, an opossum kidney cell line. J Pharma Exp Ther 1988;244:571;-a

Naftilan AJ, Gilliland GK, Eldridge CS & Kraft AS. Induction of the proto-oncogen c-jun by angiotensin II. Mol Cell Biol 1990;10:5536;

Nakade S, Rhee SK, Hamanaka H & K Mikoshiba. Cyclic AMP-dependent phosphorylation of an immunoaffinity-purified homotetrameric inositol 1,4,5-trisphosphate receptor (type I) increases Ca^{2+} flux in reconstituted lipid vesicles. J Biol Chem 1994;269:6735;

Nakamura A & Johns EJ. Effect of renal nerves on expression of renin and angiotensinogen genes in rat kidneys. Am J Physiol 1994;266:E230;

Nakamura A & Johns EJ. Influence of the renal sympathetic nerves on renal renin and angiotensinogen gene expression in spontaneously hypertensive rats during developments. J Hypertens 1995;13:301;-b

Nakamura A & Johns EJ. Renal nerves, renin, and angiotensinogen gene expression in spontaneously hypertensive rats. Hypertension 1995;25pt1:581;-a

Nash SR, Godinot N & Caron MG. Cloning and characterization of the opossum kidney cell D_1 dopamine receptor: expression of identical D_{1A} and D_{1B} dopamine receptor mRNAs in opossum kidney and brain. Mol Pharmacol 1993;44:918;

Navar LG & Rosivall L. Contribution of the renin-angiotensin system to the control of intrarenal hemodynamics. kidney Int 1984;25:857;

Navar LG, Carmines PK, Huang WC & Mitchell KD. The tubular effects of angiotensin II. Kidney Int 1987;31:(suppl20)S81;

Neer E. Heterotrimeric G proteins: organizers of transmembrane signals. Cell 1995;80:249;

Neve KA, Henningsen RA, Bunzow JR & Civelli O. Functional characterization of a rat dopamine D-2 receptor cDNA expressed in a mammalian cell line. Mol Pharmacol 1989;36:446;

Newton AC. Interaction of proteins with lipid headgroups: Lessons from protein kinase C. 1993;22:1;

Nfakaoka H, Perez DM, Baek KJ, Das T, Husain A, Misono K, Im MJ, Graham RM. G_h: a GTP-binding protein with transglutaminase activity and receptor signaling function. Science 1994;264:5165;

Ng GY, Mouillac B, George SR, Caron M, Dennis M, Bouvier M & O'Dowd BF. Desensitization, phosphorylation and palmitoylation of the human dopamine D1 receptor. Eur J Pharmacol 1994:267:7;

Nguyen TV, Kosfsky BE, Birnbaum R, Cohen BM & Hyman SE. Differential expression of c-fos and zif268 in rat striatum after haloperidol, clozapine, and amphetamine. Proc Natl Acad Sci USA 1992;89:4270;

Nichols M, Weih F, Schmid W, DeVack C, Kowenz-Leutz E, Lukow B, Boshart M & Schütz G. Phosphorylation of CREB affects its binding to high and low affinity sites: implications for cAMP induced gene transcription. EMBO J 1992;11:3337;

Nieuwland R, Van Willigen G & Akkerman JWN. 4,4'-Di-isothiocyanatostilbene-2,2'disulphonic acid ('DIDS') activates protein kinase C and Na^+/H^+ exchange in human platelets via alpha 2A-adrenergic receptors. Biochem J 1993;293:523;

Nieuwland R, Wijburg OLC, van Willigen G & Akkerman JWN. α_{2A}-Adrenergic receptors activate protein kinase C in human platelets via a pertussis toxin-sensitive G-protein. FEBS L 1994;339:79;

Niewstead J & Munkacsi I. Electron microscopic observations on the juxtamedullary efferent arterioles and arteriolae rectae in kidneys of rats. Z Zellforsch 1969;97:465;

Nishida Y, Ryan KL & Bishop VS. Angiotensin II modulates arterial baroreflex function via a central alpha(1)-adrenoceptor mechanism in rabbits. Am J Physiol 1995;38:R1009;

Nishizuka Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 1992;258:607;

Nishizuka Y. Studies and perspectives of protein kinase C. Science 1986;233:305;

Nishizuka Y. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 1988;334:661;

Nishizuka Y. The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature 1984;308:693;

O'Hare JP, Roland JM, Walters G & Corrall RJM. Impaired sodium excretion in response to volume expansion induced by water immersion in insulin dependent diabetes mellitus. Clin Sci 1986;71:403;

O'Malley KL, Harmon S, Tang L & Todd RD. The rat dopamine D4 receptor: sequence, gene structure, and demonstration of expression in the cardiovascular system. New Biol 1992;4:137;

Owens GK & Reidy MA. Hyperplastic growth response of vascular smooth muscle cells following induction of acute hypertension in rats by aortic coarctation. Cir Res 1985;57:695;

Page IH & McCubbin JW (eds). Renal Hypertension. Chicago, Year Book Medical Publishers, 1968;

Page WV, Pearlman S, Smith FG, Segar JL & Robillard JE. Renal nerves modulate kidney renin gene expression during transition from fetal to newborn life. Am J Physiol 1992:262:R459;

Peach MJ. Adrenal medullary stimulation induced by angiotensin I, angiotensi II and analogues. Circ Res 1971;28/29:II107;

Peach MJ. Renin-angiotensin system: biochemistry and mechanisms of action. Physiol Rev. 1977;57: 313;

Pease DC. Myoid features of renal corpuscles and tubules. J Ultrastruct Res 1968; 23:304;

Perez DM, DeYoung MB & Graham RM. Coupling of expressed α_{1B}-adrenergic receptors and α_{1D}-adrenergic receptors to multiple signaling pathways is both G protein and cell type specific. Mol Pharma 1993;44:784;

Perrichot R, Garcina-Ocana A, Couette S, Comoy E, Amiel C & Friedlander C. Locally formed dopamined modulates renal Na-Pi cotransport through DA_1 and DA_2 receptors. Biochem J 1995;312:433;

Petrovic T, Anderson WP & Bell C. Neuronal and nonneuronal contributions to renal catecholamine content in the dog. J Neurochem 1986;47;423;

Pfeilschifter J, Kurtz A & Bauer C. Role of phospholipase C and protein kinase C in vasoconstrictor-induced prostaglandin synthesis in cultrued renal mesangial cells. Biochem J 1986;234:125;

Piomelli D, Pilon C, Giros B. Dopamine activation of the arachidonic acid cascade as a basis for D1/D2 receptor synergism. Nature 1991;353:164;

Pitcher JA, Ingless J, Higgins JB, Arriza JL, Casey PJ, Kim C, Benovic JL, Kwatra MM, Caron MG & lefkowitz RJ. Role of βγ subunits of G proteins in targeting the ß-adrenergic receptor kinase to membrane-bound receptors. Science 1992;257:1264;

Plato CF & Osborn JL. Chronic renal neuroadrenergic hypertension is associated with increased renal norepinephrine sensibility and volume contraction. Hypertension 1996;28:1034;

Pobiner BF, Hewlett EL & Garrison JC. Role of Ni in coupling angiotensin receptors to inhibition of adenylate cyclase in hepatocytes. J Biol Chem 1985;260:16200;

Pratt WB, Scherrer LC, Huchison KA & Dalman FC. A model of glucocorticoid receptor unfolding and stabilization by a heat shock protein complex. J Steroid Biochem Mol Biol 1992;41:223;

Premont RT, Chen J, Ma HW, Ponnapalli M & Iyengar R. Two members of a widely expressed subfamily of hormone-stimulated adenylyl cyclases. Proc Natl Acad Sci USA 1992;89:9809;

Pujol MJ, Soriano M, Aligué R, Carafoli E & Bachs O. Effect of alpha-adrenergic blockers on calmodulin association with the nuclear matrix of rat liver cells during proliferative activation. J Biol Chem 1989;264:18863;

Qian JF, Wang TT, Wu XH, Wu J, Chang G, Lachance S, Carriere S & Chan JSD. Angiotensinogen gene expression is stimulated by the cAMP-responsive element binding protein in opossum kidney cells. J Am Soc Nephrol 1997;8:1072;

Rana RS, Hokin LE. Role of phosphoinositides in transmembrane signaling. Physiol Rev 1990;70:115;

Rannels SJ & Corbin JD. Characterization of small cAMP-binding fragments of cAMP-dependent protein kinases. J Biol Chem 1979;254:8605;

Rasmussen H. The calcium messenger system (Part I). N Engl J Med 1986;314:1094; -a

Rasmussen H. The calcium messenger system (Part II). N Engl J Med 1986;314:1164; -b

Re R. The myocardial intracellular renin-angiotensin system. Am J Cardiol 1987;59:56A;

Regan JW, Kobilka TS, Yang-Feng TL, Caron MG, Lefkowitz RJ & Kobilka BK. Cloning and expression of a human kidney cDNA for an α_2-adrenergic receptor subtype. Proc Natl Acad Sci USA. 1988;85:6301;

Rehfuss RP, Walton KM, Loriaux MM & Goodman RH. The cAMP-regulated enhancer-binding protein ATF-1 activates transcription in response to cAMP-dependent protein kinase A. J Biol Chem 1991;266:18431;

Reid IA. Actions of angiotensin II on the brain: Mechanisms and physiologic role. Am J Physiol 1984;246:F533;

Reid IA. The renin-angiotensin system and body function. Arch Intern Med 1985;145:1475;

Reinhart GA, Lohmeier TE & Hord CE. Hypertension induced by chronic renal adrenergic stimulation is angiotensin dependent. Hypertension Dallas 1995;25:940;

Rhee SG & Choi KD. Multiple forms of phospholipase C isozymes and their activation mechanisms. Adv Sec Mess Phosp Res 1992;26:35;-b

Rhee SG & Choi KD. Regulation of inositol phospholipid-specific phospholipase C isozymes. J Biol Chem 1992;267:12393;-a

Rhee SG, Suh PG, Ryu SH, Lee SY. Studies of inositol phospholipid-specific phospholipase C. Science 1989;244:546;

Ricci A, Collier WL, Rossodivita I & Amenta F. Dopamine receptors mediating inhibition of the cyclic adenosine monophosphate generating system in the rat renal cortex. J Auton Pharmacol 1991;11:121;

Richards JP, Bächinger HP, Goodman RH & Brennan RG. Analysis of the structural properties of cAMP-responsive element-binding protein (CREB) and phosphorylated CREB. J Biol Chem 1996;271:13716;

Rogg H, Schmid A & de Gasparo M. Identification and characterization of angiotensin II receptor subtypes in rabbit ventricular myocardium. Bioche Biophys Res Comm 1990;173:416;

Rogue P & Malviya AN. Neuroleptics differentially induce jun family genes in the rat striatum. NeuroReport 1994;5:501;

Ron D, Brasier AR & Habener JF. Transcriptional regulation of hepatic angiotensinogen gene expression by the acute-phase response. Mol Cell Endo 1990;74:C97;

Rosivall L. The renal renin-angiotensin system and its contribution to the regulation of glomerular hemodynamics. Fiziologicheskii Zhurnal Imeni I.M. Sechenova 1994;80:60;

Ruffolo, RR Jr, Nicholas AJ, Stadel JM & Hieble JP. Structure and function of α-adrenoceptors. Pharma Rev 1991 43:475;

Rump LC, Bohmann C, Schaible U, Schultzessmann W & Schollmeyer PJ. Beta-adrenergic, angiotensin II, and bradykinin receptors enhance neurotransmission in human kidney. Hypertension 1995;26:445;

Ruppert S, Cole TJ, Boshart M, Schmid E & Schutz G. Multiple mRNA isoforms of the transcription activator protein CREB: generation by alternative splicing and specific expression in primary spermatocytes. EMBO J 1992;11:1530;

Rydzewski B, Zelezna B, Sumners C & Raizada MK. Angiotensin II receptor subtype I couple to inositol phospholipid hydrolysis stimulates plasminogen activator inhibitor-1 gene expression in astraglial cell. Endocrinology 1992;130:1255;

Saitoh M, Salzman EW, Smith M & Ware JA. Activation of protein kinase C in platelets by epinephrine and A23187: correlation with fibrinogen binding. Blood 1989;74:2001;

Sanchez A, Vidal MJ, Martinez-Sierra R & Saiz J. Ontogeny of renal alpha-1 and alpha-2 adrenoceptors in the spontaneously hypertensive rat. J Pharma Exp Ther 1986;237:972;

Sandberg K, Hong J, Clark AJL, Shapiro H & Catt K. Cloning and expression of a novel angiotensin II receptor subtype. J Biol Chem 1992;267:9455;

Sassone-Corsi P, Visvader J, Ferland L, Mellone PL & Verma IM. Induction of proto-oncogene fos transcription through the adenylate cyclase pathway: characterization of a cAMP-responsive element. Genes & Dev 1988;2:1529;

Sawamura T & Nakada T. Role of dopamine in the striatum, renin-angiotensin system and renal sympathetic nerve on the development of two-kidney, one-clip goldblatt hypertension. J Urol 1996;155:1108;

Schaak S, Cayla C, Blaise R, Quinchon F & Paris H. HepG2 and SK-N-MC: two human models to study alpha-2 adrenergic receptors of the alpha-2C subtype. J Pharmacol Exp Ther 1997;281:983;

Scharschmidt LA, Douglas JG & Dunn MJ. Angiotensin II and eicosanoids in the control of glomerular size in the rat and human. Am J Physiol 1986;250:F348;

Schelling JR & Linas SL. Angiotensin II-dependent proximal tubule sodium transport requires transport requires receptor-mediated endocytosis. Am J Physiol 1994;266:C669;

Schmitz J, Graham RM, Sagalowsky A & Pettinger WA. Renal alpha$_1$ and alpha$_2$ adrenergic receptors: biochemical and pharmacological correlations. J Pharma Exp Ther 1981;219:400;

Schnermann J & Briggs J. Function of the juxtaglomerular apparatus: Local control of glomerular hemodynamics. in Seldin DW & Giebisch G (eds.): The kidney: Physiology and pathophysiology. Raven Press, New York, 1985:669;

Schunkert H, Dzau VJ, Tang SS, Hirsch AT, Apstein CS & Lorell BH. Increased rat cardiac angiotensin converting enzyme activity and mRNA expression in pressure overload left ventricular hypertrophy. Effects on coronary resistance, contractility and relaxation. J Clin Invest 1990;86:1913;

Scott JD Stofko RE, McDonald JR, Comer JD, Vitalis EA & Mangili J. Type II regulatory subunit of the cAMP-dependent protein kinase from rat skeletal muscle and mouse brain. Proc Nat Acad Sci USA 1990;84:5192;

Scott JD. Cyclic nucleotide-dependent protein kinases. Pharma Ther 1991;50:123;

Seeman P, Niznik HB, Guan HC. Link between D1 and D2 dopamine receptors is reduced in schizophrenia and Huntington diseased brain. Proc Natl Acad Sci 1989;86:10156;

Semple PF & Morton JJ. Angiotensin II and angiotensin III in rat blood. Circ Res 1976;38(suppl II):122;

Senogles SE, Siegel AM, Padrell E, Tyanzar R & Caron M. Specificity of receptor-G protein interactions. Discrimination of Gi subtypes by the D2 dopamine receptor in a reconstituted system. J Biol Chem 1990;265:4507;

Shade RE, Davis JO, Johnson JA , Gotshall RW & Spielman WS. Mechanism of action of angiotensin II and antidiuretic hormone on renin secretion. Am J Physiol 1973;224:926;

Sheikh-Hamad D, Wang YP, Jo OD & Yanagawa N. Dopamine antagonizes the actions of angiotensin II in renal brush-border membrane. Am J Physiol 1993;264:F737;

Sheng M, Thompson MA & Greenberg ME. CREB: a Ca(2+)-regulated transcription factor phosphorylated by calmodulin-dependent kinases. Science 1991;252:1427;

Short JM, Wynshao-Boris A, Short HP & Hanson RW. Characterization of the phosphoenolpyruvate carboxykinase (GTP) promoter-regulatory region. J Biol Chem 1986;261:9721;

Sibley DR & Lefkowitz RJ. Molecular mechanisms of receptor desensitization using the ß-adrenergic receptor-coupled adenylate cyclase system as a model. Nature 1985;317:124;

Sibley DR, Jeffs RA, Daniel K, Nambi P & Lefkowitz RJ. Phorbol diester treatment promotes enhanced adenylate cyclase activity in frog erythrocytes. Archs Bioche Biophy 1986;244:373;

Sibley DR, Monsma FJ Jr & Shen Y. Molecular neurobiology of dopaminergic receptors. Int Rev Neurobiol 1993;35:391;

Sibley DR, Nambi P, Perters JR & Lefkowitz RJ. Phorbol diesters promote beta-adrenergic receptor phosphorylation and adenylate cyclase desensitization in duck erythrocytes. Bioche Biophy Res Comm 1984;121:973;

Siess W, Weber PC & Lapetina EG. Activation of phspholipase C is dissociated from arachidonate metabolism during platelet shape change induced by thrombin or platelet-activating factor. Epinephrine does not induce phospholipase C activation or platelet shape change. J Biol Chem 1984;259:8286;

Silver BJ, Boker JA, Birgin JB, Vllen EA, Milsted A & Nilson JH. Cyclic AMP regulation of the human glycoprotein hormone α-subunit gene is mediated by an 18-base pair element. Proc Natl Acad Sci USA 1987;84:2198;

Simboli-Campbell M, Gagnon A, Franks DJ & Welsh J. 1,25-dihydroxyvitamin D_3 translocates protein kinase $C\beta$ to nucleus and enhances plasma membrane association of protein kinase $C\alpha$ in renal epithelial cells. J Biol Chem 1994;269:3257;

Simmoneaux V, Ebadi M & Bylund DB. Identification and characterization of α_{2D}-adrenergic receptors in bovine pineal gland. Mol Pharma 1991;40:235;

Simonds W, Goldsmith PK, Codina J, Unson CG & Spiegel AM. G_{i2} mediates α_2-adrenergic inhibition of adenylyl cyclase in platelet membranes. In situ identification with $G\alpha$ C-terminal antibodies. Proc Natl Acad Sci USA 1989;86:7809;

Sokoloff P, Giros B, Martres MP, Bouthenet ML & Schwartz JC. Molecular cloning and characterization of a novel dopamine receptor (D3) as a target for neuroleptics. Nature (Lond) 1990;347:146;

Soubrier F, Alhenc-Gelas F, Hubert C, Allegrini J, John M, Tregear G & Corvol P. Two putative active centers in human angiotensin I-converting enzyme revealed by molecular cloning. Proc Natl Acad Sci USA 1988;85:9386;

Sõzeri O, Vollmer K, Liyanage M, Frith D, Kour G, Mark III GE & Stabel S. Activation of the c-Raf protein kinase by protein kinase C phosphorylation. Oncogene 1992;7:2259;

Spiegel AM, Shenker A & Weinstein LS. Receptor-effector coupling by G proteins: implications for normal and abnormal signal transduction. Endo Rev 1992;13:536;

Stabel S & Parker PJ. Protein kinase C. Pharma Ther 1991;51:71;

Starke K. Regulation of noradrenaline release by presynaptic receptor systems. Rev Physiol Biochem Pharma 1977;77:1;

Stephenson JA & Summers RJ. Light microscopic autoradiography of the distribution of [^{3}H]rauwolscine binding to α_2-adrenoceptors in rat kidney. Eur J Pharma 1985;116:271;

Strader C, Sigal IS & Dixon RAF. Structural basis of beta-adrenergic receptor function. FASEB J 1989;3:1825;

Strange PG. New insights into dopamine receptors in the central nervous system. Neurochem Int 1993;22:223;

Swanson GN, Hanesworth JM, Sardinia MF, Coleman JK, Wright JW, Hall KL, Miller-Irving AV, Stobb JW, Cook VJ, Harding EC & Harding JW. Discovery of a distinct binding site for angiotensin II (3-8) a putative angiotensin IV receptor. Regul Pet 1992;40:409;

Sweatt JD, Connolly TM, Cragoe EJ & Limbird LE. Evidence that Na^+/H^+ exchange regulates receptor-mediated phospholipase A2 activation in human platelets. J Biol Chem 1986;261:8667;

Sweatt JD, Johnson SI, Cragoe EJ & Limbird LE. Inhibitors of Na^+/H^+ exchange block stimulus-provoked arachidonic acid release in human platelets. J Biol Chem 1985;260:12910;

Takio K, Smith SB, Krebs EG, Walsh KA & Titani K. Amino acid sequence of the regulatory subunit of bovine type II adenosine 3',5'-phosphate dependent protein kinase. Biochemistry 1984;23:4200;

Tamura K, Tanimoto K, Takahashi S, Sagara M, Fukamizu A & Murakami K. Structure and expression of the mouse angiotensinogen gene. Jpn Heart J 1992;33:113;

Tamura K, Umemura S, Ishii M, Tanimoto K, Murakami K & Fukamizu A. Molecular mechanism of transcriptional activation of angiotensinogen gene by proximal promoter. J Clin Invest 1994:93:1370;

Tanaka C & Nishizuka Y. The protein kinase C family for neuronal signaling. Annu Rev Neurosci 1994;17:551;

Tang W & Gilman AG. Adenylyl cyclases. Cell 1992;70:869;

Tang WJ & Gilman AG. Type-specific regulation of adenylyl cyclase by G protein beta gamma subunits. Science 1991;254:1500; -a.

Tang WJ, Krupinski J & Gilman AG. Expression and characterization of calmodulin-activated (type I) adenylylcyclase. J Biol Chem 1991;266:8595; -b.

Taniguchi S, Watanabe T, Nakao A, Seki G, Uwatoko S, Suzuki K & Kurokawa K. Distribution of $ß_2$-adrenergic receptor mRNA expression along the hamster nephron segment. FEBS 1993;318:65;

Taugner R, Hackenthal E, Helmchen U, Ganten D, Kugler P, Marin-Grez M, Nobiling R, Unger T, Lockwald I & Keilbach R. The intrarenal renin-angiotensin-system. An immunocytochemical study on the localization of renin, angiotensinogen and angiotensins of mouse and rat. Klin Wochenschr 1982;60:1218;

Taussig R & Gilman AG. Mammalian membrane-bound adenylyl cyclases. J Biol Chem 1995;270:1;

Taylor SJ, Chae HZ, Rhee SG, Exton JH. Activation of the beta 1 isozyme of phospholipase C by alpha subunits of the Gq class of G proteins. Nature 1991;350:516;

Tewksbury DA. Angiotensinogen: biochemistry and molecular biology. In: Laragh JH, Brenner BM, eds. hypertension: PathoPhysiology, Diagnosis and Management. New York, NY: Raven Press Publishers;1990:1197;

Tiberi M, Jarview KR, Silvia C, Falardeau P, Gingrich JA, Godinot N, Bertrand L, Yang-Feng TL, Femeau RT Jr. & Caron MG. Cloning, molecular characterization, and chromosomal assignment of a gene encoding a second D1 dopamine receptor subtype: differential expression pattern in rat brain compared with the D1A receptor. Proc Natl Acad Sci USA 1991;86:7491;

Tiberi M, Nash SR, Bertrand L, Lefkowitz RJ & Caron MG. Differential regulation of dopamine D1A receptor responsiveness by various G protein-coupled receptor kinases. J Biol Chem 1996;271:3771;

Timmermans PBMWM, Wong PC & Chiu AT. Nonpeptide angiotensin II receptor antagonists. TIPS 1991;12:55;

Tobian L. Relationship of juxtaglomerular apparatus to renin and angiotensin. Circulation 1962;25:189;

Tratner I, Ofir R & Verma IM. Alteration of a cyclic AMP-dependent protein kinase phosphorylation site in the c-Fos protein augments its transforming potential. Mole Cell Biol 1992;12:998;

Tsukada T, Fink JS, mandel G & Goodman RH. Identification of a region in the human vasoactive intestinal polypeptide gene responsible for regulation by cyclic AMP. J Biol Chem 1987;262:8743;

Tulassay T, Rascher W, Lang RE, Seyberth HW & Scharer K. Atrial natriuretic peptide and other vasoactive hormones in nephrotic syndrome. Kidney Int 1987;31:1391;

Urata H, Healy B, Stewart RW, Bumpus FM & Schambelan M. Characterization of angiotensin II receptor subtypes in the rat kidney and heart using the non-peptide antagonists DuP753 and PD123177. J Hypertens 1991;9:S224;

Vallar L, Muca C, Magni M, Alber P, Bunzow J, Meldolesi J & Civelli O. Differential coupling of dopaminergic D2 receptors expressed in different cell types. J Biol Chem 1990;265:10320;

Vallar L, Vincentini LM & Meldolesi J. Inhibition of inositol phosphate production is a late, Ca^{2+}-dependent effect of D2 dopaminergic receptor activation in rat lactotroph cells. J Biol Chem 1988;263:10127;

Van Tol HHM, Bunzow JR, Guan HC, Sunahara RK, Seeman P, Niznik HB & Civelli O. Cloning of the gene for a human dopamine D4 receptor with high affinity for the antipsychotic clozapine. Nature (Lond) 1991;350:610;

Vander AJ. Control of renin release. Physiol Rev 1967;47:359;

Von Euler US. Mechanisms of release of biogenic amines. Oxford UK: Pergamon, 1966;

Vyas SJ, Apparsundaram S, Ricci A, Amenta F & Lokhandwala MF. Biochemical, autoradiographic and pharmacological evidence for the involvement of tubular DA-1 receptors in the natriuretic response to dopexamine hydrochloride. Naunyn-Schmiedebergs Arc Pharm 1991;343:21;-a

Vyas SJ, Eichberg J & Lokhandwala MF. Identification of receptors involved in dopamine induced activation of phospholipase C in rat kidney. FASEB J 1991;5:1066;-b

Wahl MI, Daniel TO & Carpenter G. Epidermal growth factor stimulates tyrosine phosphorylation of phospholipase C-II independently of receptor internalization and extracellular calcium. Proc Natl Acad Sci USA 1989;86:1568;

Wahl MI, Sweatt JD & Carpenter G. EGF stimulates inositol trisphosphate formation in cells which overexpress the EGF receptor. Biochem Biophys Res Commun 1987;142:688;

Wang TT, Chen M, Lachance S, Delalandre A, Carriere S & Chan JSD. Isoproterenol and 8-bromo-cyclic adenosine monophosphate stimualte the expression of the angiotensinogen gene in opossum kidney cells. Kidney Int 1994;46:703;

Weber T & Hilz H. Stoichiometry of cAMP binding and limited proteolysis of protein kinase regulatory subunits RI and RII. Bioche Biophy Res Comm 1979;90:1073;

Weiner DM, Levey AI, Sunahara RK, Niznik HB, O'Dowd BF, Seeman P & Brann MR. D1 and D2 dopamine receptor mRNA in rat brain. Proc Natl Acad Sci USA 1991;88:1859;

Weinshank RL, Adham N, Macchi M, Olsen MA, Branchek TA & Hartig PR. Molecular cloning and characterization of a high affinity dopamine receptor (D1 beta) and its pseudogene. J Biol Chem 1991;266:22427;

Weinstock M, Gorodetsky E & Kalman R. Renal denervation prevents sodium retention and hypertension in salt-sensitive rabbits with genetic baroreflex impairment. Clin Sci 1996;90:287;

Willems JL, Buylaert WA, Lefevre RA & Bogaert MG. Neuronal dopamine receptors on autonomic ganglia and sympathetic nerves and dopamine receptors in gastrointestinal system. Pharm Rev 1985;37:165;

Williams JS, Dixon JE & Andrisani OM. Binding constant determination studies utilizing recombinant delta CREB protein. DNA Cell Biol 1993;12:183;

Wolfe L, Woodford TA, Francis SH & Corbin JD. Direct involvement of the cyclic nucleotide binding sites in the cyclic-nucleotide-induced charge shift of protein kinases. In: NATO ASI Series 1990;H44:133, Konlin TM Houslay MD & Van Haastert PJM (eds) Springer, Berlin;

Wolff DW, Gesek FA & Strandhoy JW. In vivo assessment of rat renal alpha adrenoceptors. J Pharmacol Exp Ther 1987;241:472;

Wong KR, Berry CA & Cogan MG. Alpha$_1$ adrenergic control of chloride transport in the rat S_1 convoluted tubule. Am J Physiol 1996;270:F1049;

Wooten MW, Seibenhener ML, Matthews LH, Zhou G & Coleman ES. Modulation of zeta-protein kinase C by cyclic AMP in PC12 cells occurs through phosphorylation by protein kinase A. J Neurochem 1996;67:1023;

Wu D, Datz A, Lee CH & Simon MI. Activation of phospholipase C by α_1-adrenergic receptors is mediated by the α subunits of G_q family. J Biol Chem 1992;267:25798;

Yamada H, Sexton PM & Chai SY. Angiotensin II receptors in the kidney. Localization and Physilogical significance. Am J Hypert 1990;3:250;

Yamamoto KK, Gonzalez GA , Biggs WH III & Montminy MR. Phsphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. Nature 1988;334:494;

Yamamoto KK, Gonzalez GA, menzel P, Rivier J & Montminy MR. Characterization of a bipartite activator domain in transcription factor CREB. Cell 1990;60:611;

Yang HYT, Erdös EG & Levin Y. A dipeptidyl carboxypeptidase that converts angiotensin I and inactivates bradykinin. Biochim Biophys Acta 1970;214:374;

Yoshimasa T, Sibley DR, Bouvier M, Lefkowitz RJ & Caron MG. Cross-talk between cellular signaling pathways suggested by phorbol-ester-induced adenylate cyclase phosphorylation. Nature 1987;327:67;

Yoshimura M & Cooper DM. Type-specific stimulation of adenylyl cyclase by protein kinase C. J Biol Chem 1993;268:4604;

Yoshimura M & Cooper DMF. Cloning and expression of a Ca(2+)-inhibitable adenylyl cyclase from NCB-20 cells. Proc Natl Acad Sci USA 1992;89:6716;

Yotsumoto H, Imai Y, Kuzuyu N, Uchimura H & Matsuzaki F. Increased levels of serum angiotensin converting enzyme activity in hyperthyroidism. Ann Intern Med 1982;96:326;

Young WS & Kahar MJ. α_2-adrenergic receptors are associated with renal proximal tubules. Eur J Pharma 1980;67:493;

Zaagsma J, Nahorski Sr. Is the adipocyte ß-adrenoceptor a prototype for the recently cloned atypical '$ß_3$-adrenoceptor'? Trends Phar Sci 1990;11:3;

Zeng D, Harrison JK, D'Angelo DD, Barber CM, Tucker AL, Lu Z & Lynch KR. Molecular characterization of a rat α_{2B}-adrenergic receptor. Proc Natl Acad Sci USA 1990;87:3102.

Appendix-1 PUBLICATIONS

1. **Wang TT**, Wu XH & Chan JSD: Glucose and angiotensinogen gene expression in opossum kidney cells (Kidney Int 1997 accepted);

2. Qian JF, **Wang TT**, Wu XH, Wu J, Ge C, Lachance S, Carrière S & Chan JSD: Angiotensinogen gene expression is stimulated by the cyclic AMP-responsive element binding protein (CREB) in opossum kidney cells (J Am Soc Nephrol 1997;8:1072);

3. **Wang TT**, Lachance S, Delalandre A, Carrière S & Chan JSD: Dopaminergic receptors and angiotensinogen gene expression in opossum kidney cells (Am J Physiol 271:R519, 1996);

4. **Wang TT**, Lachance S, Delalandre A, Carrière S & Chan JSD: Alpha-adrenoceptors and angiotensinogen gene expression in opossum kidney cells (Kidney Int. 48:139, 1995);

5. Ming M, **Wang TT**, Lachance S, Delalandre A, Carrière S & Chan JSD: Expression of the angiotensinogen gene by 8-bromo-cAMP and dexamethasone in opossum kidney proximal tubular cells (Am J Physiol 268:R105, 1995);

6. **Wang TT**, Lachance S, Delalandre A, Carrière S & Chan JSD: Isoproterenol and 8-bromo-cyclic adenosine monophsphate stimulate the expression of the angiotensinogen gene in opossum kidney cells (Kidney Int. 46:703, 1994);

Appendix-2 LIST OF ABSTRACTS

1. **Wang TT**, Wu XU, Sajedi M & Chan JSD: Le glucose et l'expression du gene de l'angiotensinogene (ANG) das les cellules rénales d'opossum (OK). XXXIXe Réunion annuelle du Club de Recherches Cliniques du Québec. Auberge Godefroy, Québec, 16-18, Oct., 1997;
2. **Wang TT**, Wu XU, Sajedi M & Chan JSD: Glucose and angiotensinogen gene expression in opossum kidney proximal tubular cells. The annual meeting of the Canadian Society of Clinical Investigation, Vancouver, B.C., Sept., 25-28, 1997;
3. **Wang TT**, Wu XU, & Chan JSD: Protein Kinase A and the cAMP-responsive element binding protein (CREB) on the expression of the Angiotensinogen (ANG) gene in opossum kidney (OK) cells. 29th Annual Meeting, Amer Soc Nephrol, New Orleans, USA, Nov., 5-8, 1996;
4. **Wang TT**, Wu XU,& Chan JSD: La Norépinéphrine (NE) et le facteur transcriptionnel CREB (cAMP responsive-element binding protein) stimulent l'expression du gène de l'angiotensinogène (ANG) dans les cellules rénale d'opossum (OK). XXXVIIIe Réunion annuelle du Club de Recherches Cliniques du Québec., Québec, Québec, 3-5, Oct., 1996;
5. **Wang TT**, Wu XU,& Chan JSD: Norepinephrine (NE) and the cAMP-responsive element binding protein (CREB) on the expression of the Angiotensinogen (ANG) gene in opossum kidney cells. The annual meeting of the Canadian Society of Clinical Investigation, Halifax, Newfoundland, Sept., 26-29, 1996;
6. Chan JSD, **Wang TT** & Carrière S: Catecholamines and Angiotensinogen (ANG) gene expression in the kidney. 12th International conference on Advanced science & technology. Chicago, Illinois, USA Apr. 6-8, 1996;
7. **Wang TT**, Lachance S, Carrière S & Chan JSD: Norepinephrine (NE) stimulates the expression of the Angiotensinogen (ANG) gene in opossum kidney (OK) cells via both beta- and alpha-adrenergic receptors (ARs). 28th Annual Meeting, Amer Soc Nephrol, San Diego, California, USA, Nov., 5-8, 1996;
8. **Wang TT**, Lachance S, Carrière S & Chan JSD: Modulation de l'expression du gène de l'angiotensinogène (ANG) dans les cellules rénale d'opossum (OK) par la norépinéphrine (NE) via les deux types de récepteuus adrénergiques β et α_2. XXXVIIe Réunion annuelle du Club de Recherches Cliniques du Québec., Bromont, Québec, 28-30, sept., 1996;
9. **Wang TT**, Lachance S, Delalandre A, Carrière S & Chan JSD: Alpha-adrenoceptors and Angiotensinogen gene expression in opossum kidney cells. 26th Annual Meeting, Amer Soc Nephrol, Orlando, USA, Oct., 22-25, 1994;
10. **Wang TT,** Carrière S & Chan JSD: Dopaminergic receptors and angiotensinogen gene expression in opossum kidney proximal tubular cells. The annual meeting of the Canadian Society of Clinical Investigation, Toronto, Ont., Sept., 14-18, 1994;
11. **Wang TT**, Lachance S, Delalandre A, Carrière S & Chan JSD: Les récepteurs adrénergiques (α) et l' l'expression du gène de l'angiotensinogène dans les cellules rénale d'opossum. XXXVIe Réunion annuelle du Club de Recherches Cliniques du Québec., Ste. Adèle, Québec, 29, sept.-1, oct., 1994;
12. **Wang TT**, Lachance S, Delalandre A, Carrière S & Chan JSD: Récepteurs dopaminergiques et l' l'expression du gène de l'angiotensinogène dans les cellules de tubules proximaux de rein d'opossum. XXXVIe Réunion annuelle du Club de Recherches Cliniques du Québec., Ste. Adèle, Québec, 29, sept.-1, oct., 1994;
13. Qian, JF, **Wang TT**, Lachance S, Delalandre A, Carrière S & Chan JSD: Evidence de la stimulation d'expression du gène de l'angiotensinogène de rat par CREB (cAMP responsive-element binding protein) dans les cellules de tubules proximaux de rein d'opossum. XXXVIe Réunion annuelle du Club de Recherches Cliniques du Québec., Ste. Adèle, Québec, 29, sept.-1, oct., 1994;

14. Chan JSD, **Wang TT**, Qian JF, Lachance S, Delalandre A, Carrière S & Chan JSD: Isoproterenol stimulates the expression of the angiotensinogen gene in opossum kidney proximal tubular cells. 15th international society of hypertension, Melbourne, Australia, Mar., 20-24, 1994;
15. **Wang TT**, Sikstrom R, Lachance S, Delalandre A, Carrière S & Chan JSD: Identification of a Phorbol 12-Myristate 13-Acetate (TPA)-responsive element (TRE) in the rat angiotensinogen (ANG) gene. 26th Annual Meeting, Amer Soc Nephrol, Boston, USA, Nov., 14-17, 1993;
16. **Wang TT**, Sikstrom R, Lachance S, Delalandre A, Carrière S & Chan JSD: Evidence de l'implication dans la protéine kinase A dans l'expression du gène de l'angiotensinogène dans les cellules de tubules proximaux de rein d'opossum. XXXVe Réunion annuelle du Club de Recherches Cliniques du Québec., Pointe-au-pic, Québec, 30, sept.-2, oct., 1993;
17. Chan JSD, **Wang TT**, Sikstrom R, Lachance S, Delalandre A, & Carrière S: Evidence for protein kinase A involvement in the expression of renal angiotensinogen gene in opossum kidney proximal tubular cells. The annual meeting of the Canadian Society of Clinical Investigation, Vancouver, B.C., Sept., 14-18, 1993;
18. Chan JSD, **Wang TT**, Ming M, Sikstrom R, Lachance S, Delalandre A, & Carrière S: Phorbol Ester (PMA) interacts with 8-bromo-cAMP (8-Br-cAMP) to stimulate the expression of the angiotensinogen gene in opossum kidney proximal tubular cells. 25th Annual Meeting, Amer Soc Nephrol, Baltimore, USA, Nov., 15-18, 1992;
19. Chan JSD, **Wang TT**, Nie ZR, Sikstrom R, Lachance S, Delalandre A, & Carrière S: Stimulation directe de l'expression du gène de l'angiotensinogène dans les cellules de tubules proximaux de rein d'opossum. XXXIVe Réunion annuelle du Club de Recherches Cliniques du Québec., Château Montebello, Québec, 8-10, oct., 1992;
20. Chan JSD, **Wang TT**, Nie ZR, Sikstrom R, Lachance S, Delalandre A, & Carrière S: 8-Bromo-cAMP (cAMP) enhances the dexamethasone stimulation of the expression of the angiotensinogen (ANG) gene in opossum kidney (OK) proximal tubular cells. The annual meeting of the Canadian Society of Clinical Investigation, Ottawa, Ont., Sept., 11-14, 1992;
21. Chan JSD, **Wang TT**, Ming M, Sikstrom R, Lachance S, Delalandre A, & Carrière S: Identification of a novel cAMP-responsive element (CRE) and a silencer element in the rat angiotensinogen gene. 25th Annual Meeting, Amer Soc Nephrol, Baltimore, USA, Nov., 15-18, 1992;
22. **Wang TT**, Nie ZR, Sikstrom R, Lachance S, Delalandre A, Carrière S & Chan JSD: L'expression du gène de l'angiotensinogène rénal dans un milieu sans sérum hormonalement défini. XXXIVe Réunion annuelle du Club de Recherches Cliniques du Québec., Château Montebello, Québec, 8-10, oct., 1992.

ɔaminergic receptors and angiotensinogen gene ression in opossum kidney cells

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g, Tian-Tian, Silvana Lachance, Aline Delalan-ɛrge Carrière, and John S. D. Chan. Dopaminergic rs and angiotensinogen gene expression in opossum cells. *Am. J. Physiol.* 271 (*Regulatory Integrative Physiol.* 40): R519–R527, 1996.—To investigate r expression of the renal angiotensinogen gene is ed by dopaminergic receptors, we used opossum kid-K 27) cells with a fusion gene containing the 5′-g regulatory sequence of the rat angiotensinogen gene with a human growth hormone (hGH) gene as a r [pOGH, angiotensinogen nucleotide (N) −1498/+18], ıently integrated into their genomes. The level of ion of pOGH (angiotensinogen N −1498/+18) in OK evaluated by the amount of immunoreactive hGH I) secreted into the culture medium. In the absence of tyl-1-methylxanthine (IBMX), addition of dopamine o 10^{-5} M) had minimal effect on the expression of the (angiotensinogen N −1498/+18) in OK 27 cells. In the ɛe of IBMX, addition of low concentrations (10^{-13} and) of dopamine stimulated the expression of pOGH ensinogen N −1498/+18) in OK 27 cells in a dose-ent manner, whereas high concentrations (i.e., $>10^{-7}$ minimal effect. The stimulatory effect of dopamine on ression of pOGH (angiotensinogen N −1498/+18) was ɛd by the presence of SCH-23390 (D_1-dopaminergic r antagonist) and spiperone (D_2-dopaminergic recep-ıgonist), but not by ketanserin ($5HT_2$/$5HT_{1c}$-serotoner-ɛptor antagonist). Moreover, the stimulatory effect of ne was inhibited by the presence of U-73122 (an ɔr of phospholipase C and phospholipase A_2) or stauro-(an inhibitor of protein kinase C) or (*R*)-*p*-adenosine clic monophosphorothioate (Rp-cAMP[S]; an inhibitor P-dependent protein kinase A I and II). Addition of low ɛrations (10^{-13} to 10^{-9} M) of SKF-82958 (D_1-dopamin-ɛceptor agonist) or PPHT (D_2-dopaminergic receptor) also stimulated the expression of pOGH (angioten-ı N −1498/+18). The stimulatory effect of SKF-82958 ıibited by the presence of SCH-23390 or Rp-cAMP[S], s the effect of PPHT was inhibited by the presence of ne or staurosporine. These studies demonstrate that ɔression of pOGH (angiotensinogen N −1498/+18) in ɛells is modulated by dopaminergic receptor agonists.

growth hormone; SCH-23390; renal proximal tubules

ENSIN II (ANG II), an octapeptide derived from ecursor protein angiotensinogen, exerts a dose-lent biphasic effect on proximal tubular sodium rption (15, 23, 31, 33, 40) and induces the growth maturing kidney (13), suggesting that ANG II lay an important role in the control of electrolyte rption in the kidney and in maintaining renal ular growth.

The existence of an intrarenal renin-angiotensin system (RAS) has now been generally accepted (11, 18). Studies by Seikaly et al. (34) and Braam et al. (5) have shown that levels of luminal ANG II in the rat renal proximal tubule are as high as 10^{-9} M, whereas levels of plasma ANG II are less than 10^{-12} M. The presence of angiotensinogen mRNA has also been localized in the renal proximal tubule by both the techniques of in situ hybridization (17) and of polymerase chain reaction (37). These studies indicated that renal ANG II is derived from the angiotensinogen synthesized in renal proximal tubules.

Angiotensinogen mRNA is expressed in mouse and rat proximal tubular cell lines (36, 43). We (7), as well as Ingelfinger et al. (16), have also demonstrated that the angiotensinogen mRNA is expressed in opossum kidney (OK) proximal tubular cells. We have also reported that thyroid hormone (L-T_3), dexamethasone, 8-bromoadenosine 3′,5′-cyclic monophosphate (8-BrcAMP), and forskolin stimulate the expression of the angiotensinogen gene in OK cells in vitro in a dose-dependent manner (7, 8, 26). More recently, we demonstrated that isoproterenol stimulates the expression of the angiotensinogen gene in OK cells (41). The effect of isoproterenol is mediated via the β_1-adrenergic receptor and could be blocked by the presence of propranolol, atenolol, and (*R*)-*p*-adenosine 3′,5′-cyclic monophosphorothioate (Rp-cAMP[S]; an inhibitor of cAMP-dependent protein kinase A I and II) (41). Our studies confirm the report of Nakamura and Johns (27) that low levels of renal nerve stimulation decrease sodium excretion and increase the level of angiotensinogen mRNA in the rat kidney in vivo but not renin mRNA. Administration of the β_1-adrenergic receptor antagonist, atenolol, blocked the effect of renal nerve stimulation. All of these studies suggest the presence of a functional relationship between the renal sympathetic nervous system and the activation of local renal RAS. Thus the local formation of renal ANG II may play an important role in the modulation of the physiology of the renal proximal tubular cells (i.e., sodium and fluid reabsorption).

Infusion of dopamine into experimental animals or humans results in an increase in renal blood flow and sodium excretion (22, 24). This increase in sodium excretion by dopamine could be an indirect tubular effect secondary to an increase in renal blood flow or it could result from a direct tubular effect on renal sodium transport. Indeed, recent studies have demonstrated that dopamine directly regulates renal tubular sodium and water transport in the proximal tubules (1, 20, 35). The action of dopamine is mediated via the

D_1-dopaminergic receptor (20) and intracellular cAMP (12). Most of the renal tubular dopamine production occurs in the proximal tubules (2, 10, 14, 35). Studies by Perrichot et al. (29) demonstrated that dopamine can be synthesized by OK cells. Furthermore, OK cells express D_1-dopaminergic receptor, which is coupled to adenylate cyclase (3, 9). Thus the stimulation of cAMP production in OK cells by dopamine (3, 9) raises the possibility that dopamine might also modulate the expression of the angiotensinogen gene in OK cells.

Therefore, in the present study, we investigated the possibility that angiotensinogen gene expression in OK cells may be modulated by dopaminergic receptors. Our results demonstrated that dopamine (10^{-13} to 10^{-5} M) has a minimal effect on the expression of the angiotensinogen gene in OK 27 cells in the absence of 3-isobutyl-1-methylxanthine (IBMX). In the presence of IBMX, however, the addition of dopamine at low concentrations (10^{-13} to 10^{-7} M) had a stimulatory effect. The stimulatory effect of dopamine can be blocked by the presence of D_1- or D_2-dopaminergic receptor antagonists.

MATERIALS AND METHODS

Materials. The fusion gene pOGH (angiotensinogen N −1498/+18), containing the 5′-flanking sequence [1498 base-pair (bp)] upstream of the transcriptional site plus 18 bp of *exon* I of the rat angiotensinogen gene fused with a human growth hormone (hGH) gene, has been described previously (6). The plasmid, pRSV-Neo, containing the coding sequence for Neomycin (Neo) with the Rous sarcoma virus (RSV) enhancer/promoter sequence fused in the 5′ end of the Neomycin gene was a gift from Dr. Teresa Wang (Dept. of Pathology, Stanford University, Stanford, CA). The plasmid, pTKGH, containing the thymidine kinase (TK) enhancer/promoter sequence fused to the 5′-end of the hGH gene was purchased from the Nichols Institute of Diagnostics (La Jolla, CA).

The radioimmunoassay kit for hGH (RIA-hGH) was a gift from National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, MD). The RIA procedure has been described previously (6). NIAMDD-hGH-I-1 (AFP-4793 B) was used for both iodination and as a hormone standard. The limit of sensitivity of the assay was 0.1 ng/ml. The intra- and interassay coefficients of variation were 10% (n = 10) and 12% (n = 10), respectively.

Dopamine hydrochloride (3-hydroxytyramine hydrochloride), 3-isobutyl-1-methylxanthine (IBMX), SKF-82958 (D_1-dopaminergic receptor agonist), (±)PPHT hydrochloride (D_2-dopaminergic receptor agonist), R(±)-SCH-23390 HCl (D_1-dopaminergic receptor antagonist), spiperone HCl (D_2-dopaminergic receptor antagonist), ketanserin ($5\text{-}HT_2/5H_{1C}$ serotonergic receptor blocker), U-73122 (an inhibitor of phospholipase C and A_2), staurosporine [protein kinase C (PKC) inhibitor], and Rp-cAMP[S] (an inhibitor of the cAMP-dependent protein kinase A I and II) were all purchased from Research Biochemicals (Natick, MA).

$Na^{125}I$ was purchased from Dupont-New England Nuclear (Boston, MA). Calcium chloride was purchased from Mallinckrodt (Montreal, Canada). Geneticin (G-418) was purchased from Bethesda Research Laboratories (GIBCO-BRL, Burlington, Canada). L-Ascorbic acid (sodium salt) was purchased from Sigma Chemical (St. Louis, MO). Other molecular biology grade reagents were obtained either from Sigma Chemical, GIBCO-BRL, Boehringer-Mannheim (Dorval, Canada), Pharmacia (Baie d'Urfe, Canada), or F Fisher (Montreal, Canada).

Cell culture. The OK proximal tubular cell line tained from the American Type Culture Collection Rockville, MD). This cell line is derived from the kic female American opossum and retains several prop proximal tubular epithelial cells in culture (21, expresses a low level of angiotensinogen mRNA (7, culture conditions of OK cells have been described pr (8, 26, 41).

OK cell stable transformants. The method for the s of OK cell stable transformants with high expre pOGH (angiotensinogen N −1498/+18) (OK 27) or (OK 13) has been described previously (41). Briefly, p pOGH (angiotensinogen N −1498/+18) or pTKGH an Neo fusion genes were cotransfected (20 μg each) cells (1×10^6 cells) utilizing calcium phosphate-n endocytosis. After transfection, the cells were cultur night in 5.0 ml of Dulbecco's modified Eagle's medium (containing 10% fetal bovine serum (FBS). Then the was replaced with fresh medium containing 10% FBS μg/ml of aminoglycoside G-418 (Geneticin, GIBC stable transformants that were able to grow in the pr G-418 and that secreted high levels of immunoreact hGH into the medium were further subcloned us method of limiting dilution. Those cells that had through at least three repetitions of limiting dilut continued to secrete high levels of ir-hGH after thre tions in the presence of G-418 were considered to b clones. We have obtained three and six clones with (angiotensinogen N −1498/+18) and pTKGH integra their genomes, respectively. In the present study, we u of these clones, clone OK 27 and OK 13, which hav (angiotensinogen N −1498/+18) and pTKGH integra their genomes, respectively.

Effect of dopamine on expression of pOGH (angioten N −1498/+18) in OK 27 cells. OK 27 cells were pla density of $1–2 \times 10^5$ cells/well in 6-well plates and in overnight in DMEM containing 10% FBS. Then cell was arrested by incubation in serum-free medium f Subsequently, various concentrations of dopamine (10^{-5} M) were added to the culture medium contai depleted FBS (dFBS) and 5 mM ascorbic acid with or 0.1 μM IBMX and incubated for 24 h. After incubation, media were taken and kept at −20°C for assays of ir-h

The dFBS was prepared by incubation with 1% a charcoal and 1% analytical grade (AG 1 × 8) ion-e resin (Bio-Rad Laboratories, Richmond, CA) for 16 h at room temperature as described by Samuels et al. (3 procedure removed endogenous steroid and thyroid hc from the FBS (32).

Effect of SCH-23390, spiperone, or ketanserin or U staurosporine, or Rp-cAMP[S] on expression of pOGH tensinogen N −1498/+18) in OK 27 cells in the pre dopamine. OK 27 cells were plated at a density of 1– cells/well in 6-well plates and incubated overnight in containing 10% dFBS. Cell growth was arrested by tion in serum-free medium for 24 h. Then, various cor tions of SCH-23390, spiperone, or ketanserin or U staurosporine, or Rp-cAMP[S] (10^{-13} to 10^{-7} M) were a the culture medium containing 1% dFBS, 5 mM ascorl 0.1 μM IBMX, and 10^{-9} M dopamine and incubated f At the end of the incubation period, media were collec kept at −20°C until assay.

Effect of SKF-82958, PPHT, or a combination of expression of pOGH (angiotensinogen N −1498/+18 27 cells. OK 27 cells were plated at a density of 1–

ell in a 6-well plate and incubated overnight in DMEM ing 10% dFBS. Then cell growth was arrested by ion in serum-free medium for 24 h. Subsequently, concentrations (10^{-13} to 10^{-5} M) of SKF-82958 or were added to the culture medium containing 1% dFBS ubated for 24 h.

larly, dopamine (10^{-9} M), SKF-82958 (10^{-11} M), PPHT M), or a combination of both SKF-82958 and PPHT was o the culture medium containing 1% dFBS and 5 mM c acid with or without 0.1 μM IBMX and incubated for he effects of SKF-82958 or PPHT or a combination of ere compared with dopamine at 10^{-9} M. At the end of ubation period, media were collected and kept at until assayed.

t of dopaminergic receptor antagonists, Rp-cAMP[S], rosporine on expression of pOGH (angiotensinogen 98/+18) in OK 27 cells in the presence of SKF-82958 or OK 27 cells were plated at a density of $1–2 \times 10^5$ ell in 6-well plates and incubated overnight in DMEM ing 10% dFBS. Then, cell growth was arrested by ion in serum-free medium for 24 h. Subsequently, concentrations (10^{-13} to 10^{-7} M) of SCH-23390, IP[S], or staurosporine were added to the culture n containing 1% dFBS in the presence of 10^{-11} M 958 and incubated for 24 h. Similarly, various concens (10^{-13} to 10^{-5} M) of spiperone, staurosporine, or erin were added to the culture medium containing 1% n the presence of 10^{-11} M PPHT and incubated for 24 h. end of the incubation period, media were collected and −20°C until assay.

t of dopamine and dopaminergic receptor agonists on ion of pTKGH in OK 13 cells. OK 13 cells were plated ensity of $1–2 \times 10^5$ cells/well in 6-well plates and ed overnight in DMEM containing 1% dFBS. Then, wth was arrested by incubation in serum-free medium n. Subsequently, dopamine (10^{-11} M) was added to the medium containing 1% dFBS, 5 mM ascorbic acid, μM IBMX, whereas SKF-82958 (10^{-11} M) or PPHT M) was added to the culture medium containing 1% vithout ascorbic acid and IBMX. At the end of a 24-h ion period, media were collected and kept at −20°C say for ir-hGH.

stical analysis. The experiments were performed at ree times in triplicate. The data were analyzed with t's *t*-test or analysis of variance. A probability level of 5 was regarded as significant.

TS

t of dopamine on expression of pOGH (angioten- n N −1498/+18) in OK 27 cells. Figure 1 shows ect of dopamine (10^{-13} to 10^{-5} M) on the expres- the pOGH (angiotensinogen N −1498/+18) in cells in the presence or absence of 0.1 μM IBMX. presence of 0.1 μM IBMX, incubation with 10^{-13} 0^{-7} M dopamine for 24 h stimulated the expres- f pOGH (angiotensinogen N −1498/+18) com- with controls (without addition of dopamine) in a ependent manner. Dopamine at concentrations r than 10^{-7} M (i.e., 10^{-5} M) had minimal stimula- fect. In the absence of 0.1 μM IBMX, dopamine to 10^{-5} M) had minimal effect on the expression GH (angiotensinogen N −1498/+18) in OK 27 These studies indicate that the expression of (angiotensinogen N −1498/+18) is stimulated concentrations of dopamine (10^{-13} to 10^{-7} M)

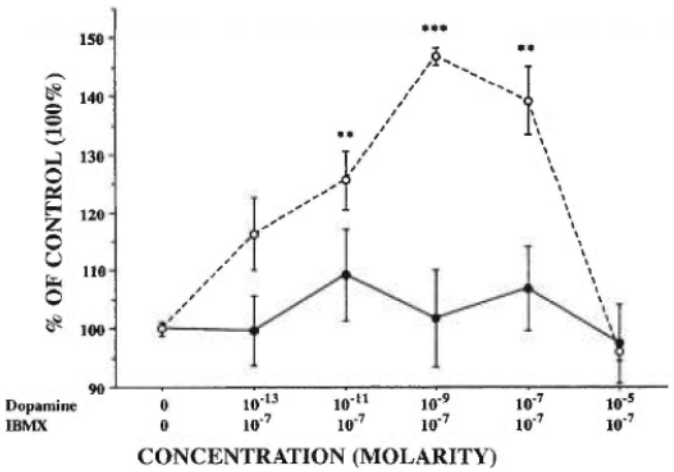


Fig. 1. Effect of dopamine on expression of pOGH (angiotensinogen N −1498/+18) in OK 27 cells in the presence or absence of 0.1 μM 3-isobutyl-1-methylxanthine (IBMX). Cells were incubated for 24 h in the presence of various concentrations of dopamine and 5 mM ascorbic acid with or without 0.1 μM IBMX. Media were harvested after 24 h of incubation and assayed for immunoreactive human growth hormone (ir-hGH). Levels of ir-hGH in the medium (with or without 0.1 μM IBMX) in the absence of dopamine are expressed as 100% (control). Each point represents mean ± SD of at least 3 dishes (**$P \leq 0.01$ and ***$P \leq 0.005$) (○, IBMX; ●, without IBMX). Similar results were obtained from 2 other experiments. The basal levels of ir-hGH in the controls were 5.4 ± 0.06 ng/ml. In subsequent experiments, levels of ir-hGH in the controls ranged from 4 to 10 ng/ml.

and required the presence of IBMX to inhibit endogenous phosphodiesterase activity.

Effect of SCH-23390, spiperone, or ketanserin on expression of pOGH (angiotensinogen N −1498/+18) in the presence of dopamine. Figure 2 shows the expression of pOGH (angiotensinogen N −1498/+18) in OK 27 cells in the presence of dopamine (10^{-9} M) and various concentrations (10^{-13} to 10^{-7} M) of SCH-23390, spiperone, or ketanserin. Addition of SCH-23390 inhibited the stimulatory effect of dopamine (10^{-9} M) on the expression of the pOGH (angiotensinogen N −1498/+18) in OK 27 cells in a dose-dependent manner. The effective dose for the inhibition of the stimulated expression (by dopamine) of the pOGH (angiotensinogen N −1498/+18) was found with 10^{-11} M of SCH-23390. Addition of spiperone (10^{-13} to 10^{-7} M) was also effective in inhibiting the effect of dopamine in a dose-dependent manner with an effective dose at a concentration of 10^{-9} M ($P \leq 0.01$). Addition of ketanserin (10^{-13} to 10^{-7} M) did not inhibit the stimulatory effect of dopamine on the expression of pOGH (angiotensinogen N −1498/+18) in OK 27 cells. These studies suggest that the stimulatory effect of dopamine on the expression of pOGH (angiotensinogen N −1498/+18) is probably mediated via both D_1- and D_2-dopaminergic receptors, but not via serotonergic receptors.

Effect of U-73122, staurosporine, or Rp-cAMP[S] on expression of pOGH (angiotensinogen N −1498/+18) in the presence of dopamine. Figure 3 shows that addition of U-73122, staurosporine, or Rp-cAMP[S] inhibited the stimulatory effect of dopamine (10^{-9} M) on the expression of pOGH (angiotensinogen N −1498/+18) in OK 27

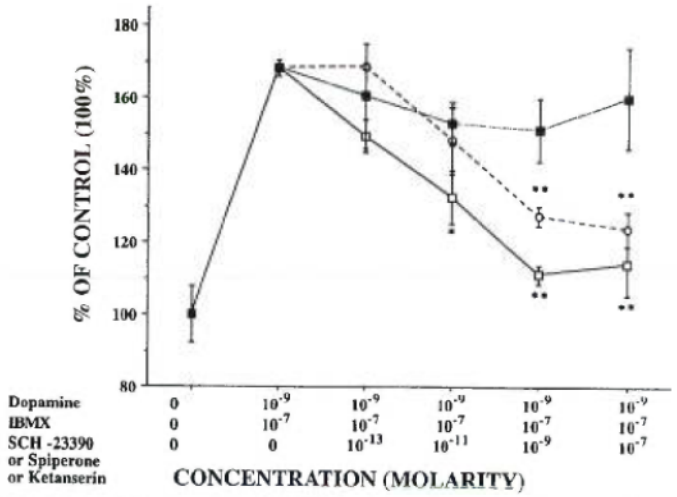


Fig. 2. Inhibitory effect of SCH-23390 or spiperone or ketanserin on expression of pOGH (angiotensinogen N −1498/+18) in OK 27 cells stimulated by dopamine. Cells were incubated for 24 h in the presence of dopamine (10^{-9} M), 5 mM ascorbic acid, 0.1 μM IBMX, and various concentrations (10^{-11} to 10^{-7} M) of SCH-23390, spiperone, or ketanserin. Levels of ir-hGH in the absence of dopamine (10^{-9} M), SCH-23390, spiperone, or ketanserin are expressed as 100% (control). The inhibitory effect of SCH-23390 or spiperone is compared with cells that were stimulated by dopamine at 10^{-9} M. Each point represents mean ± SD of at least 3 dishes (*$P \leq 0.05$ and **$P \leq 0.01$) (□, SCH-23390; ○, spiperone; ■, ketanserin). Experiments were repeated 3 times.

cells. The effective dose for the inhibition of the stimulated expression (by dopamine, 10^{-9} M) was found with 10^{-13} M of U-73122 ($P \leq 0.01$), staurosporine ($P \leq 0.05$), or Rp-cAMP[S] ($P \leq 0.01$). These studies indicate that the stimulatory effect of dopamine on the sion of pOGH (angiotensinogen N −1498/+18) ably mediated via both PKC and cAMP-dep protein kinase A I and II pathways.

Effect of SKF-82958, PPHT, or a combination on expression of pOGH (angiotensinogen N −149 in OK 27 cells. Figure 4 shows the expressior pOGH (angiotensinogen N −1498/+18) in OK in the presence of various concentrations of SKI or PPHT (10^{-13} to 10^{-5} M). SKF-82958 or P concentrations (10^{-13} to 10^{-9} M) stimulated the sion of pOGH (angiotensinogen N −1498/+18) pears that maximum stimulation of expressior pOGH (angiotensinogen N −1498/+18) was fou 10^{-11} M SKF-82958 or 10^{-11} M PPHT ($P \leq 0.0$ concentrations greater than 10^{-9} M, it appears t stimulatory effect of SKF-82958 or PPHT was minimal or inhibited.

Furthermore, it appears that, in the abs IBMX, the stimulatory effect of the additio combination of SKF-82958 (10^{-11} M) and PPH M) was significantly enhanced compared with th of SKF-82958, PPHT, or dopamine alone (P (Fig. 5*A*). In the presence of IBMX, the e dopamine was significantly higher than SKF-82 not higher than PPHT (Fig. 5*B*). Again, the stim effect of an addition of a combination of SKF-82 PPHT was significantly higher compared w effect of SKF-82958, PPHT, or dopamine alon 0.05) (Fig.5*B*). These studies indicate that t probably an additive effect of both D_1- a dopaminergic receptors on the expression of (angiotensinogen N −1498/+18) in OK 27 cells.

Effect of dopaminergic receptor antagonis cAMP[S], or staurosporine on expression of (angiotensinogen N −1498/+18) in OK 27 cell

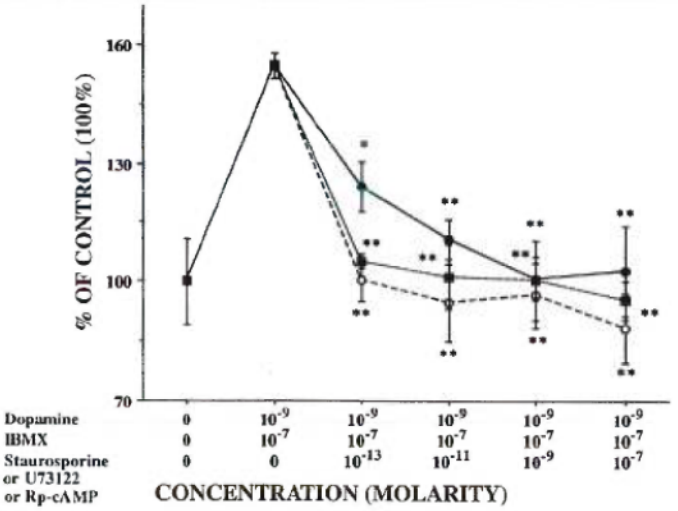


Fig. 3. Inhibitory effect of U-73122, staurosporine, or (*R*)-*p*-adenosine 3′,5′-cyclic monophosphorothioate (Rp-cAMP[S]) on expression of pOGH (angiotensinogen N −1498/+18) in OK 27 cells in the presence of dopamine. Cells were incubated for 24 h in the presence of dopamine (10^{-9} M), 1% depleted fetal bovine serum, 5 mM ascorbic acid, and 0.1 μM IBMX. Media were harvested and assayed for the level of ir-hGH. The levels of ir-hGH in the absence of dopamine, U-73122, staurosporine, or Rp-cAMP[S] are expressed as 100% (control). The inhibitory effect of U-73122, staurosporine, or Rp-cAMP[S] is compared with cells that were stimulated by dopamine. Each point represents the mean ± SD of at least 3 dishes (*$P \leq 0.05$ and **$P \leq 0.01$). Similar results were obtained from another experiment (○, U-73122; ●, staurosporine; ■, Rp-cAMP[S]).

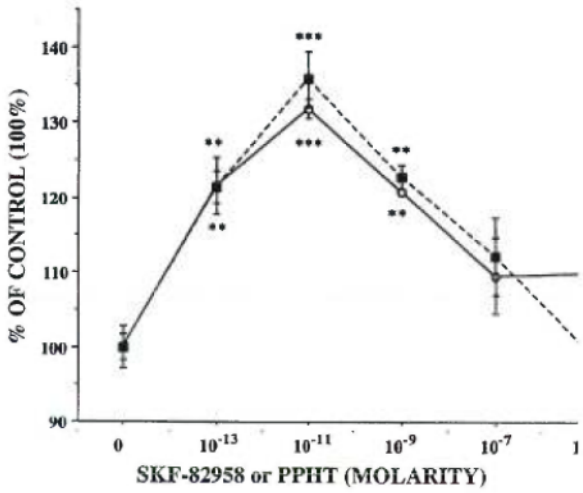


Fig. 4. Effect of SKF-82958 or PPHT on expression of pOGH tensinogen N −1498/+18) in OK 27 cells. Cells were incubat h in the presence of various concentrations (10^{-13} to 10 SKF-82958 or PPHT. Media were harvested after 24 h of in and assayed for ir-hGH. Levels of ir-hGH in the absence 82958 or PPHT are expressed as 100% (control). Each poi sents the mean ± SD of at least 3 dishes (**$P \leq 0.01$ and 0.005). Similar results were obtained from 2 other experim SKF-82958; ■, PPHT).

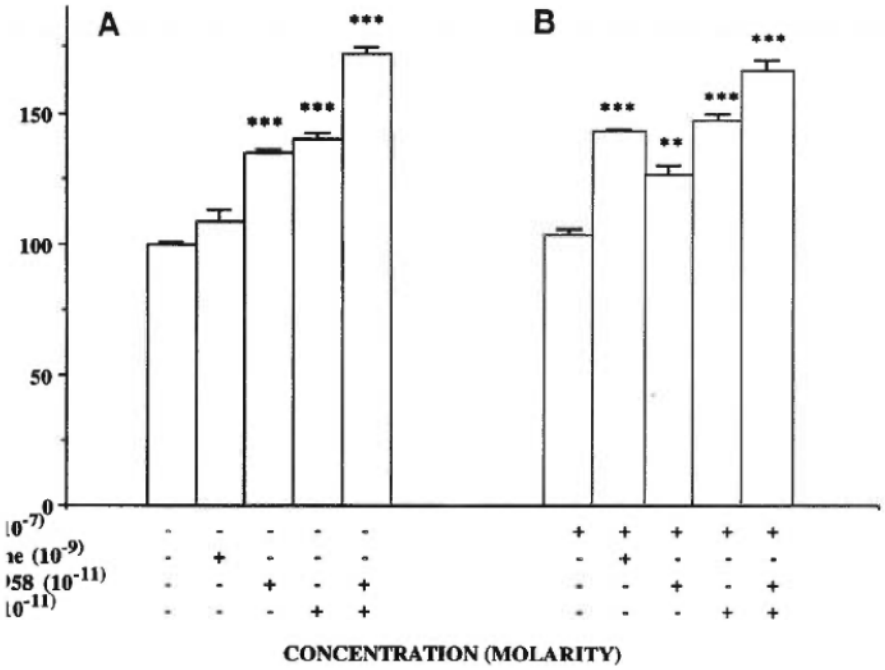
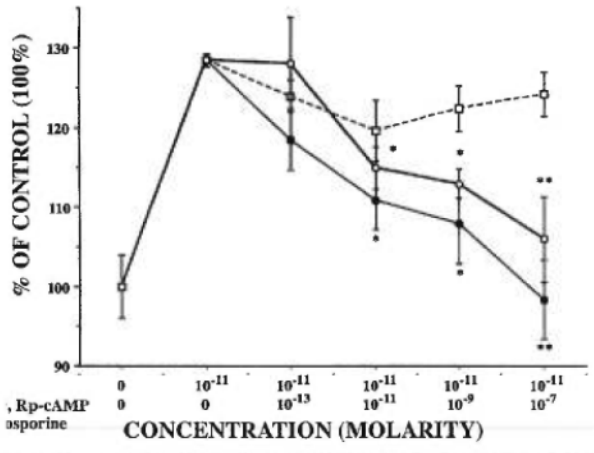


Fig. 5. Effect of dopamine, SKF-82958, PPHT, or a combination of both SKF-82958 and PPHT on expression of pOGH (angiotensinogen N −1498/+18) in OK 27 cells. Cells were incubated for 24 h in the presence of 5 mM ascorbic acid and dopamine (10^{-9} M) or SKF-82958 (10^{-11} M) or PPHT (10^{-11} M) in the absence (*A*) or presence (*B*) of 0.1 μM IBMX. The levels of ir-hGH in the absence of dopamine, SKF-82958, or PPHT are expressed as 100% (control). Each point represents mean ± SD of at least 3 dishes (**$P \leq 0.01$ and ***$P \leq 0.005$). Similar results were obtained from 2 other experiments.

ce of SKF-82958 or PPHT. Figure 6 shows that on of either SCH-23390 (10^{-13} to 10^{-7} M) or MP[S] (10^{-13} to 10^{-7} M) inhibited the stimulatory f SKF-82958 (10^{-11} M) on the expression of pOGH ensinogen N −1498/+18) in OK 27 cells in a dose-lent manner. The effective dose for the inhibition of nulatory expression (by SKF-82958) of the pOGH ensinogen N −1498/+18) was found with 10^{-11} M 3390 ($P \leq 0.05$) and 10^{-11} M Rp-cAMP[S] ($P \leq 0.05$). aximum inhibition was found with 10^{-7} M SCH-23390 or Rp-cAMP[S] ($P \leq 0.01$). Addition of staurosporine (10^{-13} to 10^{-7} M) did not significantly inhibit the stimulatory effect of SKF-82958 on the expression of pOGH (angiotensinogen N −1498/+18) in OK 27 cells. These studies suggest that the stimulatory effect of SKF-82958 is mediated via the D_1-dopaminergic receptor and cAMP-dependent protein kinase A I and II.

Similarly, Fig. 7 illustrates that the addition of spiperone or staurosporine at 10^{-13} to 10^{-5} M inhibited



nhibitory effect of SCH-23390, Rp-cAMP[S], or staurosporine pression of pOGH (angiotensinogen N −1498/+18) in OK 27 nulated by SKF-82958. Cells were incubated for 24 h in the of SKF-82958 (10^{-11} M) and various concentrations (10^{-13} to of SCH-23390 or Rp-cAMP[S]. Media were harvested and for the level of ir-hGH. Levels of ir-hGH in the absence of SKF-82958, SCH-23390, Rp-cAMP[S], or staurosporine are d as 100% (control). The inhibitory effect of SCH-23390, P[S], or staurosporine is compared with cells that were ed by SKF-82958 at 10^{-11} M. Each point represents the SD of at least 3 dishes (*$P \leq 0.05$ and **$P \leq 0.01$) (●, SCH ○, Rp-cAMP[S]; □, staurosporine). Similar results were from 2 other experiments.

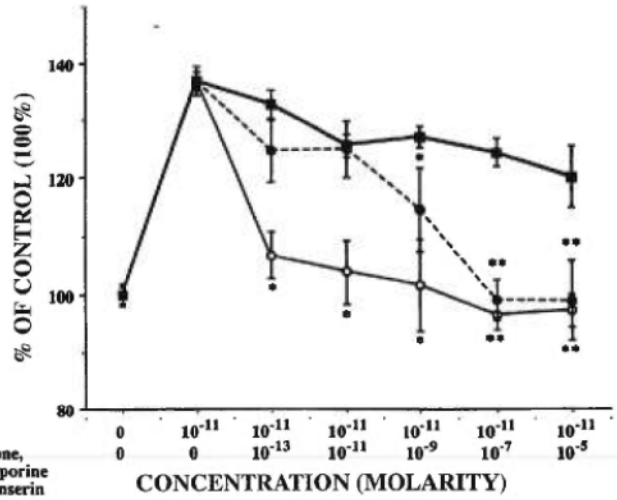


Fig. 7. The inhibitory effect of spiperone, staurosporine, or ketanserin on expression of pOGH (angiotensinogen N −1498/+18) in OK 27 cells stimulated by PPHT. Cells were incubated for 24 h in the presence of PPHT (10^{-11} M) and various concentrations (10^{-13} to 10^{-5} M) of spiperone, staurosporine, or ketanserin. Media were harvested and assayed for levels of ir-hGH. Levels of ir-hGH in the absence of 10^{-11} M PPHT, spiperone, staurosporine, or ketanserin are expressed as 100% (control). The inhibitory effect of spiperone, staurosporine, or ketanserin is compared with cells that were stimulated by PPHT at 10^{-11} M. Each point represents mean ± SD of at least 3 dishes (*$P \leq 0.05$ and **$P \leq 0.01$) (○, spiperone; ●, staurosporine; ■, ketanserin). Similar results were obtained from 2 other experiments.

the stimulatory effect of PPHT (10^{-11} M) on the expression of pOGH (angiotensinogen N −1498/+18) in OK 27 cells in a dose-dependent manner. The effective dose for the inhibition of the stimulated expression (by PPHT) of the pOGH (angiotensinogen N −1498/+18) was found with 10^{-13} M spiperone ($P \leq 0.05$) or 10^{-9} M staurosporine ($P \leq 0.05$). Addition of ketanserin (10^{-13} to 10^{-5} M) did not significantly inhibit the stimulatory effect of PPHT on the expression of pOGH (angiotensinogen N −1498/+18) in OK 27 cells. It appears that the stimulatory effect of PPHT is mediated via the D_2-dopaminergic receptor and PKC pathway.

Effect of dopamine, SKF-82958, or PPHT on expression of pTKGH in OK 13 cells. In OK 13 cells, neither dopamine (10^{-9} M), SKF-82958 (10^{-11} M), nor PPHT (10^{-11} M) stimulated the expression of pTKGH compared with the controls (absence of dopamine, SKF-82958, or PPHT) (Fig. 8).

DISCUSSION

Clones OK 27 and OK 13 are stable transformants with pOGH (angiotensinogen N −1498/+18) and pTKGH integrated into OK cellular genomes, respectively. The characteristics of these clones have been previously reported (41). Briefly, these clones have grown in the medium containing 500 μg/ml of G-418 for more than 3 mo and expressed a high amount of ir-hGH into the medium. The expression of pOGH (angiotensinogen N −1498/+18) and pTKGH in OK 27 and OK 13 cells, respectively, was time dependent. The levels of ir-hGH in cellular extracts were consistently less than 5% of those found in the media, indicating that ir-hGH is not stored in the cell.

We are surprised that the addition of dopamine in the absence of 0.1 μM IBMX (an inhibitor of phosphodiesterase) had a minimal effect on the expression of the pOGH (angiotensinogen N −1498/+18) in OK (Fig. 1). At present, we do not understand wh mine had a minimal effect on the expression of (angiotensinogen N −1498/+18) in OK 27 cell absence of IBMX. One possible explanation may the intracellular cAMP (stimulated by dopan rapidly degraded by the endogenous phospho ase. This possibility is supported by our data and the studies of Cheng et al. (9), who show dopamine was much more effective in the stim of intracellular cAMP in OK cells in the pres IBMX. On the other hand, addition of IBMX (10^{-5} M) alone did not affect the basal expres pOGH (angiotensinogen N −1498/+18) in OK (unpublished results). These data suggest th inhibition of the endogenous phosphodiesteras by IBMX is not sufficient to affect the basal exp of the angiotensinogen gene in OK 27 cells.

In the presence of IBMX, dopamine stimula expression of pOGH (angiotensinogen N −1498/ OK 27 cells at low concentrations (10^{-11} to 1 whereas higher concentrations of dopamine (i.e., than 10^{-7} M) had no effect (Fig. 1). Our studie from the studies of Cheng et al. (9), who show dopamine was effective in stimulating the cAMP tion at doses 10^{-8} to 10^{-4} M with an optimum 10^{-4} M. At present, we do not know the reasons discrepancy between our results (Fig. 1) and the of Cheng et al. (9). One possible explanation that the discrepancy is due to different metho ployed in the studies by Cheng et al. (9), who me intracellular cAMP after 10 min of stimulat dopamine in vitro, whereas we employed intact cells for the studies of gene expression. The possibility may be due to the source of OK cells our studies and in the studies by Cheng et al. (9 our OK 27 cell is a subclone and derived heterogenous population (i.e., obtained from AT is possible that OK 27 cells may be different [i.e. receptor level and the type(s) of adenylyl cycla sent in the membrane] from OK cells in the stu Cheng et al. (9). More studies are necessary to this discrepancy.

Addition of the D_1-dopaminergic receptor ant (SCH-23390) or D_2-dopaminergic receptor ant (spiperone) inhibited the stimulatory effect of mine, whereas addition of ketanserin had no effe 2). These data suggest that the effect of dopar probably mediated via both the D_1- and D_2-dopa gic receptors in OK cells and not via the serot receptors. Addition of low concentrations of sta rine or Rp-cAMP[S] (i.e., 10^{-13} to 10^{-11} M) com inhibited the stimulatory effect of dopamine (1 (Fig. 3). These studies strongly indicate that the latory effect of dopamine is via both PKC- and dependent protein kinase A I and II on the expre the pOGH (angiotensinogen N −1498/+18) in cells. The involvement of the PKC pathway is supported by the observation that U-73122 in the stimulatory effect of dopamine (Fig. 3) and

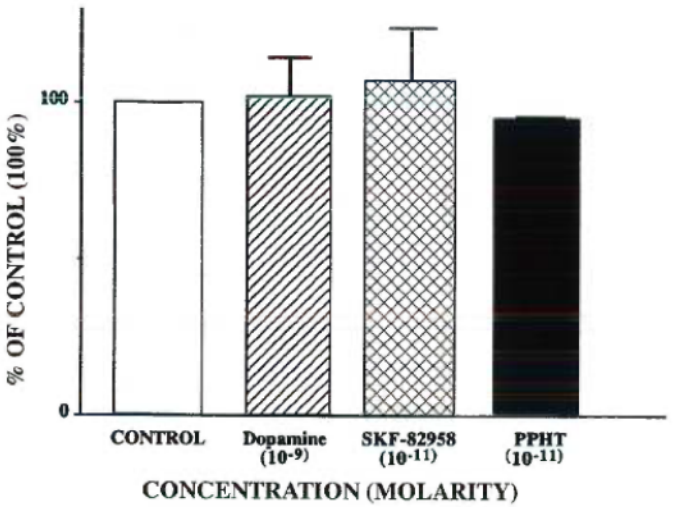


Fig. 8. Effect of dopamine, SKF-82958, and PPHT on expression of pTKGH in OK 13 cells. Cells were incubated for 24 h in the presence of dopamine (10^{-9} M), SKF-82958 (10^{-11} M), or PPHT (10^{-11} M). Media were harvested after 24 h of incubation and assayed for ir-hGH. Levels of ir-hGH in the medium without addition of dopamine, SKF-82958, or PPHT are expressed as 100% (control). Each point represents means ± SD of at least 3 dishes. Open bar at *left* is without added drugs.

)lished results). Since U-73122 is an inhibitor of
ıolipase C and phospholipase A_2, it is conceivable
e addition of U-73122 might prevent the hydroly-
hosphatidyl inositol 4,5-bisphosphate and subse-
y inhibits the activation of PKC in OK 27 cells.
, this possibility is supported by the studies of
ı et al. (25) who showed that the addition of
22 abolishes the increase in particulate PKC
y stimulated by PTH in OK cells. Nevertheless,
xperiments are warranted to demonstrate that
ine can stimulate the increase of PKC activity in
ls.

addition of SKF-82958 or PPHT alone also
ated the expression of pOGH (angiotensinogen
98/+18) in OK 27 cells (Fig. 4). These studies
· support the presence of functional D_1- and
aminergic receptors in OK 27 cells. Indeed, the
studies of Nash et al. (28) demonstrated that
paminergic receptor mRNA is expressed in OK
n the OK kidney, both D_{1A}- and D_{1B}-dopaminer-
eptor genes are expressed. On the other hand,
s no report on the presence of D_2-dopaminergic
)rs in OK cells. Thus our studies are the first to
strate functional D_2-dopaminergic receptors in
lls. Nevertheless, molecular cloning of the D_2-
inergic receptor gene from OK cells is definitely
ed to demonstrate unequivocally the presence of
-dopaminergic receptor in OK cells. At present,
not know the reasons why, at concentrations
r than 10^{-9} M (Fig. 4), SKF-82958 or PPHT had a
al effect on the expression of the pOGH (angioten-
n N −1498/+18) in OK 27 cells. One possible
ation may be that high concentrations of dopa-
gic receptor agonists may rapidly desensitize or
gulate its own dopaminergic receptors. Indeed, this
lity is supported by the studies of Bates et al. (3, 4),
owed that dopamine induced rapid downregulation
lopaminergic receptors in OK cells. Obviously,
xperiments along these lines are needed.

interesting that in the absence of IBMX, addition
'-82958 or PPHT or a combination of both SKF-
and PPHT was more effective in stimulating the
sion of pOGH (angiotensinogen N −1498/+18) in
cells compared with dopamine alone (Fig. 5*A*).
data suggest that the lack of response by dopa-
ould not be explained solely on the rapid degrada-
cAMP. It is possible that intracellular cAMP may
eased on the addition of SKF-82958 or a combina-
f both SKF-82958 and PPHT, since we had
ısly demonstrated that higher cellular levels of
are correlated with the increase of expression of
giotensinogen gene in OK 27 cells (41). It is also
e that other factors, such as binding affinity and
city of the ligand to its receptor, may also play an
ant role. Clearly, more experiments are required
ify this observation.

ıe presence of IBMX, addition of dopamine (10^{-9}
ne was more effective than SKF-82958 but not
(Fig. 5*B*). The combination of both SKF-82958
PHT, however, was more effective than either
ine or SKF-82958 or PPHT alone (Fig. 5*B*),
suggesting an additive effect of SKF-82958 and PPHT. At present, we do not understand the molecular mechanism(s) for the additive effect of SKF-82958 and PPHT. One possibility may be that there is a "cross-talk" between SKF-82958 and PPHT. That is, PPHT may enhance the effect of SKF-82958 by inducing the phosphorylation of adenylyl cyclase. Nevertheless, more experiments are warranted along this line to elucidate the additive effect of SKF-82958 and PPHT on the expression of the pOGH (angiotensinogen N −1498/+18) in OK 27 cells. Experiments are underway in our laboratory.

The effect of SKF-82958 was blocked by the presence of SCH-23390 and Rp-cAMP[S] (an inhibitor of cAMP-dependent protein kinase A I and II) (Fig. 6) but not by staurosporine, suggesting that the effect of the D_1-dopaminergic receptor agonist on the expression of pOGH (angiotensinogen N −1498/+18) is mediated via the cAMP-dependent protein kinase A I and II and not via the PKC pathway. These data support our previous results that the addition of Rp-cAMP[S] blocked the stimulatory effect of 8-BrcAMP on the expression of the angiotensinogen gene in OK 27 cells (26, 41).

It is intriguing that the D_2-dopaminergic receptor agonist (PPHT) also stimulated the expression of the pOGH (angiotensinogen N −1498/+18) in OK 27 (Figs. 4, 5, and 7). The D_2-dopaminergic receptor is known to inhibit adenylyl cyclase as well as to activate K^+ channels (38). However, recent studies by Vallar et al. (39) have demonstrated that the D_2-dopaminergic receptor may also stimulate the hydrolysis of phosphatidyl inositol 4,5-bisphosphate in Ltk^- cells, suggesting that the D_2-dopaminergic receptor can generate multiple signals by coupling to more than one effector system in different cell types. Regrettably, the characterization of these transduction pathways in OK cells is very limited. Our present studies showed that spiperone (D_2-dopaminergic receptor antagonist) or staurosporine inhibited the effect of PPHT in a dose-dependent manner (Fig. 7), suggesting that the PKC pathway is involved in mediating the effect of PPHT on the expression of pOGH (angiotensinogen N −1498/+18). Indeed, our previous studies reported that the addition of phorbol ester [phorbol 12-myristate 13-acetate (PMA)] stimulated the expression of pOGH (angiotensinogen N −1498/+18) in OK 27 cells in a dose-dependent manner and the stimulatory effect of PMA could be blocked by addition of staurosporine (42). Taken together, our studies provide evidence that PKC may be involved in the expression of the angiotensinogen gene in OK cells. Nevertheless, more experiments are necessary to elucidate the molecular mechanism(s) of action of PPHT and the isoform(s) of PKC involved in the expression of angiotensinogen gene in OK cells. Indeed, experiments are underway in our laboratory.

Finally, it appears that neither dopamine, SKF-82958, nor PPHT had any effect on the expression of pTKGH in OK 13 cells (Fig. 8). Since the expression of the pTKGH in OK 13 cells is driven by the promoter/enhancer DNA sequence of the TK gene, these studies demonstrated that the promoter/enhancer DNA se-

quence of TK gene was not responsive to addition of dopamine, SKF-82958, or PPHT. The effect of dopamine, SKF-82958, and PPHT in OK 27 cells is mediated via the 5′-flanking region of the rat angiotensinogen gene of the fusion gene pOGH (angiotensinogen N −1498/+18).

In summary, our present studies demonstrate that low levels of dopamine plus IBMX, SKF-82958, or PPHT stimulated the expression of pOGH (angiotensinogen N −1498/+18) in OK 27 cells. The stimulatory effect of SKF-82958 was blocked by the presence of SCH-23390 or Rp-cAMP[S], whereas the stimulatory effect of PPHT was blocked by the presence of spiperone or staurosporine. Our studies raise the possibility that the expression of the angiotensinogen gene in renal proximal tubules may be modulated by dopaminergic receptors in vivo. The physiological significance of this stimulation of the angiotensinogen gene expression by dopaminergic receptors in vivo, however, remains to be determined. Indeed, studies are underway in our laboratory to investigate whether dopamine could modulate the expression of renal angiotensinogen gene in vivo and its physiological relevance.

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REFERENCES

1. **Aperia, A., A. Bertorrello, and I. Seri.** Dopamine causes inhibition of Na^+-K^+-ATPase activity in rat proximal convoluted tubule segments. *Am. J. Physiol.* 252 (*Renal Fluid Electrolyte Physiol.* 21): F39–F45, 1987.
2. **Baines, A. D., P. Ho, and R. Drangova.** Proximal tubular dopamine production regulates basolateral Na-K-ATPase. *Am. J. Physiol.* 262 (*Renal Fluid Electrolyte Physiol.* 31): F566–F571, 1992.
3. **Bates, M., M. G. Caron, and J. R. Raymond.** Desensitization of DA_1 dopamine receptors coupled to adenylyl cyclase in opossum kidney cells. *Am. J. Physiol.* 260 (*Renal Fluid Electrolyte Physiol.* 29): F937–F945, 1991.
4. **Bates, M. D., C. L. Olsen, B. N. Becker, F. J. Albers, J. P. Middleton, J. G. Mulheron, S. L. Catherine-Jin, M. Conti, and J. R. Raymond.** Elevation of cAMP is required for down-regulation, but not agonist-induced desensitization of endogenous dopamine D_1 receptors in opossum kidney cells. *J. Biol. Chem.* 268: 14757–14763, 1993.
5. **Braam, B., K. D. Mitchell, J. Fox, and L. G. Navar.** Proximal tubular secretion of angiotensin II in rats. *Am. J. Physiol.* 264 (*Renal Fluid Electrolyte Physiol.* 33): F891–F898, 1993.
6. **Chan, J. S. D., A. H. H. Chan, Q. Jiang, Z.-R. Nie, S. Lachance, and S. Carrière.** Molecular cloning and expression of the rat angiotensinogen gene. *Pediatr. Nephrol.* 4: 429–435, 1990.
7. **Chan, J. S. D., A. H. H. Chan, Z.-R. Nie, R. Sikstrom, S. Lachance, S. Hashimoto, and S. Carrière.** Thyroid hormone, L-T_3, stimulates the expression of the angiotensinogen gene in cultured opossum kidney (OK) cells. *J. Am. Soc. Nephrol.* 2: 1360–1367, 1992.
8. **Chan, J. S. D., M. Ming, Z.-R. Nie, R. Sikstrom, S. L and S. Carrière.** Hormonal regulation of expressi angiotensinogen gene in cultured opossum kidney (OK) tubular cells. *J. Am. Soc. Nephrol.* 2: 1516–1522, 1992.
9. **Cheng, L., P. Precht, D. Frank, and C. Tony Lia** mine stimulation of cAMP production in cultured kidney cells. *Am. J. Physiol.* 258 (*Renal Fluid Electrolyt* 27): F877–F882, 1990.
10. **Dawson, R., R. Felheim, and M. Philipps.** Characte the synthesis and release of dopamine in LLC-PI *Physiol. Biochem.* 17: 85–100, 1994.
11. **Dzau, V. J., and J. R. Ingelfinger.** Molecular bi pathophysiology of the intrarenal renin-angiotensin s *Hypertens.* 7, *Suppl.* 7: 53–58, 1989.
12. **Felder, C. C., T. Campbell, F. Albrecht, and P.** Dopamine inhibits Na^+-H^+ exchanger activity in renal stimulation of adenylate cyclase. *Am. J. Physiol.* 2 *Fluid Electrolyte Physiol.* 28): F297–F303, 1990.
13. **Fogo, A., Y. Yoshida, A. Yared, and I. Ickikada.** Imp angiogenic action of angiotensin-II in the glomerular maturing kidneys. *Kidney Int.* 38: 1068–1074, 1990.
14. **Hagege, J., and G. Richet.** Proximal tubule dopamine rescence in renal slices incubated with L-dopa. *Kidne* 3–8, 1985.
15. **Harris, P. J., and J. A. Young.** Dose-dependent stimul inhibition of proximal tubular sodium reabsorption by sin II in rat kidney. *Pfluegers Arch.* 367: 1295–1297, 19
16. **Ingelfinger, J. R., B. Boubounes, F. F. Jun, and S.** High glucose downregulates expression of renin an system (RAS) in opossum kidney cells (Abstract). *Pediatr.* 29: 344A, 1991.
17. **Ingelfinger, J. R., W. M. Zuo, E. A. Fon, K. E. Elli V. J. Dzau.** In situ hybridization evidence for angiote mRNA in the rat proximal tubule. A hypothesis for the i renin angiotensin system. *J. Clin. Invest.* 85: 417–423,
18. **Johnston, C. I., B. Fabris, and K. Jandeleit.** I renin-angiotensin system in renal physiology and path ogy. *Kidney Int.* 44, *Suppl.* 42: 559–563, 1993.
19. **Jose, P. A., R. A. Felder, R. R. Holloway, and G. M.** Dopamine receptors modulate sodium excretion in de kidney. *Am. J. Physiol.* 250 (*Renal Fluid Electrolyte Ph* F1033–F1038, 1986.
20. **Kinoshita, S., E. Ohstein, and R. A. Felder.** Do receptors in rat proximal convoluted tubule: regulation renal dopamine. *Am. J. Physiol.* 258 (*Renal Fluid E Physiol.* 27): F1068–F1074, 1990.
21. **Koyama, H., C. Goodpasture, M. M. Miller, R. L. and A. D. Riggs.** Establishment and characterization line from the American opossum (*Didelphys virginia*). *Rockville* 14: 239–246, 1978.
22. **Lee, M. R.** Dopamine and the kidney. *Clin. Sci. I* 439–448, 1982.
23. **Liu, F. Y., and M. G. Cogan.** Angiotensin II stimula proximal bicarbonate absorption in the rat by decreasi adenosine monophosphate. *J. Clin. Invest.* 84: 83–91, 1
24. **Lokhandwala, M. F., and R. J. Barrett.** Cardi dopamine receptors: physiological, pharmacological and t tic implications. *J. Auton. Pharmacol.* 3: 189–215, 1982
25. **Martin, K. J., C. L. McConkey, A. K. Jacob, E. A. G M. Khan, and J. J. Baldassare.** Effect of U-73,122, an of phospholipase C on actions of parathyroid hormone sum kidney cells. *Am. J. Physiol.* 266 (*Renal Fluid E Physiol.* 35): F254–F258, 1994
26. **Ming, M., T. T. Wang, S. Lachance, A. Delalandre, ère, and J. S. D. Chan.** Expression of the angiotensino is synergistically stimulated by 8-bromo-cAMP and dex sone in opossum kidney cells. *Am. J. Physiol.* 268 (*Re Integrative Comp. Physiol.* 37): R105–R111, 1995.
27. **Nakamura, A., and E. J. Johns.** Effect of renal n expression of renin and angiotensinogen genes in ra *Am. J. Physiol.* 266 (*Endocrinol. Metab.* 29): E230–E24
28. **Nash, S. R., N. Godinot, and M. G. Caron.** Clo characterization of the opossum kidney cell D_1 dopami tor: expression of identical D_{1A} and D_{1B} dopamine

NAs in opossum kidney and brain. *Mol. Pharmacol.* 44: -925, 1993.

richot, R., A. Garcia-Ocana, S. Couette, E. Comoy, and Vitovsnik. Endogenous dopamine (DA) modulates Na-Pi ansport in proximal tubular cells (PTC) (Abstract). *J. Am. Nephrol.* 4: 713, 1993.

ock, A. S., D. G. Warnock, and G. J. Strewler. Parathy- hormone inhibition of Na^+-H^+ antiporter activity in a ured renal cell line. *Am. J. Physiol.* 250 (*Renal Fluid trolyte Physiol.* 19): F217–F225, 1986.

comani, G., K. D. Mitchell, and L. G. Navar. Angiotensin imulation of Na^+-H^+ exchange in proximal tubular cells. *J. Physiol.* 258 (*Renal Fluid Electrolyte Physiol.* 27): F1188–95, 1990.

uels, H. H., F. Stanley, and L. E. Shapiro. Control of rth hormone synthesis in cultured GH cells by 3,5,3′-triio-L- onine and glucocorticoids agonists and antagonists: studies he independent and synergistic regulation of the growth none response. *Biochemistry* 18: 715–721, 1979.

uster, V. L., J. P. Kokko, and H. R. Jacobson. Angiotensin rectly stimulates sodium transport in rabbit proximal convo- l tubules. *J. Clin. Invest.* 73: 507–515, 1984.

taly, M. G., B. S. Arant, F. D. Seney, L. Rutlege, and J. en. Endogenous angiotensin concentrations in specific intra- l fluid compartments of the rat. *J. Clin. Invest.* 86: 1352–7, 1990.

, I., B. C. Kone, S. R. Gullans, A. Aperia, B. M. Brenner, B. J. Ballermann. Locally formed dopamine inhibits -K^+ ATPase activity in rat renal cortical tubule cells. *Am. J. siol.* 255 (*Renal Fluid Electrolyte Physiol.* 24): F666–F673, 3.

36. **Tang, S. S., F. Jung, D. Diamant, D. Brown, D. Bachinsky, P. Hellman, and J. Ingelfinger.** Temperature-sensitive SV 40 immortalized rat proximal tubular cell line has functional renin-angiotensin system. *Am. J. Physiol.* 268 (*Renal Fluid Electrolyte Physiol.* 37): F435–F446, 1995.
37. **Terada, Y., K. Tomita, H. Nonoguch, and F. Marumo.** PCR localization of angiotensin II receptor and angiotensinogen mRNA in rat kidney. *Kidney Int.* 43: 1251–1259, 1993.
38. **Vallar, L., and J. Meldolesi.** Mechanisms of signal transduction at the dopamine D_2 receptor. *Trends Pharmacol. Sci.* 10: 74–77, 1989.
39. **Vallar, L., C. Muca, M. Magni, P. Albert, J. Bunzow, J. Meldolesi, and O. Civelli.** Differential coupling of dopaminergic D_2 receptors expressed in different cell types. *J. Biol. Chem.* 265: 10320–10326, 1990.
40. **Wang, T., and Y. L. Chan.** Mechanism of angiotensin II action on proximal tubular transport. *J. Pharmacol. Exp. Ther.* 252: 689–695, 1991.
41. **Wang, T. T., M. Chen, S. Lachance, A. Delalandre, S. Carrière, and J. S. D. Chan.** Isoproterenol and 8-bromo-cyclic monophosphate stimulate the expression of the angiotensinogen gene in opossum kidney cells. *Kidney Int.* 46: 703–710, 1994.
42. **Wang, T. T., S. Lachance, A. Delalandre, S. Carrière, and J. S. D. Chan.** Alpha-adrenoceptors and angiotensinogen gene expression in opossum kidney cells. *Kidney Int.* 48: 139–145, 1995
43. **Wolf, G., and E. G. Neilson.** Angiotensin II as a hypertrophogenic cytokine for proximal tubular cells. *Kidney Int.* 43, *Suppl.* 39: S100–S107, 1992.

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pha-adrenoceptors and angiotensinogen gene expression in opossum kidney cells

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renoceptors and angiotensinogen gene expression in opossum
. To investigate whether alpha (α)-adrenoceptor agonists have
ry effect on the expression of the angiotensinogen (Ang) gene
kidney (OK) cells, we used OK 27 cells with a fusion gene
the 5′-flanking regulatory sequence of the rat angiotensinogen
with a human growth hormone (hGH) gene as a reporter,
ig N-1498/+18), permanently integrated into their genomes.
f expression of the pOGH (Ang N-1498/+18) was quantitated
ount of immunoreactive-human growth hormone (IR-hGH)
o the medium. The addition of iodoclonidine (α_2-adrenocep-
10^{-13} to 10^{-9} M) and phorbol 12-myristate 13-acetate (PMA,
$^{-5}$ M) stimulated the expression of pOGH (Ang N-1498/+18)
dependent manner, whereas the addition of phenylephrine
eptor agonist, 10^{-13} to 10^{-5} M) had no effect. The stimulatory
doclonidine was blocked by the presence of yohimbine (α_2-
or antagonist) and staurosporine (an inhibitor of protein
ut not blocked by the presence of prazosin (α_1-adrenoceptor
or Rp-cAMP (an inhibitor of cAMP-dependent protein kinase
dition of iodoclonidine, phenylephrine or PMA had no effect
ession of pTKGH in OK 13 cells, an OK cell line, into which
ably integrated a fusion gene, pTKGH containing the promot-
DNA sequence of the viral thymidine-kinase (TK) gene fused
an growth hormone gene as a reporter. These studies demon-
the expression of the pOGH (Ang N-1498/+18) in OK 27 cells
d by α_2-adrenoceptors and the protein kinase C pathway. Our
te that OK 27 cells provide a useful model to study the
of the expression of the Ang gene *in vitro*.

stence of a local intrarenal renin-angiotensin system
s now been generally accepted [1, 2]. Evidence indicated
tensinogen (Ang) mRNA and renin mRNA are present
renal proximal tubules [3–5]. Measurement of angio-
els in the lumen of rat proximal tubules showed that the
tion of angiotensin I (Ang I) or angiotensin II (Ang II)
as 10^{-9} M, whereas the level of plasma Ang I or Ang II
n 10^{-12} M [6–8]. Moreover, recent studies also showed
vels of urinary angiotensins are independent of plasma
n levels [9]. Thus, these studies demonstrated that the
imal tubule is a major site of intrarenal Ang formation.
, as well as Ingelfinger et al [11], have demonstrated that
nRNA is expressed in opossum kidney (OK) cells. We
reported that dexamethasone and 8-bromo-cyclic aden-
osine monophosphate (8-Br-cAMP) stimulate the expression of the Ang gene in OK cells [12]. More recently, we have reported that the addition of isoproterenol stimulates the expression of the Ang gene and increases the accumulation of intracellular cAMP in OK 27 cells, an OK cell line into which has been stably integrated a fusion gene, pOGH (Ang N-1498/+18), containing the endogeneous 5′-flanking regulatory DNA sequence of the rat Ang gene fused with a human growth hormone (hGH) gene as a reporter [13]. These studies raise the possibility that the increase of intracellular cAMP, which is induced by the stimulation of β-adrenoceptors (that is, via the activation of renal nerves), may enhance the expression of the renal Ang gene. The local formation of renal Ang II might then modulate the physiology of the renal proximal tubular cells (that is, sodium and fluid absorption) [14–16]. Thus, local renal RAS might play an important role in the modulation of sodium reabsorption.

Alpha-adrenoceptors have been detected on the proximal tubules of rat kidney with the dominance of the α_2-adrenoceptors [17–20]. It is estimated that the amount of α_2-adrenoceptors is over twofold greater than α_1-adrenoceptors in the rat proximal tubules [21]. Studies have shown that renal nerves, acting through α-adrenoceptors enhance proximal tubular sodium reabsorption (anti-natriuresis) in the kidney [20, 22]. Alpha-adrenoceptors are also expressed in OK cells [23, 24]. OK cells express an α_2-adrenoceptor which has pharmacological characteristics different from both the α_{2A} and α_{2B} subtypes. It has been tentatively named α_{2C} subtype [24], and this subtype is negatively coupled to adenylate cyclase. The presence of α_2-adrenoceptor subtypes raises the question of whether the activation of this receptor subtype could inhibit or modulate the expression of the Ang gene in OK cells.

Thus, in the present studies, we investigated the possible effect of alpha (α)-adrenoceptor agonists on the expression of the Ang gene in OK 27 cells. We also examined whether the addition of phorbol 12-myristate 13-acetate (PMA, a protein kinase C stimulator) has an effect on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells. Our results indicated that the expression of pOGH (Ang N-1498/+18) fusion gene in OK 27 cells is stimulated by the addition of iodoclonidine (α_2-adrenoceptor agonist) and PMA, but not by the addition of phenylephrine (α_1-adrenoceptor agonist). Furthermore, the addition of yohimbine (α_2-adrenoceptor antagonist) and staurosporine (an inhibitor of protein kinase C) blocked the stimulatory effect of iodoclonidine.

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Methods

Materials

The fusion gene, pOGH (Ang N-1498/+18), containing the 5′-flanking sequence [1498 base-pair (bp)] upstream of the transcriptional site plus 18 bp of Exon I of the rat Ang gene fused with a hGH gene, has been described previously [25]. The plasmid, pRSV-Neo, containing the coding sequence for Neomycin (Neo) with the Rous sarcoma virus (RSV) enhancer/promoter sequence fused into the 5′-end of the Neomycin gene was a gift from Dr. Teresa Wang (Dept. of Pathology, Stanford University, Stanford, CA, USA). The plasmid, pTKGH, containing the thymidine kinase (TK) enhancer/promoter sequence fused to the 5′-end of the hGH gene was purchased from the Nichols Institute of Diagnostics (La Jolla, CA, USA).

The radioimmunoassay kit for hGH (RIA-hGH) was a gift from NIADDK (NIH, Bethesda, MD, USA). The double antibody RIA procedure was similar to that previously used for the radioimmunoassay of ovine placental lactogen (RIA-oPL) [26]. Human [^{125}I]iodo GH was prepared by a slight modification of the lactoperoxidase method of Thorell and Johannson [27]. NIAMDD-hGH-I-1 (AFP-4793 B) was used for both iodination and as a hormone standard. The limit of sensitivity of the assay was 0.1 ng/ml. The inter- and intra-assay coefficients of variation were 10% ($N = 10$) and 12% ($N = 10$), respectively.

P-iodoclonidine hydrochloride (α_2-adrenoceptor agonist), phenylephrine hydrochloride (α_1-adrenoceptor agonist), yohimbine hydrochloride (α_2-adrenoceptor antagonist), prazosin hydrochloride (α_1-adrenoceptor antagonist), staurosporine (an inhibitor of protein kinase C), phorbol 12-myristate 13-acetate (PMA; a stimulator of protein kinase C) and Rp-cAMP (an inhibitor of cAMP-dependent protein kinase A I & II) were all purchased from Research Biochemicals Inc. (RBI, Natick, MA, USA).

Na-^{125}I was purchased from Dupont, New England Nuclear (NEN; Boston, MA, USA). Calcium chloride was purchased from Mallinckrodt, Inc. (Montreal, Quebec, Canada), Geneticin (G 418) was purchased from Bethesda Research Laboratories (Gibco-BRL, Burlington, Ontario, Canada). Other molecular biology grade reagents were obtained either from Sigma Chemicals (St. Louis, MO, USA), Gibco-BRL, Boehringer-Mannheim (Dorval, Quebec, Canada), Pharmacia Inc. (Baie d'Urfe, Quebec, Canada) or Promega-Fisher, Inc. (Montreal, Quebec, Canada).

Cell culture

The opossum kidney (OK) proximal tubular cell line was obtained from the American Type Tissue Culture Collection (ATCC) (Rockville, MD, USA). This cell line is derived from the kidney of a female American opossum which retains several properties of proximal tubular epithelial cells in culture and expresses a low level of Ang mRNA [10, 28, 29].

The cells were initially grown in 100 × 20 mm plastic Petri dishes (Gibco) in Dulbecco's Modified Eagle's Medium (DMEM), pH 7.45, supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin and 50 μg/ml streptomycin. The cells were grown in a humidified atmosphere of 95% 0_2, 5% CO_2 at 37°C. For subculturing, the cells were trypsinized (0.05% trypsin and EDTA) and plated at 2.5×10^4 cells/dish (100 × 20 mm).

OK cell stable transformants

The method for the selection of OK cell stable transformants with high expression of pOGH (Ang N-1498/+18) (OK 27) or pTKGH (OK 13) has been described previously [1 plasmids pOGH (Ang N-1498/+18) or pTKGH and fusion genes were co-transfected (20 μg each) into OK 10^6 cells) utilizing calcium phosphate-mediated endocy transfection, the cells were cultured overnight in 5.0 ml containing 10% FBS. Then the medium was replaced medium containing 10% FBS and 500 μg/ml of aminog 418 (Geneticin, Gibco Inc.). The stable transformants able to grow in the presence of G418 and which sec levels of IR-hGH into the medium were further subcl the method of limiting dilution. Those cells that h through at least three repetitions of limiting dilution a ued to secrete high levels of IR-hGH after three mo presence of G 418 were considered to be stable clones obtained three and six clones with pOGH (Ang N-149 pTKGH integrated into their genomes, respective present study, we used two of these clones, clone OK 13 which have pOGH (Ang N-1498/+18) and pTKGH into their genomes, respectively.

Effect of iodoclonidine or phenylephrine or phorbol 12-13-acetate (PMA) on the expression of pOGH (Ang N- in OK 27 cells

OK 27 cells were plated at a density of 1 to 2×10^5 c six-well plates and incubated overnight in DMEM conta FBS. The growth of the cells was arrested by the inc serum-free DMEM for 24 hours. Subsequently, vario trations of iodoclonidine (10^{-13} to 10^{-5} M) or phe (10^{-13} to 10^{-5} M) or PMA (10^{-13} to 10^{-5} M) were ad culture medium containing 1% dFBS and incubated fo

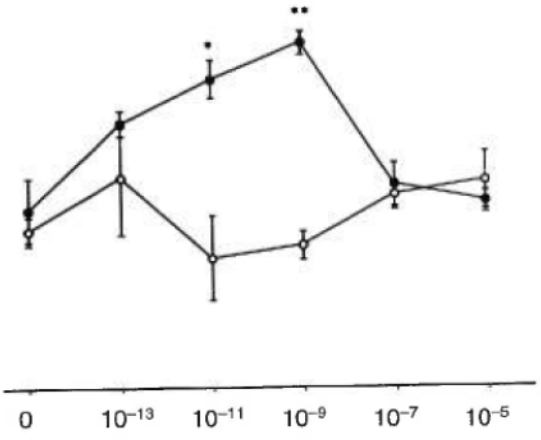
The depleted FBS was prepared by incubation wit vated charcoal and 1% analytical grade (AG 1 × 8) ion resin (Bio-Rad Laboratories, Richmond, CA, USA) fo or more at room temperature as described by Samue and Shapiro [30]. This procedure removed endogeno and thyroid hormones from the FBS, as demonstrated

Effect of yohimbine or prazosin or staurosporine or Rp- the expression of pOGH (Ang N-1498/+18) in OK 27 c presence of iodoclonidine

OK 27 cells were plated at a density of 1 to 2×10^5 c six-well plates and incubated overnight in DMEM cont dFBS. Then cell growth was arrested by incubation in s medium for 24 hours. Various concentrations of (10^{-13} to 10^{-5} M) or prazosin (10^{-13} to 10^{-5} M) or sta (10^{-13} to 10^{-5} M) or Rp-cAMP (10^{-13} to 10^{-5} M) were the culture medium and co-cultured with the iodoclon M) for 24 hours. At the end of the incubation period, m collected and kept at −20°C until assay for IR-hGH.

Effect of staurosporine on the expression of pOGH (An +18) in OK 27 cells in the presence of PMA

OK 27 cells were plated at a density of 1 to 2×10^5 c six-well plates and incubated overnight in DMEM cont dFBS. Cell growth was arrested by incubation in s medium for 24 hours. Then various concentrations of rine (10^{-13} to 10^{-5} M) were added to the culture me co-cultured with the PMA (10^{-7} M) for 24 hours. At the



ect of iodoclonidine and phenylephrine on the expression of g N-1498/+18) in OK 27 cells. Cells were incubated for up to 24 he presence of various concentrations of iodoclonidine or ine. Media were harvested after 24 hours of incubation and r immunoreactive human growth hormone (IR-hGH). The ion of IR-hGH in the absence of iodoclonidine or phenyleph- sidered as the control level. Each point represents the mean ± ast three dishes (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). e: (● iodoclonidine; ○ phenylephrine).

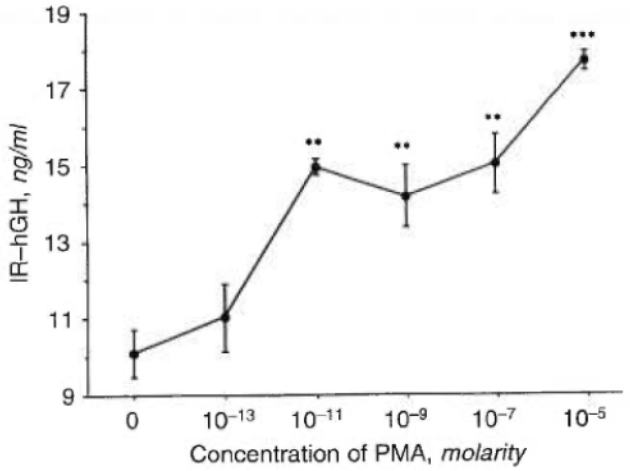


Fig. 2. *Effect of phorbol 12-myristate 13-acetate (PMA) on the expression of pOGH (Ang N-1498/+18) in OK 27 cells.* Cells were incubated for up to 24 hours in the presence of various concentrations of PMA. Media were harvested after 24 hours of incubation and assayed for IR-hGH. The concentration of IR-hGH in the absence of PMA is considered as the control level. Each point represents the mean ± SD of at least three dishes (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$).

ı period, media were collected and kept at −20°C until IR-hGH.

odoclonidine or phenylephrine or PMA on the expression of pTKGH in OK 13 cells

cells were plated at a density of 1 to 2 × 10^5 cells/well in lates and incubated overnight in DMEM containing 1% ell growth was arrested by incubation in serum-free for 24 hours. Subsequently, iodoclonidine (10^{-9} M) or ıtrine (10^{-9} M) or PMA (10^{-7} M) was added to the edium containing 1% dFBS and incubated for 16 hours. ıd of the incubation period, media were collected and 20°C until assay.

Statistical analysis

periments were performed at least three times in tripli- data were analyzed with Student's *t*-test or ANOVA A probability level of $P \leq 0.05$ was regarded as signifi-

Results

odoclonidine or phenylephrine or PMA on the expression of pOGH (Ang N-1498/+18) in OK 27 cells

1 shows the expression of the pOGH (Ang N-1498/+18) 7 cells in the presence of various concentrations of dine (10^{-13} to 10^{-5} M). A dose-dependent relationship iodoclonidine concentrations (10^{-13} to 10^{-9} M) and the ın of expression of pOGH (Ang N-1498/+18) was ob- appears that maximal stimulation of expression of the Ang N-1498/+18) was found with 10^{-9} M iodoclonidine. ntrations greater than 10^{-9} M, the stimulatory effect of dine is diminished. The addition of phenylephrine 10^{-5} M) had no stimulatory effect on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells, as shown in Figure 1.

A dose-dependent relationship between PMA concentrations (10^{-13} to 10^{-5} M) and the stimulation of expression of pOGH (Ang N-1498/+18) was also observed as shown in Figure 2. The effective and maximal stimulation of expression of the pOGH (Ang N-1498/+18) were found with 10^{-11} M ($P \leq 0.01$) and 10^{-5} M ($P \leq 0.001$) PMA. At concentrations greater than 10^{-5} M PMA, lesser amounts of IR-hGH were measured (unpublished results). This observation was due to an increased cell death in the culture.

These studies suggest that the stimulatory effect of α-adrenoceptors on the expression of pOGH (Ang N-1498/+18) in OK 27 cells is probably mediated via the α_2-adrenoceptors and protein kinase C pathway.

Effect of yohimbine or prazosin or staurosporine or Rp-cAMP on the expression of pOGH (Ang N-1498/+18) in OK 27 cells in the presence of iodoclonidine

Figure 3 shows that the addition of yohimbine (10^{-13} to 10^{-5} M) inhibited the stimulatory effect of iodoclonidine (10^{-9} M) on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells. The effective dose for inhibition of the stimulated expression (by iodoclonidine) of the pOGH (Ang N-1498/+18) was 10^{-9} M yohimbine ($P \leq 0.05$). The addition of prazosin (10^{-13} to 10^{-5} M) had no inhibitory effect on the expression of pOGH (Ang N-1498/+18) stimulated by iodoclonidine (10^{-9} M).

Similarly, the addition of staurosporine (10^{-13} to 10^{-5} M) inhibited the stimulatory effect of iodoclonidine (10^{-9} M) on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells in a dose-dependent manner (Fig. 4). The effective dose for the inhibition of the stimulated expression (by iodoclonidine) of the pOGH (Ang N-1498/+18) was 10^{-11} M staurosporine ($P \leq 0.05$; Fig. 4). The addition of staurosporine (10^{-13} to 10^{-5} M) alone had

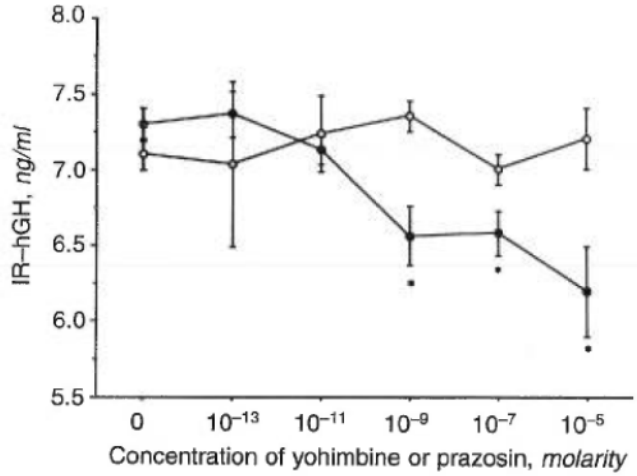


Fig. 3. *Inhibitory effect of yohimbine (α_2-adrenoceptor antagonist) or prazosin (α_1-adrenoceptor antagonist) on the expression of pOGH (Ang N-1498/+18) in OK 27 cells stimulated by iodoclonidine.* The cells were incubated for up to 24 hours in the presence of iodoclonidine (10^{-9} M) and various concentrations of yohimbine (10^{-13} to 10^{-5} M) or prazosin (10^{-13} to 10^{-5} M). Media were harvested and assayed for the level of IR-hGH. The concentration of IR-hGH in the absence of yohimbine or prazosin is considered as the control level. Each point represents the mean ± SD of at least three dishes (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). Symbols are: (● yohimbine; ○ prazosin).

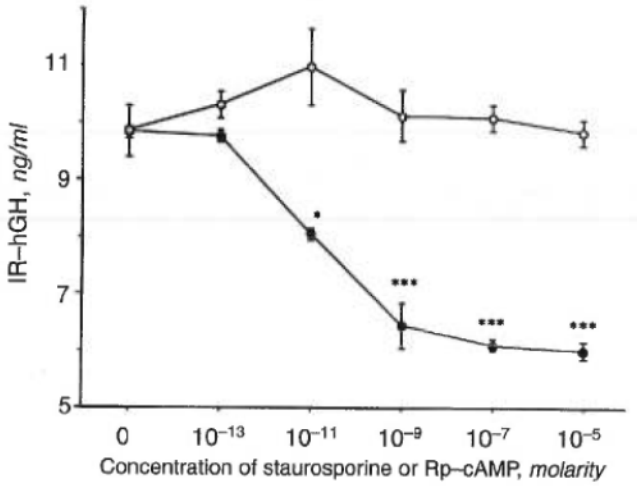


Fig. 4. *Inhibitory effect of staurosporine or Rp-cAMP on the expression of pOGH (Ang N-1498/+18) in OK 27 cells stimulated by iodoclonidine.* Cells were incubated for up to 24 hours in the presence of iodoclonidine (10^{-9} M) and various concentrations of staurosporine (10^{-13} to 10^{-5} M) or Rp-cAMP (10^{-13} to 10^{-5} M). Media were harvested and assayed for the level of IR-hGH. The concentration of IR-hGH in the presence of iodoclonidine (10^{-9} M) but in the absence of staurosporine or Rp-cAMP is the control level. Each point represents the mean ± SD of at least three dishes (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). Symbols are: (● staurosporine; ○ Rp-cAMP).

no significant effect on the expression of pOGH (Ang N-1498/+18) in OK 27 cells (unpublished data). On the other hand, the addition of Rp-cAMP (10^{-13} to 10^{-5} M) had no inhibitory effect on the expression of pOGH (Ang N-1498/+18) stimulated by iodoclonidine (10^{-9} M; Fig. 4).

Figure 5 illustrates that the addition of staurosporine (10^{-13} to

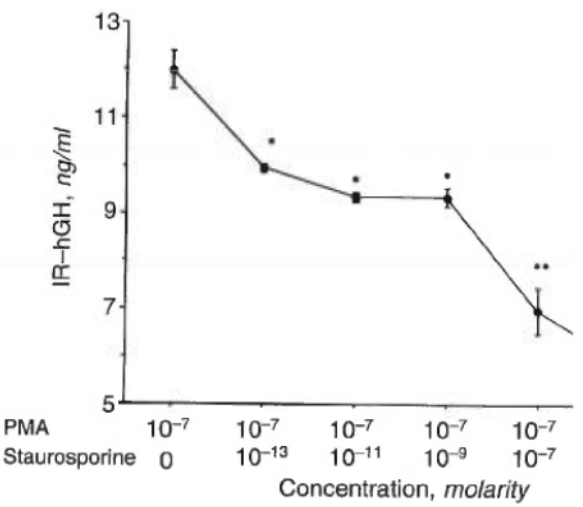


Fig. 5. *Effect of staurosporine on the expression of pOGH (A +18) in OK 27 cells stimulated by PMA.* Cells were incubated f hours in the presence of PMA (10^{-7} M) and various concer staurosporine (10^{-13} to 10^{-5} M). Media were harvested and the level of IR-hGH. The concentration of IR-hGH in the p PMA (10^{-7} M) but in the absence of staurosporine is the co Each point represents the mean ± SD of at least three dishes (**$P \leq 0.01$ and ***$P \leq 0.005$).

10^{-5} M) inhibited the stimulatory effect of PMA (10^{-7} expression of the pOGH (Ang N-1498/+18) in OK 27 dose-dependent manner. The effective dose for the inh the stimulated expression (by PMA) of the pOGH (An +18) was 10^{-13} M staurosporine ($P \leq 0.05$).

These studies further support the notion that acti α_2-adrenoceptors stimulate the expression of the fusior the protein kinase C pathway, but not via the cAMP-d protein kinase A pathway.

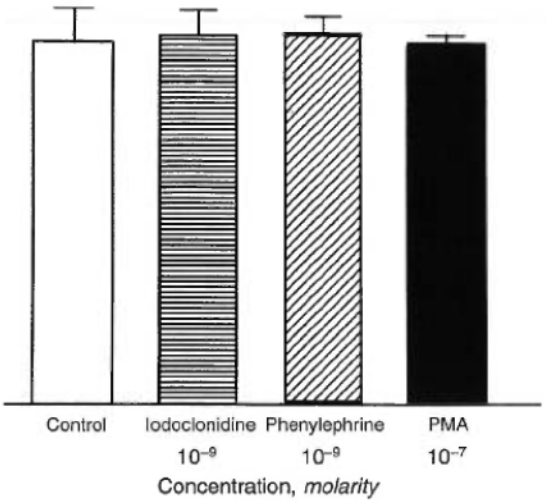
Effect of iodoclonidine or phenylephrine or PMA on the e of pTKGH in OK 13 cells

In OK 13 cells, neither iodoclonidine (10^{-9} M), phen (10^{-9} M) nor PMA (10^{-7} M) stimulated the expression o as compared to the controls (absence of iodoclonid nylephrine or PMA; Fig. 6).

Discussion

Clones OK 27 and OK 13 are stable transformants wit (Ang N-1498/+18) and pTKGH integrated into OK genomes, respectively. The characteristics of these clo been reported previously [13]. Briefly, the expression c (Ang N-1498/+18) and pTKGH in OK 27 and OK respectively, were time-dependent. The levels of IR cellular extracts were consistently less than 5% of that the culture media, suggesting that renal Ang, like hep [31], is not stored in the cell.

Renal nerves are known to influence sodium and fluid by the kidney [32–34]. The mechanism(s) whereby ren exert their effects, however, is not well understood. Recer by Nakamura and Johns [35] demonstrated that low level nerve stimulation decrease sodium excretion and incr levels of the Ang mRNA in the rat kidney *in vivo*, but r



ct of iodoclonidine, phenylephrine and PMA on the expression of OK 13 cells. Cells were incubated for 24 hours in the absence of iodoclonidine (10^{-9} M) or phenylephrine (10^{-9} M) or PMA fedia were harvested 24 hours after the incubation and assayed l. Each point represents the mean ± SD of a minimum of three concentration of IR-hGH in the absence of iodoclonidine, ine and PMA is the control level. The empty bar on the t is without added hormones. The second striped bar is from ne treated cells, the third hatched bar is from phenylephrine s and the solid black bar on the extreme right is from PMA eated cells (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$).

ı contrast, high levels of renal nerve stimulation increase ›f renin mRNA, but not Ang mRNA. Furthermore, the ıtion of the β_1-adrenoceptor antagonist, atenolol, ıe effect of renal nerve stimulation. This observation is by our recent studies that isoproterenol directly stim- expression on the Ang gene in OK cells *in vitro* [13]. The y effect of isoproterenol is blocked by the presence of ›l and the β_1-adrenoceptor antagonist, atenolol [13]. ›e studies indicate that the stimulation of renal nerve ›vate the intrarenal renin-angiotensin system (RAS) by g the expression of the Ang gene in the kidney *in vivo*. enal Ang II formation may then modulate the reabsorp- lium and fluids in the proximal tubules.

s express α_{2C}-adrenoceptor which is negatively coupled te cyclase [24]. The inhibitory effect of α_{2C}-adrenocep- MP production in OK cells raises the possibility that ceptor might also attenuate the expression of the Ang are surprised that the α_2-adrenoceptor agonist, iodo- stimulates the expression of the pOGH (Ang N-1498/ K 27 cells (Fig. 1). These results confirm the studies of ıd Bylund [23] and Blaxall et al [24], who reported the ›f α_2-adrenoceptors. At present, we do not understand r concentrations of iodoclonidine (that is, greater than ave lower stimulatory effects on the expression of the ng N-1498/+18) in OK 27 cells. One possible explana- be that the exposure of OK cells to high levels of ceptor agonists may desensitize their own adrenocep- ›d, this possibility is supported by the observations of ıd Lefkowitz [36] that α-adrenoceptors are subject to ıtion by their own agonists. Nevertheless, more experi- warranted to clarify these observations. We did not observe any significant stimulation of the pOGH (Ang N-1498/+18) by phenylephrine (α_1-adrenoceptor agonist) in OK 27 cells (Fig. 1). This might be explained by the fact that there is a low or undetectable amount of α_1-adrenoceptor in OK cells, as demonstrated by the binding studies of Murphy and Bylund [23].

Up until now, there is no report to indicate that the activation of α_2-adrenoceptor is coupled to PKC pathway in OK cells. Studies by Cotecchia et al [37], however, demonstrated that the stimulation of α_2-C10 and α_2-C4 adrenoceptors inhibits and activates the adenylate cyclase and phospholipase C activity in eukaryotic cells, respectively. Thus, these studies suggest that there are multiple second messenger pathways (such as PKA and PKC) for α-adrenergic receptors. Our present studies showed that PMA stimulates the expression of the pOGH (Ang N-1498/+18) in a dose-dependent manner (Fig. 2). Since PMA is a potent stimulator of protein kinase C [38], these studies confirm the presence of PKC in OK cells [39, 40] and raise the possibility that the route of iodoclonidine, in exerting its effect, is mediated via the protein kinase C pathway. Indeed, our results showed that yohimbine (Fig. 3) and staurosporine (a potent inhibitor of protein kinase C [41]) (Fig. 4) inhibit the expression of the pOGH (Ang N-1498/+18) stimulated by iodoclonidine in OK 27 cells in a dose-dependent manner. These data further support the hypothesis that the effect of α_2-adrenoceptors on the expression of the Ang gene is probably via the protein kinase C pathway and not via the protein kinase A pathway, since Rp-cAMP (an inhibitor of cAMP-dependent protein kinase A [42]) did not inhibit the effect of iodoclonidine on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells (Fig. 4).

Furthermore, our studies showed that the addition of staurosporine blocks the effect of PMA on the expression of pOGH (Ang N-1498/+18) in OK 27 cells (Fig. 5). These data further confirm that protein kinase C is involved in the expression of the Ang gene in OK cells.

Prazosin has a Ki which is approximately 80-fold less potent than yohimbine in competition for the displacement of ^{3}H-rauwolscine in OK cell membrane preparation [24]. At present, we do not understand why prazosin at concentrations as high as 10^{-5} M does not inhibit the stimulatory effect of iodoclonidine (Fig. 4). One possible explanation may be that the discrepancy is due to different methods employed in the studies of Blaxall et al [24], who used OK cellular membranes for binding studies, whereas we employed intact OK cells for studies in gene expression. The second possibility may be due to the source of OK cells used in our studies and in the studies of Blaxall et al [24]. Since our OK 27 cell is a subclone and derived from a heterogenous population (obtained from ATCC), it is possible that OK 27 cells may be different (at the receptor level) from OK cells used in the studies of Blaxall et al [24]. Nevertheless, more studies are necessary to clarify this discrepancy.

The OK 13 cell is a cell line into which has been stably integrated a fusion gene: pTKGH containing the promoter/enhancer DNA sequence of the viral thymidine kinase gene fused with a human growth hormone gene as a reporter. The expression of the pTKGH in OK 13 cells is driven by the promoter/enhancer DNA sequence of the thymidine kinase (TK) gene. Therefore, we used OK 13 cells as control cells to examine the effect of adrenoceptor agonists. We did not observe any significant stimulation of expression of the pTKGH by neither iodoclonidine (10^{-9} M), phenylephrine (10^{-9} M) nor PMA (10^{-7} M) in OK 13 cells

(Fig. 6). These data demonstrate that the promoter/enhancer DNA sequence of TK gene is not responsive to the addition of iodoclonidine, phenylephrine nor PMA. On the other hand, our studies demonstrated that the effect of iodoclonidine and PMA on the expression of pOGH (Ang N-1498/+18) in OK 27 cells is gene-specific and mediates via the 5′-flanking regulatory sequences of the rat Ang gene and not mediated via the DNA sequence of the hGH reporter gene.

At present, we do not understand the molecular mechanism(s) of protein kinase C on the expression of the Ang-GH fusion gene. Our previous studies [25], however, have shown that there is DNA sequence (nucleotides N-758 to N-752, TGACTAC) in the 5′-flanking regulatory sequence of the rat Ang gene which is almost identical to the consensus AP-1 sequence (TGACTCA) [43], with the exception that the last two nucleotides are in reverse order. This raises the possibility that the activation of protein kinase C might induce the expression of early-immediate protooncogenes, that is, c-*Fos* and c-*Jun* genes. These protooncogenes form an heterodimer complex (AP-1 complex) and then bind to the putative AP-1 DNA sequence (Ang N-758 to N-752). Subsequently, the AP-1 complex enhances the expression of the Ang gene. Indeed, more studies are warranted to investigate this possibility.

Another possibility may be that the activation of protein kinase C phosphorylates the cAMP-responsive element binding protein (CREB) or CREB-like nuclear protein(s), since CREB contains the site of phosphorylation by protein kinase C [44]. The phosphorylated CREB then binds with the putative cAMP-responsive element (CRE) of the rat Ang gene (TGACGTAC, nucleotides N-795 to N-788) [25] and subsequently enhances the expression of the Ang gene. This possibility is supported by our preliminary studies that the cloned CREB is able to stimulate directly the expression of the pOGH (Ang N-1498/+18) in OK 27 cells (unpublished results). Nevertheless, more studies are warranted to elucidate the molecular mechanism(s) of protein kinase C activation and the expression of the Ang gene in OK cells.

It is intriguing that both β-adrenoceptor agonist (isoproterenol) [13] and iodoclonidine stimulate the expression of pOGH (Ang N-1498/+18) in OK 27 cells. Since α_2-adrenoceptor is known to uncouple the adenylate cyclase system [23] in OK cells, this raises the question of whether α_2-adrenoceptor could modulate the effect of β-adrenoceptors on the expression of Ang gene or vice versa. Indeed, studies are underway in our laboratory to investigate this possibility.

In summary, our studies show that the addition of iodoclonidine directly stimulates the expression of the Ang-GH fusion gene in OK 27 cells. Furthermore, the stimulatory effect of iodoclonidine could be blocked by the presence of yohimbine and staurosporine. These studies demonstrate that there are putative transactivating factors in OK cells which interact with the 5′-flanking sequence of the Ang gene to promote its expression. The effect of α_2-adrenoceptors on the expression of the renal Ang gene may be mediated via the protein kinase C pathway. Our studies indicate that the activation of renal nerves may stimulate the expression of the renal Ang gene via α_2-adrenoceptors *in vivo*. The local formation of renal AII might then modulate the physiology of the renal proximal tubular cells (that is, sodium and fluid absorption). Thus, local renal RAS might play a significant role in the modulation of sodium reabsorption.

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References

1. Dzau VJ, Ingelfinger JR: Molecular biology and pathop the intrarenal renin-angiotensin system. *J Hypertens* 7(supp 1989
2. Johnston CI, Fabris B, Jandeleit K: Intrarenal renin-system in renal physiology and pathophysiology. *Kidney I* 42):559–563, 1993
3. Ingelfinger JR, Zuo WM, Fon EA, Ellison KE, Dzau hybridization evidence for angiotensinogen mRNA in the r tubule. A hypothesis for the intrarenal renin angiotensin sy *Invest* 85:417–423, 1990
4. Terada T, Tomita K, Nonoguch H, Marumo F: PCR loc angiotensin II receptor and angiotensinogen mRNA in *Kidney Int* 43:1251–1259, 1993
5. Chen M, Harris MP, Rose D, Smart A, He XE, Kr Briggs JP, Schnermann J: Renin and renin mRNA i tubules of the rat kidney. *J Clin Invest* 94:237–243, 1994
6. Seikaly MG, Arant BS, Seney FD, Rutlege L, Green enous angiotensin concentrations in specific intrarenal flui ments of the rat. *J Clin Invest* 86:1352–1357, 1990
7. Braam B, Mitchell KD, Fox J, Navar LG: Proxin secretion of angiotensin II in rats. *Am J Physiol* 264:F891–
8. Navar LG, Lewis L, Hymel A, Braam B, Michell K fluid concentrations and kidney contents of angiotensins anesthesized rats. *JASN* 5:1153–1158, 1994
9. Vos PF, Boer P, Braam B, Koomans HA: The origin angiotensins in humans. *JASN* 5:215–223, 1994
10. Chan JSD, Chan AHH, Nie Z-R, Sikstrom R, La Hashimoto S, Carrière S: Thyroid hormone, L-T_3, stir expression of the angiotensinogen gene in cultured opos (OK) cells. *JASN* 2:1360–1367, 1992
11. Ingelfinger JR, Boubounes B, Jun FF, Tang S: Hi downregulates expression of renin angiotensin system opossum kidney cells. (abstract) *Pediatr Nephrol* 29:344A,
12. Chan JSD, Ming M, Nie Z-R, Sikstrom R, LaChance S, S: Hormonal regulation of expression of the angiotensino cultured opossum kidney (OK) proximal tubular cells. *JA* 1522, 1992
13. Wang TT, Chen M, LaChance S, Delalandre A, C Chan JSD: Isoproterenol ad 8-Bromo-cyclic adenosine phate stimulate the expression of the angiotensinogen ge sum kidney cells. *Kidney Int* 46:703–710, 1994
14. Schuster VL, Kokko JP, Jacobson HR: Angiotensin stimulates sodium transport in rabbit proximal convolute *Clin Invest* 73:507–515, 1984
15. Liu FY, Cogan MG: Angiotensin II stimulates early prox bonate absorption in the rat by decreasing cyclic adenos phosphate. *J Clin Invest* 84:83–91, 1989
16. Saccomani G, Mitchell KD, Navar LG: Angiotensin II of Na^+-H^+ exchange in proximal tubular cells. *Am J P* F1188–F1195, 1990
17. Insel PA, Snavely MD, Healy DP, Munzel PA, Po Nord EP: Radiologic binding and functional assays d post-synaptic α_2-receptors on proximal tubules of rat kidney. *J Cardiovasc Pharmacol* 7(Suppl 8):S1–S8, 1985

ɜER WA, UNEMURA S, SMYTH DD, JEFFRIES WB: Renal noceptors and the adenylate cyclase-cAMP system: biochem- physiological interactions. *Am J Physiol* 252:F199–F208, 1987

ɛESAN PR, BARAC-NIETO M, STAMBO GW, KELVIE SL: Glo- · and tubular α_1- and α_2-adrenoceptors in the rat kidney: tion in basolateral and brush border membranes of tubular *Cardiovasc Pharmacol* 13:16–24, 1989

FA, STRADHOY JW: Dual interactions between α_2-adrenocep- nists and the proximal Na^+-H^+ exchanger. *Am J Physiol* 6–F642, 1990

RE, BERNDT WO: Characterization of adrenergic receptors of ıl tubular basolateral membranes. *Life Sci* 43:1473–1478, 1988

AD, HO P: Specific α_1-, α_2, and β-responses to norepinephrine /ate-perfused rat kidneys. *Am J Physiol* 252:F170–F176, 1987

Y TJ, BYLUND DB: Characterization of alpha$_2$ adrenergic rs in the OK cell, an opossum kidney cell line. *J Pharmacol Exp* 4:571–578, 1988

L HS, MURPHY TJ, BAKER JC, RAY C, BYLUND DB: Charac- ɔn of the alpha-2c adrenergic receptor subtype in the opossum and in the OK cell line. *J Pharmacol Exp Ther* 259:323–329,

SD, CHAN AHH, JIANG Q, NIE Z-R, LACHANCE S, CARRIÈRE :cular cloning and expression of the rat angiotensinogen gene. *Nephrol* 4:429–435, 1990

SD, ROBERTSON HA, FRIESEN HG: Maternal and fetal con- on of ovine placental lactogen measured by radioimmunoas- *docrinology* 102:1606–1613, 1978

L JI, JOHANNSON BG: Enzymatic iodination of polypeptides I of high specific activity. *Biochim Biophys Acta* 251:363–369,

A H, GOODPASTURE C, MILLER MM, TEPLITZ RL, RIGGS AD: ihment and characterization of a cell line from the American n (Didelphys virginia). *In Vitro* 14:239–246, 1978

K AS, WARNOCK DG, STREWLER GJ: Parathyroid hormone ɔn of Na^+-H^+ antiporter activity in a cultured renal cell line. *hysiol* 250:F217–F225, 1986

S HH, STANLEY F, SHAPIRO LE: Control of growth hormone is in cultured GH cells by 3,5,3′-triiodo-L-thyronine and rticoid agonists and antagonists: Studies on the independent ergistic regulation of the growth hormone response. *Biochem-* 715–721, 1979

R E, BOUHNIK J, COEZY E, CORVOL P, MENARD J: Synthesis ease of immunoreactive angiotensinogen by rat liver slices. *inology* 112:1188–1193, 1983

32. DIBONA, GF: Neural control of renal tubular sodium reabsorption and renin secretion. *Fed Proc* 44:2816–2822, 1985
33. RUDD AM, GRIPPO RS, ARENDSHORST WJ: Acute renal denervation produces a diuresis and natriuresis in young SHR but not WKY rats. *Am J Physiol* 251:F655–F661, 1986
34. BENSCATH PL, ASZTALOS B, SZALAY L, TAKACE L: Renal handling of sodium after chronic sympathectomy in the anesthetized rat. *Am J Physiol* 236:F513–F518, 1979
35. NAKAMURA A, JOHNS EJ: Effect of renal nerves on expression of renin and angiotensinogen genes in rat kidneys. *Am J Physiol* 266:E230–E241, 1994
36. KUROSE H, LEFKOWITZ RJ: Differential desensitization and phosphorylation of three cloned and transfected α_2-adrenergic receptor subtypes. *J Biol Chem* 269:10093–10099, 1994
37. COTECCHIA S, KOBILKA BK, DANIEL KW, NOLAN RD, LAPETINA EY, CARON MG, LEFKOWITZ RJ, REGAN JW: Multiple second messenger pathways of α-adrenergic receptor subtypes expressed in eukaryotic cells. *J Biol Chem* 265:63–69, 1990
38. CASTAGNA M, TAKAI Y, KAIBUCHI K, SANO K, KIKKAWA U, NISHIZUKA Y: Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J Biol Chem* 257:7847–7851, 1982
39. MIDDLETON JP, KHAN WA, COLLINSWORTH G, HANNAN YA, MEDFORD RM: Heterogeneity of protein kinase C-mediated rapid regulation of Na/K-ATPase in kidney epithelial cells. *J Biol Chem* 268: 15958–15964, 1993
40. VRTOVSNIK F, JOURDAIN M, CHERQUI G, LEFEBVRE J, FRIEDLANDER G: Glucocorticoid inhibition of Na-Pi cotransport in renal epithelial cells is mediated by protein kinase C. *J Biol Chem* 269:8872–8877, 1994
41. PERSAUD SJ, JONES PM, HOWELL SL: Staurosporine inhibits protein kinases activated by Ca^{2+} and cyclic AMP in addition to inhibiting protein kinase C in rat islets of Langerhans. *Mol Cell Pharmacol* 94:55–60, 1993
42. LANDSBERG CR, JASTORFF B: In vitro phosphorylation of microtubule-associated protein 2: differential effects of cyclic AMP analogues. *J Neurochem* 45:1212–1222, 1985
43. RAUSCHER FJ II, SAMBUCETTI LC, CURRAN T, DISTEL RJ, SPIEGELMAN BM: Common DNA binding site for Fos protein complexes and transcription factor AP-1. *Cell* 52:471–480, 1988
44. GONZALEZ GA, YAMAMOTO KK, FISCHER WH, KARR D, MENZEL P, BIGGS W III, VALE WW, MONTMINY MR: A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. *Nature (London)* 337:749–752, 1989

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ect of glucose on the expression of the angiotensinogen gene opossum kidney cells

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t of glucose on the expression of the angiotensinogen gene in m kidney cells. To investigate whether D(+)-glucose has a stimu- effect on the expression of the angiotensinogen (Ang) gene in n kidney (OK) cells, we used OK cells with a fusion gene ing various lengths of the 5′-flanking regulatory sequence of the rat ne fused with the human growth hormone (hGH) gene as a r, stably integrated into their genomes. The level of expression of on gene was quantified by the amount of immunoreactive-human hormone (IR-hGH) secreted into the medium. The addition of lucose stimulated the expression of pOGH (Ang N-1498/+18) in cells in a dose-dependent manner (5 to 25 mM), whereas the n of D-mannitol, L-glucose and 2-deoxy-D-glucose (25 mM) had no The stimulatory effect of D(+)-glucose (25 mM) was blocked by the e of staurosporine or H7 (an inhibitor of protein kinase C) or (an inhibitor of phospholipase C and A_2) but not blocked by the e of Rp-cAMP (an inhibitor of cAMP-dependent protein kinase addition of D(+)-glucose (25 mM) also stimulated the expression H (Ang N-960/+18) and pOGH (Ang N-688/+18) in OK 960 and cells, respectively. It had no stimulatory effect, however, on the ion of pOGH (Ang N-280/+18) and pOGH (Ang N-35/+18) in and OK 35 cells, respectively. The addition of D(+)-glucose also effect on the expression of pTKGH in OK 13 cells, an OK cell line, ich had been stably integrated a fusion gene, pTKGH containing moter/enhancer DNA sequence of the viral thymidine-kinase (TK) ised with a human growth hormone gene as a reporter. These demonstrate that the stimulatory effect of high D(+)-glucose ration (25 mM) on the expression of the angiotensinogen-growth ie fusion genes in OK cells is mediated via the 5′-flanking region of iotensinogen gene and the protein kinase C signal transduction y. Our data indicate that a high glucose concentration may activate in-angiotensin system in the renal proximal tubular cells.

lies have shown that the mRNA components of the renin- ensin system (RAS), including angiotensinogen (Ang), re- ngiotensin-converting enzyme (ACE) and angiotensin-II or (AT_1-receptor) are expressed in murine (mouse and rat) talized proximal tubular cell lines [1–4]. More recently, we eported that the Ang protein is secreted from rat immor- proximal tubular cells as measured by a specific radioim- ssay for rat Ang [5]. We [6] as well as Ingelfinger et al [7] lso demonstrated that the Ang mRNA is expressed in opossum kidney (OK) proximal tubular cells. These studies indicate that the intrarenal angiotensin II (Ang II) is probably derived from the Ang that is synthesized within the renal proximal tubular cells.

We have previously reported that isoproterenol and iodoclonidine stimulate the expression of the Ang gene in OK cells *in vitro* in a dose-dependent manner [8, 9]. The effect of isoproterenol is mediated via the β_1-adrenoceptor and cAMP-dependent protein kinase A (PKA) pathway [8], whereas the effect of iodoclonidine is mediated via the α_2-adrenoceptor and protein kinase C (PKC) pathway [9]. Our studies confirm the reports of Nakamura and Johns [10] that low levels of renal nerve stimulation increase the Ang mRNA levels in the rat kidney *in vivo*. Our studies and those of Nakamura and Johns [8–10] together indicate the presence of a functional relationship between the renal sympathetic nervous system and the activation of local intrarenal RAS. Thus, the local formation of renal Ang II may play an important role in the physiology of the renal proximal tubular cells (that is, sodium and fluid reabsorption [11–14]).

Hyperglycemia is an important etiologic factor in the development of diabetic nephropathy [15]. However, the molecular mechanisms for the development of nephropathy in diabetes are not completely understood. Studies have shown that the incubation of mesangial cells in a high glucose medium increases cellular proliferation, extracellular matrix protein synthesis and the expression of mRNA of extracellular matrix proteins in mesangial cells [16–19]. Experimental evidence also indicates that the effect of elevated glucose concentration is probably mediated via the activation of the protein kinase C pathway in mesangial cells [20–24]. Furthermore, tubular basement membrane thickening, accumulation of glycogen droplets in proximal tubular epithelium and tubulointerstitial fibrosis are often observed in diabetic patients [25]. These studies indicate that the elevated glucose concentration may directly or indirectly be responsible for the development of diabetic nephropathy.

We have previously demonstrated that the addition of phorbol 12-myristate 13-acetate (PMA, a protein kinase C stimulator) has a stimulatory effect on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells [9], indicating that the PKC signal transduction pathway plays a regulatory role on the expression of the Ang gene in OK cells. In the present studies, we investigated the possible effect of glucose on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells. Our results showed that the expression of pOGH (Ang N-1498/+18) fusion gene in OK 27

rds: angiotensinogen gene, glucose, opossum kidney cells, growth ie, renin-angiotensin system.

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cells is stimulated by high concentrations (25 mM) of D(+)-glucose, but not by D-mannitol, L-glucose or 2-deoxy-D-glucose. Furthermore, the addition of staurosporine or H-7 (an inhibitor of protein kinase C) and U73122 (an inhibitor of phospholipase C and A_2) blocked the stimulatory effect of glucose. Finally, the addition of high glucose (25 mM) had a stimulatory effect on the expression of pOGH (Ang N-960/+18) and pOGH (Ang N-688/+18) in OK 960 and OK 688 cells, respectively. The addition of glucose had no effect, however, on the expression of pOGH (Ang N-280/+18), pOGH (Ang N-35/+18) and pTKGH in OK 280, OK 35 and OK 13 cells, respectively.

METHODS

D(+)-glucose, L-glucose, D-mannitol and 2-deoxy-D-glucose were purchased from Sigma Chemicals (St. Louis, MO, USA). Staurosporine (an inhibitor of protein kinase C), H-7 (an inhibitor of protein kinase C), U73122 (an inhibitor of phospholipase C and A_2) and Rp-cAMP (an inhibitor of the cAMP-dependent protein kinase AI and II) were purchased from Research Biochemicals Inc. (RBI, Natick, MA, USA).

The plasmid, pRSV-Neo, containing the coding sequence for Neomycin (Neo) with the Rous Sarcoma Virus (RSV) enhancer/promoter sequence fused in the 5′-end of the Neomycin gene was a gift from Dr. Teresa Wang (Dept. of Pathology, Stanford University, Stanford, CA, USA). The plasmid, pTKGH, containing the thymidine kinase (TK) enhancer/promoter sequence fused to the 5′-end of the hGH gene was purchased from the Nichols Institute of Diagnostics (La Jolla, CA, USA).

The radioimmunoassay kit for hGH (RIA-hGH) was a gift from NIADDK, NIH, USA. The RIA procedure has been described previously [26]. NIAMDD-hGH-I-1 (AFP-4793 B) was used for both iodination and as a hormone standard. The limit of sensitivity of the assay was 0.1 ng/ml. The inter- and intra-assay coefficients of variation were 10% (n-10) and 12% (n-10), respectively.

$Na^{125}I$ was purchased from Dupont, New England Nuclear (NEN, Boston, MA, USA). Calcium chloride was purchased from Mallinckrodt, Inc. (Montreal, Quebec, Canada), Geneticin (G 418) was purchased from Bethesda Research Laboratories (Gibco-BRL, Burlington, Ontario, Canada). Other molecular biology grade reagents were obtained either from Sigma Chemicals, Gibco-BRL, Boehringer-Mannheim, Pharmacia Inc. (Baie d'Urfe, Quebec, Canada), or Promega-Fisher, Inc. (Montreal, Quebec, Canada).

Construction of fusion genes

The method of construction of the Ang-GH fusion genes, pOGH (Ang N-1498/+18) and pOGH (Ang N-35/+18), has been described previously [26]. To construct pOGH (Ang N-960/+18) and pOGH (Ang N-280/+18), we simply transferred the DNA fragments, Ang N-960/+18 and Ang N-280/+18, from the plasmids pOCAT (Ang N-960/+18) and pOCAT (Ang N-280/+18) [27], respectively, into the pOGH vector.

Cell culture

The opossum kidney (OK) proximal tubular cell line was obtained from the American Tissue Culture Collection (ATCC, Rockville, MD, USA). This cell line is derived from the kidney of a female American opossum and retains several properties of proximal tubular epithelial cells in culture [28, 29] and expresses a low level of Ang mRNA [6, 7]. The culture conditions cells have been described previously [8, 9, 30].

Opossum kidney cell stable transformants

Opossum kidney 27 and OK 13 cells are stable transfo with pOGH (Ang N-1498/+18) and pTKGH integrated in cellular genomes, respectively. The characteristics of thes have been previously reported [8, 9]. Briefly, OK 27 and cells that had passed through at least three repetitions of l dilution and continued to secrete high levels of immunore hGH (IR-hGH) after three months in the presence of G41 considered to be stable clones.

By similar procedures, we have obtained stable transfo OK 960, OK 688, OK 280 and OK 35 with pOGH N-960/+18), pOGH (Ang N-688/+18), pOGH (Ang N-28 and pOGH (Ang N-35/+18) integrated into OK cellular ge respectively. The expression of the Ang-GH fusion genes i cells was time-dependent. The levels of IR-hGH in c extracts were consistently less than 5% of those found culture media, suggesting that renal Ang is not stored in tl

Effect of D(+)-glucose on the expression of angiotensinog growth hormone fusion genes in opossum kidney 27 cell s transformants

Opossum kidney 27 cells were plated at a density of 1 to 2 cells/well in six-well plates and incubated overnight in D containing 10% FBS. Cell growth was then arrested by incu in serum-free and a low D(+)-glucose concentration (medium for 24 hours. Subsequently, various concentrati D(+)-glucose (final concentration 5 to 25 mM) were added culture medium containing 1% depleted fetal bovine (dFBS) and incubated for 24 hours. Since glucose chang tonicity of the media, the media were supplemented w mannitol until the final concentration was reached equival mM (that is, 5 mM D(+)-glucose plus D-mannitol until equ 30 mM, etc.). At the end of the incubation period, medi collected and kept at −20°C until assayed for IR-hGH.

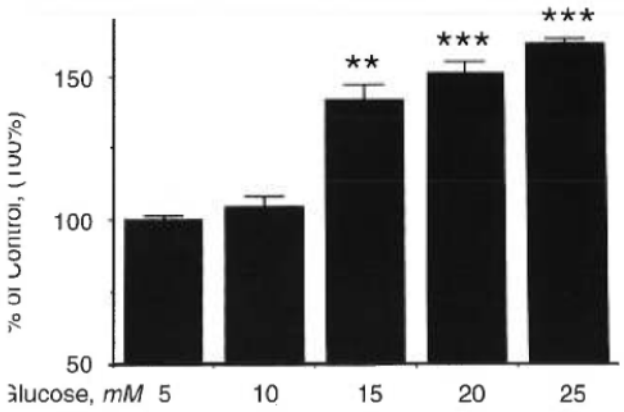
To determine the specificity of D(+)-glucose, 5 or 25 L-glucose or D-mannitol or 2-deoxy-glucose was added culture medium and incubated for 24 hours. Then, the medi collected and kept at −20°C until assay for IR-hGH.

To compare the inhibitory effect of staurosporine, H-7, U and Rp-cAMP on the expression of pOGH (Ang N-1498/+ OK 27 cells, various concentrations (10^{-13} to 10^{-7} M) inhibitors were co-cultured with the D(+)-glucose (25 mM) hours. At the end of the incubation period, media were co and kept at −20°C until assay for IR-hGH.

The depleted FBS was prepared by incubation with 1% vated charcoal and 1% AG 1 × 8 ion-exchange resin (Bi Laboratories, Richmond, CA, USA) for 16 hours or more a temperature as described by Samuels, Stanley and Shapir This procedure removed endogenous steroid and thyroi mones from the FBS as demonstrated by Samuels et al [3

Statistical analysis

The experiments were performed at least three to four ti triplicate. The data were analyzed with Student's *t*-test or a of variance (ANOVA). A probability level of $P \leq 0.0$ regarded as significant.



Effect of D(+)-glucose on the expression of pOGH (Ang N-1498/ i opossum kidney (OK) 27 cells. Cells were incubated for up to 24 i the presence of various concentrations of D(+)-glucose. Media ollected after 24 hours of incubation and assayed for immuno- : human growth hormone (IR-hGH). The concentration of IR- the medium containing low D(+)-glucose (5 mM) (that is, 1.43 ± nl) is considered as the control level. Each point represents the : SD of at least three dishes (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq$ Experiments were repeated three times.

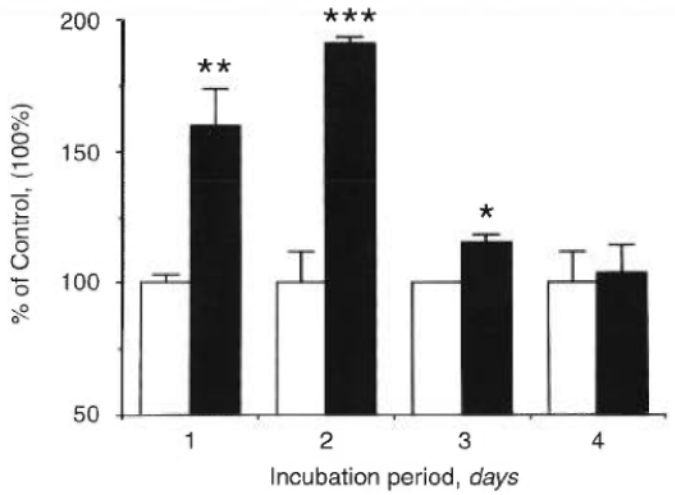


Fig. 2. Effect of incubation period on the expression of pOGH (Ang N-1498/+18) in opossum kidney (OK) 27 cells. Cells were incubated for one to four days in the presence of 5 mM or 25 mM of D(+)-glucose. Media were harvested after various periods of incubation and assayed for IR-hGH. The concentration of IR-hGH in the medium containing low D(+)-glucose (5 mM) (that is, Day 1, 1.32 ± 0.1 ng/ml; Day 2, 1.61 ± 0.15 ng/ml; Day 3, 1.86 ± 0.15 ng/ml; Day 5, 1.91 ± 0.1 ng/ml) is considered as the control level. Each point represents the mean ± SD of at least three dishes (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). Similar results were obtained from two other experiments.

LTS

of D(+)-glucose on the expression of angiotensinogen- hormone fusion gene in opossum kidney cell stable ormants

re 1 shows the expression of the pOGH (Ang N-1498/+18) 27 cells in the presence of various concentrations (5 to 25 f D(+)-glucose after 24 hours of incubation. A dose- lent relationship between D(+)-glucose concentrations e stimulation of expression of pOGH (Ang N-1498/+18) served at 5 to 25 mM. The maximal stimulation of expres- the pOGH (Ang N-1498/+18) was found with 25 mM of glucose.

re 2 shows the expression of the pOGH (Ang N-1498/+18) 27 cells in the presence of 5 mM or 25 mM D(+)-glucose at nt time periods. The maximal expression of the pOGH J-1498/+18) with 25 mM D(+)-glucose was found after two incubation. The stimulatory effect of 25 mM D(+)-glucose gnificantly reduced following three days of incubation. was no significant difference between the stimulatory effect ed with 25 mM D(+)-glucose at four days of incubation red to the 5 mM D(+)-glucose. For subsequent studies, we ly performed the experiments following 24 hours of incu- in the presence of low or high glucose levels.

re 3 shows that the effect of the addition of 5 mM or 25 mM glucose on the expression of the pOGH (Ang N-1498/+18) 27 cells when OK 27 cells were pre-incubated with 25 mM glucose (Fig. 3A) or 5 mM (D+)-glucose in the presence of phorbol 12-myristate, 13-acetate (PMA) (Fig. 3B) for 24 It is apparent that the pre-incubation with 25 mM of glucose or 10^{-5} M PMA for 24 hours abolished the stimu- effect of 25 mM of D(+)-glucose compared to the 5 mM glucose level.

re 4 compares the effect of the addition of 5 mM or 25 mM)-glucose, D-mannitol, L-glucose or 2-deoxy-D-glucose on pression of the pOGH (Ang N-1498/+18) in OK 27 cells

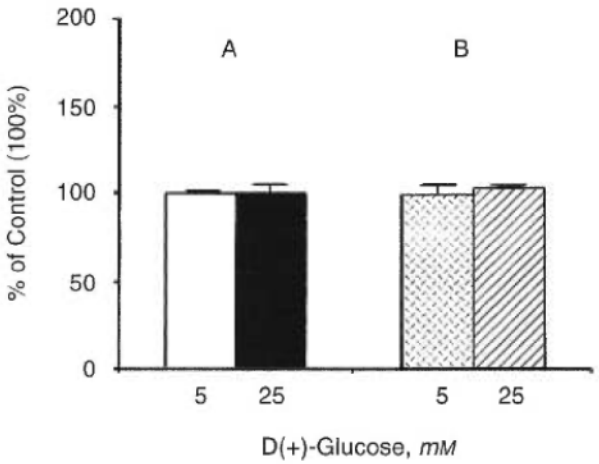


Fig. 3. Effect of the addition of D(+)-glucose on the expression of the pOGH (Ang N-1498/+18) in opossum kidney (OK) 27 cells when the cells were pre-incubated with a high level of glucose or phorbol 12-myristate 13-acetate (PMA). Cells were incubated for 24 hours with 25 mM D(+)-glucose (*A*) or 5 mM D(+)-glucose in the presence of 10^{-5} M PMA (*B*). Then, the media were replaced with the fresh media containing 5 mM or 25 mM D(+)-glucose and incubated further for 24 hours. Subsequently, the media were havested and assayed for immunoreactive-human growth hormone (IR-hGH). The concentration of IR-hGH in the medium containing 5 mM D(+)-glucose in A or B (that is, 1.80 ± 0.1 ng/ml or 1.51 ± 0.2 ng/ml) are expressed as 100% (control). Each point represents the mean ± SD of at least three dishes (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq$ 0.005). Similar results were obtained from two other experiments.

after 24 hours of incubation with or without the supplementation of D-mannitol. In contrast to D(+)-glucose, the addition of 25 mM D-mannitol, L-glucose or 2-deoxy-D-glucose had no significant

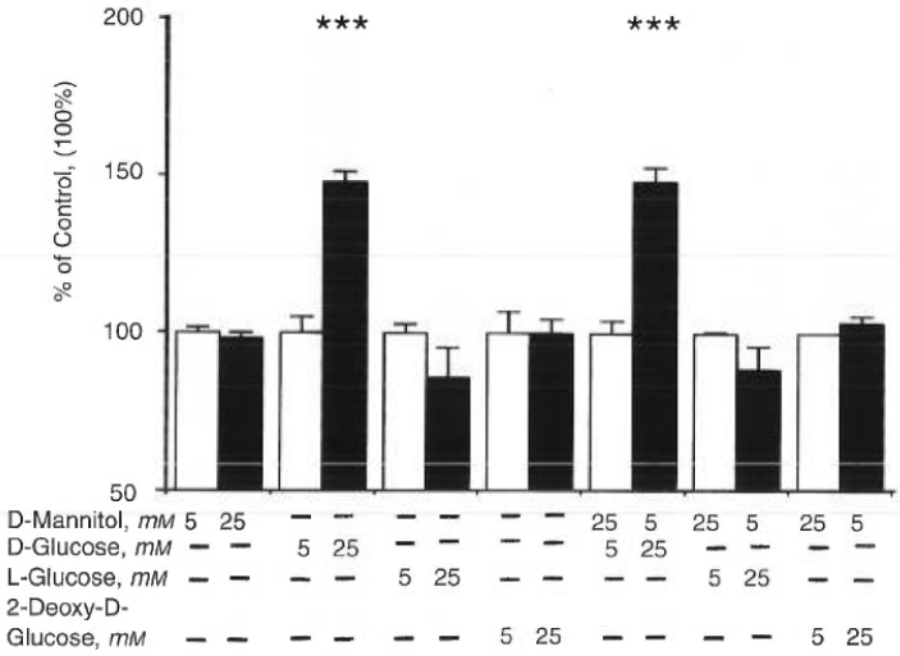


Fig. 4. Effect of D(+)-glucose, D-manni glucose and 2-deoxy-D-glucose on the expression of pOGH (Ang N-1498/+18) opossum kidney (OK) 27 cells. Cells wer incubated for up to 24 hours in the pres low (5 mM) or high (25 mM) of D(+)-gl D-mannitol, L-glucose or 2-deoxy-D-gluc with or without supplementation of D-m Media were harvested after 24 hours of incubation and assayed for immunoreact human growth hormone (IR-hGH). The concentration of IR-hGH in the medium containing 5 mM D(+)-glucose (4.71 ± ng/ml), 5 mM D-mannitol (2.53 ± 0.05 n or 5 mM L-glucose (3.32 ± 0.06 ng/ml), 2-deoxy-D-glucose (1.42 ± 0.17 ng/ml), 5 D(+)-glucose plu 25 mM mannitol (2.84 ng/ml), 5 mM L-glucose plus 25 mM man (2.58 ± 0.02 ng/ml) or 5 mM 2-deoxy-D- plus 25 mM mannitol (1.61 ± 0.01 ng/ml considered as the control level. Each poi represents the mean ± SD of at least thr dishes ($*P \leq 0.05$, $**P \leq 0.01$ and $***P$ 0.005). Similar results were obtained fro other experiments.

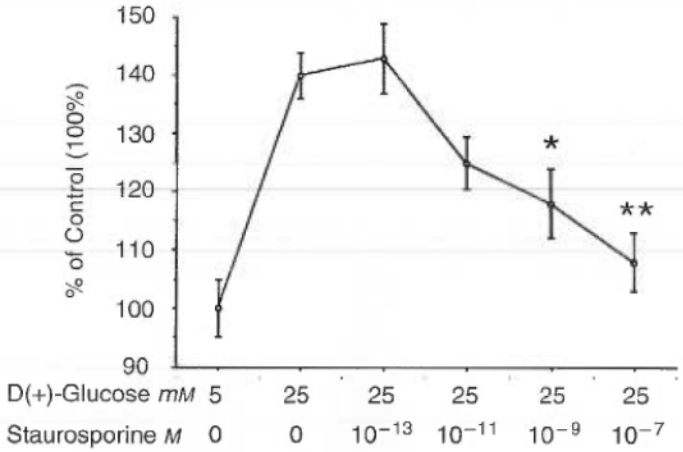


Fig. 5. Inhibitory effect of staurosporine on the expression of pOGH (Ang N-1498/+18) in OK 27 cells in the presence of 25 mM D(+)-glucose. Cells were incubated for 24 hours in the presence of 5 mM or 25 mM D(+)-glucose. Media were harvested and assayed for the level of IR-hGH. The levels of IR-hGH in the medium containing low D(+)-glucose (5 mM) (that is, 1.69 ± 0.15 ng/ml) are expressed as 100% (control). The inhibitory effect of staurosporine is compared with cells that were incubated in 25 mM D(+)-glucose (without the presence of staurosporine). Each point represents the mean ± SD of at least three dishes ($*P \leq 0.05$, $**P \leq 0.01$ and $***P \leq 0.005$). Similar results were obtained from another experiment.

effect on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells compared to those at 5 mM.

Effect of staurosporine, H-7, U73122 or Rp-cAMP on the expression of pOGH (Ang N-1498/-18) in OK 27 cells in the presence of D(+)-glucose

Figure 5 shows that the addition of staurosporine (10^{-13} to 10^{-5} M) inhibited the stimulatory effect of D(+)-glucose (25 mM) on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells in a dose-dependent manner. The effective inhibitory d inhibition of the stimulated expression (25 mM D(+)-gluc the pOGH (Ang N-1498/+18) was at 10^{-9} M staurosporin 0.05). At 10^{-7} M staurosporine, the stimulatory effect of glucose (25 mM) on the expression of the pOGH (Ang N +18) was completely abolished.

Similarly, the addition of H-7 or U73122 (10^{-7} M) com inhibited the stimulatory effect of D(+)-glucose on the exp of the pOGH (Ang N-1498/+18) in OK 27 cells (Fig. 6). other hand, the addition of Rp-cAMP (10^{-7} M) had no inh effect on the expression of pOGH (Ang N-1498/+18) stim by D(+)-glucose (25 mM; Fig. 6).

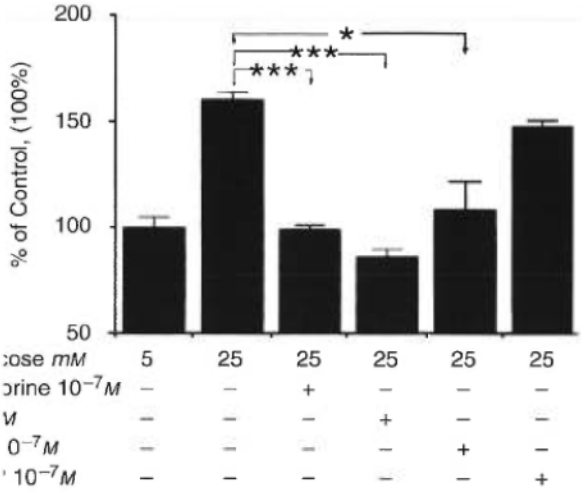
Effect of D(+)-glucose on the expression of the angiotensinogen-growth hormone fusion genes and pTKG opossum kidney cells

Figure 7 shows that the addition of 25 mM D(+)-g stimulated the expression of pOGH (Ang N-1498/+18), (Ang N-960/+18) and pOGH (Ang N-688/+18) in OK 27, and OK 688 cells compared to the 5 mM D(+)-glucose, tively. The addition of 25 mM D(+)-glucose had no stim effect on the expression of pOGH (Ang N-280/+18) and (Ang N-35/-18) in OK 280 and OK 35 cells compared to th D(+)-glucose, respectively.

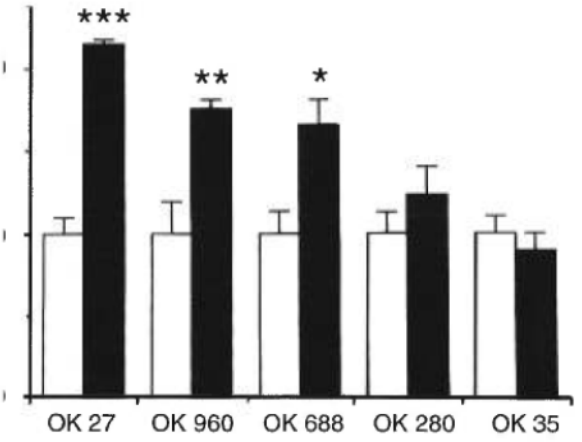
Figure 8 shows that the addition of D(+)-glucose (5 to 2 had no stimulatory effect on the expression of the pTKGH 13 cells.

DISCUSSION

Studies *in vitro* on cultured murine proximal tubular cel high glucose-containing medium (that is, ≥ 25 mM) showe the high glucose levels stimulated the hypertrophy of the pr tubular cells [32]. It appears that the hypertrophic effect glucose is mediated via the autocrine induction of transf growth factor (TGF-β) [33]. These observations were con



hibitory effect of staurosporine, H-7, U73122 or Rp-cAMP on sion of pOGH (Ang N-1498/+18) in OK 27 cells stimulated by +)-glucose. Cells were incubated for 24 hours in the presence of 25 mM D(+)-glucose plus 10^{-7} M of staurosporine or H-7 or Rp-cAMP. Media were harvested and assayed for the level of active-human growth hormone (IR-hGH). Levels of IR-hGH in n containing the low D(+)-glucose (5 mM) (that is, 3.72 ± 0.18 the absence of staurosporine, H-7, U73122 or Rp-cAMP are as 100% (control). The inhibitory effect of staurosporine, H-7, Rp-cAMP is compared with cells that were stimulated by 25 glucose. Each point represents the mean ± SD of at least three ≤ 0.05, $^{**}P \leq 0.01$ and $^{***}P \leq 0.005$). Similar results were rom two other experiments.



ect of D(+)-glucose on the expression of angiotensinogen-rmone (Ang-GH) fusion genes in opossum kidney (OK) cells. incubated for up to 24 hours in the presence of 5 mM or 25 mM ose. Media were harvested after 24 hours of incubation and r IR-hGH. The concentration of IR-hGH in the medium low glucose (5 mM) (that is, OK 27 cells, 1.42 ± 0.08 ng/ml; OK 0.1 ng/ml; OK 688, 1.43 ± 0.1 ng/ml; OK 280, 1.28 ± 0.1 ng/ml, 0 ± 0.12 ng/ml) is considered as the control level. Each point the mean ± SD of at least three dishes ($^{*}P \leq 0.05$, $^{**}P \leq 0.01$ ≤ 0.005). Similar results were obtained from two other ts.

studies, where it was found that the renal hypertrophy ssociated with the increased renal expression of the 1 spontaneously diabetic Bio-Breeding (BB) rats and 2 diabetic rats [34].

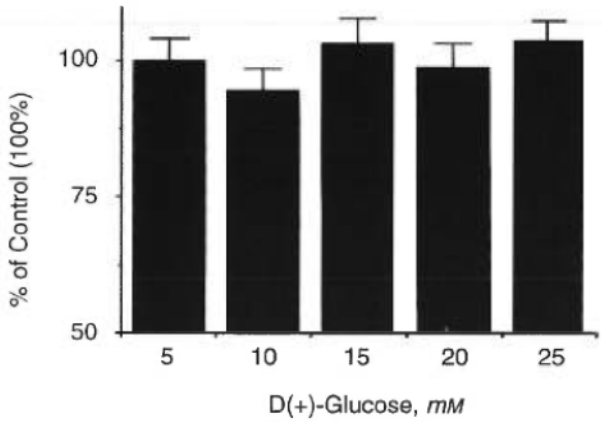


Fig. 8. Effect of D(+)-glucose on the expression of pTKGH in opossum kidney (OK) 13 cells. Cells were incubated for up to 24 hours in the presence of various concentrations of D(+)-glucose. Media were harvested after 24 hours of incubation and assayed for immunoreactive-human growth hormone (IR-hGH). The concentration of IR-hGH in the medium containing low glucose (5 mM) (that is, 1.19 ± 0.08 ng/ml) is considered as the control level. Each point represents the mean ± SD of at least three dishes ($^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.005$). Similar results were obtained from two other experiments.

Studies *in vitro* also showed that the addition of Ang II stimulates the hypertrophy of mouse proximal tubular cells (MCT) and LLC-PK_1 (porcine proximal tubular cells) [2, 35–37]. The hypertrophic effect of Ang II also depends on the autocrine induction of TGF-β gene expression [38, 39]. However, studies by Wolf et al [40] demonstrated that the Ang II-mediated hypertrophy of proximal tubular cells occurs only in the presence of high concentrations of glucose. Thus, it appears that Ang II has an additive effect on the hypertrophy of the murine proximal tubular cells only in the presence of high glucose levels in the medium.

Conflicting results have been reported concerning the expression of the renin and Ang genes in the kidney in experimental diabetes mellitus. For example, studies of Kaylinyak et al [41] showed that there is no significant difference in renal renin and Ang mRNA levels in rats two weeks after the induction of diabetes compared with controls. Studies of Correa-Rotler, Hostetter and Rosenberg [42], however, reported that the levels of renal and liver Ang mRNA are lower in the diabetic group. In contrast, the studies of Everett et al [43] showed that there is an increase in immunoreactive Ang in the renal proximal tubules while there is no significant increase in renal proximal ANG mRNA in rats 4 to 8 months after induction of diabetes. The studies of Anderson, Jung and Ingelfinger [44] demonstrated that a small but significant increase is observed in renal renin and Ang mRNA expression in rats six to eight weeks after induction of diabetes with the administration of streptozotocin. Thus, until now it has been uncertain whether high glucose levels may modulate the expression of the renal Ang gene during the course of diabetes mellitus.

Our present studies showed that the addition of D(+)-glucose stimulated the expression of the fusion gene, pOGH (Ang N-1498/+18) in OK 27 cells in a dose-dependent manner (Fig. 1). Moreover, the stimulatory effect of a high D(+)-glucose level is time-dependent (Fig. 2). The highest stimulatory effect of D(+)-glucose was observed during two days of incubation (Fig. 2). The stimulatory effect was diminished with greater than two days of

incubation. We had measured the levels of glucose in the medium at various times of incubation (unpublished results). Our results showed that the levels of glucose were 0.1 mM and 15.7 mM after four days of incubation. These studies indicate that the lack of stimulatory effect by the high level of glucose following four days of incubation was not due to the depletion of glucose in the medium. We have also performed experiments by changing the old media after 48 hours of incubation with fresh media (unpublished results). The addition of fresh media did not displayed any stimulatory effect of 25 mM D(+)-glucose on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells compared to the addition of 5 mM D(+)-glucose. At present, we do not understand why longer incubation periods (that is, > two days) diminished or abolished the stimulatory effect of high D(+)-glucose on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells. One possible explanation may be that the prolonged exposure of OK cells to high D(+)-glucose may desensitize the protein kinase C signal transduction pathway. Indeed, our results (Fig. 3) showed that the 24 hours pre-incubation of OK 27 with 25 mM D(+)-glucose (Fig. 3A) or 5 mM D(+)-glucose in the presence of 10^{-5} M phorbol 12-myristate 13-acetate (PMA) (Fig. 3B) abolished the stimulatory effect of high D(+)-glucose (25 mM) on the expression of the fusion gene in OK 27 cells. Nevertheless, more experiments are warranted to clarify these observations. We did not observe any significant stimulation of the pOGH (Ang N-1498/+18) by L-glucose, D-mannitol or 2-deoxy-D-glucose (Fig. 4). These studies indicate that the effect of high D(+)-glucose levels on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells is probably mediated via the metabolized products of D(+)-glucose to stimulate the PKC pathway.

Our present studies showed that the addition of staurosporine (an inhibitor of protein kinase C) blocked the stimulatory effect of D(+)-glucose on the expression of pOGH (Ang N-1498/+18) in OK 27 cells in a dose-dependent manner (Fig. 5). Furthermore, the addition of H-7 (an inhibitor of protein kinase C) or U73122 (an inhibitor of phospholipase C and A_2) 10^{-7} M also completely blocked the stimulatory effect of high D(+)-glucose levels on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells (Fig. 6). These data support the hypothesis that the effect of high D(+)-glucose levels on the expression of the Ang gene is mediated via the protein kinase C pathway and not via the protein kinase A pathway, since Rp-cAMP (an inhibitor of cAMP-dependent protein kinase A) did not inhibit the effect of high D(+)-glucose levels on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells (Fig. 6). Indeed, the involvement of PKC on the expression of the Ang gene in OK cells are confirmed by our previous studies where it was reported that the addition of PMA stimulates the expression of the pOGH (Ang N-1498/+18) in OK 27 cells [9]. This stimulatory effect of PMA is blocked in the presence of staurosporine [9].

Our data show that the addition of high glucose (25 mM) stimulated the expression of pOGH (Ang N-1498/+18), pOGH (Ang N-960/+18) and pOGH (Ang N-688/+18) in OK 27, OK 960 and OK 688 cells, respectively (Fig. 7). The addition of high levels of glucose, however, had no effect on the expression of pOGH (Ang N-280/+18) and pOGH (Ang N-35/+18) in OK 280 and OK 35 cells, respectively. These studies indicate that the glucose-responsive element is probably localized within nucleotides N-1498 to N-280 in the 5′-flanking region of the rat Ang gene. At present, we have not identified the precise DNA sequence of the glucose-responsive element in the 5′-flanking region c Ang gene. Studies are underway in our laboratory to id putative glucose-responsive element in the rat angiot gene.

Opossum kidney 13 is a cell line into which has be integrated a fusion gene: pTKGH containing the p enhancer DNA sequence of the viral thymidine kinase g with the human growth hormone gene as a reporter. T we used OK 13 cells as control cells to examine the D(+)-glucose. We did not observe any significant stim expression of the pTKGH by D(+)-glucose at various c tions (5 to 25 mM) in OK 13 cells (Fig. 8). These data de that the promoter/enhancer DNA sequence of the TK g responsive to the addition of D(+)-glucose. On the ot our studies demonstrated that the effect of D(+)-gluco expression of pOGH (Ang N-1498/+18) in OK 27 cell specific and is mediated via the 5′-flanking regulatory s of the rat Ang gene and not mediated via the DNA se the hGH reporter gene.

At present, we do not understand the exact molecul nism(s) of D(+)-glucose and protein kinase C on the e of the pOGH (Ang N-1498/+18) in OK 27 cells. One p may be that the D(+)-glucose may stimulate the *de novo* of diacylglycerol (DAG) from the metabolized glucos polyol pathway which then increases the protein kinase [45]. Surprisingly, our studies also showed that the ac U73122 completely blocked the stimulatory effect glucose (Fig. 6). Since U73122 is an inhibitor of phosph and A_2, these studies suggest that the glucose may increase the phospholipase C activity in OK cells undefined mechanism(s). Indeed, studies are underw laboratory to explore this possibility.

Once the PKC is activated, it is possible that the prot C may phosphorylate the cAMP-responsive elemen protein (CREB) or CREB-like nuclear protein(s), sin contains the site of phosphorylation by protein kinase C recent studies by Kreisberg et al [47] have shown that high glucose levels stimulate the phosphorylation of CI phosphorylated CREB then binds to the putative cAM sive element (CRE) of the rat Ang gene (TGACGTAC tides N-795 to N-788) [26] and subsequently enhances th sion of the Ang gene. This possibility is supported by c studies [48] whereby the cloned CREB is able to stimula the expression of the pOGH (Ang N-1498/+18) in OK Nevertheless, more studies are warranted to elucidate t ular mechanism(s) of D(+)-glucose and PKC activatio expression of the Ang gene in OK cells.

In summary, our studies show that the high D(+)-gluc directly stimulate the expression of the Ang-GH fusion OK cells. The stimulatory effect of high D(+)-glucose c tions was blocked by the presence of staurosporine, U73122. Our studies suggest that the expression of the gene may be stimulated during hyperglycemia *in vivo*. formation of renal Ang II might then modulate the phy the renal proximal tubular cells (that is, sodium and f sorption, as well as the induction of the hypertropl proximal tubular cells). Thus, local renal RAS mig significant role in the development of diabetic nephrop

NOWLEDGMENTS

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requests to Dr. John S.D. Chan, University of Montreal, Maisonmont Hospital, Research Center, 5415 Boulevard de l'Assomption, Quebec, Canada H1T 2M4.

DIX

ations used in this article are: ACE, angiotensin converting ng, angiotensinogen; Ang II, angiotensin II; ANOVA, analysis ; MCT, mouse proximal tubular cells; AT_1-receptor, angiotenptor; CRE, cAMP-responsive element; CREB, cAMP-respon- nt binding protein; DAG, diacylglycerol; dFBS, depleted fetal um; hGH, human growth hormone; IR-hGH, immunoreactive- wth hormone; LLC-PK_1, porcine proximal tubular cells; Neo, OK, opossum kidney; PKA, protein kinase A; pOGH (Ang 8) and pOGH (Ang N-35/+18), angiotensinogen-growth hor- on genes; PKC, protein kinase C; PMA, phorbol 12-myristate RAS, renin-angiotensin system; RIA-hGH, radioimmunoassay growth hormone; Rp-cAMP, an inhibitor of cAMP-dependent nase A; RSV, Rous Sarcoma Virus; TGF-β, transforming tor; TK, thymidine kinase.

ENCES

INGER JR, ZUO WM, FON EA, ELLISON KE, DZAU DJ: In situ ization evidence for angiotensinogen mRNA in the rat proximal A hypothesis for the intrarenal renin angiotensin system. *J Clin* 85:417–423, 1990

A TK, TOMITA K, NONOGUCH H, MARUMO F: PCR localization otensin II receptor and angiotensinogen mRNA in rat kidney. *Int* 43:1251–1259, 1993

G, NEILSON EG: Angiotensin II as a hypertrophogenic cytokine ximal tubular cells. *Kidney Int* 43(Suppl 39):S100–S107, 1992

SS, JUNG F, DIAMANT D, BROWN D, BACHINSKY D, HELLMAN P, INGER JR: Temperature-sensitive SV 40 immortalized rat al tubule cell line has functional renin-angiotensin system. *hysiol* 268:F435–F436, 1995

L, LEI CL, ZHANG SL, ROBERTS KD, TANG SS, INGELFINGER AN JSD: Synergistic effect of dexamethasone and isoproterenol expression of angiotensinogen in immortalized rat proximal cells. *Kidney Int* 53:287–295, 1998

JSD, CHAN AHH, NIE Z-R, SIKSTROM R, LACHANCE S, MOTO S, CARRIÈRE S: Thyroid hormone, L-T_3, stimulates the ion of the angiotensinogen gene in cultured opossum kidney ells. *J Am Soc Nephrol* 2:1360–1367, 1992

INGER JR, BOUBOUNES B, JUN FF, TANG SS: High glucose gulates expression of renin angiotensin system (RAS) in m kidney cells. (abstract) *Pediatr Nephrol* 29:344A, 1991

TT, CHEN M, LACHANCE S, DELALANDRE A, CARRIÈRE S, JSD: Isoproterenol and 8-Bromo-cyclic adenosine monophosstimulate the expression of the angiotensinogen gene in opossdney cells. *Kidney Int* 46:703–710, 1994

TT, LACHANCE S, DELALANDRE A, CARRIÈRE S, CHAN JSD: adrenoceptors and angiotensinogen gene expression in opossdney cells. *Kidney Int* 48:139–145, 1995

IURA A, JOHNS EJ: Effect of renal nerves on expression of renin giotensinogen genes in rat kidney. *Am J Physiol* 266:E230–1994

11. HALL JE, GUYTON AC, TRIPPODO NC, LOHMEIER TE, MCCREA RE, COWLEY ALW JR: Intrarenal control of electrolyte excretion by angiotensin II. *Am J Physiol* 232:F538–F544, 1977
12. SCHUSTER VL, KOKKO JP, JACOBSON HR: Angiotensin II directly stimulates sodium reabsorption in the kidney. *J Clin Invest* 73:506–515, 1984
13. LIU FY, COGAN MG: Angiotensin II stimulates early proximal bicarbonate absorption in the rat by decreasing cyclic adenosine monophosphate. *J Clin Invest* 84:83–91, 1989
14. WANG T, CHAN YL: Mechanism of angiotensin II action on proximal tubular transport. *J Pharmacol Exp Ther* 252:689–695, 1990
15. RASKIN P, ROSENSTOCK J: Blood glucose control and diabetic complications. *Ann Intern Med* 105:254–263, 1986
16. AYO SH, RADNIK RA, CLASS WF II, GARONI JA, RAMPT ER, APPLING DR, KREISBERG JI: Increased extracellular matrix synthesis and mRNA in mesangial cells grown in high-glucose medium. *Am J Physiol* 260:F185–F191, 1991
17. NAHMAN JR NS, LEONHART KL, COSIO FG, HEBERT CL: Effects of high glucose on cellular proliferation and fibronectin by cultured human mesangial cells. *Kidney Int* 41:396–402, 1992
18. WOLF G, SHARMA K, CHEN Y, ERICKSEN M, ZIZADEH FN: High glucose-induced proliferation in mesangial cells is reversed by autocrine TGF-β. *Kidney Int* 42:647–656, 1992
19. ZIYADEH FN, SHERMA K, ERICKSEN M, WOLF G: Stimulation of collagen gene expression and protein synthesis in murine mesangial cells by high glucose is mediated by autocrine activation of transforming growth factor-β. *J Clin Invest* 93:536–542, 1994
20. FUMO P, KUNCIO GS, ZIYADEH FN: PKC and high glucose stimulate collagen α1 (IX) transcriptional activity in a reporter mesangial cell line. *Am J Physiol* 267:F632–F638, 1994
21. AYO SH, RADNIK R, GARONI J, TROYER DA, KREISBERG JI: High glucose increases diacylglycerol mass and activates protein kinase C in mesangial cell culture. *Am J Physiol* 261:F571–F577, 1991
22. STUDER RK, CRAVEN PA, DERUBERTIS FR: Role for protein kinase C in the mediation of increased fibronectin accumulation by mesangial cells grown in high-glucose medium. *Diabetes* 42:118–126, 1993
23. CHATZILIAS A, WHITESIDE CI: Cellular mechanisms of glucose-induced myoinositol transport upregulation in rat mesangial cells. *Am J Physiol* 267:F459–F466, 1994
24. KREISBERG JI, KREISBERG SH: High glucose activates protein kinase C and stimulates fibronectin gene expression by enhancing a cAMP response element. *Kidney Int* 48(Suppl 51):S3–S11, 1995
25. IBRAHIM HN, HOSTETTER TH: Diabetic nephropathy. *J Am Soc Nephrol* 8:487–493, 1997
26. CHAN JSD, CHAN AHH, JIANG Q, NIE Z-R, LACHANCE S, CARRIÈRE S: Molecular cloning and expression of the rat angiotensinogen gene. *Pediatr Nephrol* 4:429–435, 1990
27. MING M, WANG TT, LACHANCE S, DELALANDRE A, CARRIERE S, CHAN JSD: Expression of the angiotensinogen gene is synergistically stimulated by 8-Br-cAMP and DEX in opossum kidney cells. *Am J Physiol* 268:R105–R111, 1995
28. KOYAMA H, GOODPASTURE C, MILLER MM, TEPLITZ RL, RIGGS AD: Establishment and characterization of a cell line from the American opossum (Didelphys virginia). *In Vitro* 14:239–246, 1978
29. POLLOCK AS, WARNOCK DG, STREWLER GJ: Parathyroid hormone inhibition of Na^+-H^+ antiporter activity in a cultured renal cell line. *Am J Physiol* 250:F217–F225, 1986
30. CHAN JSD, MING M, NIE Z-R, SIKSTROM R, LACHANCE S, CARRIÈRE S: Hormonal regulation of expression of the angiotensinogen gene cultured in opossum kidney (OK) proximal tubular cells. *J Am Soc Nephrol* 2:1516–1522, 1992
31. SAMUELS HH, STANLEY F, SHAPIRO LE: Control of growth hormone synthesis in cultured GH cells by 3,5,3′-triiodo-L-thyronine and glucocorticoid agonists and antagonists: Studies on the independent and synergistic regulation of the growth hormone response. *Biochemistry* 18:715–721, 1979
32. ZIYADEH FN, SNIPES ER, WATANABE M, ALVAREZ RJ, GOLDFARB S, HAVERTY TP: High glucose induces cell hypertrophy and stimulates collagen gene transcription in proximal tubule. *Am J Physiol* 259: F704–F714, 1990
33. ROCCO M, CHEN Y, GOLDFARB S, ZIYADEH FN: Elevated glucose stimulates TGF-β gene expression and bioactivity in proximal tubule. *Kidney Int* 41:107–114, 1992

34. SHARMA K, ZIYADEH FN: Renal hypertrophy is associated with up-regulation of TGF-β gene expression in diabetic BB rats and NOD mice. *Am J Physiol* 267:F1094–F1101, 1994
35. WOLF G, NEILSON EG: Angiotensin II induces cellular hypertrophy in cultured murine proximal tubular cells. *Am J Physiol* 259:F768–F777, 1990
36. WOLF G, NEILSON EG: Angiotensin II as renal growth factor. *J Am Soc Nephrol* 3:1531–1540, 1993
37. WOLF G, ZAHNER G, MONDORF U, SCHOEPPE W, STAHL RAK: Angiotensin II stimulates cellular hypertrophy of LLC-PK1 cells through the AT_1 receptor. *Nephrol Dial Transplant* 8:128–133, 1993
38. WOLF G, KILLEN PD, NEILSON EG: Intracellular signalling of transcription and secretion of type IV collagen after angiotensin II-induced cellular hypertrophy in cultured proximal tubular cells. *Cell Regul* 2:219–227, 1991
39. WOLF G, MUELLER E, STAHL RAK, ZIYADEH FN: Angiotensin-II induced hypertrophy of cultured murine proximal tubular cells is mediated by endogenous transforming growth factor-β. *J Clin Invest* 92:1366–1372, 1993
40. WOLF G, NEILSON EG, GOLDFARB S, ZIYADEH FN: The influence of glucose concentration on angiotensin II-induced hypertrophy of proximal tubular cells in culture. *Biochem Biophys Res Commun* 176:902–909, 1991
41. KALINYAK JE, SECHI LA, GRIFFIN CA, DON BR, TAVANGAR K, KRAEMER FB, HOFFMAN AR, SCHAMBELAN M: The renin angiotensin system in streptozotocin-induced diabetes mellitus in the r *Nephrol* 4:1337–1345, 1993
42. CORREA-ROTTER R, HOSTETTER TH, ROSENBERG ME: angiotensinogen gene expression in experimental diabet *Kidney Int* 41:796–804, 1992
43. EVERETT A, SCOTT J, WILFONG N, MARINO B, ROSEN INAGAMI T, GOMEZ RA: Renin and angiotensinogen during the evolution of diabetes. *Hypertension* 19:70–78,
44. ANDERSON S, JUNG FF, INGELFINGER JR: Renal renin-system in diabetes: Functional, immunohistochemical, an biological correlations. *Am J Physiol* 265:F477–F486, 199
45. TILTON RG, BAIER D, HARLON JE, SMITH SR, OSTRON E SON JR: Diabetes-induced glomerular dysfunction: Link reduced ratio of $NADH^+/NAD^+$. *Kidney Int* 41:778–788,
46. BRINDLE P, NAKAJIMA T, MONTMINY M: Multiple protei regulated events are required for transcriptional inductio *Proc Natl Acad Sci USA* 92:10521–10525, 1995
47. KREISBERG JI, RADNIK RA, KREISBERG SH: Phospho cAMP-responsive element binding protein after treatmen gial cells with high glucose plus TGF-β or PMA. *Kidney* 810, 1996
48. QIAN JF, WANG TT, WU X-H, LACHANCE S, DELALANDR ERE S, CHAN JSD: cAMP-responsive element binding prot stimulates the expression of the angiotensinogen gene kidney cells. *J Am Soc Nephrol* 8:1072–1079, 1997