Université de Montréal

Regulations of G-protein Expression and Adenylyl Cyclase Activity in One kidney One Clip (1K-1C) Hypertension: Role of Angiotensin II

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To my parents, and

to my husband,

for their profound support and love.

To my future,

for which my efforts mean.

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Finally, I want to express my thankfulness to the faculty and staff of the Department of Physiology, in particular, Drs. Pierre Gauthier and Christianne Malo for their time of support.

RESUME

Les protéines G jouent un rôle important dans la régulation d'une variété de systèmes de transduction du signal incluant le système AMPc-adénylyl cyclase. Des altérations des taux de protéines G et de l'activité de l'adénylyl cyclase ont été démontrées chez les rats spontanément hypertendus (SHR) et chez les rats rendus hypertendus expérimentalement. Des études de notre laboratoire ont aussi montré des taux augmentés de Giα-2 et Giα-3 dans les cœurs et les aortes de rats SHR et de rats rendus hypertendus par la désoxycorticostérone et le sel (DOCA). Il a été rapporté qu'une variété d'hormones telles que l'angiotensine II (AII), l'ANP, l'endothéline, etc., dont les taux sont augmentés dans l'hypertension artérielle, peuvent contribuer à l'augmentation de l'expression des protéines Gi. Des études récentes dans notre laboratoire ont démontré que le traitement des cellules musculaires lisses vasculaires (CMLV) par l'AII augmentait l'expression des protéines Gi dans les CMLV. Puisque les taux d'AII sont augmentés dans l'hypertension un rein-un clip (1K-1C), il est possible que cela puisse résulter en une expression augmentée des protéines Gi dans ce modèle d'hypertension. Par conséquent, ces études ont été entreprises pour examiner cette possibilité et aussi pour étudier les mécanismes possibles responsables de la surexpression des protéines Gi induite par l'AII dans les CMLV.

L'expression des protéines G a été déterminée par les taux de protéines et d'ARNm en utilisant les techniques d'immunoblot et de Northern blot avec des anticorps spécifiques et des sondes d'ADNc, respectivement pour différentes isoformes de protéines G. Les taux des proteines Giα-2 et Giα-3 ainsi que les taux d'ARNm étaient significativement augmentés dans le cœur et l'aorte des rats hypertendus 1K-1C en comparaison des rats

contrôles, alors que les taux de Gsα étaient inchangés. Le GTPγS, l'isoprotérénol, le glucagon, le fluorure de sodium (NaF) et la forskoline (FSK) stimulaient l'activité de l'adénylyl cyclase dans les cœurs et les aortes des rats hypertendus et des rats contrôles à des degrés variés. Ces stimulations étaient significativement diminuées chez les rats hypertendus en comparaison des rats contrôles. L'effet inhibiteur de faibles concentrations de GTPγS sur l'activité de l'adénylyl cyclase stimulée par la forskoline était significativement augmenté dans les cœurs et les aortes des rats hypertendus 1K-1C en comparaison des rats contrôles. De plus, l'effet inhibiteur du C-ANF$_{4-23}$ sur l'adénylyl cyclase était augmenté et celui de l'AII était diminué dans les cœurs des rats hypertendus 1K-1C, alors que les effets inhibiteurs du C-ANF$_{4-23}$ et de l'AII étaient significativement diminués dans les aortes des rats hypertendus 1K-1C en comparaison des rats contrôles. Le captopril, un inhibiteur de l'enzyme de conversion de l'angiotensine, restaurait les taux augmentés de protéines Gi dans les cœurs de rats hypertendus et aussi la stimulation diminuée de l'adénylyl cyclase par le GTPγS et les hormones stimulatrices. Ces données suggèrent que les taux de protéines Giα et leurs fonctions associées sont augmentés chez les rats hypertendus 1K-1C lesquels peuvent être attribués aux taux augmentés d'AII dans ce modèle d'hypertension.

Dans la seconde partie du projet, le mécanisme responsable de l'expression augmentée des protéines Gi par l'AII a été examiné dans les CMLV. Le traitement par l'AII augmentait les taux des protéines Giα-2 et Giα-3 et l'actinomycine D, un inhibiteur de la synthèse de l'ARN, atténuait l'augmentation induite par l'AII de l'expression des protéines Giα-2 et Giα-3. De plus, le wortmannin, un inhibiteur de la phosphatidylinositol 3-kinase (PI-3-K), la rapamycine, un inhibiteur de p70^{S6K}, et le PD 098059, un inhibiteur de

la protéine kinase activée par les mitogènes (MAPK) étaient capables d'inhiber l'augmentation induite par l'AII de l'expression des protéines Giα-2 et Giα-3 à des degrés divers. A la concentration de 50 μM, le PD 098059 était capable d'atténuer les taux augmentés de Giα-2 et Giα-3 induit par le traitement par l'AII. Ces données suggèrent que l'expression augmentée des protéines Gi par le traitement par l'AII peut être attribuée à la synthèse accrue d'ARN des protéines Gi, et la MAPK kinase, la PI-3-kinase et $p70^{S6K}$ peuvent être impliqués dans l'expression augmentée induite par l'AII des protéines Gi dans les CMLV.

SUMMARY

G-proteins play an important role in the regulation of variety of signal transduction systems including adenylyl cyclase /cAMP system. Alterations in G-protein levels and adenylyl cyclase acticity have been demonstrated in spontaneously (SHR) and experimentally induced hypertensive rats. Studies in our lab have also shown an augmented levels of Giα-2 and Giα-3 in hearts and aorta from SHR and deoxycorticosterone (DOCA) salt hypertensive rats. It has been reported that various hormones such as angiotensin II (AII), ANP, endothelin etc. whose levels are increased in hypertension may contribute to the enhanced expression of Gi proteins. Recent studies in our lab have demonstrated that AII treatment of vascular smooth muscle cells (VSMC) enhanced the expression of Gi proteins in VSMC. Since the levels of AII are augmented in one kidney one clip (1K-1C) hypertension, it may be possible that this will result in enhanced expression of Gi-proteins in this model of hypertension. The present studies were therefore undertaken to investigate this possibility and also to study the possible mechanisms responsible for AII-induced overexpression of Gi proteins in VSMC.

The expression of G proteins was determined at protein and mRNA levels by immunoblotting and Northern blotting techniques with specific antibodies and cDNA probes respectively for different isoform of G-proteins. The levels of Giα-2 and Giα-3 proteins as well as mRNA were significantly increased in heart and aorta from 1K-1C hypertensive rats compared to control rats, whereas the levels of Gsα were unchanged. GTPγS, isoproterenol, glucagon, sodium fluoride (NaF) and forskolin (FSK) stimulated the adenylyl cyclase activity in hearts and aorta from hypertensive and control rats to various degrees. These stimulations were significantly decreased in hypertensive rats as compared to control rats. The inhibitory effect of low concentrations of GTPγS on FSK-stimulated adenylyl cyclase activity was significantly enhanced in hearts and aorta from 1K-1C

hypertensive rats as compared to control rats. In addition, the inhibitory effect of C-ANF$_{4-23}$ on adenylyl cyclase was increased and of AII was decreased in hearts from 1K-1C hypertensive rats, whereas the inhibitory effects of C-ANF$_{4-23}$ and AII were significantly decreased in aorta from 1K-1C hypertensive rats as compared to control rats. Captopril, an angiotensin-converting enzyme inhibitor restored the augmented levels of Gi proteins in hearts from hypertensive rats and also the diminished stimulation of adenylyl cyclase by GTPγS and stimulatory hormones. These data suggest that the levels of Giα proteins and associated functions are increased in 1K-1C hypertensive rats also which may be attributed to the enhanced levels of AII in this model of hypertension.

In the second part of the project, the mechanism responsible for AII-induced enhanced expression of Gi proteins was investigated in VSMC. AII treatment of VSMC increased the levels of Giα-2 and Giα-3 proteins and actinomycin D, an inhibitor of RNA synthesis attenuated the AII-evoked enhanced expression of Giα-2 and Giα-3 proteins. In addition, wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI-3-K); rapamycin, an inhibitor of p70^{S6K} and PD 098059, an inhibitor of mitogen-activated protein kinase (MAPK) were able to inhibit AII-induced enhanced expression of Giα-2 and Giα-3 to various degrees. The attenuation of AII enhanced levels of Giα-2 and Giα-3 by PD 098059 was concentration dependent. At 50 μM, PD 098059 was able to attenuate the enhanced levels of Giα-2 and Giα-3 caused by AII treatment. These data suggest that the enhanced expression of Gi-proteins by AII treatment may be attributed to increased RNA synthesis of Gi-proteins, and MAPK kinase, PI-3-Kinase and p70^{S6K} may be involved in AII-mediated increased expression of Gi-proteins in VSMC.

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LIST OF ABBREVIATIONS

1K-1C	One kidney one clip hypertensive rat
2K-1C	Two kidneys one clip hypertensive rat
1K-1C+CAP	One kidney one clip hypertensive rat treated with captopril
ACE	Angiotensin II converting enzyme
ADC	Adenylyl cyclase
A II	Angiotensin II
ANP	Atrial natriuretic peptides
ANS	Autonomic nervous system
AT_1	Angiotensin II type 1 receptor
AT_2	Angiotensin II type 2 receptor
c-AMP	Cyclic adenosine monophosphate
C-ANF $_{4-23}$	Ring-deleted analogue of atrial natriuritic factor
cDNA	Complementary deoxyribonucleic acid
cGMP	Cyclic guanine monophosphate
CNS	Central nervous system
CT	Cholera toxin
CTA1	Active A subunit type 1 of cholera toxin
DMEM	Dulbecco's modified Eagle's medium
DOCA-salt	Deoxycorticosterone-salt rats
DTT	Dithpiothreitol
EDRF	Endothelium derived relaxing factor
FSK	Forskolin
Gi	Inhibitory guanine nucleotide regulatory protein
Gi 1, 2, 3	Isoforms of inhibitory guanine nucleotide regulatory protein
GDP	Guanosine diphosphate

GLUC	Glucagon
Go	α-o guanine nucleotide regulatory protein
Gs	Stimulatory guanine nucleotide regulatory protein
GTP	Guanosine tripphosphate
GTPγS	Guanosine 5'-O-thiotriphosphate
IP3	1,4,5-Inositoltrisphosphate
ISO	Isoproterenol
L-NAME	N^{w}-nitro-L-arginine
MAP	Mitogen activated protein
MAPK	Mitogen activated protein kinase
MEK	MAPK /extracellular signal related kinase
NaF	Sodium Fluoride
NECA	5'-N-Ethyl-carboxamido-adenosine
$P70^{S6K}$	70-kD form of S6 kinase
PI-3	Phosphatidylinositol 3-OH
PKA	Protein kinase A
PKC	Protein kinase C
PRA	Plasma renin activity
PSN	Parasympathetic nervous system
PT	Pertussis toxin
PLC	Phospholipase C
PLD	Phospholipase D
RAS	Renin-angiotensin system
RSK	Ribosomal protein-S6 kinase or 85-96 kDa S6 kinase
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SHR	Spontaneously hypertensive rats
SNS	Sympathetic nervous system

VSMC	Vascular smooth muscle cells

CHAPTER 1

INTRODUCTION

Review of Literature

I. Intracellular Signaling Pathways

A. Introduction

The transmembrane signal transduction plays a fundamental role in cellular functions (Alberts et al. 1989; Rodbell 1994). Generally, external signals arrive at receptors in the plasma membrane and activate a closely related family of transducer proteins (i.e. G_S, G_i or G_O) that activate primary effector enzymes (i.e. adenylyl cyclase, phospholipase C, phospholipase A_2). These enzymes produce a second messenger (i.e. c-AMP, IP_3, diacylglycerol, or arachidonic acid). A second messenger activates a secondary effector (i.e. c-AMP-dependent protein kinase, protein kinase C, or 5-lipoxygenase etc.) or acts directly on a target (regulatory) protein. The c-AMP signal system is one of the best-characterized signal transduction pathways. The other systems are known as the guanylate cyclase/cGMP system, the phosphoinositide/Ca^{2+}/protein kinase C system, growth factor and signaling by ion-channels (Alberts et al. 1989).

B. Adenylyl Cyclase/c-AMP System

The adenylyl cyclase/c-AMP signaling system has been implicated in the maintenance of various components of the human physiology such as vascular tone, cardiac contractility and neurohormonal transmission (Krupinski 1991; Tang and Gilman 1992). Alterations in this system have been implicated in various diseases such as heart failure (Feldman et al. 1988) and diabetes (Lynch et al. 1989). The adenylyl cyclase system is composed of three distinct units: 1) receptors for hormones that regulate c-AMP synthesis; 2) regulatory GTP binding proteins (G-proteins); 3) the family of enzymes, the adenylyl cyclase. Each unit performs a vital role to ensure that an extracellular signal is received, transmitted and interpreted correctly. Failure at any step is sufficient to jeopardize the well-being of the cell and perhaps even the whole organism.

Extracellular signals are received by the cell-surface receptors through direct interaction between a signaling molecule (ligand) and receptor binding site. The ligand/receptor binding favors the activation of the associated G-protein, which couples the receptor to the adenylyl cyclase enzyme. In this way, the hormone does not need to enter the cell to elicit a response because all of the necessary components can be reached by way of the plasma membrane. This chain of events begins with the binding of ligand to the receptor and end with the elicited response, which is called a cascade reaction (Figure 1).

B.1. G-protein Linked Cell Surface Receptors

B.1.1. Structure

Extracellular signaling molecules elicit their metabolic response within the cell by interacting either with one or a combination of the classes of cell-surface receptors present on the plasma membrane. Most of cell surface receptors are G-protein linked receptors (Gudermann and Nürnberg 1995). Up to now more then 300 G-protein-linked receptors have been cloned; the total number is assumed to exceed 1000. By resolving the primary structures of various G-protein-linked receptors, including the light receptor rhodopsin, it has become clear that all of these receptors are designed according to a common structural principle. It has been proposed that G-protein-linked receptors belong to a superfamily of integral membrane proteins which are characterized by seven hydrophobic stretches of 20-25 amino acids, predicted to form transmembrane α-helices, connected by alternating extracellular and intracellular loops. The N-terminus of these "heptahelical" or "serpentine" receptors is located extracellularly; the C-terminus extends into the cytoplasm (Figure 2). All G-protein-linked receptors known so far are glycoproteins, and they have at least one consensus sequence for N-linked glycosylation in the extracellular domains.

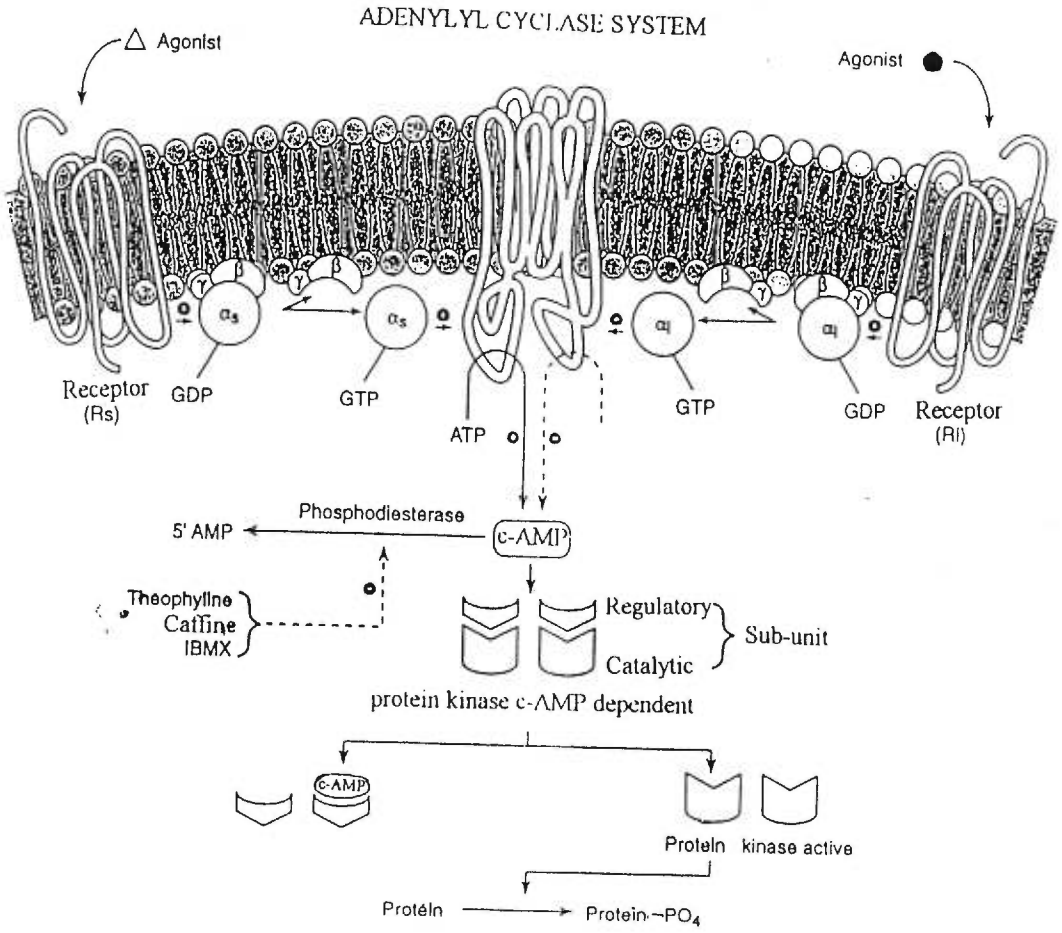

Figure 1: The adenylyl cyclase /c-AMP system

Krupinski J (1991): "The adenyly cyclase family." Mol. Cell. Biochem. **104**: 73-79.

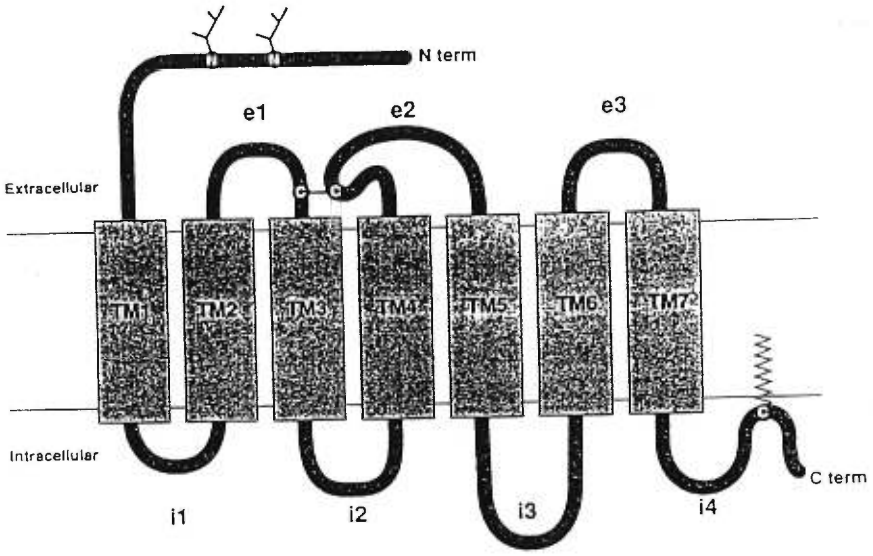

Figure 2 Schematic two-dimensional representation of a generic G protein-coupled receptor. The amino-terminus (N term) is located extracellularly and is shown with two N-linked glycosylation sites. The seven putative membrane-spanning α helices are shown as vertical rectangles and are numbered TM1–TM7. The three extracellular and the three intracellular loops are labeled e1–e3 and i1–i3, respectively. A disulfide linkage of cysteines in e1 and e2 is shown, as is a palmitoylated cysteine in the intracellular carboxy-terminus (C term) predicted to create a fourth intracellular loop (i4). Spiegel AM. Annu. Rev. Physiol. 1995. 58 : 143-70.

B.1.2. Function and Desensitization

Receptors, such as G-protein-coupled receptors, have their specific binding domains (Gudermann and Nürnberg 1995), that interact with their specific ligands. However, different binding domains of receptor subtypes can bind the same ligand. There are a wide variety of signaling ligands for those binding domains. These ligands include amines, amino acids and their derivatives, peptides and proteins, nucleotides, fatty acid derivatives, phopholipid derivatives, multistructural odorants and retinal rhodopsin. After ligand binding, G-protein-coupled receptors need to communicate with G-proteins to direct the flow of information to effectors. The structure of determinants for G-protein coupling are also distinct. For example, determinants for G-protein recognition are proved in the domain encompassing transmembrane helices 5 and 6 and the connecting cytoplasmic loop (Dohlman et al. 1991). An exceptional consequence shared among many G-protein-coupled receptors is an Asp-Arg-Tyr triplet, which is a key structure for the receptor to signal to a G-protein.

Desensitization of receptors is a process in which a receptor can become refractory to later application of the same ligand (Kandel et al. 1991). Although many mechanisms produce diminished responsiveness, desensitization has been shown in several instances to result from protein phosphorylation. For example, the β-adrenergic receptor is phosphorylated in the cytoplasmic domains of the receptor molecule that interact with Gs by a specific c-AMP-dependent β-adrenergic receptor kinase as well as by both the c-AMP-dependent protein kinase and protein kinase C. During the phosphorylation of the receptor, 2-3 mol of phosphate per mol of receptor are incorporated into the receptor protein, and the degree of the desensitization correlates with the extent of phosphorylation. Phosphorylation by the c-AMP-dependent kinase or by β-adrenergic receptor kinase slows the ability of the receptor to activate Gs. But the chief inhibitory effect of β-adrenergic

receptor kinase is to promote the binding of an inhibitory protein to the phosphorylated receptor. This inhibitor is similar to arrestin, a protein that regulates the function of rhodopsin in the retina.

C. G-proteins

C.1. Structure

Heterotrimeric regulatory guanine nucleotide-binding proteins (G-proteins) belong to the superfamily of GTP-binding proteins or GTPases that play a key regulatory role in the transduction of extracellular signals to intracellular effector proteins such as adenylyl cyclase. G-proteins are composed of three distinct subunits: α, β, and γ subunits. Upon activation of a G-protein in solution under non-denaturing conditions, α subunits readily dissociate from the $\beta\gamma$ complex. Each α subunit of G-protein is distinct, and may determine the specificity of a G-protein. On SDS-PAGE, known α subunits vary in size from about 39 to 52 kDa. The stimulation and inhibition of the adenylyl cyclase is mediated through Gsα (stimulatory G-protein) and Giα (inhibitory G-protein) respectively (Figure 3) (Gilman 1984; Stryer and Bourne 1986). Recently molecular cloning has revealed four different forms of Gsα resulting from the differential splicing of one gene (Bray et al. 1986; Robishaw et al. 1986), and three distinct forms of Giα; Giα-1, Giα-2 and Giα-3 encoded by the three different genes (Itoh et al. 1986; Jones and Reed 1987; Itoh et al. 1988). The β subunit migrates as a 36 kDa protein on SDS-PAGE, and is similar if not identical in each G-protein (Spiegel 1987). A more rapidly migrating, approximately 35 kDa species is observed in some tissues. The γ subunits are relatively low molecular weight proteins (about 8-11 kDa on SDS-PAGE). The γ subunit of transducin is distinct from other G-protein γ subunits, but the relationship among the γ subunits of G-proteins other than transducin is not clear.

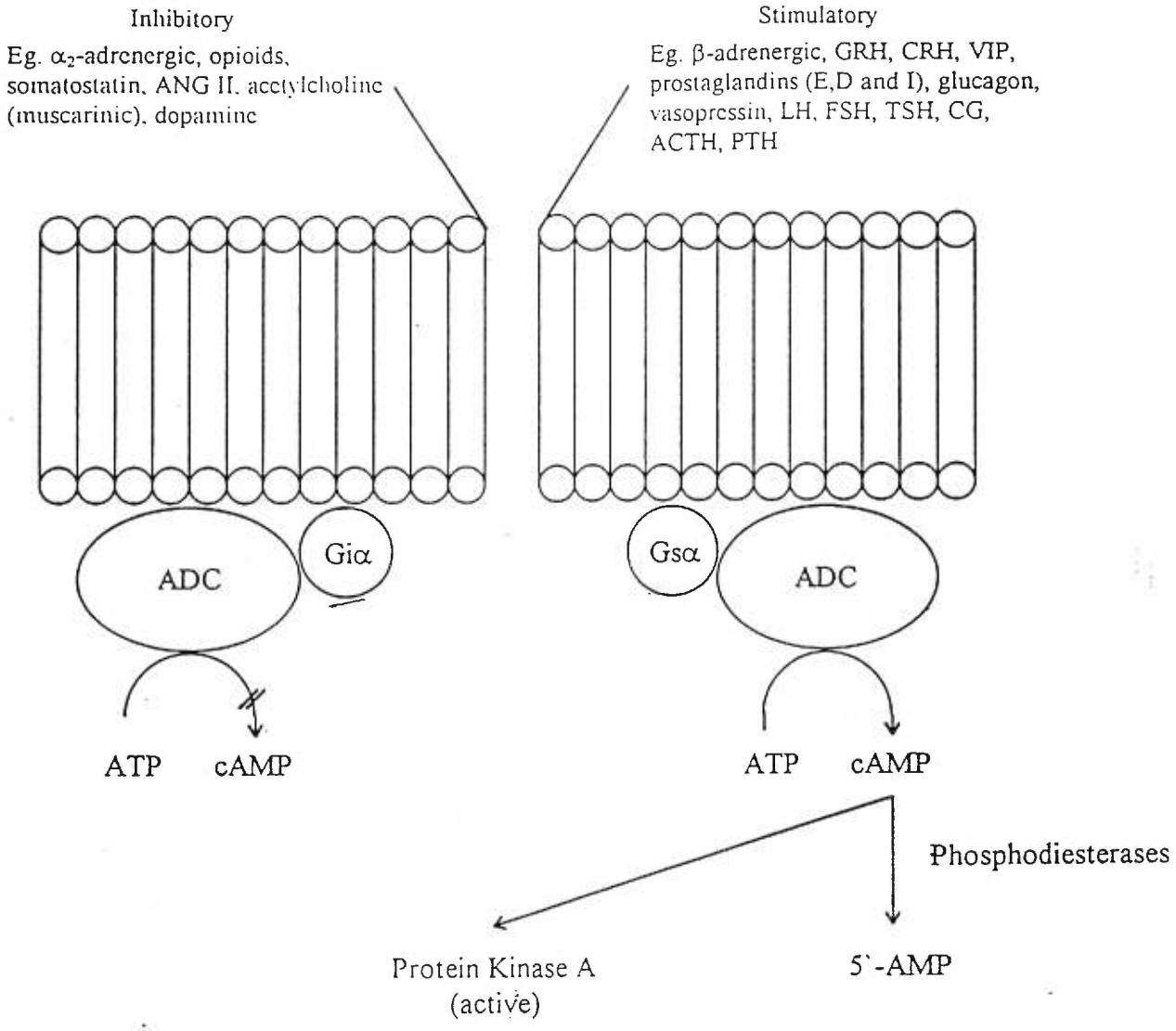

Figure 3 : The hormonal stimulation and inhibition of adenylyl cyclase are mediated by two G-proteins : stimulatory (Gs) and inhibitory (Gi).

Gilman AG. (1984) "G-proteins and dual control of adenylate cyclase." Cell **36**: 577-579.

C.2. Function

G-proteins function as signal transducers by acting as on-off switches. Interaction of G-proteins with receptors activated by external signals to the cell enables GTP to bind to and activate the G-protein. Activated G-protein in turn interacts with effector which transmits intracellular signals. G-protein activation and interaction with effector is terminated by the relatively low GTPase activity of the α subunit. GDP-bound G-protein, no longer capable of interacting with effector, must reassociate with activated receptor to begin another cycle.

In solution, G-protein activation is accompanied by dissociation of α from $\beta\gamma$ subunits. G-protein activation causes a change in α subunit conformation, reflected in altered susceptibility to tryptic proteolysis, changes in exposure of sulfhydryl groups, and reduced affinity for the $\beta\gamma$ complex. Mg^{2+} concentration is a critical determinant of protein activation. At sufficiently high Mg^{2+} concentration (50mM), α subunit dissociation may occur without addition of activators such as GTPγS (Spiegel 1987). Resolved, activated α subunits are capable of interacting with and activating their corresponding effector. Hydrolysis of GTPα to GDP returns the α subunit to a state of higher affinity for the $\beta\gamma$ complex.

The $\beta\gamma$ complex has been regarded as an "inhibitor", keeping the G-protein in its inactive form. Binding of GTP, promoted by activated receptor, is necessary to overcome inhibition by $\beta\gamma$. The $\beta\gamma$ complex, however, may be required for association between G-protein and receptor. GDP-bound α subunits, incapable by themselves of interacting with receptor, would represent a "dead-end" in the activation-deactivation GTPase cycle. Reassociation with $\beta\gamma$ subunits promotes receptor interaction and thereby binding of GTP to begin another round of the cycle.

It is worth to note that G-protein affinity for guanine nucleotides is seemingly much greater than required given the high concentrations of GTP in the cell. Such high affinity dictates a relatively slow spontaneous dissociation rate for bound GDP. Analysis of G-protein α subunits, and of *ras* gene products, indicates that they co-purify with tightly bound GDP. Thus, spontaneous activation of G-proteins does not occur. Instead, GTP binding is dependent on interaction of G-protein with activated receptor. The relatively low GTPase activity, moreover, permits GTP-bound G-protein to activate effector before hydrolysis to GDP occurs.

C.3. Mechanism of Activation

The activation/inactivation cycle of G-proteins is illustrated in Figure 4. When GTP is bound to the α-subunit, it is depicted to be active; when GDP is bound to the α-subunit, it is depicted as being inactive. Conversion between these two activity states is governed by the cell surface receptor with which the G-protein is associated to. Upon receptor activation, the receptor's intracellular domain undergoes a conformational change which favors the coupling of the inactive G-protein to the receptor. Once coupled to the receptor, the GDP bound to the G-protein's α-subunit is replaced by GTP. The active G-protein disassociates itself from the receptor which favors the release of α- and $\beta\gamma$-subunits. The α-and $\beta\gamma$-subunits are then free to interact with effector proteins. The return to an inactive conformation is achieved by the intrinsic GTPase activity of the α-subunit which hydrolyzes GTP to GDP. Once GTP is hydrolyzed and the inorganic phosphate released, the α-subunit and $\beta\gamma$ dimer re-associate to form the inactive G-protein heterotrimer. This cycle continues until the ligand is either removed or degraded from the receptor or if the receptor is internalized, thus preventing any more interaction with signaling molecules.

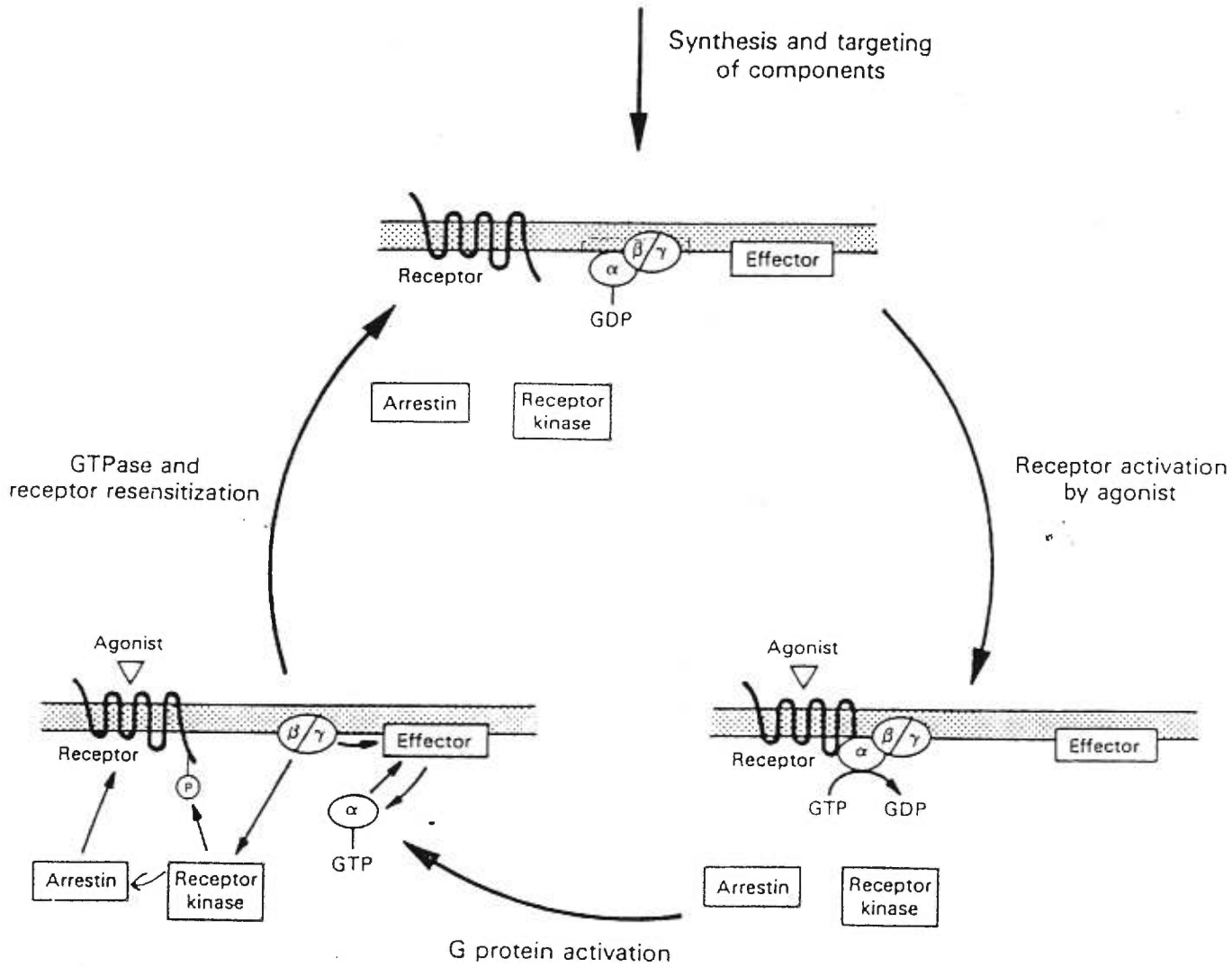

Figure 4. The G protein GTPase cycle and agonist-dependent desensitization: potential sites for disease-causing abnormalities (see text for complete description). In each panel, the stippled region denotes the plasma membrane with extracellular above and intracellular below. Arrows between α subunit and effector indicate regulation of effector activity by α subunit and regulation of α subunit GTPase activity by certain effectors. Receptor kinase and arrestin are shown as cytosolic proteins that translocate to the membrane upon binding to agonist-activated receptor. Arrows drawn from $\beta\gamma$ dimer denote its potential regulation of effector activity, as well as its action to facilitate membrane binding of certain receptor kinases. A presumptive receptor phosphatase (not shown) removes phosphate residues (shown here on the carboxy terminus, but may also occur on the third intracellular loop) from receptor upon resensitization. Spiegel et a. J. Clinc. Inv.(1990) 92 : 1119-1125.

C.4. Interaction of G-protein with Receptors

A single receptor may interact with a single or several G-proteins, and multiple receptors may interact with a specific G-protein or compete for a common pool. Evidence suggests the different interactions between receptor and G-protein may depend in part upon the expression and on the sub-cellular localization of the proteins.

Evidence obtained from expression of α_2-adrenergic receptors demonstrates that the receptor interacts with Gi-1/2 and Gi3 to inhibit adenylyl cyclase /c-AMP system (ADC) (Eason et al. 1992). Evidence even demonstrated the competition of 2 different receptor types for a common pool of G-proteins (Wielant et al. 1992).

C.5. Interaction of G-protein with Effectors

G-proteins act through a wide variety of receptors and are capable of activating a number of effector proteins (Table 1). α-subunit has been shown to interact with a wide variety of effectors. For example, when serotonin receptors were stably expressed in Hela-cells, it was demonstrated that the HT_{1A} receptors preferentially interacted with Giα-3 and in turn Giα-3 inhibited adenylyl cyclase /c-AMP (ADC) system while simultaneously activated PLC (Forgin et al. 1991).

It has been demonstrated that the interaction of the α-subunit with the effector protein may increase the GTPase activity resulting in a shorter time of activity. However, more attention is paid towards the presence of GTPase inhibitors and their role in cellular signaling. GTPase inhibitors such as phosducin, inhibit the hydrolysis of GTP to GDP by both the α-subunit and effector protein resulting in a greater duration of the active form of the G-protein. Though assumed to be involved exclusively with phototransduction, phosducin and /or its analogs were found in bovine brain and the recombinant form has

Family and subunits	Mass (kDa)	% AA identity	Toxin	Receptors	Effectors
Gs					
αs (short, S)	44.2	100	C	β-AR, A_2 adenosine, glucagon, etc	ADC, Ca^{+2} channels, Na^{+} channels
αs (long, L)	45.7		C		K^{+} channel
αolf	55.7	88	C	Odorant	ADC
Gi/o/t					
iα1	40.3	100	P	α_2-AR, opoid, 5-HT, C5a, etc	K^{+} channel, Ca^{+2} channels, ADC, PLC, PLA_2
iα2	40.5	88	P		
iα3	40.5	94	P	m_2-AChR, somatostatin	
αo1	40.0	73	P		
αo2	40.1	73	P		
αt1	40.0	68	C+P	Rhodopsin	cGMP-PDE
αt2	40.1	68	C+P	Cone opsin	cGMP-PDE
αg	40.5	67			cAMP-PDE?
αz	40.9	60			ADC?
Gq					
αq	42.0	100		m_1-AChR, α_1-AR, TRH, etc.	PLC-βs+others?
α11	42.0	88			PLC?
α14	41.5	79		IL-8 and others?	PLC?
α15	43.0	57		IL-8 and others?	?
α16	43.5	58		IL-8, C5a and others?	PLC-β2+others?
G12					
α12	44.0	100		?	?
α13	44.0	67		?	?

Table 1 : Interactions of G-protein with receptors and effectors.

Gilman AG (1987): "G-protein: transducers of receptor generated signals." Annu. Rev. Biochem. **56**: 615-449.

been determined to inhibit the GTPase activity of a number of G-proteins (Carson et al. 1986).

Transmission of signals through G-proteins has been known to be achieved with either α or $\beta\gamma$ subunits (Hepler and Gilman 1992). The $\beta\gamma$ dimers have been shown to be implicated in the regulation of some types of adenylyl cyclase (Tang and Gilman 1991) and phospholipase C (Camps et al. 1992). More precisely, adenylyl cyclase type II and IV have been shown to be regulated by the $\beta\gamma$-subunit in the presence of stimulated α_2 subunits (Tang et al. 1991). In addition, Katada et al.(1984) have shown that $\beta\gamma$-subunits stimulate the mouse lymphoma S49 *cyc*$^-$ cell membrane adenylyl cyclase. It has been also shown that phospholipase C from HL-60 cells and neutrophils can be stimulated by $\beta\gamma$ (Camps et al. 1992).

C.6. Modification of G-protein by Toxins

It is well known that some toxins can prolong the activation period of G-proteins by specific ADP-ribosylation of the α-subunit. Pertussis toxin (also known as islet-activating protein) is a bacterial toxin produced by some *Bordeterlla pertussis* strains and is composed of an A and B component. These types of toxin are called A-B toxins where the A subunit is enzymatically active and the B subunit serves to bind the toxin to the cell surface so the A subunit may enter (Gill 1978). Once reached inside the cell, the A and B subunits dissociate and the ADP-ribosyltransferase and NAD^+ glycohydrolase activities are expressed (Moss et al. 1983; Kaslow and Burns 1992). Among the substrates of ADP-ribosylation by the A subunit of the pertussis toxin are the members of the inhibitory guanine nucleotide-binding regulatory protein family (Giα). The ADP-ribosylation of Gi occurs at a conserved cysteine residue occurring at the fourth position from the carboxy termini of the α-subunit. The ADP-ribosylation functionally uncouples Gi proteins from

receptors (Gilman 1987), and attenuates the GTP-dependent and receptor-mediated inhibitory adenylyl cyclase response.

The cholera toxin, a secretory product of the bacterium *Vibrio cholera*, is responsible for the significant fluid and electrolyte loss in cholera (Kelly 1986). Like pertussis toxin, cholera toxin is an A-B type toxin composed of one enzymatically-active A subunit and five B subunits responsible for binding to the cell surface. Once entered the cell, the A-B subunits dissociate and the A subunit undergoes a further proteolytic cleavage in order to be enzymatically active (CTA1). The enzymatically active A subunit (CTA1) is believed to be responsible for the ADP-ribosylation effect of the toxin on cells. The major substrate of the CTA1 subunit is the α-subunit of the stimulatory regulatory guanine nucleotide-binding protein family (Gsα) (Birnbaumer et al. 1990). The ADP-ribosylation of Gs by the cholera toxin occurs at a conserved arginine residue occurring at position 201 of the α-subunit (Gilman 1987; Landis et al. 1989). Cholera toxin-catalyzed ADP-ribosylation inhibits the intrinsic GTPase activity of the α-subunit and thereby prolongs its active state (Cassel and Selinger 1977; Navon and Fung 1984). Effects of pertussis toxin and cholera toxin on G-protein are shown in Figure 5.

D. Adenylyl Cyclase

D.1. Structure

Krupinski and co-workers (1992) were able to purify a sufficient amount of calmodulin-sensitive adenylyl cyclase from bovine brain to obtain partial amino acid sequence and thereby isolate cDNA clone encoding the full-length protein; which was termed the type I isoform. Application of low stringency hybridization and

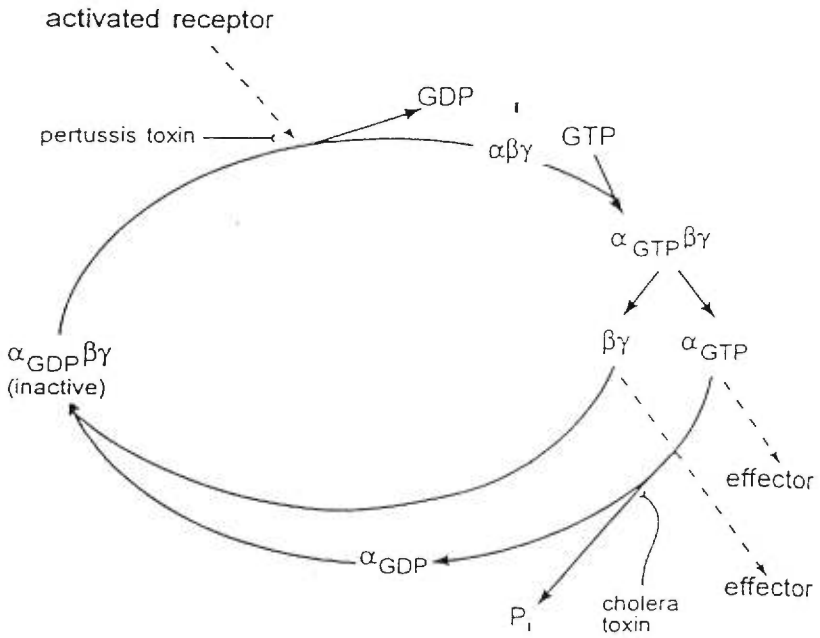

Figure 5 : Activation/Inactivation cycle of G proteins. α, β, and γ represent the Gα, Gβ, and Gγ subunits of the G protein. Pertussis toxin functionally uncouples the G protein from the receptor whereas cholera toxin abolishes the GTPase activity of the G protein Nürnberg et al. J. Mol. Med. (1995) 73 : 123-132.

polymerase chain reaction techniques has now permitted isolation of seven additional full-length clones: types II-VI and VIII (Bakalyar and Reed 1990; De Vivo and Iyengar 1994).

Adenylyl cyclase has molecular weight of roughly 120,000 (1064-1248 amino acid residues) and a complex topology within the membrane. There are at least three general classes of membrane-bound adenylyl cyclases. The most common motif in higher eukaryotes includes a short amino-terminal region (N) and two ~40 kDa cytoplasmic domains (C_1 and C_2), punctuated by two intensely hydrophobic stretches (M_1 and M_2); each of the latter is hypothesized to contain six transmembrane helices (Figure 6). It is this complex and widely distributed group of adenylyl cyclase that is responsive to stimulatory and inhibitory regulation by hormones and neurotransmitters, acting via the intermediary of both receptors (generally with seven transmembrane spans) and G-proteins (Gilman 1987). Different isoformes of adenylyl cyclase have been identified (Krupinski et al. 1992), and its distribution in different tissues has been determined by immunoblot and cloning studies (Table-2). All isoforms of adenylyl cyclase appear to be expressed in the brain, apparently in region-specific pattern. The type I enzyme is largely restricted to the nervous system, while type III is found predominantly in olfactory neuroepithelium, only two types, types V and VI have been identified in heart, aorta and brain (Premont et al. 1992).

D.2. Regulation of Adenyly Cyclase Activity

All isoforms of adenylyl cyclase are activated by both forskolin and the GTP-bound α subunit of the stimulatory G-protein Gs. All are inhibited by certain adenosine analogs termed P-site inhibitors; 2"-deoxy-3"-AMP is particularly potent. However, all of the isoforms of adenylyl cyclase are further regulated in type-specific patterns by other inputs, particularly including those that depend on Ca^{2+} or that arise from other (non-Gsα) G-protein subunits, and phosphorylation (Tang and Gilman 1992; Taussig and Gilman 1995).

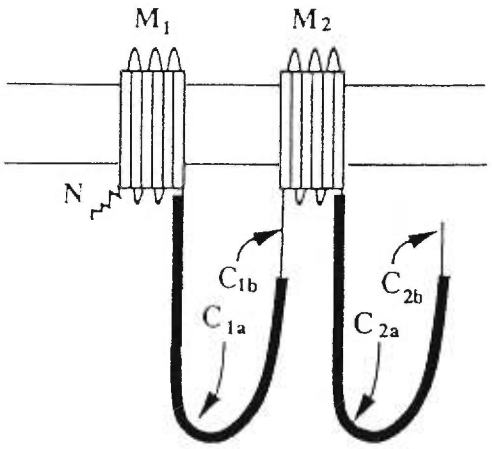

Figure 6 : The structures of adenylyl cyclase. Wei-Jen Tang et al. Cell. (1992) 70 : 869- 872.

Table 2 : Different isoformes and properties of adenylyl cyclase.

Properties of Eukaryotic Adenylyl Cyclases

Type[a]	Amino Acid Residues	Expression	Effect of G Proteins		Ca^{2+} Calmodulin	Forskolin
			$G_s\alpha$	$\beta\gamma$		
I	1134	Brain	+[b]		+	+
II	1090	Brain, lung	+	+	0	+
III	1144	Olfactory	+	0	+	+
IV	1064	Brain, others	+	+	0	+
V	1184	Heart, brain, others	+	0	0	+
VI	1165	Heart, brain, others	+	0	0	+
rutabaga	2249	Mushroom body	+	0	+	+
AC-A	1407	During aggregation	?[c]	?	?	0
AC-G	858	Fruiting body	?[d]	?[d]	?[d]	0
CYR1	2026	Constitutive	0[e]	0	0	0

[a] Adenylyl cyclase types I–VI are mammalian; rutabaga is from Drosophila; AC-A and AC-G are from Dictyostelium; CYR1 is from Saccharomyces cerevisiae. References: I (Krupinski et al., 1989), II (Feinstein et al., 1991), III (Bakalyar and Reed, 1990), IV (Gao and Gilman, 1991), V (Ishikawa et al., 1992), VI (Premont et al., 1992; Katsushika et al., 1992), rutabaga (Levin et al., 1992), AC-A and AC-G (Pitt et al., 1992), and CYR1 (Kataoka et al., 1985).

[b] +, stimulates; –, inhibits; 0, no effect.

[c] AC-A is activated by GTPγS; however, a homolog of $G_s\alpha$ has not been detected in Dictyostelium.

[d] Unlikely to have an effect; GTPγS does not activate.

[e] Activated by ras.

Taussig R, Gilman AG (1995): "Mammalian membrane-bound adenyly cyclases." J Biol. Chem. **270**(1): 1-4.

Different types of adenylyl cyclase are activated by different regulators; adenylyl cyclase type II and IV are activated by Gβγ in the presence of Gsα, type I is inhibited by Gβγ and type III, V and VI do not appear to be directly regulated by Gβγ (Toro et al. 1987; Taussig et al. 1993). (Figure 7 and Table 2).

II. Hypertension

It is estimated that about 23,000,000 North Americans have hypertension. The hypertension was somewhat defined arbitrarily as persistent elevation of arterial pressure above 140/90 mm Hg. The high blood pressure shortens life because of its adverse effects on blood vessels and on cardiac, renal, and cerebral function (Kaplan 1998).

A. Arterial Blood Pressure

In most mammals, mean arterial blood pressure is maintained at relatively constant levels between 75 and 100 mm Hg. Arterial pressure changes continuously throughout each cardiac cycle. The highest pressure reached during systole is called systolic arterial pressure and the lowest pressure reached during diastole is called diastolic arterial pressure.

B. Regulation of Arterial Blood Pressure

The mean arterial pressure is mainly determined by cardiac output (CO) and total peripheral vascular resistance (PR) (Guyton 1991). The regulation of a proper arterial blood pressure is essential in order to maintain a healthy supply of nutrients to the tissues. A hierarchy of mechanisms are in place to effectively respond to different conditions that the maintenance of arterial pressure may encounter.

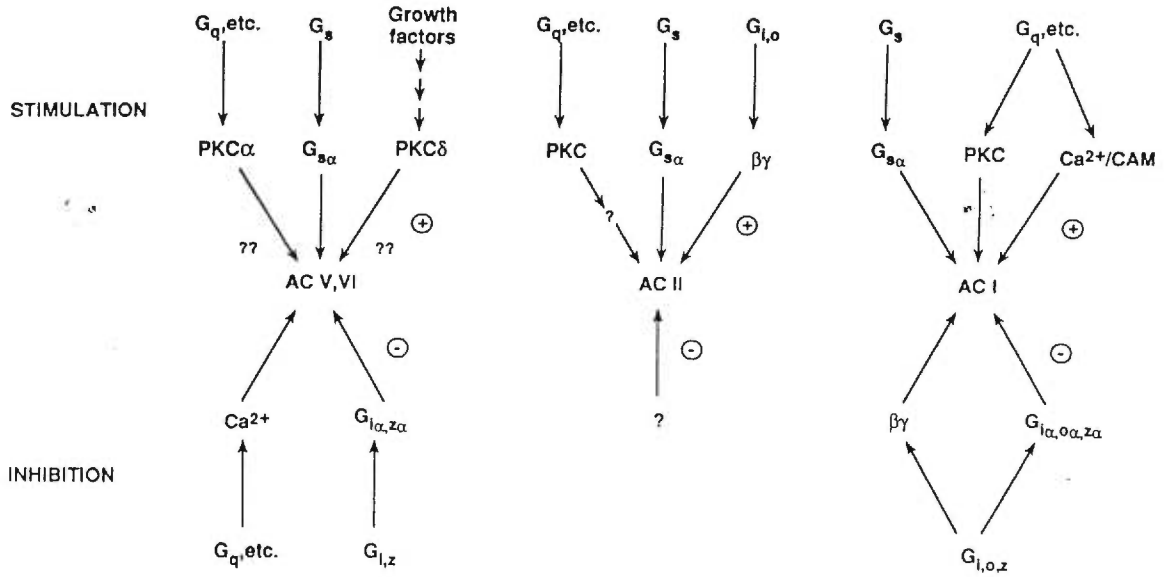

Figure 7 : **Patterns of regulation of adenylyl cyclase activity.** *PKC*, protein kinase C; *CAM*, calmodulin; *AC*, adenylyl cyclase. See text for discussion.

Ronald and Gilman. J. Biol. Chem. (1995) 270 : No.1, Issue of January 6.pp.1-4.

B.1. Feedback-Reflex Mechanisms

B.1.1. The Baroreceptor Reflex

The reflex is initiated by stretch receptors called baroreceptors, which are located in the wall of the carotid sinus and the wall of the aortic arch (Guyton 1991). The baroreceptors transmit to the central nervous system (CNS) of any changes in arterial blood pressure and feedback signals are then sent through the autonomic nervous system (ANS) to the periphery to correct the changes. Signals from the carotid sinus are transmitted through the small *Hearing's nerve* to the *glossopharyngeal nerve* and finally to the *tractus solitaris* in the medullar area of the brain stem, whereas, signals from the aortic arch are sent through the *vagus nerve* to the same area of medulla. Upon signal reception, secondary signals from the *tractus solitari* inhibit the vasoconstrictor center of the medulla and excites the vagal center. This in turn produces a vasodilation of the veins and the arterioles throughout the periphery as well as a decrease in both heart rate and force of contraction. Therefore, excitation of the baroreceptors by an increase of pressure produces a decrease in arterial blood pressure because of a reduction of peripheral resistance and a decrease in cardiac output. Conversely, a sudden drop in arterial blood pressure has opposite effects, producing a rise in arterial blood pressure.

Though an effective control in transient changes in arterial blood pressure, the baroreceptor reflex does not exert any important role in the long term regulation of arterial blood pressure. This is reflected in the ability of the baroreceptors to "reset" themselves when an increase in arterial blood pressure is sustained over a prolonged period of time.

B.1.2. The Chemoreceptor Reflex

The chemoreceptors are located in carotic bodies near the inside wall of bifurcation of each common carotid (Guyton 1991). The chemoreceptors reflex functions similarly to

that of the baroreceptor reflex except that instead of responding to a change in stretch by pressure, it responds to a reduction of arterial oxygen ions, to lesser extent to elevations of carbon dioxide ions or an excess of hydrogen in blood. The chemoreceptor discharge frequency begins to rise when arterial oxygen level falls or carbon dioxide increases, and results mainly in increase of ventilation and peripheral vasoconstriction. These changes cause high ventilation and increase of arterial pressure and chiefly improve blood perfusion into the tissue and increase oxygen and carbon dioxide exchanges in lung.

B.2. Autonomic Nervous System

The autonomic system consists of the sympathetic system and the parasympathetic system (Ross 1982). It has afferent fibers carrying impulses toward the central nervous system (CNS) and efferent fibers conveying impulses away from the CNS to the effector. The sympathetic system influences the circulation via two routes: one is specific sympathetic nerves innervating the viscera and the heart and the other is the spinal nerves innervating the peripheral vasculature. Most blood vessels only have sympathetic nerve innervation. The sympthetic nerve controls the blood pressure by altering muscle tone and the diameter of small arterial vessels on one hand, and reduces circulatory blood volume by making constriction of large vessels, especially veins. The sympathetic system also innervates the heart. It increases heart rate, conduction, and myocardial force, which increases cardiac output. On contrary, the parasympathetic vagus nerve slows the heart, depresses conduction, and decreases cardiac output. The degree of the sysmpathetic regulation depends on neural factors and other factors such as metabolic factors (Guyton 1991). The neural factors include the density of innervation, amount of transmitter released, width of "synaptic cleft" and activity of corresponding receptors (such as α receptors).

B.3. Central Nervous System

The CNS is higher control center over the autonomic one (Ross 1982). It has different levels or regions. The lower brain stem (medulla and lower pons) is the most important area for integrating autonomic afferent impulses of relevance to the circulation and for producing appropriate outputs to regulate cardiovascular functions. It has the vasomotor center located bilaterally in its reticular subtance and lower third of the pons. Two important areas are located in this center. One area, called vasoconstrictor area (C-1), exists in the anterolateral part of the upper medulla, which releases norepinephrine through its fibers distributed through the spinal cord. The secreted norepinephrine excites the vasoconstrictor neurons, and in turn results in constriction of peripheral vessels and stimulates the heart. The second area, called the vasodilator area (A-1), is present in bilateral and anterolateral parts of the lower half of the medulla. The fibers from this area project to the vasoconstrictor area and inhibit the vasoconstrictor activity in that area, causing vasodilation. In addition, a sensory area or A-2 is located bilaterally in the posterolateral parts of the medulla and lower pons. This area connects with C-1 and A-1, to help to control the activity of both the vasoconstrictor and vasodilator areas, thus providing "reflex" controls of baroreceptor and chemoreceptor reflexes as described previously.

Several specific groups of neurons in the lower brainstem also form a network of "common centers". This network is influenced by neurons in the hypothalamus, cerebral cortex, limbic system, and cerebellum. The hypothalamus is responsible for integrating some of the more complex cardiovascular responses. Electrical stimulation of hypothalamic sites in certain animals increases heart rate and arterial pressure and redistributes blood flow. The cerebral cortex clearly can produce profound circulatory changes, such as emotional fainting and tachycardia. It is also involved in regulating cardiovascular

adjustments to exercise. The exercise response is vasodilation in the muscle and vasoconstriction elsewhere. This kind of conflicting needs requires complex interactions between the medullary, hypothalamic and cortical cardiovascular centers, with the need to facilitate some circuits and to inhibit others so that the optimal response can be made. In some complicated situation like optimal thermoregulation, there are needs for appropriate vascular adjustments

B.4. Renin-Angiotensin System

Figure 8 (Laragh 1992) overviews the renin-angiotensin system: its major components, the regulators of renin release, and the primary effects of AII.

Renin plays a critical role in the pathogenesis of hypertension (Laragh 1992; Kaplan 1998). Renin is synthesized and stored in an inactivated form called prorenin in the juxtaglomerular cells of the kidney and splits and releases renin in certain circumstances, such as falls in the arterial pressure. Renin is an aspartyl proteinase. It acts on angiotensinogen to release a 10- amino acid peptide, angiotensin I, which has mild vasoconstrictor effect but not enough to affect circulatory function. Within a few seconds of its formation, angiotensin I splits into 8-amino acid peptide, angiotensin II(AII) in the small vessels of the lungs, a reaction catalyzed by the enzyme either converting enzyme or chymase (Erdös 1990). AII is an extremely powerful vasoconstrictor that is more effetive in circulation, and is inactivated by angiotensinase in the blood.

Angiotensin-converting enzyme (ACE) is bound to the luminal surface of vascular endothelial cells, where it is in contact with circulating plasma and, hence, constant supply of angiotensin II (Ehlers and Riordan 1990; Riordan 1995). The somatic form of ACE contains 1277 amino acids and is heavily glycosylated.

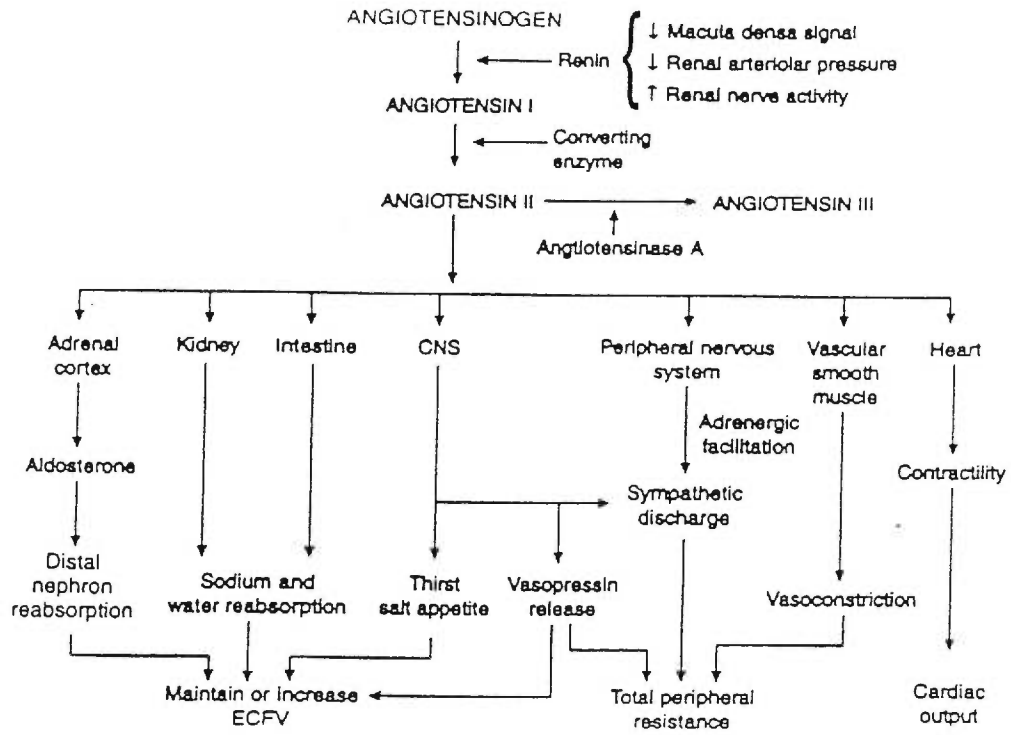

Figure 8 : Renin-agiotensin system : effects of angiotensin II.

Laragh JH (1992): "The renin system and four lines of hypertension research." Hypertension **20**: 267-279.

From cDNA analysis, it is apparent that it consists of two homologous domains. Some ACE is present in soluble form in plasma, but this appears to be a minor component of total ACE activity and arises from proteolytic release of the enzyme from the endothelial cell surface. AII is the final product of this system, and it is physiologically important. AII is inactivated by a series of peptidase enzymes, called angiotensinases, which are present in most tissues and in high concentrations in red blood cells (Griendling 1997).

B.5. **Angiotensin II (AII):** Receptors, Signal Transduction Mechanisms and Physilogical Effects

B.5.1. Effects of AII

The potent effector peptide of the renin-angiotensin system, angiotensin II, regulates a wide variety of physiological responses including fluid homeostasis, blood pressure, adrenal and renal function, and vascular tone. AII is generated locally in the vascular wall (Dzau 1988) and has been shown to stimulate a hypertrophic growth response in cultured vascular smooth muscle cells (VSMC)(Geisterfer et al. 1988; Berk, Vekshtein et al. 1989; Krug and Berk 1992) . AII regulates vascular contractility by binding to specific receptors on the VSMC plasma membrane (Murphy et al. 1991; Sasaki et al. 1992) and stimulating intracellular signalling events. The signal transduction events stimulated by AII are similar to those stimulated by growth factors and include activation of phospholipase C (Griendling et al. 1986) , inositol trisphosphate formation (Griendling et al. 1986), calcium mobilization (Brock et al. 1985), activation of protein kinase C (Griendling et al. 1989), induction of proto-oncogenes (Naftilan et al. 1989; Taubman et al. 1989) and protein tyrosin phosphorylation (Tsuda et al. 1992) .

In VSMC, the principle of AII functioning process is to induce contraction (Marsden and Riordan 1994). Myosin light chain kinase, stimulates phosphorylation of myosin light chains. In the presence of actin, these myosin chains can now cleave bound ATP and this results in cross-bridge formation between actin and myosin and contraction of myofibrils. In renal tubular cells AII stimulates fluid reabsorption by activating Na^+/H^+ exchange and basolateral Na^+/CO_3^- cotransport, likely mediated through inhibition of adenylate cyclase. It is also thought to activate phospholipase A_2 in the proximal tubules which leads to the hydrolysis of the fatty acid, usually arachidonic acid, from the 2-position of the glycerol moiety of membrane phospholipids. Arachidonic acid is subsequently converted to prostaglandins and prostacyclin. It may be that AII does not activate phospholipase C in these renal cells. In adrenal glomerulosa AII increases aldosterone secretion and stimulates steroid biosynthesis by increasing intracellular calcium. It also has a trophic effect on the adrenal glomerulosa and stimulates catecholamine release from the medulla.

AII acts to stimulate contraction of vascular smooth muscle cells, to enhance sodium retention and to increase extracellular volume through water retention by the kidney, and to increase aldosteron secretion and catecholamine release from the adrenals. The AT_1 receptor is coupled to a G-protein transduction mechanism. In their inactive state, these heterotrimeric proteins consist of a GDP-binding α-subunit. The nature of α-subunit is the principle determinant of the receptor response. With AII, response is typically through a member of the $G_q/_{11}$ family which activates phospholipase C to mobilize intracellular calcium. Binding of AII to its AT_1 receptor causes the α-subunit of the G-protein trimer to undergo a conformational change that results in an exchange of GTP for GDP and allows the α-subunit to dissociate from the $\beta\gamma$-subunits. The dissociated α-subunit activates its downstream target, in this case phospholipase C and, most likely, phospholipase D and A_2 as well. Dissociation of the α-subunit somehow converts the receptor from a high affinity to a low affinity state thus dissociating AII.

B.5.2. AII receptors

AII exerts its effects by acting through cell surface receptors, and these effects have important consequences for hypertension. The response varies with the particular organ or specific location within a given organ, likely due in part to the type of angiontensin II receptor and to the signalling pathway coupled to the receptor, such as adenylate cyclase (Riordan 1995) . There are at least two types of AII receptor-binding affinity, thiol sensitive (AT_1) and low affinity, thiol insensitive, or thiol activated (AT_2) (Riordan 1995; Ubger et al. 1996) . Table 3 shows the AII receptors functions and distributions. Almost all of the known biological actions of AII are now believed to be mediated by the AT_1 receptor, but a few are mediated by AT_2. Both receptors have been identified and characterized by expression cloning in the COS-7 cell line which is normally devoid of AII receptors (Bernstein and Berk 1993) . The two forms of the AT_1, AT_1a and AT_1b, have been recognized in rat, mouse and human. Both forms evoke a rapid increase in phosphatidylinositol hydrolysis and cytosolic calcium but at high concentrations of AII (>1μM) the response of AT_1b are inhibited while those of AT_1a are not. The presence of AT1 receptor subtype has been shown in rat vascular tissues, however, a small proportion of AT2 receptor is also present in rat aorta (Chang and Lotti 1991; Viswanathan et al. 1991)

B.5.2.1. AT_1 receptor signaling

AT_1 receptors have been identified in VSMC, brain, liver, kidney, heart, and adrenal granulosa cells (Timmermans et al. 1993) . They have been found throughout the body and a variety of species. The AT_1 receptors belongs to the superfamily of seven transmembrane domain, G-protein-coupled receptors. The AT_1 receptors interact with various G-proteins and is coupled to one of the two heterotrimeric G-proteins: $G_{q\alpha}$ or $G_{i\alpha}$. Binding of AII to specific sites of the AT_1 receptors results in the release of the α-subunit of the G-protein

Table 3 : Summary of the characteristics of the angiotensin II receptor subtypes AT_1 and AT_2

	AT_1 receptor	AT_2 receptor
Distribution	Adult vasculature, kidney, adrenal gland, heart, liver, brain	Fetal tissues, adult brain adrenal, gland, ovary, uterus, endothelial cells
Function	Vasoconstriction, cardiac contractility, aldosterone and arginine vasopressin release, renal blood flow, vascular hypertrophy, cardiac hypertrophy?	Inhibition of cell proliferation possible role in cell differentiation and development
Structure	7-transmembrane-receptor G-protein coupling	7-transmembrane-receptor G-protein coupling?
Ligands	Losartan, valsartan, irbesartan, candesartan	PD 123177, PD 123319, CGP 42112
Isoforms	AT_{1a}, AT_{1b}	
Gene localization	AT_{1A}: chromosome 17 rat AT_{1b}: chromosome 2 rat AT_1: chromosome 3 human	X-chromosome

Unger T, et al. Angiotensin II receptors. J. Hypertens. 1996. 14(suppl 5) : S95-S103.

and the subsequent stimulation of the phospholipase C via G_q or inhibition of adenylyl cyclase via G_i, respectively. The action of phospholipase C results in the generation of 1,4,5-inositoltrisphosphate (IP_3) and diacyglycerol. IP_3 releases calcium from intracellular stores; diacylglycerol stimulates protein kinase C, leading to an influx of extracellular calcium via L-type calcium channels. Thus, both pathways contribute to an increase in intracellular calcium concentrations (Timmermans et al. 1993) .

AII via its AT_1 receptors has been shown not only to mediate vasoconstriction but also to promote cell growth of a variety of cells such as vascular smooth muscle cells, fibroblasts and cardiac myocytes (Geisterfer et al. 1988; Aceto and Baker 1990; Papuet et al. 1990) . Protein kinase C as well as elevated intracellular calcium levels can promote the expression of growth-related inducible transcription factors such as *c-fos, c-myc* and *c-jun* (Naftilan et al. 1989; Lyall et al. 1992) . The proteins encoded by these genes act as transcription factors for various target genes which may be involved in the stimulation of mitogenesis. Stimulation of AT_1 receptors also induces the transcription of platelet-derived growth factor-A chain and transforms growth factors-b1 and, via this mechanism, is directly coupled to the expression of growth factors (Naftilan et al. 1989; Stouffer and Owens 1992) . Marrero et al. (1995) recently reported that stimulation of AT_1 receptors in vascular smooth muscle cells induces a rapid phosphorylation of tyrosine in the intracellular kinase Jak2 and Tyk2 and that this phosphorylation is associated with an increased activity of Jak2. This pathway has been proposed as the signaling mechanism used by cytokines responsible for transcriptional activation of early growth response genes (Damell et al. 1994) . This pathway may thus play an additional role in AT_1-mediated growth.

B.5.2.2 . AT_2 receptor signaling

The AT_2 receptor has been cloned and it contains 363 amino acids of which 34% have sequence identity with AT_1. The AT_2 receptor also belongs to the family of seven transmembrane receptors. It is still controversial how the AT_2 receptor signals and whether or not it is coupled to G-proteins. Kambayashi et al. (1993) reported that AT_2 inhibits a phosphotyrosine phosphatase in COS-7 cells stably expressing the cloned receptor, an effect which was dependent on a pertussis toxin-sensitive, G-protein-coupled mechanism. Further evidence in support of AT_2 receptors coupling to G-proteins has been provided by Kang et al. (1994), who demonstrated that the G-protein Gi (but not G_0) is involved in AT_2 receptor-mediated modulation of potassium channels in rat primary cultures of neuronal origin. On the other hand, Mukoyama et al. (1993) reported that the rat AT_2 receptor shares a seven transmembrane topology which may belong to a unique class of receptors for which G-protein coupling has not yet been demonstrated. In their studies, the AT_2 receptor failed to increase IP_3 or intracellular calcium, and no apparent effects on cyclic AMP and cyclic GMP levels and phosphotyrosine phosphatase activity could be observed.

The relationship between AT_2 receptor-mediated signaling and tyrosine phosphorylation (Bottari et al. 1992; Kambayashi et al. 1993) suggests that this receptor plays a role in physiological processes involving cellular growth, differentiation and adhesion. It was futher supported by the fact that the AT_2 subtype is highly and transiently expressed in fetal tissues followed by a dramatic drop in most organs just after birth (Grady et al. 1991).

B.5.3. AII-mediated signal transduction in VSMC

AII-mediated signal transduction pathways in VSMC has been shown in Figure 9. Binding of AII to the G-protein couple receptor AT_1 in VSMC stimulates at least two independent pathways. Activation of phospholipase C (PLC) (Taubman et al. 1989) stimulates the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol trisphophate and 1.2.-diacylglycerol (DAG). DAG and Ca^{2+} activate protein kinase (PKC) (Tsuda et al. 1992) that has been shown to phosphorylate various proteins including inhibitory G-binding proteins Gi (Katada et al. 1985; Tsuda and Alaxander 1990) . The phosphorylation of Gi regulatory protein uncouples the inhibitory hormone receptor from adenylyl cyclase and thereby attenuates the hormone-mediated inhibition of adenylyl cyclase. In addition, DAG and Ca^{2+} activate protein kinase C, which also has been shown to feed into the MAP kinase cascade and activate the phosphorylation of ribosomal S6 protein by the S6 kinase ($p70^{S6K}$). Angiotensin II also activates the MAP kinase pathway which consists of Raf-1 kinase (RAF) or the MEK kinase (MEKK), and the MAP kinase phosphatase (Gautier et al. 1991) .

C. Antihypertensive Drugs

Drug treatment of hypertension is applied when the use of lifestyle modification can not lower the arterial pressure (Kaplan 1998). There are at least five types of antihypertensive drugs. They are diuretics, adrenergic-inhibiting drugs (α and β blockers), direct vasodilaters, calcium channel blockers, ACE inhibitors, AII receptor blockers. According to therapeutic approach, they are classified into first line and second line drugs. The first line ones include diuretic, adrenergic receptor-inhibiting agents (β blockers) and calcium channel blockers. In combination with nonmedical therapy, the first line therapy is initially used because of their lower price and less significant side effects. However, second line drugs are increasingly used due to their less frequent side effects in long-term treatment

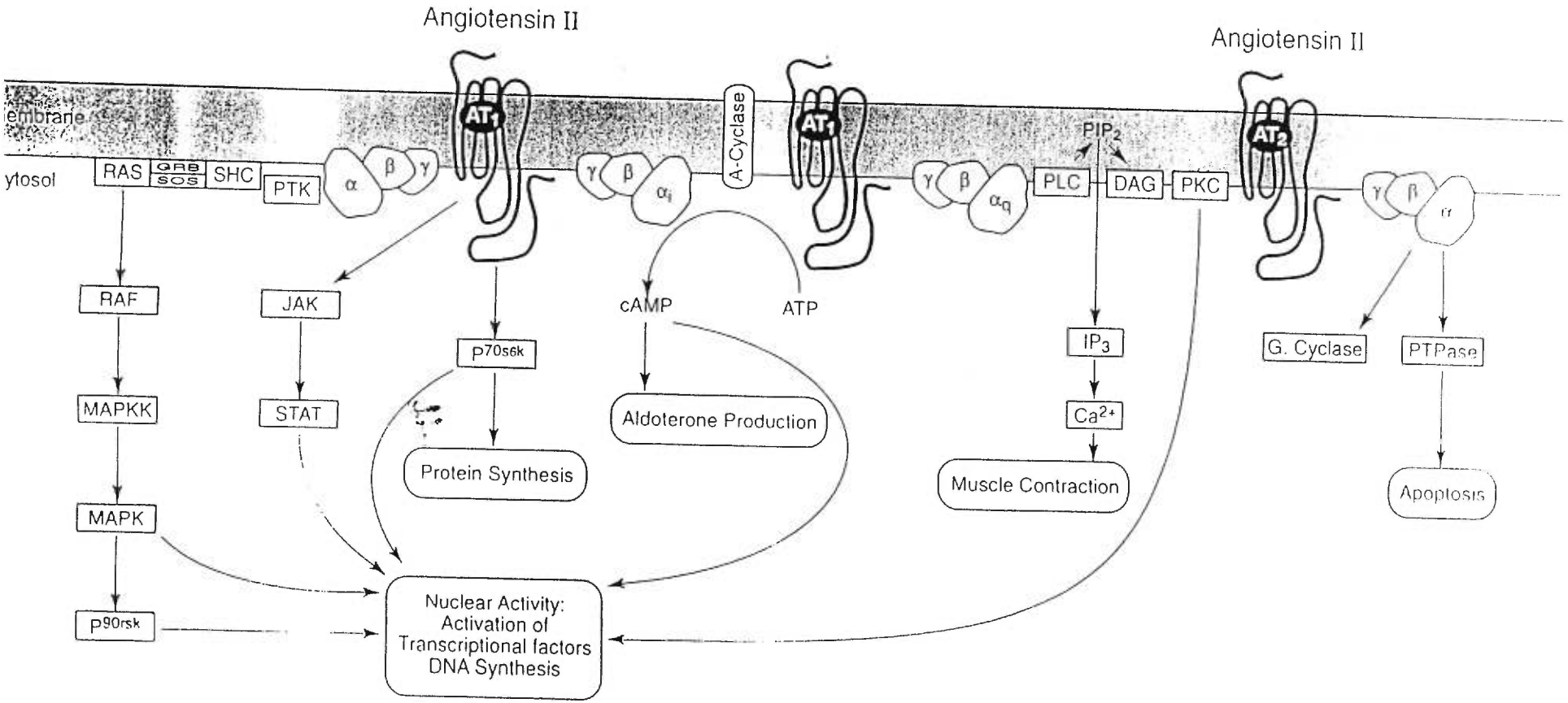

Figure 9 : AII-mediated signaling trasduction in VSMC.

Made by Dr. Anand-Srivastava, MB (1998)

of hypertension. ACE inhibitors to which many physicians prefer have their effect in large population of patients, good tolerability, and specific benefits in subsets of patients, such as the hypertensive patients with congestive heart failure. ACE inhibitors, reduce blood pressure primarily by decreasing total peripheral resistance (Burris JF, 1995).

C.1. ACE Inhibitor

The first peptide of ACE inhibitors were discovered from the venom of the Brazilian viper bothrops jararaca (Burris 1995). Their mechanisms include: inhibition of the conversion of angiotensin I to AII in circulation, potentiation of the vasodilator kalikrein-kinin system, alteration in prostanoid metabolism, suppression of aldosterone and thereby of sodium retention, a variety of sympatho-inhibitory effects, and possibly by inhibition of tissue renin-angiotensin systems (Bauer 1990).

Captopril is one of the ACE inhibitors, the most obvious action of captopril is to markedly reduce the circulating levels of AII, thereby removing the direct vasoconstriction induced by this peptide. At the same time, the activity of ACE within vessel walls and multiple tissues, including brain and heart, is inhibited by captopril.

C.2. AII Receptor Antagonists

AII receptor antagonists are agents, which displace AII from its specific AT_1 receptor and resulting in a dose-dependent fall in peripheral resistance and little change in heart rate or cardiac output. Losartan is the first of a new class of AII receptor antagonists to be introduced for the treatment of hypertension. It is an orally active, long-lasting, and well tolerated drug.

D. Experimental Hypertensive Models

Many hypertensive rat models have been used in the studies of hypertension. They include spontaneously hypertensive rats (SHR) (Okamoto and Aoki 1963), Dahl salt-sensitive hypertensive rats (Rapp 1987), Milan hypertensive rats (Ferrai et al. 1987) , Lyon hypertensive rats (Vincent and Sassard 1987), New Zealand hypertensive rats (Phelan and Simpson 1987) , deoxycorticosterone acetate (DOCA)-salt hypertensive rats (Grollman et al. 1940; de Champlain et al. 1969), one kidney one clip hypertensive rats (1K-1C) and two kidneys one clip hypertensive rats (2K-1C).

D.1. One Kidney One Clip (1K-1C) Hypertensive Rat Model

When one kidney is removed and a constrictor is placed on the renal artery of the remaining kidney, the immediate effect is a greatly reduced pressure in the renal artery beyond the constrictor. Then, within a few minutes the systemic arterial pressure begins to rise and continues to rise for several days. The pressure usually rises rapidly for the first hour or so and this is followed by a slower rise over a period of several days to a much higher pressure level. When the systemic arterial pressure reaches its new stable pressure level, the renal arterial pressure returns either all the way or almost all the way back to normal. The hypertension produced in this way is called "one-kidney" Gold-blatt hypertension in honor of Goldblatt, who first studied the important quantitative features of hypertension caused by renal artery constriction.

D.2. Mechanisms of 1K-1C Hypertension

The early rise in arterial pressure in Goldblatt hypertension is caused by the renin-angiotensin vasoconstrictor mechanism. Because of the poor blood flow through the kidney after acute reduction of renal arterial pressure, large quantities of renin are secreted by the kidney, and this causes angiotensin to be formed in the blood; the angiotensin in turn

raises the arterial pressure acutely. The secretion of renin rises to a peak in a few hours but returns all the way back to normal within 5 to 7 days because the renal arterial pressure by that time has also risen back to normal, so that the kidney is no longer ischemic.

The second rise in arterial pressure is caused by fluid retention; within 5 to 7 days the fluid volume has increased enough to raise the arterial pressure to its new sustained level. The quantitative value of this sustained pressure level is determined by the degree of constriction of the renal artery. That is , the aortic pressure must now rise to a much higher than normal level in order to make the renal arterial pressure distal to the constrictor rise high enough to cause normal urinary output.

Note especially that one-kidney Goldblatt hypertension has two phases. The first phase is vasoconstrictor type of hypertension caused by the angiotensin. The second stage is a volume-loading type of hypertension. However, it is often very difficult to tell that this second stage is true volume-loading hypertension because neither the blood volume nor the cardiac output is significantly elevated. Instead, the total peripheral resistance is increased. In the pure volume-loading hypertension blood volume and cardiac output are elevated only the first few days during the onset; volume-loading hypertension is a high resistance hypertension exactly as seen in the second stage of one-kidney Goldblatt hypertension.

D.3. Two Kidneys One Clip (2K-1C) Hypertensive Rats Model

Hypertension also often results when the artery to one kidney is constricted, while the artery to the other kidney is still normal. This hypertension results from the following mechanism: The constricted kidney retains salt and water because of decreased renal arterial pressure in this kidney. Also, the "normal" kidney retains salt and water because of renin produced by the ischemic kidney. This renin causes the formation of angiotensin, which circulates to the opposite kidney and results in the retention of salt and water. Thus, both

kidney, but for different reasons, become salt and water retainers. Consequently, hypertension develops.

III. Adenylyl Cyclase /c-AMP System in Hypertension

Alterations in adenylyl cyclase/cAMP system have been implicated in various diseases such as hypertension (Triner et al. 1972), heart failure (Feldman et al. 1988; Feldman et al. 1989), and diabetes (Lynch et al. 1989).

The elevation of blood pressure in essential hypertension is due to a general increase in the resistance of peripheral vessels. A part of this heightened peripheral resistance has been attributed to structural changes in the vessels, abnormalities in Ca^{2+} movements, and aberrations in cyclic nucleotide metabolism. It has been suggested that the adenylyl cyclase /c-AMP system is one the biochemical mechanism which participates in the regulation of arterial tone and reactivity (Triner et al. 1972). Decreased cAMP levels in cardiovascular tissues have been implicated in the pathogenesis of hypertension. Various studies have reported increased (Triner et al. 1972), decreased (Ramanathan and Shubata 1974) or unaltered (Amer 1973) adenylyl cyclase activity in SHR. A diminished stimulation of adenylyl cyclase by different agonistes like isoproterenol, adenosine, epinephrine, glugacon, dopamine, secretin and NaF has been reported in hearts (Bhalla and Ashley 1978; Limas and Limas 1978; Chatelain et al. 1979; Palmer and Greenberg 1979; Chiu 1981; Anand-Srivastava 1988) and aorta (Amer 1973; Bhalla and Ashley 1978; Anand-Srivastava 1988; Anand-Srivastava 1992) from SHR, from Goldblatt rats (Amer et al. 1974; Bhalla and Ashley 1978) and DOCA-salt hypertensive rats (Anand-Srivastava et al. 1993) compared to their control rats, respectively. Similar observations have also been shown in arteries of SHR (Amer et al. 1974; Triner et al. 1975), in the cortex and capillaries of cerebrum, (Palmer and Greenberg 1979) in hypothalamus of SHR (Schmidt and Thonberry 1978).

On the other hand, an augmented stimulation of adenylyl cyclase by isoproterenol, NECA, (Anand-Srivastava 1993) and prostaglandin E1 (PGE1) was observed in the platelets and spleen from SHR as compared to their control WKY (Hamet et al. 1978; Anand-Srivastava 1993). In addition, the inhibition of adenylyl cyclase activity by ANF, oxotremorine and AII were significantly augmented in heart sarcolemma and aorta from SHR and DOCA-salt hypertensive rats as compared to their WKY and control rats (Anand-Srivastava 1992; Anand-Srivastava 1993) . This altered responsiveness of adenylyl cyclase to hormones has been shown to be associated with altered receptor number or altered affinity (Limas and Limas 1978); changes in the levels of G-proteins; and a defective coupling and /or impaired catalytic subunit of adenylyl cyclase system (Anand-Srivastava 1988).

An enhanced expression of Giα-2 and Giα-3 at protein and mRNA levels and its relation with adenylyl cyclase regulation in heart and aorta from SHR and DOCA-salt hypertensive rats, has been recently reported, whereas the levels of Gsα were not altered in SHR but decreased in DOCA-salt hypertensive rats (Anand-Srivastava et al. 1991; Anand-Srivastava 1992; Thibault and Anand-Srivastava 1992; Anand-Srivastava et al. 1993). The enhanced expression of Giα-2 and Giα-3 precedes the development of blood pressure in SHR as well as in DOCA-salt hypertensive rats (Marcil et al. 1997; Marcil et al. 1998) . The role of various hormones in the regulation of G-protein levels has been reported (Parsons and Stiles 1987; Bageot et al. 1989; Langlois et al. 1990) . The levels of catecholamines and various vasoactive peptides such as atrial natriuretic peptides (ANP), vasopressin and AII have been shown to be increased in different models of hypertension, which may be responsible for the altered expression of G-protein in hypertensive rats (Schiffrin and St-louis 1987; de Champlain et al. 1989; Jennemaitre et al. 1992; Trinder et al. 1992) . Recent studies by Anand-Srivastava et al. (1997) indicated that exposure of VSMC with AII resulted in the augmentation of the levels of Giα-2 and Giα-3 proteins and mRNA, whereas the pretreatment of the cells with ANP_{4-23} resulted in the reduction of

levels of Giα-2 and Giα-3. These data indicate that AII and ANP modulate the expression of Gi-protein in a different manner (Anand-Srivastava et al. 1997). Taken together, it may be suggested that the enhanced levels of Giα protein observed in hypertension may be attributed to the augmented levels of AII and not to ANP (Anand-Srivastava et al. 1997).

IV. Objectives

1. To investigate whether the expression of G-protein and adenylyl cyclase activity is altered in 1K-1C hypertension, which is associated with increased levels of AII, and whether captopril an angiotensin-converting enzyme (ACE) inhibitor can reverse the changes in G-protein expression and adenylyl cyclase activity.

2. To examine the role of some of the AII-mediated signaling pathways such as PI-3-kinase, MAP kinase and $p70^{S6K}$ in AII-induced enhanced expression of Giα-2 and Giα-3 proteins in VSMC.

CHAPTER 2

Altered Expression of G-protein and Adenylyl Cyclase Activity in Hearts and Aorta from One Kidney One Clip Hypertentive Rats : Effect of Captopril

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ABSTRACT

We have previously shown augmented levels of Giα-2 and Giα-3 protein but not of Gsα in heart and aorta from spontaneously and experimentally induced hypertensive rats. We have recently demonstrated that angiotensin II treatment of vascular smooth muscle cells increased Giα-2 and Giα-3 protein levels. Since the angiotension II (AII) levels are augmented in one kidney one clip (1K-1C) hypertension, it may be possible that this will result in enhanced expression of G-protein in this model of hypertension. The present studies were therefore undertaken to investigate this possibility.

The expression of G-proteins was determined at protein and mRNA levels by immunoblotting and Northern blotting techniques with specific antibodies and cDNA probes respectively for different isoform of G-proteins. The levels of Giα-2 and Giα-3 proteins as well as mRNA were significantly increased in heart and aorta from 1K-1C hypertensive rats compared to control rats, whereas Gsα levels were unchanged. GTPγS, isoproterenol, glucagon, NaF and forskolin (FSK) stimulated the adenylyl cyclase activity in hearts and aorta from control and hypertensive rats to various degree, however, the stimulations were significantly decreased in hypertensive rats as compared to control rats. On the other hand, The inhibitory effect of low concentrations of GTPγS on FSK-stimulated adenylyl cyclase activity was significantly enhanced in hearts and aorta from 1K-1C hypertensive rats.. However, the inhibitory effect of C-ANF$_{4-23}$ on adenylyl cyclase was increased and of AII was decreased in hearts from 1K-1C hypertensive rats, whereas these were significantly decreased in aorta from 1K-1C hypertensive rats as compared to control rats. Captopril, an angiotensin-converting enzyme inhibitor restored the augmented levels of Gi proteins in hearts from hypertensive rats and also the diminished stimulation of adenylyl cyclase by GTPγS and stimulatory hormones. These data suggest that the

enhanced expression of Giα proteins and associated functions in 1K-1C hypertensive rats may be attributed to the enhanced levels of AII in this model of hypertension.

Key Words: Adenylyl cyclase; G-proteins; Heart; Aorta; One kidney one clip hypertensive rats (1K-1C); Angiotensin II (AII); Captopril.

INTRODUCTION

The adenylyl cyclase/c-AMP system is composed of three components, receptor, catalytic subunit and guanine nucleotide regulatory proteins (G-proteins). The stimulatory and inhibitory response of hormones on adenylyl cyclase are mediated via the stimulatory (Gs) and inhibitory (Gi) proteins respectively (Gilman 1984; Stryer and Bourne 1986) . G-proteins exist as a heterotrimer of α, β, γ subunits (Gilman 1987) . Molecular cloning revealed four different isoforms of Gsα originating from the differential splicing of one gene (Bray et al. 1986; Robishaw et al. 1988) , and three distinct isoforms of Giα; Giα–1, Giα–2 and Giα–3 encoded by three distinct genes (Itoh et al. 1986; Jones and Reed 1987) . On the other hand, five different isoforms of β and at least six distinct isoforms of γ have been determined, which could produce at least 30 different combinations. The Gβγ subunit has been shown to regulate various effector proteins such as adenylyl cyclase, phospholipase Cγ, phospholipase A2, phosphoinositide 3-kinase, and β-adrenergic receptor kinase (Neers 1995).

Alterations in the adenylyl cyclase/c-AMP system pathway have been widely documented in genetic SHR (Anand-Srivastava et al. 1991; Anand-Srivastava 1992; Thibault and Anand-Srivastava 1992; Anand-Srivastava 1993) and experimentally-induced hypertensive rat models (DOCA-salt) (Anand-Srivastava et al. 1993; Bohm et al. 1993) . An enhanced expression of Giα protein and Giα mRNA has been reported in SHR and DOCA-salt induced hypertension, (Anand-Srivastava 1992; Thibault and Anand-Srivastava 1992; Bohm et al. 1993) whereas Gsα levels remained unchanged in SHR (Anand-Srivastava 1992; Thibault and Anand-Srivastava 1992) or decreased in DOCA-salt hypertensive rats (Anand-Srivastava et al. 1991) . The elevated levels of Giα were shown to be associated with altered responsiveness of adenylyl cyclase to hormonal stimulation or inhibition (Anand-Srivastava et al. 1991; Anand-Srivastava 1992; Thibault and Anand-

Srivastava 1992; Anand-Srivastava et al. 1993). Vasoactive peptides and other hormones have been reported to modulate the expression of G-proteins (Parsons and Stiles 1987; Bageotet al. 1989; Langlois et al. 1990). Recently, Palaparti and Anand-Srivastava (1994) have shown that AII treatment of vascular smooth muscle cells resulted in an enhanced expression of Giα-2 and Giα-3 protein and mRNA. Since AII levels are increased in one kidney one clip (1K-1C) hypertensive rats, we have undertaken the present studies to investigate if G-protein expression and adenylyl cyclase activity are altered in this model of hypertensive rats and whether captopril an angiotensin-converting enzyme (ACE) inhibitor can reverse the changes in G-protein expression and adenylyl cyclase activities.

MATERIALS AND METHODS

Materials

ATP, cyclic AMP and isoprenaline (isoproterenol), oxotremorine, glucagon were purchased from Sigma (St.Louis, MO, U.S.A.). Creatine kinase (EC 2.7.3.2), myokinase (EC 2.7.4.3), guanosine triphosphate (GTP), guanosine 5'-[3'-thio] triphosphate (GTPγS) and adenosine deaminase (EC 3.5.4.4), were from Boehriger-Mannheim (Montreal, Que.,Canada). Atrial natriuretic factor (99-126-peptide) (ANF) was acquired from the peninsula laboratories Inc. (San Carlos. CA, USA). [α-^{32}P] NAD^+ from DuPont Canada (Mississauga, Ont., Canada). PT was from List Biochemicals (C-AMPbell, CA, U.S.A.), [α-^{32}P]ATP, [α-^{32}P]dCTP (3,000 Ci/mmol) and [γ-^{32}P]ATP (3,000 Ci/mmol) and the Western Blotting detection kit were from Amersham (Oakville, Ont, Canada). AS/7, EC/2, RM/1 antibodies were from Dupont (Mississauga, Ontario, Canada). Plasmids containing rat cDNAs encoding Giα-2, Giα-3 and Gsα were kindly obtained from Dr. Randall Reed from the Johns Hopkins University, and Dr. Hiroshi Itoh from the University of Tokyo. Chemicals necessary for total RNA extraction and Northern blot analysis were obtained from Sigma (St-Louis, MO, USA), except quanidinium thiocyanate which was from Research Organics Inc. (Cleveland, OH, USA), glyoxal from BDH Ltd (ST. Laurent, Que., Canada), and β-actin probe was kindly given by Dr. Angelino Calderone from University of Montreal.

Animal experiments

Animal experiments were performed following the recommendation of the Canadian Council for Animal Care and were approved by the Animal Care Committee of the Clinical Research Institute of Montreal. Sprague-Dawley rats were purchased from Charles River

(St. Constant, Quebec, Canada). They were housed under conditions of constant temperature (22 ^{0}C) and humidity (60%), exposed to a 12-h dark-light cycle, and fed normal Purina Rat chow (Ralston Purina International, Ontario, Canada) constaining 0.28% sodium and 1.08% potassium. When rats weighed 200g at age 5-6 weeks, they were anesthetized with pentobarbital (40mg/kg, Somnotol, MTS Pharmaceuticals, Cambridge, Ontario), and their renal hypertension was induced as previously described (Deng and Schiffrin 1991) by applying a silver clip with 0.2-mm lumen to the right renal artery. To induce 1K-1C hypertension a left nephrectomy was also performed. Control normotensive rats were prepared only for 1K-1C rats by performing a unilateral nephrectomy. After the animals were warmed with a heating pad at 37 ^{0}C and under slight restrain in a Plexiglas cage, systolic blood pressure was taken weekly by the tail-cuff method. All 1K-1C rats develop hypertension within 2 weeks (systolic blood pressure > 150 mmHg), one group of 1K-1C hepertensive rats were also treated with captopril. Captopril was given in drinking water at a dose level of 1g·litre^{-1} (equivalent to about 150 mg·kg^{-1} body weight daily). After 2 weeks of treatment, rats were killed by decapitation after the last blood pressure measurement.

Preparation of heart-washed particles

Heart washed particles were prepared as described previously (Marcil et al. 1997). Frozen hearts were quickly pulverized to a fine powder using a mortar and pestle cooled in liquid nitrogen, and were homogenezed in a Teflon/glass homogenizer containing 10 mM Tris-HCl and 1mM EDTA (pH 7.5). The homogenate was centrifuged at 1000 g for 10 min. The supernatant fraction was discarded and the pellet was finally suspended in 10 mM Tris-HCl and 1 mM EDTA (pH 7.5) and used for adenylyl cyclase activity and immunoblotting studies.

Preparation of aorta washed particle

Aorta washed particles were prepared as described previously (Anand-Srivastava 1992) . The dissected aorta were quickly frozen in liquid nitrogen and stored at -80^{0}C until assayed. The frozen aortae were pulverized to a fine powder in a mortar cooled in liquid nitrogen. The aorta powder was homogenized in a Teflon/glass homogenizer in a buffer containing 10 mM Tris-HCl and 1 mM EDTA, pH 7.5. The homogenate was centrifuged at 16,000g for 15 minutes at 4^{0}C. The supernatant was discarded and the pellet was suspended in Tris-EDTA buffer, pH 7.5 and used for determination of adenylyl cyclase activity and immunoblotting studies.

Adenylyl cyclase activity determination

Adenylyl cyclase activity was determined by measuring [^{32}P] c-AMP formation from [α^{32}-P]ATP, as described previously (Anand-Srivastava, 1988, 1992). Typical assay medium contained 50 mM glycylglycine pH 7.5, 0.5 mM MgATP, 5 mM $MgCl_2$, 0.5 mM c-AMP, 5U/mL ADA (or otherwise as indicated), 100 mM NaCl, 1 mM 3-isobutyl-1-methylxanthine (or otherwise as indicated), 0.1 mM EGTA, 10 mM GTP (or otherwise as indicated), [α-^{32}P]ATP (1-1.5x10^6 CPM) and an ATP-regenerating system consisting of 2 mM creatine phosphate, 0.1 mg/ml creatine kinase and 0.1 mg/ml myokinase in a final volume of 200ml. Incubations were initiated by the addition of reaction mixture to the membranes which had been thermally equilibrated for 2 minutes at 37°C. The reactions conducted in triplicate for 10 minutes at 37°C were terminated by the addition of 0.6 ml of 120 mM zinc acetate containing 0.5 mM unlabelled c-AMP. c-AMP was purified by coprecipitation of other nucleotide with $ZnCO_3$ and subsequent chromatograghy by the double column system as described by Salomon et al. (1974). The unlabelled c-AMP served to monitor the recovery of the [^{32}P] c-AMP by measuring absorbance at 259 nm. Under the assay conditions used, adenylyl cyclase activity was linear with respect to protein concentration and time of incubation. Protein concentration was determined by Lowry et al. (1951) with crystalline bovine serum albumin (BSA) as standard.

Immunoblotting

After SDS-PAGE, the seperated proteins were electrophoretically transferred to nitrocellulose paper (Schleicher & Schuell) using a semidry transblot apparatus (Bio-Rad) at 15 Volt for 45 minutes. After transfer, the membranes were washed twice in phosphate-buffered saline (PBS) and were incubated in PBS containing 3% dehydrated milk at room temperature for 2 hours. The blots were then incubated with antisera against G-proteins in PBS containing 1.5% dehydrated milk and 0.1% Tween 20 at room temperarure overnight. The antigen-antibody complexes were detected by incubating the blots with goat anti-rabbit IgG (Bio-Rad) conjugated with horseadish peroxidase for 3 hours at room temperature. The blots were washed three times with PBS before reacting with enhanced-chemiluminescence (ECL) Western blotting detection reagents from Amersham.

The autoradiograms were quantified by densitometric scanning using an enhanced laser densitometer (LKB Ultroscan XL, Pharmacia, Quebec, Canada) and gel scan XL evolution software (version 2.1, Pharmacia). The scanning was one dimensional and scanned the entire area of protein bands in autoradiogram.

PT-catalysed ADP-ribosylation

ADP-ribosylation of heart membranes by PT was performed as described previously (Anand-Srivastava et al.1987) . The heart membranes from control and 1K-1C hypertensive rats were incubated in 25 mM-glycylglycine buffer, pH 7.5, containing 15 mM-[α-^{32}P] NAD+ (20 mCi /ml), 0.4 mM-ATP, 0.4 mM-GTP, 15mM-thymidine, 10 mM-dithiothreitol and ovalbumin (0.1 mg/ml) with or without PT (5 mg/ml) for 30 min at 37 ^{o}C in a total volume of 100 ml. The reaction was terminated by addition of 20 ml of "stop" mixture containing 5% SDS and 50% β- mercaptoethanol. The contents were heated for 10 min in a boiling-water bath. The labelled proteins were analysed by subjecting the samples to SDS/PAGE by the method of Laemmli (1970) with 12% polyacrylamide gels.

After electrophoresis, the gels were fixed, stained, destained, dried and autoradiographed by exposure to Kodak XAR-5 film, as described previously (Anand-Srivastava et al. 1987) They were calibrated by using molecular-mass standards (Pharmacia): phosphorylase *b* (94000 Da) , albumin (67000 Da), ovalbumin (43000 Da), carbonic anhydrase (30000 Da), Trypsin inhibitor (20000 Da) and β-lactalbumin (14400 Da).

Total RNA extraction

Total RNA was isolated as described earlier (Thibaut and Anand-Srivastava, 1992) by the method of Chomczynski et al. (1987). Briefly, frozen heart ventricles were homogenized in a denaturing solution (solution D) containing 4M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarkosyl and 0.1 M 2-mercaptoethanol. The homogenates were extracted once with 1 vol. of phenol and 0.2 vol. of chloroform-isoamylalcohol (49:1) in the presence of 0.2 M sodium acetate, pH 4.0, and once with 1 vol. of chloroform-isoamylalcohol (49:1). Total RNA was then precipitated with isopropanol. Following a second precipitation in solution D and isopropanol (v:v), total RNA was washed in 70% ethanol and the pellets were resuspended in diethyl-pyrocarbonate (DEPC) treated H_2O, and the optical density was determined at 260 and 280 nm. Typically a ratio of 1.6 to 1.8 was obtained.

Radio-labeling of the probes

cDNA inserts encoding for Giα-2, Giα-3 and Gsα were radiolabeled with [α-^{32}P]dCTP by random priming essentially as described by Feinberg et al (1983). Specific activities of the labeled probes ranged from 1 to 3 $\cdot 10^8$ cpm/μg of DNA. The β-actin was radiolabeled and used to rehybridize the membranes to check the amounts of loaded RNA.

Northern analysis

Total RNA samples (20μg) were ethanol precipitated and respended in 20 μl denaturing solution containing 1x running buffer (20 mM 3-[N-morpholino] propane-silfonic acid (MOPS) (pH 7.0), 6 mM sodium acetate, 1mM EDTA), 6% formaldehyde, and 50% formamide and heated for 15 min at 65°C before loading. RNA samples were run on 1.3% agarose gel containing 1x running buffer for 4-5 h. The samples were transferred from the gel to a nylon membrane (Genescreen Plus; Dupont-NeN) by vacuum blotting (Model 785; Bio-Rad laboratories; Hercule, calif.) with 10x SSC (Salt Sodium Citrate) containing 1.5 M NaCl, 0.15 M sodium citrate. After transfer, the filters were dried by baking at 80°C for 2h.

The membranes, after prehybridization at 60°C in hybridization oven for 2 h in 400mM sodium phosphate buffer (pH 7.2) containing 5% SDS, 1 mM EDTA, 0.1 % bovin serum albumin, and 50% formamide were hybridized overnight in hybridization solution containing dextran sulphate (10% w/v) and the cDNA probe at $1\text{-}3\cdot10^6$ cpm/ml as described by Singh et al. (1984). Filters were then washed twice (15 minutes, room temperature) with 300 mM NaCl/30 mM trisodium citrate (pH 7.0) and 0.1% SDS and twice (15 minutes, 60^0C) with 30 mM Nacl/3mM trisodium citrate (pH 7.0) and 0.1% SDS. Autoradiography was performed with X-ray films at -70°C. In order to assess the possibility of any variations in the amounts of total RNA in individual samples applied to the gel, each filter was re-hybridized with β-actin. The blots which had been probed with the G-protein cDNA were de-hybridized by washing for 15 minutes at 65°C in 0.1% SDS and 1mM EDTA and rinsed in DEPC-treated H_2O and re-hybridized overnight at room temperature with the β-actin. Quantitive analysis of the hybridization of probes bound was performed by densitometric scanning of the autoradiographs employing the enhanced laser densitometer, LKB Ul-troscan XL and quantified using the gel scan XL evaluation software (version 2.1) from Pharmacia (Que., Canada).

Analysis of data

Data are presented as mean ± S.E.M. Comparison between groups were made using either Student's t-test or Analysis of Variance (ANOVA) where appropriate. The results were considered significantly different if $p<0.05$.

RESULTS

The physiological parameters of rats are shown in Table-1. The arterial blood pressure in 1K-1C hypertensive rats was significantly increased as compared to control rats (210.5±17.3 as compared to 116.5±6.5), whareas in 1K-1C treated with captopril rats (1K-1C+CAP), the arterial blood pressure was decreased as compared to 1K-1C hypertensive rats (180.7±16.6 as compared to 210.5±17.3). The heart/body ratio was significantly increased in 1K-1C hypertensive rats as compared to control rats (5.93±0.7 as compared to 3.89±0.3), whereas in 1K-1C +CAP rats, the ratio was unaltered as compared to 1K-1C hypertensive rats, but significantly increased as compared to control rats, suggesting that the 1K-1C hypertensive rats exhibit cardiac hypertrophy, which was not altered by captopril treatment.

Effects of GTPγS on adenylyl cyclase activity

We have previously shown that the ability of guanine nucleotides such as guanosine 5'-0-(3-thiotriphosphate) (GTPγS) to stimulate adenylyl cyclase was altered in both SHR and experimental models of hypertensive rats (DOCA-salt rats) which was associated with altered levels of Gi-protein (Anand-Srivastava et al. 1991; Anand-Srivastava, 1992; Anand-Srivastava et al. 1993) . Since 1K-1C hypertension is associated with an increased levels of AII, it was of interest to investigate if the G-proteins are impaired in 1K-1C hypertensive rats and the impairment is due to the enhanced levels of AII. For this, we determined the ability of GTPγS to stimulate adenylyl cyclase activity in hearts and aorta from 1K-1C hypertensive rats, their sham-operated control (CTL), and 1K-1C rats treated with captopril (1K-1C+CAP). The results depicted in Figure 1A, demonstrate that GTPγS stimulated adenylyl cyclase activity in a concentration-dependent manner in all three groups. However, the extent of stimulation was significantly decreased in hearts from 1K-1C hypertensive rats as compared to control rats, whereas in captopril treated hypertensive

rats, the stimulation was partially restored towards control levels. At 10^{-5}M, GTPγS augmented the adenylyl cyclase activity by about 10-fold in CTL rats, and about 1.8-fold in 1K-1C hypertensive rats. However, in captopril treated 1K-1C hypertensive rats, GTPγS augmented the enzyme activity by about 3-fold. Similar results were also observed in aorta, as shown in Figure 1B, however, the restoration of decreased stimulation of adenylyl cyclase by GTPγS was greater as compared to heart. These data indicate that like other models of hypertensive rats, G-proteins may also be impaired in hearts and aorta from 1K-1C hypertensive rats, which may partly be due to increased levels of AII.

G-protein levels

To further investigate if the decreased stimulation of adenylyl cyclase activity by GTPγS in 1K-1C was due to an increased levels of Gi or decreased levels of Gs or both, we determined the expression of G-proteins in hearts from hypertensive rats at the level of ADP ribosylation, protein and mRNA by PT-catalyzed ADP-ribosylation, immunoblotting using specific antibodies AS/7 against Giα-1 and Giα-2, EC/2 against Giα-3, RM/1 against Gsα and Northern blotting using specific cDNA probes encoding Giα-2, Giα-3 and Gsα respectively.

As shown in Figure 2A, PT in the presence of [α-^{32}P] NAD catalyzed the ADP-ribosylation of a single protein band of Gi 40/41 kD in hearts from both control and hypertensive rats, however, the labeling of this band was significantly increased by 40.5 ± 1.2% (n=3) in 1K-1C rats as compared to control rats. These results suggest that hearts from 1K-1C hypertensive rats exhibit enhanced expression of Gi/Go proteins. These studies were further extended to examine the expression of G-protein at protein and mRNA levels.

The results shown in Figure 2B, indicate that AS/7 antibody recognized a single protein of 40 kDa referred as Giα-2 (Giα-1 is absent in heart, Jones and Reed, 1987) however, the relative amounts of immunodetectable Giα-2 were significantly increased in

hypertensive rats by 68,9±10.1%, (n=3) as compared to control rats as determined by densitometric scanning (Figure 3). Captopril treatment of hypertensive rats restored the enhanced levels of Giα-2 protein towards control level by 90.1±9.9%, (n=3), as determined by densitometric scanning (Figure 3A). Similarly, EC/2 antibody recognized a single protein of 41 kDa referred to as Giα-3 in hearts, however, the relative amount of immunodetectable Giα-3 as determined by densitometric scanning (Figure 3B), was also significantly increased in 1K-1C hypertensive rats by 101.9±17.7%, (n=3), as compared to their CTL rats, and in 1K-1C+CAP rats, the levels of Giα-3 were restored towards control level by 97.7±15.6%, (n=3).

Figure 2B shows that RM/1 antibody recognized three Gsα isoforms, $Gs\alpha_{45}$, $Gs\alpha_{47}$, and $Gs\alpha_{52}$, in hearts from CTL rats, 1K-1C rats and 1K-1C+CAP rats. However, no significant alterations in the relative amount of immunodetectable $Gs\alpha_{45}$, $Gs\alpha_{47}$, and $Gs\alpha_{52}$ in hearts from three groups as determined by densitomeric scanning were observed. The Gsα protein levels were (in arbitrary units): $Gs\alpha_{45}$, CTL: 0.244±0.03; 1K-1C: 0.262±0.04; 1K-1C+CAP: 0.257±0.03; $Gs\alpha_{47}$, CTL: 0.192±0.023; 1K-1C: 0.189±0.012; 1K-1C+CAP: 0.187±0.08; $Gs\alpha_{52}$, CTL: 0.631±0.05; 1K-1C: 0.674±0.13; 1K-1C+CAP: 0.621±0.04; (n=3).

The mRNA levels of G-protein were also determined by Northern blot analysis, using specific cDNA probes encoding for Gsα, Giα-2 and Giα-3 to examine if they also change concomitantly with corespondent protein levels. As shown in Figure 2C, the Giα-2 probe detected a message of 2.3 kilobase in hearts from CTL, 1K-1C rats and 1K-1C+CAP rats, however, the amount of Giα-2 mRNA as determined by densitometric scanning (Figure 3) was significantly increased by about 77.6±9.5%, (n=3) as compared to CTL group, and was restored towards control level by 82.6±14% (n=3) by captopril treatment. In addition, the Giα-3 probe detected a message of 3.5 kilobase in hearts from CTL rats, 1K-1C rats and 1K-1C+CAP rats (Figure 2C), however, the levels of Giα-3 mRNA were significantly

increased in 1K-1C hypertensive rats by 118.5±13.1% (n=3) as compared to control rats, and were restored towards control levels by 87.2±7.8% (n=3) by captopril treatment (Figure 3). The alterations in Giα-2 and Giα-3 mRNA levels in heart from 1K-1C rats may not be attributed to the variations in the amounts of total RNA in individual samples applied to the gels, because of the fact that the hybridization with the β-actin showed a similar amount of β-actin loaded from all three groups on the gels. In addition, the Gsα cDNA probe detected a message of 1.8 kilobase in hearts from CTL rats, 1K-1C hypertensive rats and 1K-1C treated with captopril rats (Figure 2C), however, densitometric analysis revealed that the amount of Gsα mRNA was not different between the three groups.

We have also determined the G-protein levels in aorta from control and hypertensive rats by immunoblotting using specific antibodies against Giα-2, Giα-3 and Gsα. Similar results were observed in aorta (Figure 2D). The levels of Giα-2 and Giα-3 proteins were significantly increased in 1K-1C hypertensive rats as compared to control rats by about 63.5±7.7% (n=3) and 62.6±10.4% (n=3) respectively, however, no difference in Gsα protein levels between CTL and 1K-1C was observed.

Gi-protein functions

G-proteins couple the hormone receptors to adenylyl cyclase and mediate the stimulatory and inhibitory responses of hormones on adenylyl cyclase (Gilman 1984; Stryer and Bourne 1986) . Since the levels of Giα-2 and Giα-3 were increased in hearts and aorta from 1K-1C hypertensive rats, it was of interest to investigate if the altered expression of Giα is reflected in Gi-functions. For this reason, the receptor-independent and -dependent functions of Gi-proteins were examined by studying the effect of low concentrations of GTPγS on FSK-stimulated adenylyl cyclase activity as well as by studying the hormonal inhibition of adenylyl cyclase activity.

As shown in Figure 4, the GTPγS inhibited FSK-stimulated adenylyl cyclase activity in control and hypertensive rats, however the inhibition was significantly enhanced in both heart and aorta from hypertensive rats as compared to control rats. For example, GTPγS, at 10^{-9} M inhibited forskolin-stimulated enzyme activity by about 18.2±2.1% in heart from control rats and about 35±2.4% in hearts from 1K-1C hypertensive rats. In addition, in aorta, GTPγS at 10^{-9} M, inhibited FSK-stimulated enzyme activity by about 11.5±1.3% in control rats and about 34.7±2.4% in 1K-1C hypertensive rats (Figure 4B).

Figure 5A shows the effect of inhibitory hormones on adenylyl cyclase activity in hearts from control, 1K-1C hypertension and captopril treated hypertensive rats. C-ANF_{4-23}, and AII inhibited adenylyl cyclase activity in hearts from both control and hypertensive rats, however, the extent of inhibition by C-ANF_{4-23} was significantly increased and by AII was significantly decreased in hearts from 1K-1C hypertensive rats (Figure 5A). On the other hand, in aorta, C-ANF_{4-23} and AII inhibited adenylyl cyclase activity in 1K-1C and CTL rats to various degrees, however, the extent of inhibition was significantly decreased in 1K-1C hypertensive rats as compared to control rats (Figure 5B).

Hormonal stimulation of adenylyl cyclase

Since the levels of Gsα were not altered and the levels of Giα-2 and Giα-3 were increased in hearts and aorta from 1K-1C hypertensive rats, it was of interest to investigate if the increased expression of Giα-2 and Giα-3 proteins can modulate the Gs-mediated functions. Therefore the effects of isoproterenol and glucagon on adenylyl cyclase activity in hearts and aorta from control and 1K-1C hypertensive rats were examined. As shown in Figure 6A and Figure 6C, isoproterenol (50μM) and glucagon (1μM) stimulated adenylyl cyclase activity in hearts and aorta from control and hypertensive rats to various degrees, however, the extent of stimulation by isoproterenol and glucagon was significantly decreased in hypertensive rats as compared to control rats by 24.2±2.1% (n=3) and

26.4±3.1% (n=3), respectively in hearts and by 22.3±3.3% (n=3) and 24.2±1.7% (n=3) respectively in aorta. Captopril treatment restored partially the decreased stimulation of adenylyl cyclase by 53.1±6.3% (n=6) and 26.3±4.3% (n=6) by isoproterenol and glucagon respectively.

Effect of FSK on adenylyl cyclase activity

FSK interacts directly with catalytic subunit of adenylyl cyclase and stimualtes the enzyme acticity. To investigate if catalytic subunit of adenylyl cycalse is impaired in 1K-1C hypertensive rats, we examined the effects of FSK on adenylyl cyclase activity in hearts and aorta from control, 1K-1C hypertensive and captopril-treated hypertensive rats. As shown in Figure 6B, FSK (50μM) stimulated adenylyl cycalse activity in all three groups, however, the stimulatory effect of FSK on adenylyl cycalse activity was significantly decreased in hearts and aorta from 1K-1C hypertensive rats by 31.8±2.3% (n=4) and 30.3±3.4% (n=4) respectively as compared to control rats. The decreased stimulation of adenylyl cyclase by FSK in hypertensive rats was restored towards control level by 78.9±12.7% (n=4) by captopril treatment. In addition, the effect of sodium fluoride (NaF) on adenyly cyclase activity was also examined in control, 1K-1C and 1K-1C hypertensive rats treated with captopril. As shown in Figure 6, NaF (10mM) stimulated adenylyl cyclase activity in all three groups, however, the extent of stimulation was significantly decreased in hearts (Figure 6A) and aorta (Figure 6B) from 1K-1C hypertensive rats by 33.9±4.9% (n=4) and 17.0±2.8% (n=4) respectively as compared to control rats, and was restored towards control level by 50.1±6.4% (n=4) in hearts by captopril treatment.

DISCUSSION

We and others (Anand-Srivastava, 1988; Anand-Srivastava, 1992; Bohm et al., 1994), have previously reported that a decreased sensitivity of adenylyl cyclase to hormonal stimulation in hearts and aorta from several models of hypertension was associated with the enhanced expression of Gi proteins. Several hormones and vasoactive peptides such as ANP and AII have been reported to modulate the expression of Gi-proteins (Parsons and Stiles, 1987; Bageot et al., 1989; Langlois et al., 1990; Anand-Srivastava et al., 1997) . We have recently reported that AII treatment of vascular smooth muscle cells resulted in an enhanced expression of Gi proteins at protein and mRNA levels (Palaparti and Anand-Srivastava, 1994; Anand-Srivastava et al., 1997) .

In the present studies, we demonstrate that 1K-1C hypertensive rats that have high levels of AII exhibit enhanced expression of Giα-2 and Giα-3 proteins and mRNA in hearts and aorta, whereas the levels of Gsα were unaltered. The enhanced expression of Giα-2 and Giα-3 was restored significantly towards control levels by captopril treatment, suggesting the contribution of AII in exhibiting augmented levels of Giα proteins in 1K-1C hypertensive rats. A significant attenuation of the responsiveness of adenylyl cyclase activity to GTPγS stimulation in hearts and aorta from 1K-1C rats as compared to control rats may not be due to Gsα protein, because the levels of Gsα were not altered, and may be contributed to the increased levels of Giα. This was further supported by the fact that captopril treatment that restored the enhanced levels of Giα towards control levels was also able to restore partially the decreased stimulation of adenylyl cyclase by GTPγS in hearts and almost completely in aorta.

The increased expression of Giα proteins in hearts and aorta from SHR, DOCA-salt and 1K-1C hypertensive rats Anand-Srivastava 1992; Anand-Srivastava, 1993; Ge and

Anand-Srivastava 1997), and the restoration of enhanced levels of Giα proteins by losartan and captopril in hearts from SHR has been shown (Pandey and Anand-Srivastava 1996) .

The decreased responsiveness of adenylyl cyclase to isoproterenol and glucagon in hearts and aorta from 1K-1C hypertensive rats is in agreement with the studies reported earlier in SHR and other models of hypertensive rats (Anand-Srivastava et al. 1983; Anand-Srivastava 1988; Anand-Srivastava 1992; Anand-Srivastava et al. 1993) , and may be attributed to the down regulation of hormone receptors (Limas and Limas 1978) , defective coupling and/or impaired catalytic subunit of adenylyl cyclase system (Anand-Srivastava 1988) . The down regulation of β-adrenergic receptors in hypertension as one of the mechanisms responsible for the diminished sensitivity of adenylyl cyclase to β-adrenergic agonists has been demonstrated in various cardiovascular diseases including hypertension (Limas and Limas 1984; Bohm et al. 1994) . Since no alterations in the levels of Gsα proteins were observed in 1K-1C hypertensive rats, the decreased stimulation of adenylyl cyclase by isoproterenol and glucagon can not be explained by Gsα. This is further substantiated by the results that captopril treatment restored the attenuated stimulation of adenylyl cyclase by isoproterenol and glucagon towards control levels in the absence of a change in Gsα protein levels. Thus, captopril-mediated modulation of hormonal responses may occur at the level of receptor, Giα protein or catalytic subunit or at all of the levels. Since Giα-2 and Giα-3 levels were significantly increased in hypertensive rats as compared to control rats, the decreased responsiveness of adenylyl cyclase to isoproterenol and glugacon in hypertensive rats can be attributed to increased levels of Giα proteins. This notion is supported by the partial correction of the responsiveness of adenylyl cyclase to isoproterenol and glucagon with restoration of enhanced levels of Giα-2 and Giα-3 by captopril treatment

Increased inhibition of FSK-stimulated adenylyl cyclase activity by low concentrations of GTPγS (an index of Gi protein function) in hearts and aorta from hypertensive rats as

compared to control rats, suggests that Gi functions are also increased in hypertensive rats that may be due to the enhanced levels of Giα-2 and Giα-3 proteins in hypertensive rats. The enhanced functions of Giα protein were also reflected in an enhanced inhibition of adenylyl cyclase by C-ANF$_{4-23}$ in hearts as well as by the results on captopril treatment that restored the levels of Giα protein also restored the enhanced inhibition towards control levels. On the other hand, AII-mediated inhibition of adenylyl cyclase that was attenuated in hypertensive rats as compared to control rats may be explained by the downregulation of AII-receptors due to increased levels of AII in 1K-1C hypertensive rats (Amiri and Garcia 1997) . This notion is further supported by the fact that captopril treatment that reduces the levels of AII was also able to restore the AII-mediated inhibition towards control level. However, C-ANF$_{4-23}$ and AII-mediated inhibition of adenylyl cyclase that were attenuated significantly in aorta from hypertensive rats, may be attributed to the downregulation of ANP-C and AII receptors (Gutkind et al. 1987; Marcil and Anand-Srivastava 1995) . The down regulation of ANP-C and AII receptors in various tissues from various models of hypertension has been demonstrated (Swithers et al. 1987; Gauquelin et al. 1991).

Our results on the decreased stimulation of adenylyl cyclase by FSK and NaF in 1K-1C hypertensive rats are in agreement with earlier studies on SHR and DOCA-salt hypertensive rats (Anand-Srivastava 1988; Anand-Srivastava et al. 1993) , and may be due to the defective catalytic subunit per se or the overexpression of Giα or both. The involvement of Gi in FSK-mediated stimulation has been also shown (Anand-Srivastava et al. 1987). In addition, the requirement of Gsα and guanine nucleotide for FSK-activation of adenylyl cyclase has been reported (Hilderbrandt et al. 1982). Since the present studies do not demonstrate any attenuation in Gsα, the diminished stimulation of adenylyl cyclase by FSK in 1K-1C hypertensive rats can not be attributed to the impaired Gsα levels. Thus, the overexpression of Gi proteins in 1K-1C hypertensive rats may contribute to the diminished responsiveness of adenylyl cyclase to FSK-stimulation. This is further

substantiated by the fact captopril treatment that restored the enhanced levels of Giα proteins towards control level also restored the sensitivity of FSK to stimulate adenylyl cyclase towards control levels.

In conclusion, we have demonstrated that 1K-1C hypertensive rats exhibit enhanced expression of Giα-2 and Giα-3 proteins which are reflected in enhanced functions. The stimulatory effects of hormones on adenylyl cyclase were diminished in hearts and aorta from 1K-1C hypertensive rats. Captopril treatment of hypertensive rats restored the enhanced expression of Gi proteins and diminished sensitivity of adenylyl cyclase to hormonal stimulation towards control levels. It may be suggested that AII whose levels are increased in 1K-1C hypertensive rats may be responsible for the enhanced levels of Gi proteins, which may be one of the factors responsible for the pathogenesis of hypertension.

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Abbreviations: Gs, stimulatory guanine nucleotide regulatory protein; Gi, inhibitory guanine nucleotide regulatory protein; Giα-1, Giα-2, Giα-3, isoforms of inhibitory guanine nucleotide regulatory protein encode by these distinct genes; SHR, spontaneously hypertensive rats; mRNA, messenger ribonucleic; 1K-1C, one kidney one clip hypertensive rats; AII, angiotensin II; C-ANF$_{4-23}$, ring-deleted analogue of atrial natriuritic factor; PT, pertussis toxin; GTPγS, guanosine 5' -[γ-thio] triphosphate.

Table 1. Body weight, heart weight, body weight to heart weight ratio and blood pressure of CTL, 1K-1C and 1K-1C+CAP

Group	Body weight (g)	Heart weight (mg)	Heart weight/ body weight (mg/g)	Systolic blood pressure (mmHg)
CTL	333.4 ± 14.8	1296.4 ± 76.1	3.89 ± 0.3	116.5 ± 6.5
1K-1C	337.1 ± 19.4	2000.2 ± 130.1	5.93 ± 0.7 ***	210.5 ± 17.3 ***
1K-1C+CAP	339.7 ± 16.3	2030.3 ± 138.4	5.97 ± 0.6	180.7 ± 16.6 ††

CTL = control rat; 1K-1C = one kidney one clip hypertensive rat; 1K-1C+CAP = one kidney one clip hypertensive rat treated with captopril. Values are mean ± S. E. M. from 10 different rats from control and 1K-1C groups and 6 different rats from 1K-1C+CAP group. *** $P < 0.001$ (compared with CTL); †† $P < 0.01$ (compared with 1K-1C).

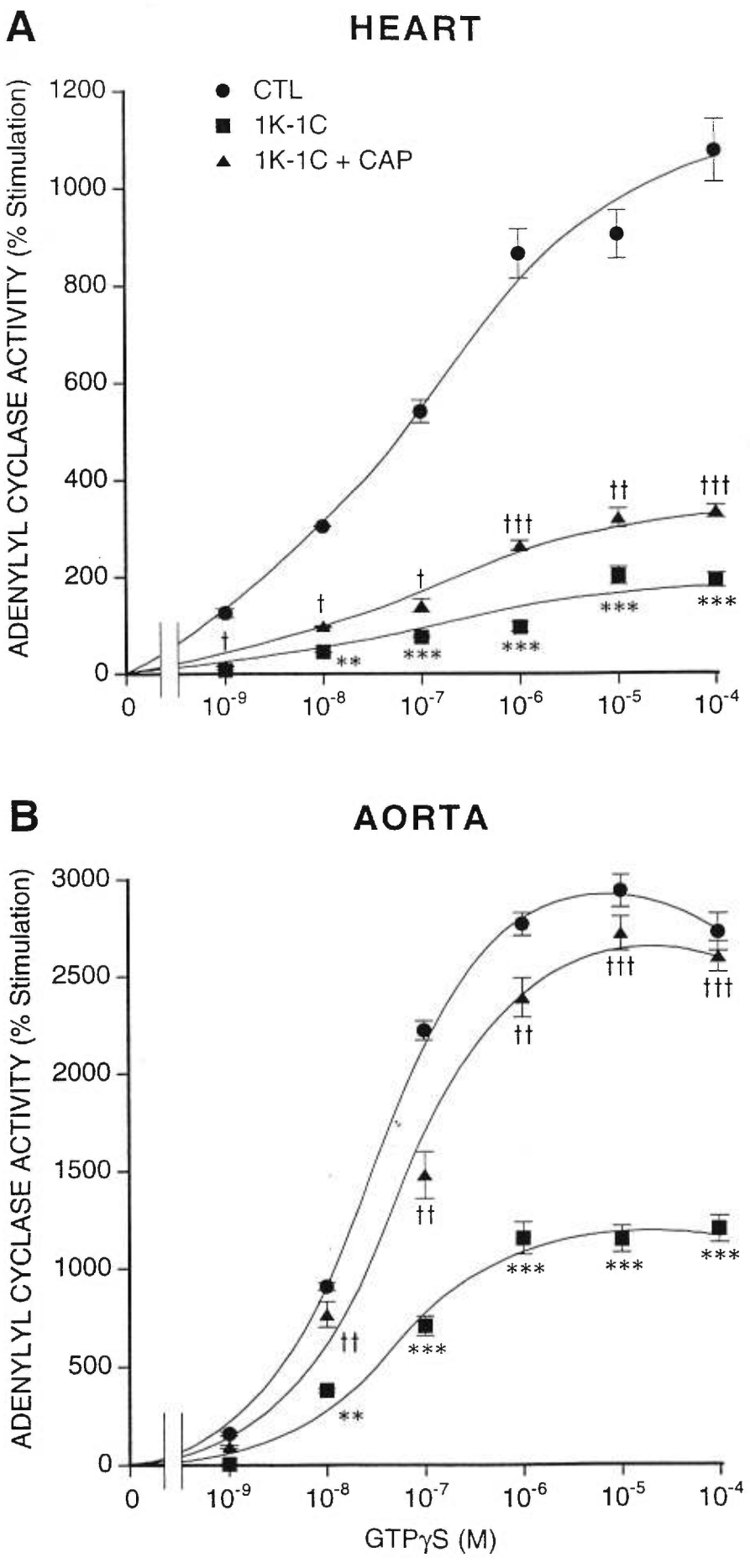

A
HEART
CTL
1K-1C
1K-1C + CAP
ADENYLYL CYCLASE ACTIVITY (% Stimulation)
1200
1000
800
600
400
200
0
0
10-9
10-8
10-7
10-6
10-5
10-4
B
AORTA
ADENYLYL CYCLASE ACTIVITY (% Stimulation)
3000
2500
2000
1500
1000
500
0
GTPγS (M)

Figure Legends:

Figure 1. Effect of GTPγS on adenylyl cyclase activity in hearts (Figure 1A) and aorta (Figure 1B) from control rats (CTL), one kidney one clip hypertensive rats (1K-1C) and one kidney one clip hypertensive treated with captopril rats (1K-1C+CAP).

Adenylyl cyclase activity was determined in the presence and absence of increasing concentrations of GTPγS (10^{-9}M--10^{-4}M) as described in "Materials and Methods". The values are the means ± SEM. of three separate experiments. Basel adenylyl cyclase activities in hearts from control (CTL), one kidney one clip hypertensive rats (1K-1C) and one kidney one clip hypertensive treated with captopril rats (1K-1C+CAP) were: 37.5±4.1, 29.7±1.2, and 35.1±2.3 pmol of c-AMP/10 min per mg of proteins respectively; and in aotra from control (CTL), one kidney one clip hypertensive rats (1K-1C) and one kidney one clip hypertensive treated with captopril rats (1K-1C+CAP) were 34.2±2.9, 28.9±0.3, 30.7±2.5 pmol of c-AMP/10 min per mg of proteins respectively. *$p< 0.05$, **$p< 0.01$, ***$p< 0.001$(compared with control); †$p<0.05$, ††$p<0.01$, †††$p<0.001$ (compared with 1K-1C).

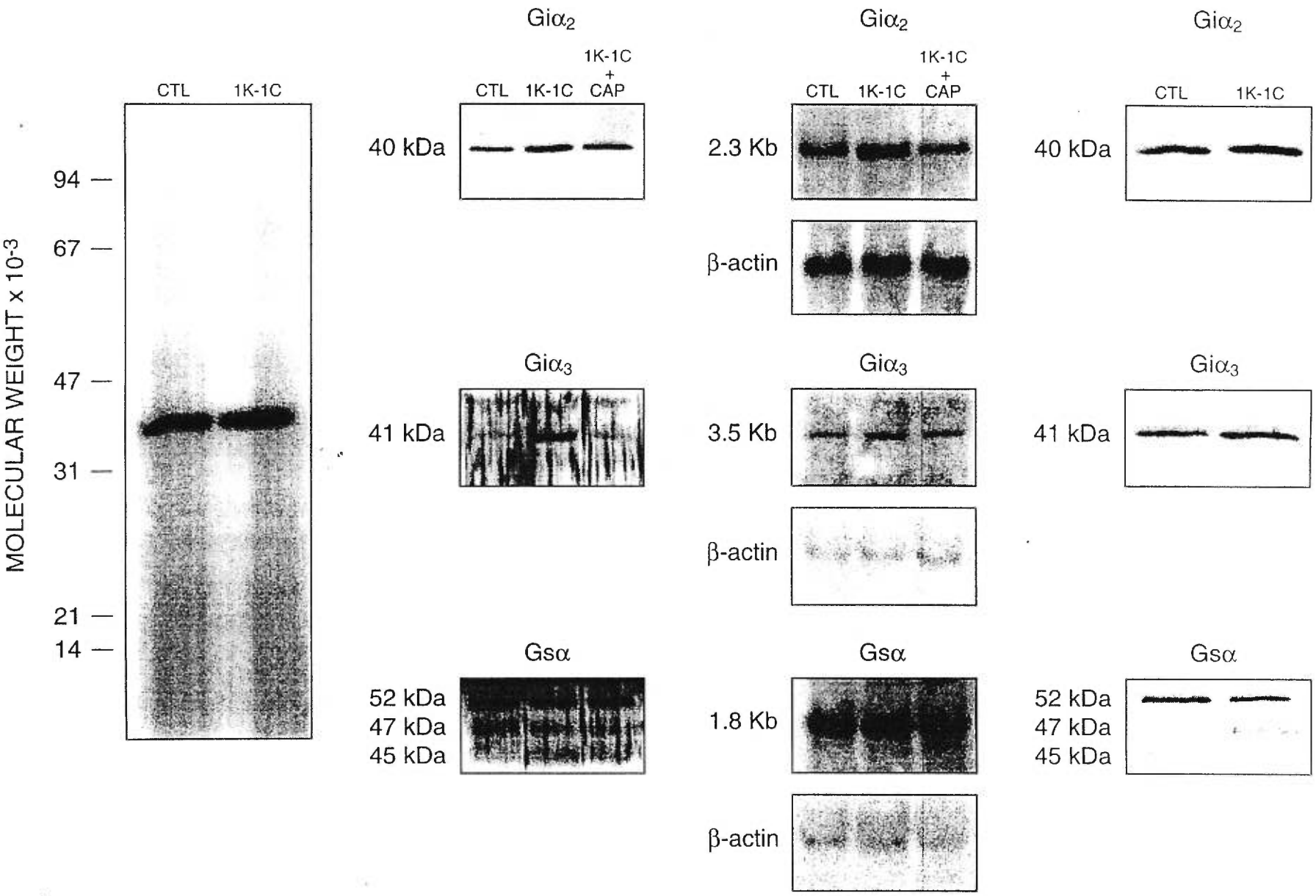

MOLECULAR WEIGHT x 10-3
94
67
47
31
21
14
CTL
1K-1C
Giα2
1K-1C + CAP
40 kDa
2.3 Kb
β-actin
Giα3
41 kDa
3.5 Kb
Gsα
52 kDa
47 kDa
45 kDa
1.8 Kb

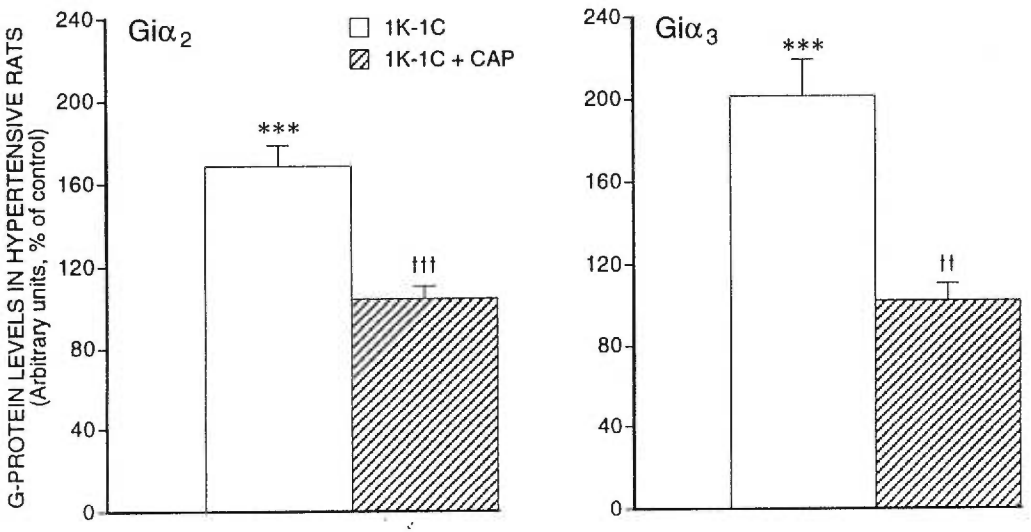
Figure 2. G-protein levels as determined by PT-catalysed ADP-ribosylation (Figure 2A), immunoblotting (Figure 2B), and Northern blotting (Figure 2C) in hearts and immunoblotting in aorta (Figure 2D) from control (CTL), one kidney one clip hypertension (1K-1C) and one kidney one clip treated with captopril rats (1K-1C+CAP).

Figure 2A: Pertussis toxin-catalyzed ADP-ribosylation of hearts membranes incubated with [α-^{32}p] NAD in presence of 5 μg/ml PT from control and 1K-1C hypertensive rats. The ^{32}p-labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography as described in "Materials and Methods".

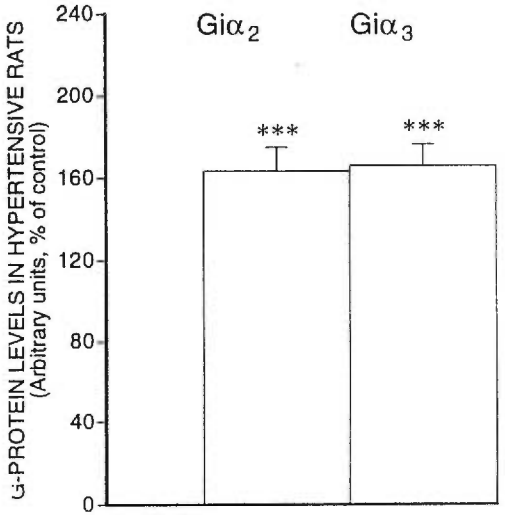
Figure 2B and 2D: The hearts and aorta membranes were prepared as described in "Materials and Methods". The membrane proteins (50μg) were separated on SDS-PAGE and transferred to nitrocellulose which was then immunoblotted using antibody AS/7 for Giα-1 and Giα-2, antibody EC/2 for Giα-3, and antibody RM/1 for Gsα as described in "Materials and Methods". The detection of G-protiens was performed by using the chemiluminescence (ECL) Western blotting detection reagents from Amersham. The autoradiograms shown are representative of three separate experiments.

Figure 2C: The G-protein mRNA levels determined by Northern blotting in hearts from control rats (CTL), one kidney one clip hypertensive rats (1K-1C) and one kidney one clip treated with captopril rats (1K-1C+CAP). Total RNA extracted from hearts was subjected to 1.3% agarose gel electrophoresis and transferred to nylon membrane as described in "Materials and Methods". The blots were then probed with a full length cDNA probe complimentary to Giα-2, Giα-3 and Gsα, and then reprobed with β-actin as described in "Materials and Methods". The autoradiograms shown are representive of three separate experiments.

HEART



AORTA



HEART

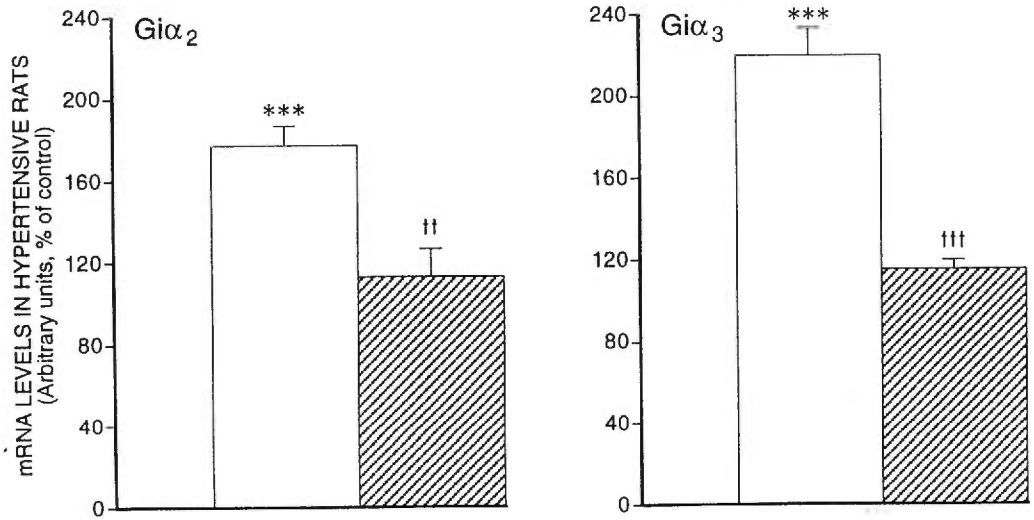


Figure 3. Summary of the quantification of immunoblots and Northern blot by densitometric scanning.

The membrane proteins (50μg) were separated on a SDS/PAGE and transferred to nitrocellulose which was then immunoblotted by using Giα-2, Giα-3 and Gsα antibodies as described in "Materials and Methods". The total RNA (20μg) was resolved on 1.3% agarose gels and transferred to nylon membranes, which then Northern blotted by hybridizing with cDNA probes of Giα-2, Giα-3 and Gsα, and re-hybridized with β-actin as described in "Materials and Methods". The quantification of G-proteins was performed by desitometric scanning using an enhanced laser densitometer (LKB). The values are means ± SEM of 3 different experiments. ***$p<0.001$ (compared with control); ††$p<0.01$, †††$p<0.001$ (compared with 1K-1C).

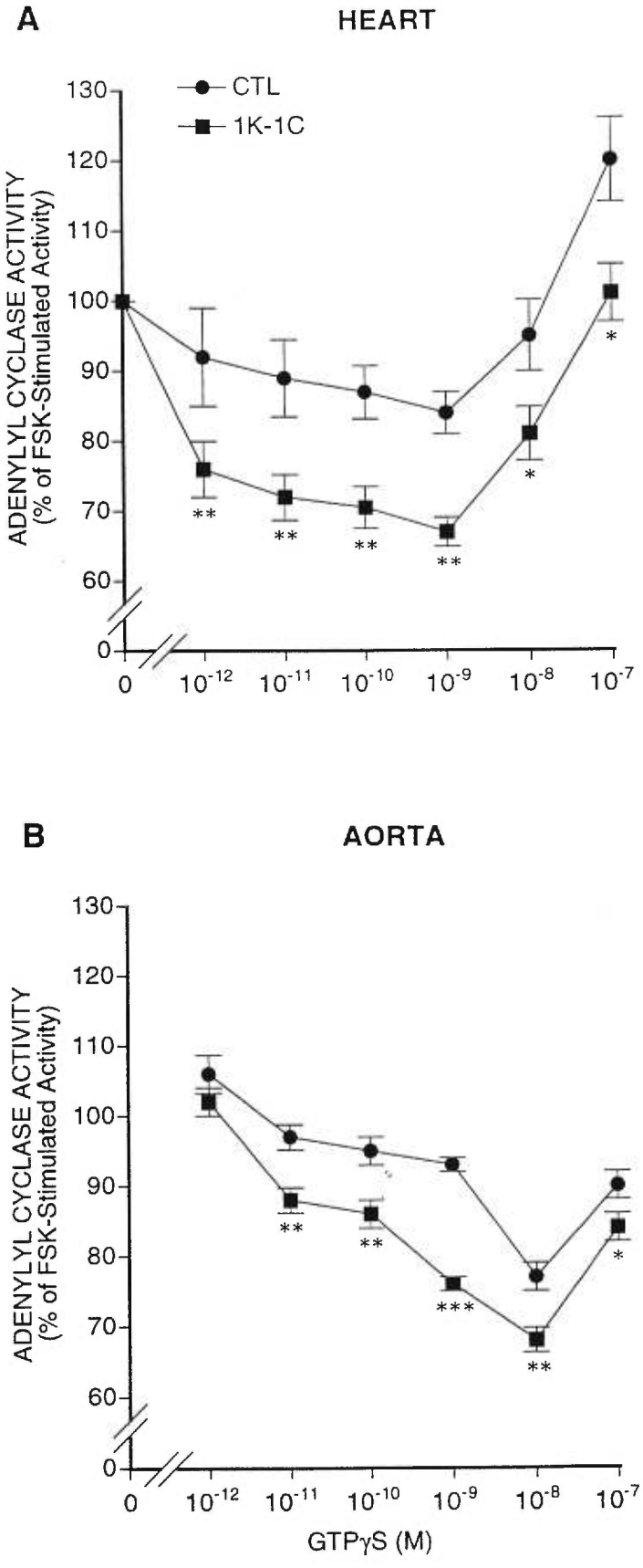

A
HEART
CTL
1K-1C
ADENYLYL CYCLASE ACTIVITY
(% of FSK-Stimulated Activity)
130
120
110
100
90
80
70
60
0
0
10-12
10-11
10-10
10-9
10-8
10-7
**
**
**
**
*
*
B
AORTA
ADENYLYL CYCLASE ACTIVITY
(% of FSK-Stimulated Activity)
130
120
110
100
90
80
70
60
0
0
10-12
10-11
10-10
10-9
10-8
10-7
**
**
***
**
*
GTPγS (M)

Figure 4 . Effect of low concentrations of GTPγS on forskolin stimulated adenylyly cyclase activity in hearts (Figure 4A) and aorta (Figure 4B) from control (CTL) and one kidney one clip hypertensive rats (1K-1C).

Adenylyl cyclase activity was determined in the absence (basal) or presence of increasing concentration of GTPγS (10^{-12}M-10^{-7}M) as described in "Materials and Methods". The values are the means ± SEM of triplicate determinations from one of three, typical separate experiments. The basal adenylyl cyclase activities in control (CTL) and one kidney one clip hypertensive rats (1K-1C) were 41.1±0.8 and 25.7±2.3 pmol of c-AMP/10 min per mg of proteins in hearts and 38.9±3.9 and 31.5± 5.2 pmol of c-AMP/10 min per mg of proteins in aorta respectively. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

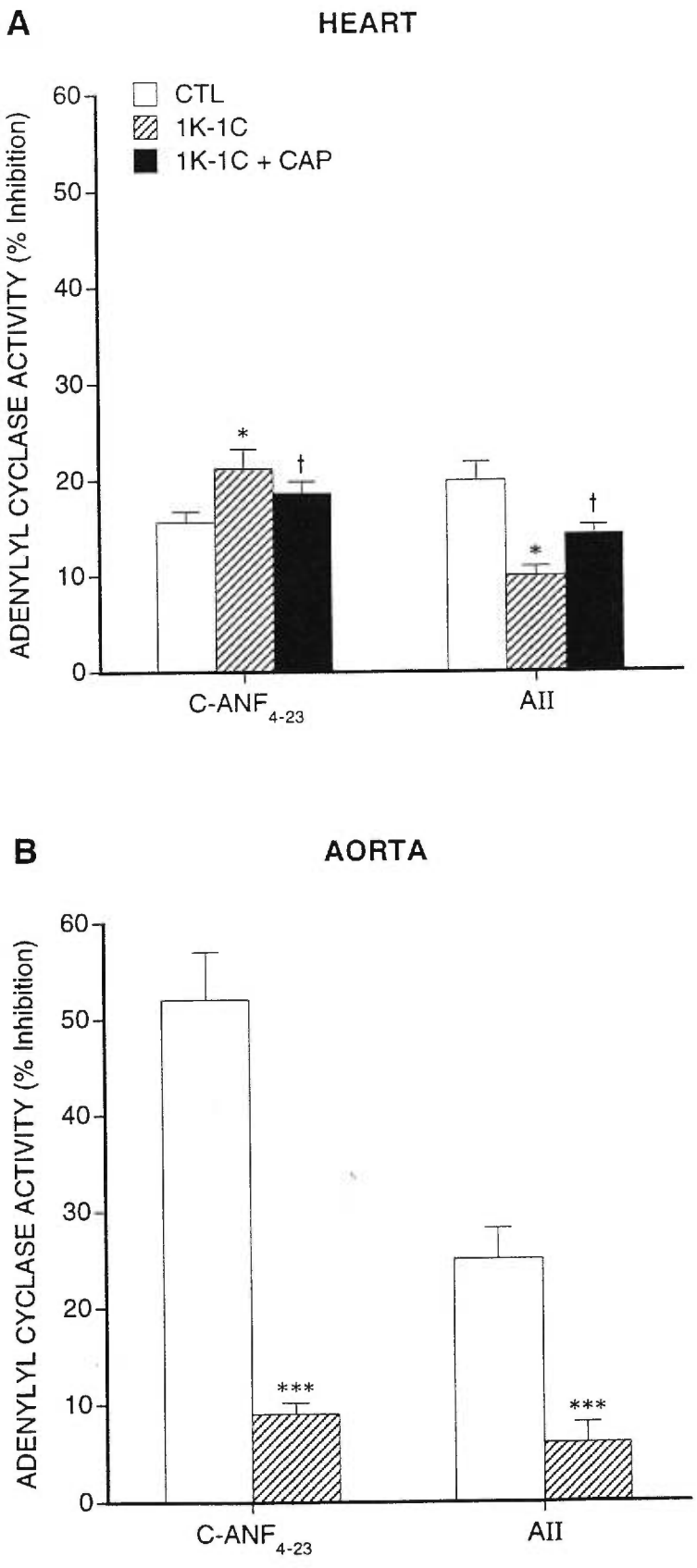

A
HEART
CTL
1K-1C
1K-1C + CAP
ADENYLYL CYCLASE ACTIVITY (% Inhibition)
60
50
40
30
20
10
0
*
†
*
†
C-ANF4-23
AII
B
AORTA
ADENYLYL CYCLASE ACTIVITY (% Inhibition)
60
50
40
30
20
10
0
***
***
C-ANF4-23
AII

Figure 5 . Inhibitory effects of c-ANF_{4-23}, and AII on adenylyl cyclase activities in hearts (Figure 5A) and aorta (Figure 5B) from control (CTL) one kidney one clip hypertension (1K-1C) and one kidney one clip hypertensive treated with captopril rats (1K-1C+CAP).

Adenylyl cyclase activities were determoned in absence (basal) or presence of C-ANF_{4-23} (10^{-7}M) and AII (10^{-5}M) as described in "Materials and Methods". The values are means ± SEM of three separate experiments. Basal enzyme activities in the presence of 10μM GTPγS (basal) in hearts from control (CTL), one kidney one clip hypertensive rats (1K-1C) and captopril treated hypertensive rats (1K-1C+CAP) were: 482.3±4.9, 380.2±28.9 and 435.7±21.1 pmol of c-AMP/10 min per mg of proteins respectively. Basal enzyme activities in the presence of 10μM GTPγS in aorta from control (CTL) and one kidney one clip hypertensive rats (1K-1C) were: 307.0±27.9 and 362.4±15.6 pmol of c-AMP/10 min per mg of proteins respectively. **$p<0.01$; ***$p<0.001$.

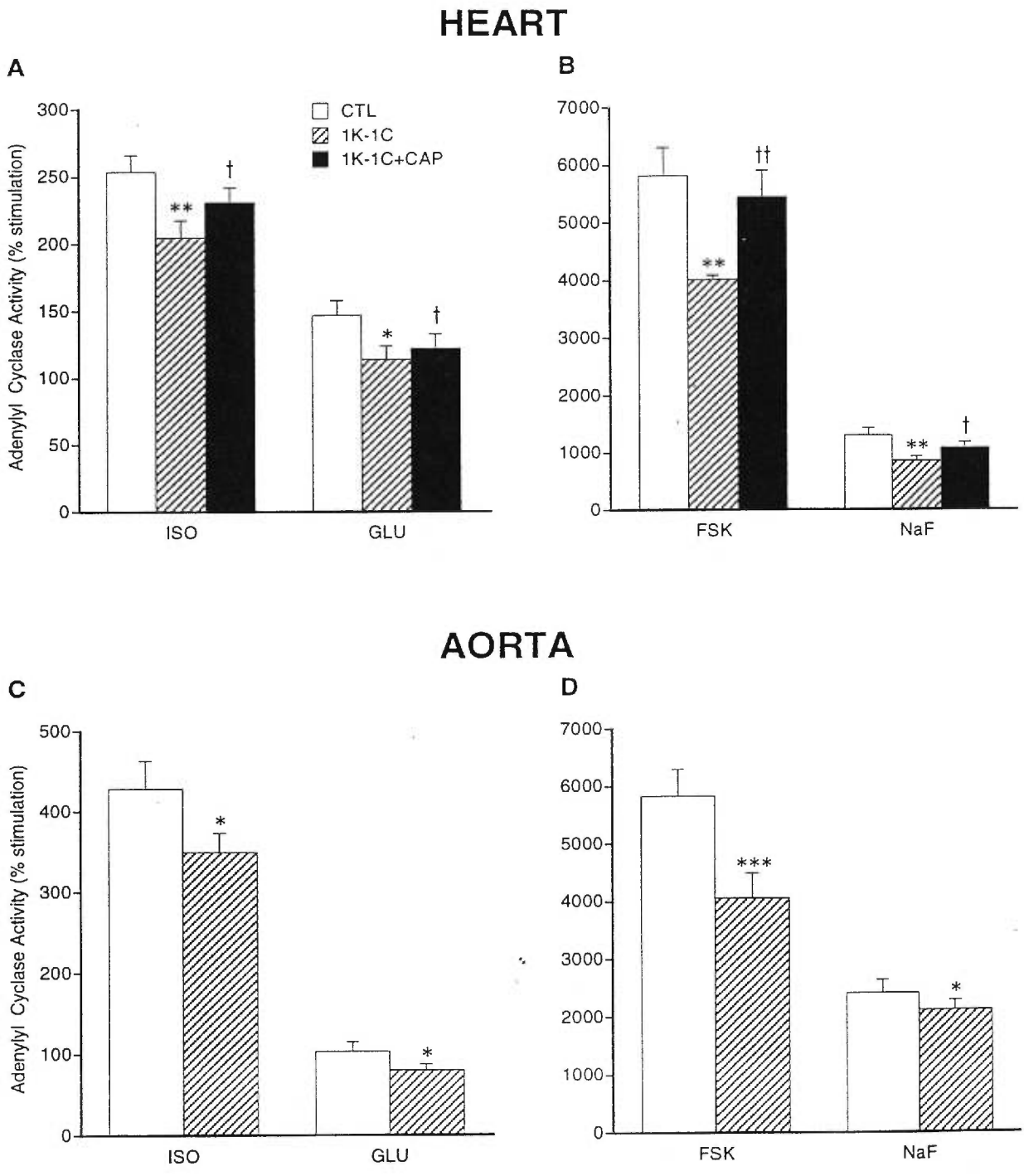

HEART
A
B
CTL
1K-1C
1K-1C+CAP
Adenylyl Cyclase Activity (% stimulation)
300
250
200
150
100
50
0
**
†
*
†
ISO
GLU
7000
6000
5000
4000
3000
2000
1000
0
**
††
**
†
FSK
NaF
AORTA
C
D
Adenylyl Cyclase Activity (% stimulation)
500
400
300
200
100
0
*
*
ISO
GLU
7000
6000
5000
4000
3000
2000
1000
0
***
*
FSK
NaF

Figure 6. Effect of stimulatory hormones on adenylyl cyclase activity in hears (Figure 6A, 6B) and aorta (Figure 6C, 6D) from control (CTL), one kidney one clip hypertension (1K-1C) and one kidney one clip rats treated with captopril (1K-1C +CAP).

Adenylyl cycasle activities were determined in the absent (basal) or presence of 50μM isoproterenol (ISO) and 1μM glucagon (GLU); 10 μM Sodium Fluoride (NaF) or 50μM Forskolin (FSK) as described in "Materials amd Methods". The values are the means ± SEM of three separate experiments. Basal enzyme activities in the presence of 10μM GTP in hearts from control (CTL), one kidney one clip hypertensive rats (1K-1C) and one kidney one clip hypertensive teated with captopril rats (1K-1C+CAP) were: 43.8±4.0, 36.2±2.2 and 36.9±3.3 pmol of c-AMP/10 min per mg of proteins respectively. In aorta from control (CTL) and one kidney one clip hypertensive rats (1K-1C) the basal enzyme activities were 32.5±2.9 and 20.6±1.5 pmol of c-AMP/10 min per mg of proteins respectively. $p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ (compared with control); $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, (compared with 1K-1C).

CHAPTER 3

Involvement of Phosphatidylinositol 3-Kinase and Mitogen-Activated Protein Kinase Pathways in AII-Mediated Enhanced Expression of Gi Proteins in Vascular Smooth Muscle Cells*

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volvement of Phosphatidylinositol 3-Kinase and itogen-Activated Protein Kinase Pathways All-Mediated Enhanced Expression of Gi oteins in Vascular Smooth Muscle Cells[1]

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e have previously demonstrated that angiotensin II eased Giα-2 and Giα-3 expression at both protein mRNA levels in vascular smooth muscle cell MC). The present study was undertaken to investi- the mechanisms responsible for AII-induced en- ced expression of Gi proteins. The levels of Gi pro- were determinated by immunoblotting techniques g specific antibodies against Giα-2 and Giα-3. AII tment of VSMC increased the levels of Giα-2 and 3 proteins and actinomycin D, an inhibitor of RNA hesis attenuated the AII-evoked enhanced expres- of Giα-2 and Giα-3 proteins. In addition, wortman- an inhibitor of phosphatidylinositol 3-kinase (PI-3- apamycin, an inhibitor of $p70^{S6K}$ and PD 098059, an bitor of mitogen-activated protein kinase (MAPK) se were able to inhibit AII-induced enhanced ex- sion of Giα-2 and Giα-3 to various degrees. The at- ation of AII-evoked enhanced levels of Giα-2 and 3 by PD 098059 was concentration dependent. At 50 PD 098059 was able to completely attenuate the nced levels of Giα-2 and Giα-3 caused by AII treat- t. These data suggest that the enhanced expression i-proteins by AII treatment may be attributed to eased RNA synthesis of Gi-proteins, and MAPK ki- , PI-3-Kinase and $p70^{S6K}$ may be involved in AII- ated increased expression of Gi-proteins in VSMC.
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whom correspondence should be addressed.

reviations: Gi, inhibitory guanine nucleotide regulatory pro- AII, angiotensin II; AT_1, angiotensin II type 1 receptor, AT_2, ensin II type 2 receptor, DMEM, Dulbecco's modified Eagle's m; MAPK, mitogen activated protein kinase; $P70^{S6K}$, 70 kDA f S6 kinase; VSMC, vascular smooth muscle cells.

Guanine nucleotide regulatory proteins (G-proteins) play an important role in the regulation of variety of signal transduction mechanisms that mediate the physiological responses of the hormones. Several abnormalities in adenylyl cyclase activities and c-AMP levels in cardiovascular tissues of different models of hypertension have been implicated in the pathogenesis of hypertension (1-4). The alterations in expression of Gsα or Giα have been demonstrated in various pathophysiological conditions such as heart failure, diabetes and hypertension (5-7). We have recently shown an enhanced expression of Giα-2 and Giα-3 at protein and mRNA levels and its relation with adenylyl cyclase regulation in heart and aorta from different hypertensive rat models such as SHR and DOCA-salt hypertensive rats (8-11).

Several hormones have been shown to modulate the expression of G-proteins (12-14). AII and ANP have been reported to augment or decrease the levels of Giα-2 and Giα-3 in VSMC respectively (15). An enhanced expression of Giα-2 and Giα-3 was observed in AII-treated vascular smooth muscle cells (15, 16). AII has also been reported to stimulate vascular smooth muscle cell growth that is associated with increased protein synthesis (17). The cellular effects of AII have been shown to be augmented via various mechanisms including activation phospholipase C (18), calcium mobilization (19), activation of protein kinase C (20), induction of pro-oncogenes (21), protein tyrosine phosphorylation (22) and activation of MAPK kinase (23, 24). We undertook the present studies to examine the contribution of some of signaling pathways mediated by AII which may be responsible for AII-induced enhanced expression of Gi-proteins in VSMC. For this reason, we examined the effects of wortmannin, an inhibitor of PI-3 kinase, PD 098059, an inhibitor of MAPKkinase, and rapamycin, an inhibitor of $p70^{S6K}$

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II-mediated enhanced expression of Gi-proteins in C.

ERIALS AND METHODS

erials. Angiotensin II was obtained from Peninsula Laboratories Inc., CA, USA. Actinomycin D and wortmannin (> 97% purity) urchased from Sigma. Rapamycin and PD 098059 were purchased from the Research Biochemicals International, U.S.A.

culture and incubation. Pure VSMC (A-10) from embryonic ic aorta of rat was obtained from American Type Culture tion (ATCC), Rockville, MA, USA. The cells were plated in 7.5 asks and incubated at 37°C in 95% and 5% CO_2 humidified phere in Dulbecco's modified Eagle's medium (DMEM) (with e, 1-glutamine, and sodium bicarbonate) containing antibiotics 0% heat-inactivated fetal calf serum (FCS). The cells were ed upon reaching confluence with 0.5% trypsin containing EDTA and utilized between passages 5 and 15. Confluent cell es were starved by incubation for 3 h in DMEM without FCS at These cells were then incubated with AII (10^{-7}M) alone or in ation with inhibitors for 24 h at 37° C as described previously he cells were treated with actinomycin D (5 μM), wortmannin M) rapamycin (100 nM), PD 098059 (50 μM) or at different trations (1 μM-20 μM) respectively for 30 min prior to AII ent. After incubation, cells were washed twice with ice-cold enization buffer (10 mm Tris-HCl, pH 7.5 containing 1 mM). The VSMC were scraped into ice-cold homogenization buffer a rubber policeman, and collected by centrifugation at 4°C for a at 600 ×g. The cells were then homogenized in Dounce enizer (10 strokes) and the homogenate was used for immuing.

unoblotting. Immunoblotting of G-proteins was performed ribed previously (9). After SDS-PAGE, the separated proteins lectrophoretically transferred to nitrocellulose paper (Schlei- Schuell) using a semidry transblot apparatus (Bio-Rad) at 15 r 45 minutes. After transfer, the membranes were washed n phosphate-buffered saline (PBS) and were incubated in PBS ing 3% dehydrated milk at room temperature for 2 hours. The ere then incubated with antisera against G proteins in PBS ing 1.5% dehydrated milk and 0.1% Tween 20 at room temre overnight. The antigen-antibody complexes were detected ibating the blots with goat anti-rabbit IgG (Bio-Rad) conjuwith horseradish peroxide for 3 hours at room temperature. ots were washed three times with PBS before reacting with ed-chemiluminescence (ECL) Western blotting detection re- from Amersham.

autoradiographs were quantified by densitometric scanning an enhanced laser densitometer (LKB Ultroscan XL, Pharmaebec, Canada) and gel scan XL evolution software (version armacia). The scanning was one dimensional and scanned the area of protein bands in autoradiogram.

ILTS

of Actinomycin D on AII-Evoked Giα Protein els

vious studies demonstrated that AII treatment of ascular smooth muscle cells for 24 hours end the expression of Giα-2 and Giα-3 proteins (16). vestigate whether the enhanced levels of Gi proas due to the increased RNA synthesis, the effect inomycin D, an inhibitor of RNA synthesis was ined on G-protein expression induced by AII in C. As shown in Fig. 1A antibodies AS/7, that react

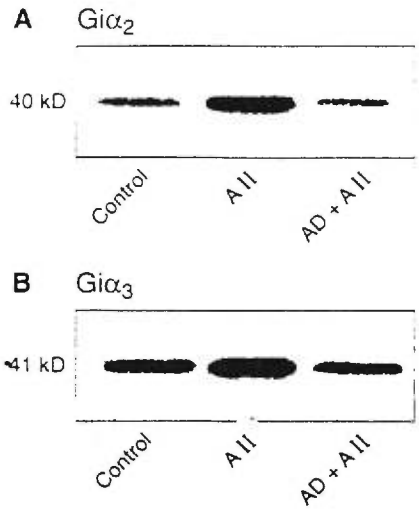
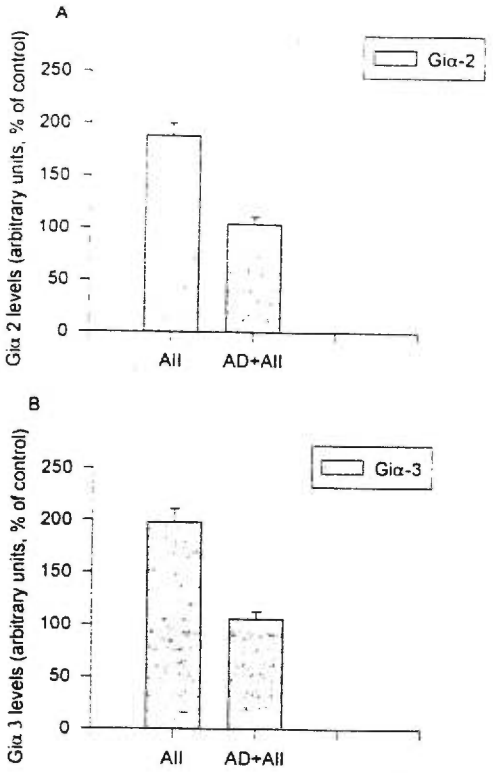


FIG. 1. Effect of actinomycin D on AII-induced expression of Giα-2 and Giα-3 proteins in vascular smooth muscle cells (VSMC). VSMC were preincubated with 10^{-7} M AII in the absence (control) or presence of 5μM actinomycin D for 24 hours at 37°C as described in "Materials and Methods". Membranes were prepared as described in "Materials and Methods". Membrane proteins (50mg) were separated on SDS/PAGE and transferred to nitrocellulose which was then immunoblotted by using Giα-2 (Figure 1A) and Giα-3 (Figure 1B) antibodies as described in "Materials and Methods". The detection of G-proteins was performed by using the chemiluminescence (ECL) Western blotting detection reagents from Amersham. The autoradiograms shown are representative of 3 separate experiments.

with both Giα-1 and Giα-2 (26) recognized a single protein of approximately 40 kDa referred to as Giα-2, (Giα-1 is absent in VSMC (27) while antibodies EC/2 detected a single protein of 41 kDa referred to as Giα-3 (Fig. 1B) on immunoblots of VSMC from control, AII-treated and actinomycin D-treated cells. However, the relative amounts of immunodetectable Giα-2 (Fig.2A) and Giα-3 (Fig. 2B) as determined by densitometric scanning were significantly increased by 188.0 ± 13.3% and 197.6 ± 17.5% respectively in AII-treated cells, and actinomycin D treatment attenuated the AII-mediated increased levels of Giα-2 and Giα-3 by 94.3 ± 13.2% and 93.8 ± 10.9% respectively (Fig. 2). These results indicate that the AII-evoked increases in Giα-2 and Giα-3 protein levels may be due to increased Gi RNA synthesis.

Effect of Wortmannin, Rapamycin and PD 098059 on AII-Evoked Giα Protein Levels

It has been suggested that the phosphorylation of 40S ribosomal protein is correlated with the efficiency of protein synthesis. AII induces increase cardiac hypertrophy by activating $p70^{S6K}$ (28). To investigate if the activation of $p70^{S6K}$ is involved in AII-mediated increased Gi protein synthesis, we examined the effect of rapamycin, a specific inhibitor of $p70^{S6K}$ on AII-induced Gi protein expression. As shown in Fig. 3 and



. 2. Summary of the quantification of immunoblots by den-
tric scanning. The membrane proteins (50μg) were separated
DS/PAGE and transferred to nitrocellulose which was then
noblotted by using antibodies AS/7 for Giα-2 (Figure 2A) and
or Giα-3 (Figure 2B) as described in "Materials and Methods".
ification of G-proteins was performed by densitometric scan-
sing an enhanced laser densitometer (LKB). The values pre-
are means ± SEM of 3 different immunoblots.

4, rapamycin at 100 nM partially inhibited the
nduced increased expression of Giα-2 and Giα-3 by
± 4.3% and 17.8 ± 2.1% (n = 3) respectively.
e results indicate that the activation of $p70^{S6K}$
be involved in AII-evoked Gi protein synthesis.
investigate the involvement of PI-3-K in AII-
ılated Gi protein synthesis, the effect of wortman-
a selective inhibitor of PI-3-K, was examined on
otein expression in VSMC. As shown in Fig. 3 and
, treatment of VSMC with wortmannin (200 nM)
to AII treatment reduced AII-mediated increased
ssion of Giα-2 and Giα-3 proteins by 71.7 ± 4.9%
37.1 ± 1.2% (n = 3) respectively in VSMC as
mined by densitometric scanning.
nilarly, PD 098059, an inhibitor of MAPK kinase
M) resulted in a complete attenuation of AII-
lated Gi-protein expression (Fig. 3 and Fig. 4). In
ion, the effect of various concentrations of PD
9 was also investigated on AII-evoked increased
of Giα-2 and Giα-3. Results shown in Fig. 5 and
3 indicate that PD 098059 inhibited the AII-
ed enhanced expression of Giα-2 and Giα-3 in a
ntration-dependent manner. At 20 μM, PD 098059 inhibited the AII-induced enhanced expression of Giα-2 and Giα-3 by 65.2 ± 7.4 and 100% (n = 3) respectively.

DISCUSSION

We and others have previously shown an enhanced expression of Giα-2 and Giα-3 proteins in genetic (SHR) and experimentally-induced hypertensive rats, such as DOCA-salt and one kidney one clip hypertensive rats (9, 11, 29). The expression of Giα-2 and Giα-3 was enhanced before the development of blood pressure in SHR (30) and in DOCA-salt hypertensive rats (31), suggesting that the increased levels of Giα proteins may be one of the contributing factors responsible for the pathogenesis of hypertension. The increased expression of Gi proteins in hypertensive rats may be attributed to the enhanced levels of catecholamines, AII, ANP and vasopressin observed in hypertension (32-35, 15). In this regard, AII has been shown to augment the levels of Giα-2 and Giα-3 in VSMC (16). In the present studies, we

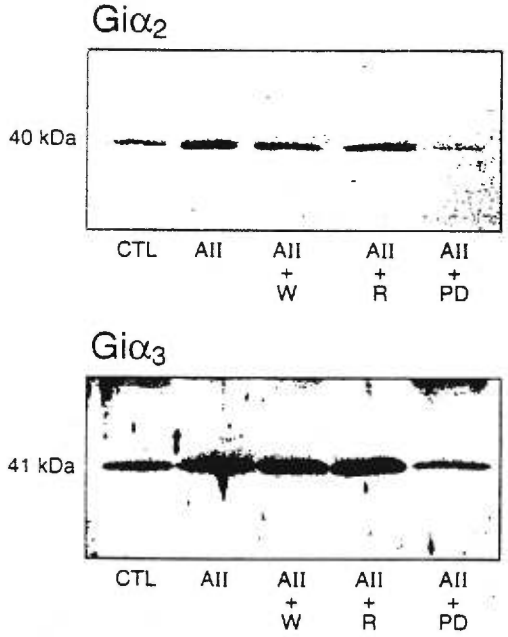


FIG. 3. Effects of wortmannin, rapamycin or PD 098059 on AII-induced enhanced levels of Giα-2 and Giα-3 proteins in vascular smooth muscle Cells (VSMC). VSMC were pretreated with 10-7M AII in the absence (control) or presence of 200 nM wortmannin, or 100nM rapamycin and 50mM PD 098059 for 24 hours at 37°C as described in "Materials and Methods". Membranes were prepared as described in "Materials and Methods". Membrane proteins (50μg) were separated on SDS/PAGE and transferred to nitrocellulose which was then immunoblotted by using antibodies AS/7 for Giα-2 and EC/2 for Giα-3 as described in "Materials and Methods". The detection of G-proteins was performed by using the chemiluminescence (ECL) Western blotting detection reagents from Amersham. The autoradiograms shown are representative of 3 separate experiments.

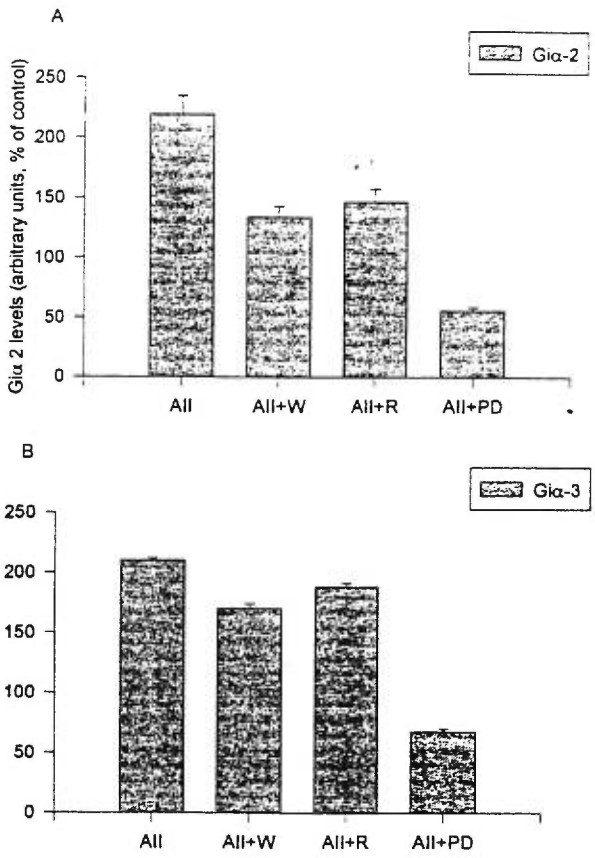


FIG. 4. Summary of the quantification of immunoblots by densitometric scanning. The membrane proteins (50μg) were separated by SDS/PAGE and transferred to nitrocellulose which was then immunoblotted by using antibodies AS/7 for Giα-2 (Figure 4A) and for Giα-3 (Figure 4B) as described in "Materials and Methods". Quantification of G-proteins was performed by densitometric scanning using an enhanced laser densitometer (LKB). The values presented are mean ± SEM of 3 different immunoblots.

shown that the AII-evoked enhanced expression of Giα-2 and Giα-3 may be attributed to the enhanced RNA synthesis of Giα-proteins, because of the fact that actinomycin D that inhibits RNA synthesis was also able to inhibit completely the AII-induced enhanced levels of Giα-2 and Giα-3 proteins. In this regard, AII-induced hypertrophy in VSMC has been shown to be due to increased protein synthesis and not due to cell proliferation (36).

Activation of MAPK kinase cascade has been widely associated with cell proliferation, hypertrophy and new protein synthesis (24, 37, 38). AII has been reported to stimulate MAPK pathway (39). Role of MAPK kinase activation in AII-induced protein synthesis has been demonstrated (38). In the present studies, we have shown that PD 098059, a specific inhibitor of MAPK pathway was able to attenuate completely the AII-induced enhanced expression of Giα-2 and Giα-3 proteins, suggesting a role of MAPK activation in AII-induced Gi-protein expression. In addition, we also provide an evidence that rapamycin, a specific inhibitor of $p70^{S6K}$ also inhibited partially the AII-induced Gi-protein synthesis, suggesting an involvement of $p70^{S6K}$ in this process. Our results are in agreement with the studies of Sadoshima and Izumo (40), who demonstrated the activation of RSK by AII and inhibition of AII-induced activation of $p70^{S6K}$ and protein synthesis by rapamycin in cardiac myocytes. In addition, we have also shown that wortmannin, a specific inhibitor of PI-3-kinase was also able to inhibit AII-

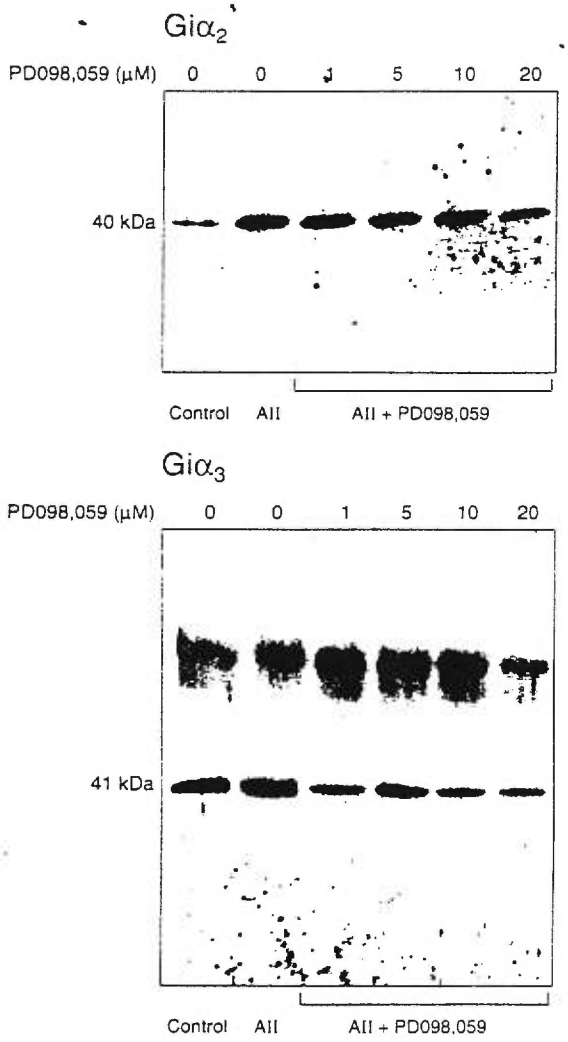
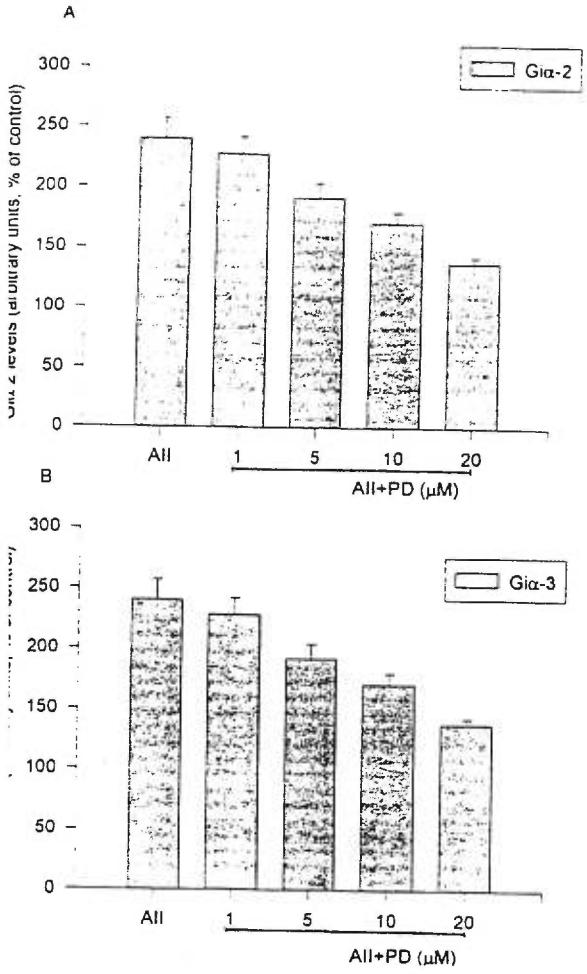


FIG. 5. Effect of various concentrations of PD 098059 on AII-evoked increased expression of Giα-2 and Giα-3 in vascular smooth muscle cells (VSMC). VSMC were pretreated with 10^{-7}M AII in the absence (control) or presence of various concentrations (1μM-20μM) of PD 098059 for 24 hours at 37°C as described in "Materials and Methods". Membranes were prepared as described in "Materials and Methods". Membrane proteins (50μg) were separated on SDS/PAGE and transferred to nitrocellulose which was then immunoblotted by using antibodies AS/7 for Giα-2 and EC/2 for Giα-3 as described in "Materials and Methods". The detection of G-proteins was performed by using the chemiluminescence (ECL) Western blotting detection reagents from Amersham. The autoradiograms shown are representative of 3 separate experiments.



6. Summary of the quantification of immunoblots by densitometric scanning. The membrane proteins (50μg) were separated by SDS/PAGE and transferred to nitrocellulose which was then immunoblotted by using antibodies AS/7 for Giα-2 (Figure 6A) and AS/6 for Giα-3 (Figure 6B) as described in "Materials and Methods". Densification of G-proteins was performed by densitometric scanning an enhanced laser densitometer (LKB). The values presented are means ± SEM of 3 different immunoblots.

ed enhanced expression of Giα-2 and Giα-3, suggesting a role of PI-3-kinase in AII-induced Gi-protein thesis.

onclusion, we have shown for the first time the ement of PI-3-kinase, MAPK kinase and $p70^{S6K}$ -induced synthesis of Giα-2 and Giα-3 proteins in

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CHAPTER 4

DISCUSSION AND CONCLUSIONS

DISCUSSION

Discussion is divided into two parts. In the first part, I discuss the results obtained on "G-protein expression and adenylyl cyclase in 1K-1C hypertension", and in second part, the mechanism responsible for AII-mediated enhanced expression of Giα proteins is discussed.

I- Gi protein expression and adenylyl cyclase in 1K-1C hypertension

In the present studies we demonstrate that 1K-1C hypertensive rats that have been shown to have high levels of AII exhibit an increased expression of Giα-2 and Giα-3 at protein and mRNA levels, whereas the expression of Gsα was unaltered. Our results are in agreement with the previous studies in SHR and DOCA-salt models (Anand-Srivastava 1988; Anand-Srivastava 1992; and Bohm et al. 1994).

The increased expression of Giα-2 and Giα-3 were significantly restored towards control levels by captopril treatment, suggesting a contribution of AII in the augmentation of Giα expression in 1K-1C hypertensive rats. In this regard AII-mediated enhanced expression of Giα protein in cultured vascular smooth muscle cells has been reported (Palaparti and Anand-Srivastava, 1994). A significant attenuation of the responsiveness of adenylyl cyclase to GTPγS observed in hearts and aorta of 1K-1C rats in comparison to control rats may not be due to Gsα protein expression because the levels of Gsα were not altered, and may be attributed to the increased levels of Giα. This was supported by the fact that captopril treatment that restored the enhanced levels of Giα towards control and GTPγS stimulated adenylyl cyclase activity was also able to restore the decreased stimulation of adenylyl cyclase by GTPγS in hearts and almost completely in aorta. These results are in accordance with previous studies showing a restoration of enhanced Gi protein expression and GTPγS-stimulated adenylyl cyclase activity in SHR by AT_1 receptor

antagonist; losartan (Anand-Srivastava and Palaparti 1998) and captopril (Pandey and Anand-Srivastava 1996).

A decreased responsiveness of adenylyl cyclase to isoproterenol and glucagon in hearts and aorta from 1K-1C hypertensive rats is consistent with the studies reported earlier in SHR and other models of hypertensive rats (Anand-Srivastava et al. 1983; Anand-Srivastava 1988; Anand-Srivastava 1992; Anand-Srivastava et al. 1993) and may be attributed to the down regulation of hormone receptors (Limas and Limas 1978), defective coupling and/or impaired catalytic subunit of adenylyl cyclase system (Anand-Srivastava 1988). Since the levels of Gsα were not altered, the attenuated responsiveness of isoproterenol and glucagon can not be explained by Gsα and may be due to the enhanced levels of Giα. This assumption is supported by the partial correction of the responsiveness of adenylyl cyclase to isoproterenol and glucagon with restoration of enhanced levels of Giα-2 and Giα-3 by captopril treatment.

The increased expression of Giα-2 and Giα-3 was reflected in enhanced functions of Gi as demonstrated by increased inhibition of C-ANF $_{4-23}$-mediated attenuation of adenylyl cyclase in heart and of FSK-stimulated adenylyl cyclase activity by low concentration of GTPγS in 1K-1C hypertensive rats as compared to control rats. However, AII-mediated inhibition of adenylyl cyclase that was attenuated in hypertensive rats as compared to control rats, may be explained by the down-regulation of AII-receptors due to increased levels of AII in 1K-1C hypertensive rats (Amiri and Garcia 1997). On the other hand, the attenuated inhibition of adenylyl cyclase by C-ANF $_{4-23}$ and AII in aorta from hypertensive rats may be attributed to the down regulation of ANP-C and AII receptors (Gutkins et al. 1987; Marcil and Anand-Srivastava 1995; Swithers et al. 1987; Gauquelin et al. 1991).

Our results on the decreased stimulations of adenylyl cyclase by FSK and NaF in 1K-1C hypertensive rats are consistent with earlier on SHR and DOCA-salt hypertensive rats

(Anand-Srivastava 1988; Anand-Srivastava 1993). This decreased stimulation may be due to the defective catalytic subunit percent or to the overexpression of Giα or both. The contribution of Gi in decreased FSK-stimulated adenylyl cyclase activity in 1K-1C hypertensive rats is supported by the fact captopril treatment that restored the enhanced levels of Giα proteins towards control levels also restored the sensitivity of FSK to stimulate adenylyl cyclase activity.

II- Mechanism responsible for AII-mediated enhanced expression of Giα protein

We have shown that AII increased the expression of Giα-2 and Giα-3 protein and mRNA levels in VSMC (Palaparti and Anand-Srivastava 1994). In the present studies, we demonstrate that AII-induced increased expression of Giα-2 and Giα-3 may be due to the increased RNA synthesis of Giα proteins, because actinomycin D, an inhibitor of RNA synthesis was able to completely attenuate the AII-induced increased levels of Giα-2 and Giα-3 proteins.

Activation of MAPK kinase cascade has been widely associated with cell proliferation, hypertrophy and new protein synthesis (Tsuda et al. 1992; Berk and Corson 1997; Takahashi et al. 1997) . AII has been reported to stimulate MAPK pathway (Duff et al. 1995) . Role of MAPK kinase activation in AII-induced protein synthesis has been demonstrated (Takahashi et al. 1997). In the present studies, we have shown that PD 098059, a specific inhibitor of MAPK pathway was able to attenuate completely the AII-induced enhanced expression of Giα-2 and Giα-3 proteins, suggesting a role of MAPK activation in AII-evoked Gi-protein expression. In addition, we also provide an evidence that rapamycin, a specific inhibitor of $p70^{S6K}$ also inhibited partially the AII-induced Gi-protein synthesis, suggesting an involvement of $p70^{S6K}$ in this process. Our results are in agreement with the studies of Sadoshima and Izumo (1995), who demonstrated the activation of RSK by AII and inhibition of AII-induced activation of $p70^{S6K}$ and protein

synthesis by rapamycin in cardiac myocytes. In addition, we have also shown that wortmannin, a specific inhibitor of PI-3-kinase was also able to inhibit AII-induced enhanced expression of Giα-2 and Giα-3, suggesting a role of PI-3-kinase in AII-induced Gi-protein synthesis.

In conclusion, we have shown for the first time the involvement of PI-3-kinase, MAPK kinase and $p70^{S6K}$ in AII-induced synthesis of Giα-2 and Giα-3 proteins in VSMC.

CONCLUSIONS

1. The heart and aorta from 1K-1C hypertensive rats exhibit enhanced expression of Giα-2 and Giα-3 proteins, but there was no changes in expression of Gsα. The enhanced expression of Giα was associated with enhanced functions of Gi. The stimulatory effects of GTPγS, stimulatory hormones, FSK and NaF on adenylyl cyclase activity were inhibited in hearts and aorta from 1K-1C hypertensive rats which were restored towards control levels by captopril treatment. In addition, captopril treatment of 1K-1C hypertensive rats also restored the enhanced expression of Giα proteins and Gi-mediated inhibition of adenylyl cyclase towards control levels. These results suggest a role of AII in exhibiting enhanced expression of Giα in 1K-1C hypertensive rats, that may be one of the factors responsible for the pathogenesis of hypertension.

2. AII treatment resulted in an increased levels of Giα-2 and Giα-3 proteins in VSMC. AII-induced enhanced levels of Giα proteins were inhibited by actinomycin D, wortmannin, rapamycin and PD 098059. These results suggest that AII-mediated enhanced expression of Giα proteins may be due to the increased RNA synthesis of Gi proteins. In addition, it is the first study showing the involvement of PI-3-kinase, MAPK kinase and $p70^{S6K}$ in AII-induced enhanced expression of Giα-2 and Giα-3 proteins in VSMC.

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PUBLICATIONS

1. **C.Ge** and M.B. Anand-Srivastava (1998). Involvement of Physphatidylinositol 3-Kinase and Mitogen-Acvivated Protein Kinase Pathways in AII-Mediated Enhanced Expression of Gi proteins in Vascular Smooth Muscle Cells. Biochemical and Biophysical Research Communication. 251 (2):570-575 (See Chapter 3)

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ıgiotensin II Enhances the Expression of Giα A10 Cells (Smooth Muscle): Relationship th Adenylyl Cyclase Activity[1]

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. the present studies, we have investigated the ef- of angiotensin II (AII) on guanine nucleotide reg- ory protein (G protein) expression and functions .10 smooth muscle cells. AII treatment of A10 cells anced the levels of inhibitory guanine nucleotide ılatory protein (Gi) as well as Gi mRNA and not of ıulatory guanine nucleotide regulatory protein in a concentration-dependent manner as deter- ed by immunoblot and Northern blot analysis, re- :tively. AII-evoked increased expression of Giα-2 Giα-3 was inhibited by actinomycin D treatment A synthesis inhibitor). The increased expression of 2 and Giα-3 by AII was not reflected in functions, ıuse the GTPγS-mediated inhibition of forskolin- ulated adenylyl cyclase and the receptor-medi- inhibition of adenylyl cyclase by AII and NP$_{4-23}$ [des(Gln^{18}, Ser^{19}, Gln^{20}, Leu^{21}, Gly^{22}) ANP$_{4-23}$-] were not augmented but attenuated in AII- ted A10 cells. The attenuation was prevented by rosporine (a protein kinase C inhibitor) treat- t. On the other hand, AII treatment did not affect expression and functions of stimulatory guanine .eotide regulatory protein (Gs), however, the stim- ory effects of 5′-*O*-(3-thiotriphosphate), isoproter- , and *N*-ethylcarboxamide adenosine (NECA) on ıylyl cyclase activity were inhibited to various de- s by AII treatment. Staurosporine reversed the :voked attenuation of isoproterenol- and NECA- ulated enzyme activity. From these results, it can uggested that AII, whose levels are increased in hypertension, may be one of the possible contributing factors responsible for exhibiting an enhanced expression of Gi protein in hypertension. © 1999 Academic Press

Key Words: **angiotensin II; Giα proteins; adenylyl cyclase; A10 smooth muscle cells.**

Angiotensin II (AII),[4] a vasoactive peptide and a key component of the renin–angiotensin system, elicits a wide variety of biological responses, including vasoconstriction, stimulation of aldosterone secretion, and renal sodium reabsorption (1). In addition, AII is a growth-promoting factor for several cell types such as fibroblasts, adrenocortical cells, cardiac myocytes, and vascular smooth muscle cells (2, 3). AII induces cell hypertrophy in cultured aortic smooth muscle cells as a result of increased protein synthesis (4, 5), which is associated with increased expression of the growth-associated nuclear proto-oncogenes, c-*fos,* c-*jun,* and c-*myc* (6, 7). AII also stimulates tyrosine phosphorylation of multiple substrates (8), including mitogen-activated protein kinase (8–10).

AII elicits its physiological effects by interacting with two distinct receptor subtypes designated as AT_1 and AT_2 (11) based on their interaction with nonpep-

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[4] Abbreviations used: ANP, atrial natriuretic peptide (99–126), C-ANP$_{4-23}$, a ring deleted analog of atrial natriuretic factor; C-ANP$_{4-23}$, [des(Gln^{18}, Ser^{19}, Gln^{20}, Leu^{21}, Gly^{22}) ANP$_{4-23}$-NH$_2$]; Gi, inhibitory guanine nucleotide regulatory protein; Gs, stimulatory guanine nucleotide regulatory protein; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); AII, angiotensin II; PKC, protein kinase C; NECA, *N*-ethylcarboxamide adenosine; PLC, phospholipase C; PLD, phospholipase D; PLA$_2$, phospholipase A$_2$; DAG, diacylglycerol; IP$_3$, inositol triphosphate; SHR, spontaneously hypertensive rats; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; CT, cholera toxin; ACE, angiotensin-converting enzyme; DOCA, deoxycorticosterone acetate.

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tide antagonists losartan and PD123177, respectively (12). The presence of AT_1 receptor subtype has been shown in rat vascular tissues; however, a small proportion of AT_2 receptors is also present in rat aorta (13, 14). Most of the physiological effects of AII are mediated by AT_1 receptors. AT_1 receptors are coupled to several second-messenger systems such as stimulation of phospholipase C (PLC) (15), D (PLD) (16), and A_2 (PLA_2) (17), inhibition of adenylyl cyclase/cAMP (18–20), and plasma membrane calcium channels (21). The activation of PLC results in the formation of two second messengers, inositol trisphosphate (IP_3), and diacylglycerol (DAG). DAG activates protein kinase C (PKC), which has been shown to phosphorylate various proteins including inhibitory G-binding proteins (Gi; 22, 23). The phosphorylation of Gi regulatory protein uncouples the inhibitory hormone receptor from adenylyl cyclase and thereby attenuates the hormone-mediated inhibition of adenylyl cyclase.

The adenylyl cyclase/cAMP system is composed of three components: receptor, catalytic subunit, and guanine nucleotide regulatory proteins (G proteins). The G proteins act as transducers and, in the presence of guanine nucleotides, transmit the signal from the hormone-occupied receptor to the catalytic subunit. The hormonal stimulation and inhibition of adenylyl cyclase are mediated through the stimulatory (Gs) and inhibitory (Gi) guanine nucleotide protein, respectively (24, 25), resulting in the increased or decreased formation of cAMP, respectively. G proteins are heterotrimeric, consisting of α, β, and γ subunits. The α subunits bind and hydrolyze GTP and confer specificity in receptor and effector interactions (25). Four different isoforms of Gs have been identified which appear to be products of alternate splicing of a common precursor (26, 27). On the other hand, three distinct forms of Giα, Giα1, Giα2, and Giα3, have been identified and have been shown to be products of three different genes (28, 29).

Genetic linkage between AII gene and hypertension has been established (30). Levels of AII have been reported to be elevated in hypertensive human beings (31). Transgenic mice overexpressing AII have also been shown to have elevated blood pressure (32, 33). In addition, AII has been shown to be differentially regulated in tissues involved in blood pressure regulation (34). We have recently demonstrated increased expression of Giα protein and Giα mRNA and associated functions in spontaneously hypertensive (SHR) and deoxycorticosterone acetate (DOCA)–salt hypertensive rats as compared to their control rats (35, 36). The enhanced expression of Giα and associated functions in SHR were restored toward control levels by angiotensin-converting enzyme (ACE) inhibitor (37), which inhibits the conversion of AI to AII and thereby decreases the levels of AII. Taken together, it is possible that the enhanced levels of AII reported in hypertension (31) may be responsible for the observed enhanced expression of Gi proteins in hypertensive rats (35, 36). To examine this possibility, the present studies were undertaken to examine the effect of AII treatment on the expression of G proteins and adenylyl cyclase activity in A10 cells (smooth muscle). This rat embryonal thoracic aorta cell line has been shown to demonstrate characteristics similar to those of vascular smooth muscle cells (38) and has been a useful model to study vascular cellular processes (39, 40).

MATERIALS AND METHODS

Materials

ATP, cAMP, and other chemicals necessary for total RNA extraction and Northern blot analysis were obtained from Sigma Chemical Co. (St. Louis, MO). Creatine kinase (EC 2.7.3.2), myokinase (EC 2.7.4.3), GTP, and GTPγS were purchased from Boehringer-Mannheim, Canada. 3-Isobutyl-1-methylxanthine (IBMX) was purchased from Aldrich Chemical Corporation (Milwaukee, WI). [α-^{32}P]ATP, and [α-^{32}P]dCTP were purchased from Amersham Corp. (Oakville, Ontario, Canada). Angiotensin II and C-ANP_{4-23} were from Peninsula Laboratories, Inc. (CA).

Methods

Cell culture and incubation. The A10 cell line from embryonic thoracic aorta of rat was obtained from American Type Culture Collection (Rockville, MA). The cells were plated in 7.5-cm^2 flasks and incubated at 37°C in 95% air and 5% CO_2 humidified atmosphere in Dulbecco's modified Eagle's medium (DMEM) (with glucose, L-glutamine, and sodium bicarbonate) containing antibiotics and 10% heat-inactivated calf serum (FCS). The cells were passaged upon reaching confluence with 0.5% trypsin containing 0.2% EDTA and utilized between passages 5 and 15. Confluent cell cultures were starved by incubation for 3 h in DMEM without FCS at 37°C. These cells were then incubated with different concentrations of AII for 24 h at 37°C as described previously (41). To study the effect of PKC, the cells were pretreated with staurosporine (1 nM) for 30 min in the absence of AII and thereafter with AII (10^{-7} M) for 24 h as described earlier (42). After incubation, cells were washed twice with ice-cold homogenization buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5). The A10 cells were scraped into ice-cold homogenization buffer using a rubber policeman and collected by centrifugation at 4°C for 10 min at 600*g*. The cells were then homogenized in a Dounce homogenizer (10 strokes) and the homogenate was used for adenylyl cyclase assay and immunoblotting.

Adenylyl cyclase activity determination. Adenylyl cyclase activity was determined by measuring [^{32}P]cAMP formation from [α-^{32}P]ATP, as described previously (35, 36). Briefly, the assay medium containing 50 mM glycylglycine, pH 7.5, 0.5 mM MgATP, [α-^{32}P]ATP (1.5×10^6 cpm), 5 mM $MgCl_2$ (in excess of the ATP concentration), 100 mM NaCl, 0.5 mM cAMP, 1 mM IBMX, 0.1 mM ethylene glycol bis(β-aminoethyl ether) *N, N'*-tetraacetic acid, 10 μM GTPγS, and an ATP-regenerating system consisting of 2 mM phosphocreatine, 0.1 mg of creatine kinase/ml, and 0.1 mg of myokinase/ml in a final volume of 200 μl. Incubations were initiated by the addition of the membrane preparations (20–30 μg) to the reaction mixture, which had been thermally equilibrated for 2 min at 37°C. The reactions conducted in triplicate for 10 min at 37°C, were terminated by the addition of 0.6 ml of 120 mM zinc acetate. cAMP was purified by coprecipitation of other nucleotides with $ZnCO_3$, by addition of 0.5 ml of 144 mMNa_3CO and subsequent chromatography by the double-

nn system, as described by Salomon *et al.* (43). Under the assay tions used, adenylyl cyclase activity was linear with respect to in concentrations and time of incubation. Protein was deter- d essentially as described by Lowry *et al.* (44) with bovine serum nin as standard.

munoblotting. Immunoblotting was performed as described ously (36, 45). After SDS–PAGE, the separated proteins were rophoretically transferred to nitrocellulose paper (Schleicher Schuell) with a semidry transblot apparatus (Bio-Rad) at 15 V 5 min. After transfer, the membranes were washed twice in phate-buffered saline (PBS) and were incubated in PBS contain- % dehydrated milk at room temperature for 2 h. The blots were incubated with antisera against G proteins in PBS containing ehydrated milk and 0.1% Tween 20 at room temperature for 2 h. antibody–antigen complexes were detected by incubating the with goat anti-rabbit IgG (Bio-Rad) conjugated with horserad- eroxidase for 2 h at room temperature. The blots were washed times with PBS before reaction with enhanced chemilumines- (ECL) Western blotting detection reagents from Amersham. ıtitative analysis of the G proteins was performed by densito- ic scanning of the autoradiographs employing the enhanced densitometer (LKB Ultroscan XL) and quantified using the gel XL evaluation software (version 2.1) from Pharmacia (Quebec, ıda).

tal RNA extraction. Total RNA was extracted from A10 cells as ibed earlier (45, 46) by the guanidinium thiocyanate–phenol– oform method described by Chomczynski and Sacchi (47).

diolabelling of the probes. cDNA inserts encoding for Giα2, , and Gsα were radiolabeled with [α-^{32}P]dCTP by random prim- ssentially as described by Feinberg *et al.* (48). Specific activities e labeled probes ranged from 1 to 3 × 10^8 cpm/μg of DNA. The er oligonucleotide recognizing the 28 S rRNA was end-labeled [γ-^{32}P]ATP using T4 polynucleotide kinase as described by ɔrook *et al.* (49).

rthern analysis. Dimethyl sulfoxide/glyoxal-treated total RNA esolved on 1% agarose gels and transferred to nylon membrane escribed previously (45, 46). Filters, after prehybridization at for 6 h in hybridization solution (600 mM NaCl, 8 mM EDTA, nM Tris, pH 7.4, 0.1% sodium pyrophosphate, 0.2% SDS, 500 heparin), and were then hybridized overnight in hybridization ion containing dextran sulfate (10%, w/v) and the cDNA probe -3 × 10^6 cpm/ml as described previously (45, 46). Filters were rinsed at 65°C for 2 × 30 min in 300 mM NaCl, 4 mM EDTA, 60 Tris, pH 7.4, and 0.2% SDS and 1 × 30 min in 150 mM NaCl, 2 EDTA, 30 mM Tris, pH 7.4, and 0.1% SDS. Autoradiography performed with X-ray films at −70°C. In order to assess the bility of any variations in the amounts of total RNA in individ- amples applied to the gel, each filter was hybridized with the ^{32}P abeled oligonucleotide, which recognizes a highly conserved n of 28 S ribosomal RNA. The blots, which had been probed with protein cDNA, were dehybridized by washing for 1 h at 65°C in formamide, 300 mM NaCl, 4 mM EDTA, and 60 mM Tris, pH ınd rehybridized overnight at room temperature with the oligo- eotide. Quantitative analysis of the hybridization of probes d was performed by densitometric scanning of the autoradio- hs employing the enhanced laser densitometer (LKB Ultroscan ınd quantified using the gel scan XL evaluation software (ver- 2.1) from Pharmacia.

ULTS

ct of AII Pretreatment on GTPγS-Stimulated denylyl Cyclase Activity

'e have recently reported a decreased responsive- s of adenylyl cyclase to guanine nucleotide stim-

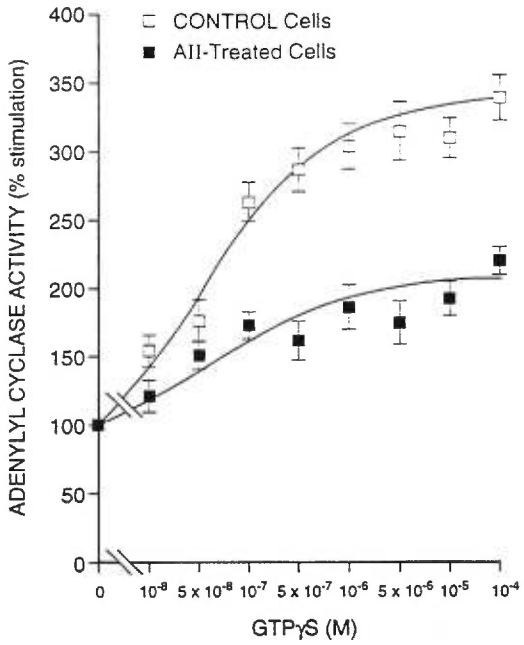
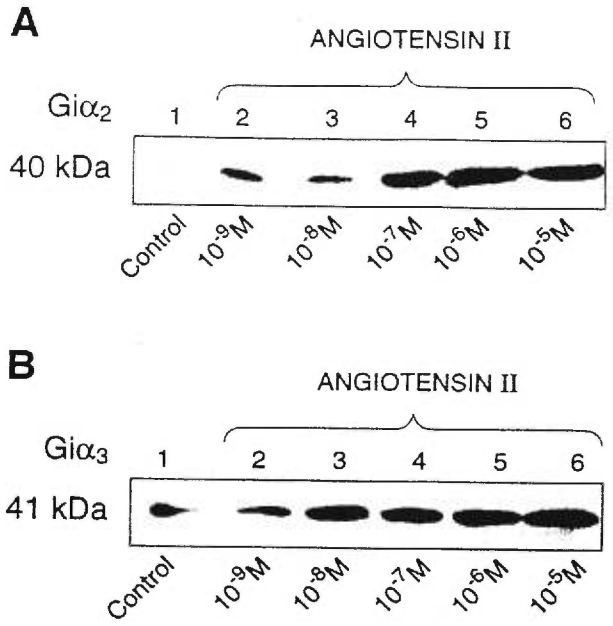


FIG. 1. Effect of AII treatment on GTPγS-mediated stimulation of adenylyl cyclase activity in A10 smooth muscle cells. A10 smooth muscle cells were incubated in the absence (control, □) or presence of 10^{-7} M angiotensin II (AII, ■) for 24 h as described under Materials and Methods. Membranes were prepared as described under Materials and Methods. Adenylyl cyclase activity was determined in the membranes in the absence or presence of various concentrations of GTPγS as described under Materials and Methods. Basal enzyme activities in the control and AII-treated membranes were 81.5 ± 12 and 50.3 ± 5 pmol cAMP/10 min/mg of protein, respectively. Values are means ± SE of three separate experiments performed in triplicate.

ulation in hearts from SHR (35) which was restored toward control levels by captopril treatment (37). These results indicate a possible involvement of AII in the observed decreased response. To investigate this possibility, the effect of AII treatment on GTPγS-mediated stimulation of adenylyl cyclase was investigated in A10 cells. As shown in Fig. 1, GTPγS stimulated adenylyl cyclase activity in control and AII-treated cells in a concentration-dependent manner; however, the extent of stimulation was significantly diminished in AII-treated cells. For example, at 10 μM, GTPγS stimulated adenylyl cyclase activity by about 300% in control cells and about 200% in AII-treated cells. These results indicate that AII treatment may result in the impairement of G proteins and thereby decrease the stimulatory effect of GTPγS on adenylyl cyclase.

Effect of AII Treatment on G Protein Expression

To investigate if the decreased stimulation of adenylyl cyclase by GTPγS in response to AII treatment is attributed to its ability to increase the levels of Gi or to



'IG. 2. (A and B) Effect of AII treatment on Giα-2 and Giα-3 rotein expression in A10 smooth muscle cells. A10 smooth muscle ells were incubated in the absence (control) or presence of various oncentrations of angiotensin II (AII) (10^{-9} to 10^{-5} M) for 24 h as escribed under Materials and Methods. Membranes were prepared s described under Materials and Methods and were used for immuoblotting. The membrane proteins were resolved by sodium dodecyl ulfate–polyacrylamide gel electrophoresis and transferred to nitroellulose, which was then immunoblotted using AS/7 antibody for iα1 and Giα-2 (A) or EC/1 antibody for Giα3 (B) and were detected y using ECL Western blotting technique as described under Mate-.als and Methods. The autoradiograms are representatives of three eparate experiments.

ecrease the levels of Gs or both, we determined the ffect of AII treatment (at various concentrations) on ıe levels of G proteins by immunoblotting using spe-ific antibodies against different G proteins. As shown ı Fig. 2A, antibody AS/7, which reacts with both Giα1 nd Giα2 (50), recognized a single protein of approxiıately 40 kDa referred to as Giα2 (Giα-1 is absent in ıt vascular smooth muscle cells (28)), while antibody C/2 detected a single protein of 41 kDa, referred to as iα-3, on immunoblots from control and AII-treated ells (Fig. 2B). However, the relative amounts of imıunodetectable Giα2 and Giα3 were significantly in-eased in a concentration-dependent manner in AII-eated cells. For example, AII treatment at 10^{-6} and 0^{-5} M enhanced the levels of Giα-2 by 495.1 ± 29.3 nd 431.5 ± 43.6% (n = 3), respectively, and the levels Giα3 by 315.3 ± 31.9 and 398.6 ± 26.3% (n = 3), espectively, compared to the control cells as deterıined by densitometric scanning. In addition, the RNA levels of Giα-2 and Giα-3 were also increased by 2.5 ± 2.2 and 21.7 ± 1.5%, respectively, in cells eated with AII at 10^{-6}M, whereas in cells treated with 10^{-5} M AII, the increase was 35.8 ± 3.2 and 40.6 ± 2.6%, respectively, as determined by densitometric scanning of Northern blots (Fig. 3). On the other hand, AII treatment of the cells did not alter the levels of Gsα proteins and Gs mRNA (Fig. 4).

We have further investigated the effect of actinomycin D (an inhibitor of RNA synthesis) on G-protein expression in the presence of AII and the results are shown in Fig. 5. AII treatment augmented the levels of Giα-2 (A) and Giα-3 proteins (B) which were inhibited by actinomycin D treatment. These results indicate that the AII-evoked increases in Giα-2 and Giα-3 protein levels may be due to increases in RNA synthesis.

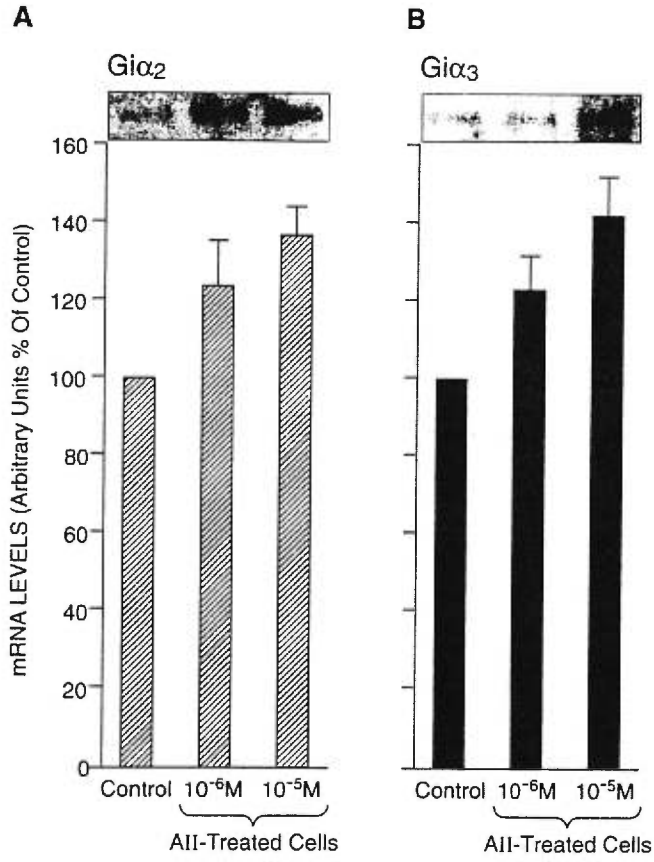
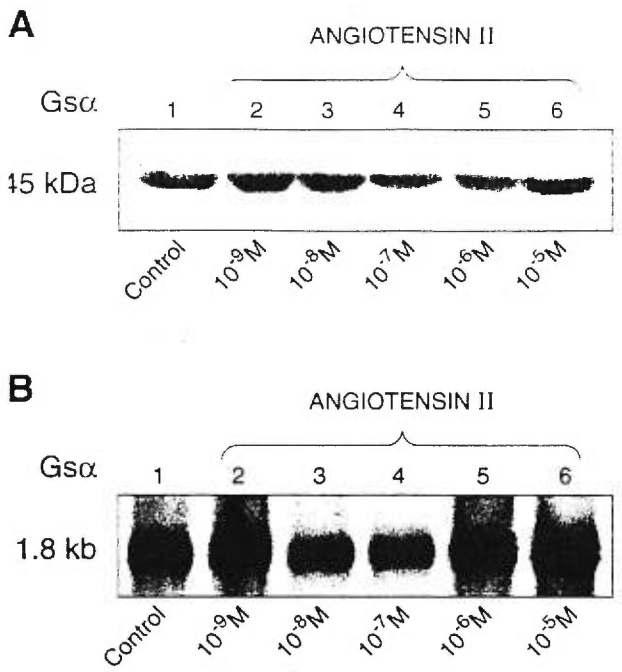


FIG. 3. Effect of AII treatment on Giα-2 and Giα-3 mRNA levels in A10 vascular smooth muscle cells. (Top) Total RNA from A10 smooth muscle cells incubated in the absence (control) or presence of 10^{-6} and 10^{-5} M angiotensin II (AII) were subjected to 1% agarose gel electrophoresis and transferred to nylon membranes. The blots were then probed with full-length radiolabeled Giα-2 cDNA probe (A) or with a full-length radiolabeled Giα-3 cDNA probe (B) as described under Materials and Methods. (Bottom) Summary of quantification of Northern blots by densitometric scanning using an enhanced laser densitometer as described under Materials and Methods. Values obtained from densitometric scans of the resulting autoradiographs were normalized relative to the transcripts detected in control cells which were arbitrarily assigned the value of 100%. Results represent means ± SE of three separate experiments conducted with different preparations of total RNA.



. 4. (A) Effect of AII treatment on Gsα protein expression in smooth muscle cells. A10 smooth muscle cells were incubated in absence (control) or presence of various concentrations of angiotin II (AII) (10^{-9} to 10^{-5} M) for 24 h as described under Materials Methods. Membranes were prepared as described under Mate- and Methods and were used for immunoblotting. The mem- ne proteins were resolved by sodium dodecyl sulfate–polyacryl- de gel electrophoresis and transferred to nitrocellulose, which then immunoblotted using RM/1 antibody, and were detected by g the ECL Western blotting technique as described under Ma- als and Methods. The autoradiograms are representatives of e separate experiments. (B) Effect of AII treatment on Gsα NA levels in A10 smooth muscle cells. Total RNA from A10 oth muscle cells incubated in the absence (control) or presence of ous concentrations of angiotensin II (AII) (10^{-9} and 10^{-5} M) was ected to 1% agarose gel electrophoresis and transferred to nylon abranes. The blots were then probed with full-length radiola- d Gsα cDNA probe as described under Materials and Methods. autoradiograms are representatives of three separate experi- ts.

ect of AII Treatment on Gi Protein Functions

o investigate whether the augmentation of Giα2 l Giα3 levels by AII treatment is also reflected in Gi ctions, the receptor-dependent functions were ex- ined by studying the effects of AII and C-ANP_{4-23}, ich have been reported to inhibit adenylyl cyclase ough Gi proteins (18, 20, 51, 52) on adenylyl cyclase ivity in control and AII-treated A10 cells. As shown Fig. 6, AII and C-ANP_{4-23} inhibited adenylyl cyclase ivity by about 30 and 40%, respectively, in control) cells, which was completely attenuated by AII atment (10^{-7} M). The attenuation of AII- and ANP_{4-23}-mediated inhibition of adenylyl cyclase by was dependent on the concentration of AII used for treatment (Fig. 7). AII treatment of A10 cells at 10^{-8} M attenuated the C-ANP_{4-23}-mediated inhibition of adenylyl cyclase completely, whereas AII-mediated inhibition was attenuated by about 35%. However, at higher concentrations, C-ANP_{4-23}- and AII-mediated inhibition were completely attenuated. These results suggest that AII treatment causes the densensitization of both AII as well as ANP-C-receptor-mediated response.

In addition, the effect of AII treatment on receptor-independent Gi functions were also examined and the results are shown in Fig. 8. Lower concentrations of GTPγS inhibited FSK-stimulated adenylyl cyclase activity in a concentration-dependent manner in control cells, whereas the inhibition was attenuated in AII-treated cells. GTPγS at 10^{-10} M inhibited FSK-stimulated adenylyl activity by about 45% in control cells and about 20% in AII-treated cells. These results suggest that receptor independent Gi functions were also

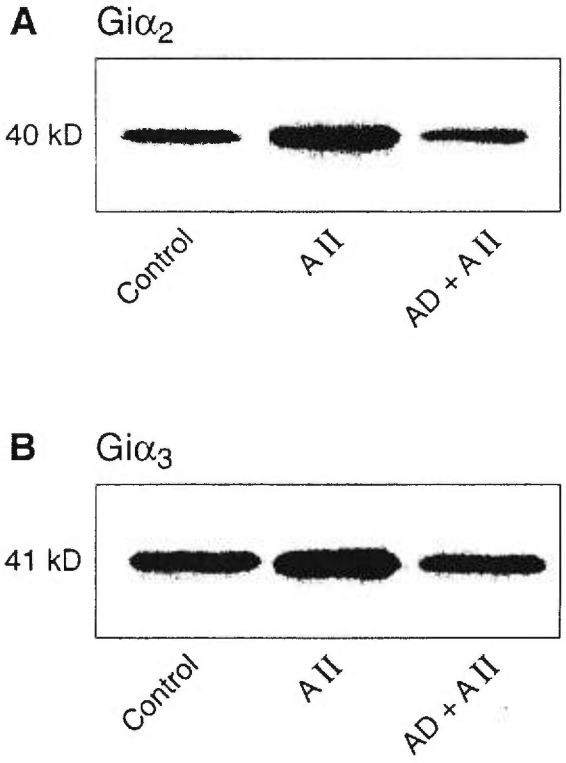


FIG. 5. Effect of actinomycin D on AII-evoked Giα protein levels. A10 smooth muscle cells were incubated in the absence (control) or presence of angiotensin II (AII) alone or in combination of actinomycin D (5 μM) for 24 h as described under Materials and Methods. Membranes were prepared as described under Materials and Methods. Membranes were used for immunoblotting. The membrane proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose, which was then immunoblotted using AS/7 antibody for Giα-1 and Giα-2 (A) or EC/1 antibody for Giα-3 (B), and were detected by using the ECL Western blotting technique as described under Materials and Methods. The autoradiographs are representative of three separate experiments.

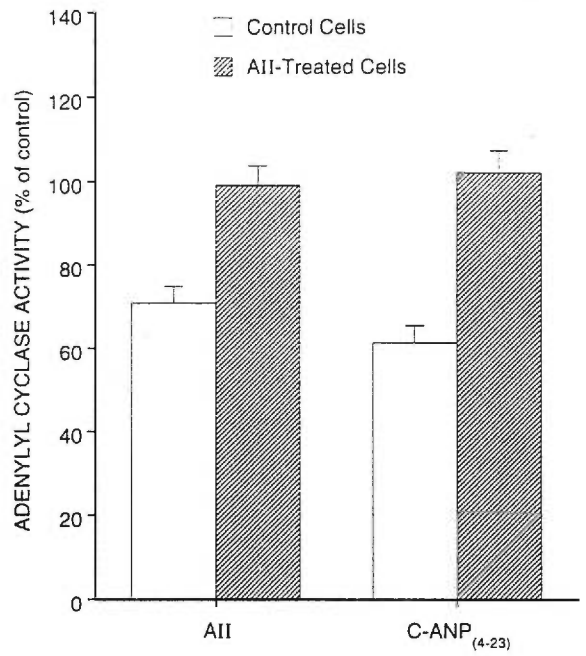


FIG. 6. Attenuation of AII- and C-ANP$_{4-23}$-mediated inhibition of adenylyl cyclase by AII pretreatment of A10 smooth muscle cells. A10 smooth muscle cells were incubated in the absence (□) or the presence of 10^{-5}M AII (▨) for 24 h as described under Materials and Methods. Membranes were prepared as described under Materials and Methods. Adenylyl cyclase activity was determined in the membranes in the absence (basal) or presence of 10 μM AII or 1 μM C-ANP$_{4-23}$ as described under Materials and Methods. Basal adenylyl cyclase activity in the presence of GTPγS in control membranes was 382.5 ± 18.5 and in AII-treated membrane was 223 ± 10.6 pmol cAMP/mg protein/10 min. Values are means ± SE of three separate experiments performed in triplicates.

inhibited but not completely abolished by AII treatment.

Effect of AII Treatment on Gs Functions

To corroborate our results of Gsα levels with Gsα functions, the effect of cholera toxin (CT) on the GTP-sensitive adenylyl cyclase was examined in control and AII-treated cells. CT treatment stimulated adenylyl cyclase activity in both control and AII-treated cells; however, the extent of stimulation was not significantly different in both groups [control cells, basal + GTP (10 μM) 150.5 ± 10.2, CT 2107 ± 130 (% stimulation = 1300 ± 120%, n = 4); AII-treated cells, basal + GTP 100.5 ± 7.1; CT, 1250 ± 55 pmol cAMP (mg protein · 10 min)$^{-1}$ (% stimulation = 1150 ± 135%, n = 4)]. These results indicate that Gs functions were also not altered by AII treatment.

AII has been shown to modulate the stimulatory responses of hormones on adenylyl cyclase in vascular smooth muscle cells and adrenal cortical cells (53, 54). To investigate if AII treatment of A10 cells for 24 h also results in the altered responsiveness of adenylyl cyclase to stimulatory hormones, we examined the effects of isoproterenol and NECA on adenylyl cyclase activity in control and AII-treated cells. As shown in Fig. 9, isoproterenol at 50 μM (A) and NECA at 10 μM (B) stimulated adenylyl cyclase activity by about 500 and 200% in control cells, which were inhibited by AII treatment in a concentration-dependent manner. At 10^{-5}M, isoproterenol- and NECA-mediated stimulations of adenylyl cyclase were inhibited by about 50 and 40%, respectively.

Effect of Protein Kinase C Inhibitor on AII-Evoked Attenuation of Hormone-Sensitive Adenylyl Cyclase Activity

Since AII also stimulates the PLC/PKC signal transduction pathway, we investigated the implication of PKC in AII-mediated attenuation of inhibitory and stimulatory inputs on adenylyl cyclase. For this, the effect of staurosporine, a potent inhibitor of protein kinase C, on AII-evoked inhibition of adenylyl cyclase

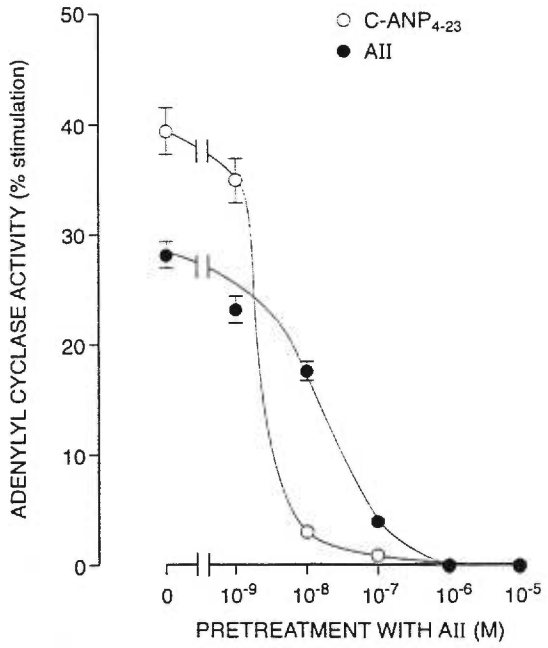
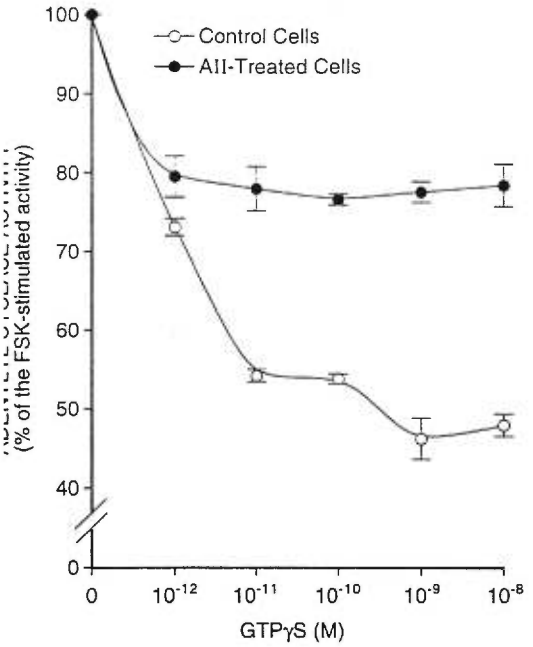


FIG. 7. Effect of various concentrations of AII pretreatment on AII- and C-ANP$_{4-23}$-mediated inhibition of adenylyl cyclase activity in A10 smooth muscle cells. A10 smooth muscle cells were incubated in the absence (control) or in the presence of various concentrations of angiotensin II (AII) (10^{-9} to 10^{-5} M) for 24 h as described under Materials and Methods. Membranes were prepared as described under Materials and Methods. Adenylyl cyclase activity was determined in the membranes in the absence (basal) or in the presence of 10 μM AII (●) or 1 μM C-ANP$_{4-23}$ (○) as described under Materials and Methods. Basal adenylyl cyclase activity in the presence of 10 μM GTPγS in control membranes was 382.5 ± 18 pmol cAMP/10 mg/min protein. Values are means ± SE of three separate experiments performed in triplicates.



Effect of GTPγS on FSK-stimulated adenylyl cyclase activ-
ıtrol and AII-treated A10 smooth muscle cells. A10 smooth
ells were incubated in the absence (control) or in the pres-
10^{-5} M angiotensin II (AII) for 24 h as described under
s and Methods. Membranes were prepared as described
aterials and Methods. Adenylyl cyclase activity was deter-
ı the membranes in the absence or presence of 100 μM
(FSK) alone or in combination with various concentrations
S as described under Materials and Methods. Basal enzyme
s in the absence and presence of 100 μM FSK were 62.4 ± 12
8.5 ± 65, respectively, in control cells and 69.0 ± 10 and
59 pmol cAMP (mg protein · 10 min^{-1}) respectively in AII-
cells. Values are means ± SEM of three separate experi-
erformed in triplicates.

amined and the results are shown in Fig. 10.
atment of the cells with 1 nM staurosporine for
ı did not alter basal adenylyl cyclase activity
255 ± 10 pmol (mg protein · 10 min^{-1}) stauro-
e (1 nM); 240 ± 5 pmol cAMP]; however, it at-
ed completely the AII-evoked AII and C-ANP_{4-}
ated inhibitions as well as isoproterenol- and
-mediated stimulations of adenylyl cyclase activ-

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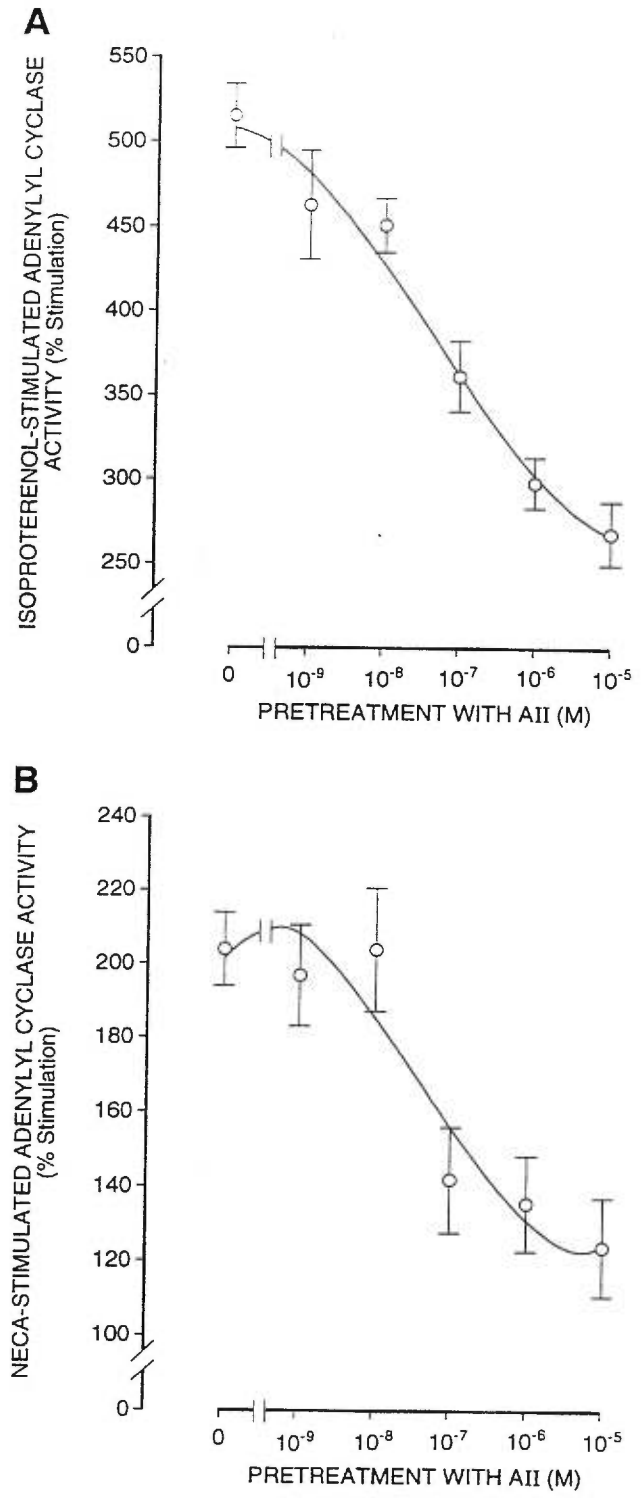
present studies demonstrate that AII treatment
cells for 24 h enhanced the expression of Giα-2
α-3 proteins, whereas the expression of Gsα was
ıged. These results are in agreement with the
eported earlier (55), where systemic infusion of
sulted in the augmented levels of Giα-2 and
proteins in glomerular and mesenteric vascular
ı muscle membranes. The levels of Giα-2 and
nRNA were also increased by AII treatment but not to the same extent as that of proteins. The reason for the lack of correlation between AII-mediated increases in Gi mRNA and protein levels is not clear. However, it may be possible that AII, in addition to regulate Gi expression at transcriptional level, also exerts its effects at the translational level and may involve an increased efficiency of translation of Gi protein mRNA or a decreased degradation rate of Gi proteins. The increased levels of Giα-2 and Giα-3 mRNA by AII may not be due to the variation in the amounts of total RNA loaded in individual samples applied to the gels, because of the fact that the hybridization with an oligonucleotide that recognizes a highly conserved region of the 28 S RNA shared a similar amount of 28S RNA loaded from control and AII-treated cells (data not shown). These results suggest that the genes for Giα-2 and Giα-3 enhanced by AII treatment may be responsible for the observed increase in protein levels. In addition, the inhibition of AII-evoked enhanced expression of Giα-2 and Giα-3 proteins by actinomycin D further supports that AII-mediated increases in Giα proteins are at transcriptional levels.

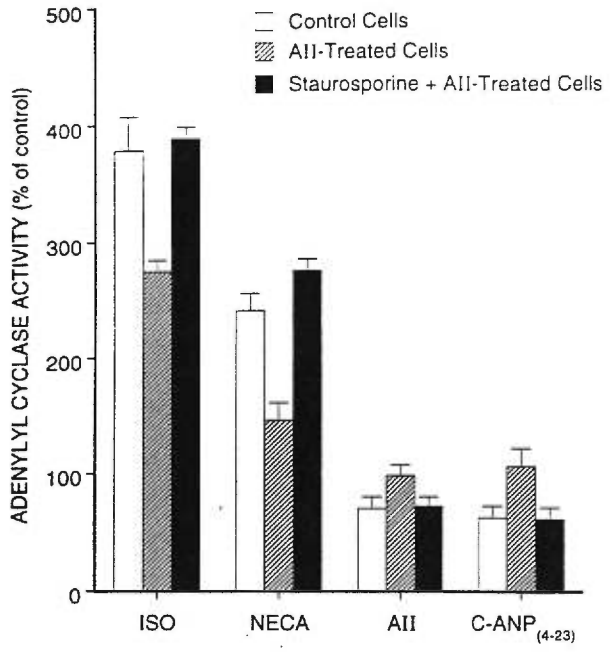
A relationship between the levels of Gi protein and functions has been reported by several investigators (35, 56). An increased levels of Giα proteins and increased responsiveness of adenylyl cyclase to ANP, oxotremorine, and AII inhibition in aorta and heart from SHR and DOCA–salt hypertensive rats has recently been shown (35, 36). Similarly decreased levels of Giα-2 and complete attenuation of ANP-receptor-mediated inhibition of adenylyl cyclase in platelets from SHR has also been reported (56). However, in the present studies, AII-evoked increased expression of Giα-2 and Giα-3 was not reflected in increased Gi functions; however, on the other hand, the receptor-independent Gi functions were inhibited to some extent and $C\text{-}ANP_{4\text{-}23}$ and AII receptor-mediated inhibitions of adenylyl cyclase were completely attenuated by AII treatment. Several mechanisms may be involved in the observed attenuation of adenylyl cyclase inhibition by $C\text{-}ANP_{4\text{-}23}$ and AII. One of the possibilities may be that AII treatment for 24 hrs has resulted in the downregulation of ANP-C as well as AII receptors in A10 cells also as has been reported in vascular smooth muscle cells (57, 58). Alternatively the Gi-protein levels although enhanced by AII treatment may have been phosphorylated by AII-mediated activation of PKC, another signalling pathway of AII action. The phosphorylation and thereby inactivation of Gi protein may therefore result in the uncoupling of receptors from adenylyl cyclase and thereby a complete attenuation of ANP-C- and AII-receptor-mediated inhibition of adenylyl cyclase. The phosphorylation and thereby inactivation of Giα proteins and the uncoupling of inhibitory hormone receptors from adenylyl cyclase by PKC has been reported (23). Taken together, it may be pos-



G. 9. Effect of various concentrations of angiotensin II pretreat-ent on isoproterenol- and NECA-mediated stimulation of adenylyl clase activity in A10 smooth muscle cells. A10 smooth muscle cells re incubated in the absence (control) or in the presence of various ncentrations of angiotensin II (AII) (10^{-9} to 10^{-5} M) for 24 h as scribed under Materials and Methods. Membranes were prepared described under Materials and Methods. Adenylyl cyclase activity as determined in the membranes in the absence (basal) or in the presence of 50 μM isoproterenol (A) or 10 μM NECA (B) as described under Materials and Methods. Basal adenylyl cyclase activity in control membranes in the presence of 10 μM GTP was 207 ± 18 pmol cAMP/10 min/mg protein. Values are means ± SE of three separate experiments performed in triplicates.

sible that the observed attenuation of ANP-C- and AII-receptor-mediated inhibition of adenylyl cyclase caused by AII treatment may be attributed to the downregulation of the receptors or/and to the phosphorylation of Giα protein, which may be responsible for the uncoupling of these receptors from adenylyl cyclase.

Furthermore, the diminished responsiveness of adenylyl cyclase to GTPγS stimulation by AII treatment may not be attributed to Gsα protein, because the levels as well as functions of Gsα were not altered by AII treatment. In addition, the AII-evoked decreased stimulation of adenylyl cyclase by GTPγS may not be explained by the augmented levels of Giα-2 and Giα-3 because the functions of Giα were attenuated and not enhanced by AII treatment. However, the contribution of the βγ subunit in eliciting the diminished stimulation of adenylyl cyclase by guanine nucleotides cannot be ignored, since the βγ subunit has been reported to inhibit adenylyl cyclase directly (59) or by interacting with and inactivating Gsα-GTP (60). In addition, our data showing the attenuation of NECA- and isoproterenol-stimulated adenylyl cyclase activity by AII treatment are not in agreement with the results of other investigators who have shown the potentiation of the stimulatory effects of isoproterenol and adenosine by AII on cAMP levels in vascular smooth muscle cells (53) and of ACTH in bovine adrenal cortical cells (54). These discrepancies may be attributed to the difference in the cell type (A10 cells versus vascular smooth muscle and adrenal cortical cells) as well as in the duration of treatment of the cells with AII (1 min and 1 h exposure of vascular smooth muscle cells and adrenal cortical cells, respectively, compared to 24 h in our studies). However, the fact that ACE inhibitor captopril reverses the diminished isoproterenol-mediated stimulation of adenylyl cyclase activity in SHR (37) and enhances β1 adrenergic receptor density in patients with idiopathic dilated cardiomyopathy (61) implicates the inhibitory input of AII on β-adrenergic-stimulated adenylyl cyclase activity and β-adrenergic receptors. Thus, taken together, it may be possible that AII-induced attenuated responsiveness of NECA and isoproterenol to stimulate adenylyl cyclase may not be attributed to the enhanced levels of Gi proteins and may be due to the downregulation of adenosine and β-adrenergic receptors. Or, alternatively, adenosine and β-adrenergic receptors may be phosphorylated by PKC, which may result in the uncoupling of receptors



G. 10. Effect of staurosporine on AII-evoked attenuation of stim-ıtory and inhibitory effects of hormones on adenylyl cyclase activ- in A10 smooth muscle cells. A10 smooth muscle cells were incu-ted in the absence (control) or presence of 10^{-7} M angiotensin II II) alone or in combination with staurosporine (10^{-9} M) for 2 h as scribed under Materials and Methods. Membranes were prepared described under Materials and Methods. Adenylyl cyclase activity ıs determined in the presence of 10 μM GTPγS alone (basal) or in nbination with 1 μM C-ANP$_{4-23}$ or 10 μM AII or 10 μM GTP alone ısal) or in combination with 50 μM isoproterenol (ISO) or 10 μM ethylcarboxamide adenosine (NECA) in control (□), AII-treated), and AII + staurosporine-treated (■) vascular smooth muscle ls as described under Materials and Methods. Basal adenylyl clase activities in the presence of 10 μM GTPγS or 10 μM GTP re 432 ± 15 and 197.5 ± 5.4 pmol cAMP (mg protein · 10 min^{-1}), spectively, in control cells, 269 ± 12 and 227 ± 3 pmol cAMP (mg otein · 10 min^{-1}), respectively, in AII-treated cells, and 227.8 ± 10 d 239 ± 13 pmol cAMP (mg protein 10 min^{-1}), respectively, in I + staurosporine-treated cells. Values are means ± SE of four parate experiments performed in triplicates.

om adenylyl cyclase. The phosphorylation of β-adrengic receptor by PKC and its uncoupling from adenylyl clase has also been reported earlier (62). This notion further substantiated by our studies showing that aurosporine, a PKC inhibitor, resulted in the restotion of AII-induced attenuated isoproterenol- and lenosine-stimulated adenylyl cyclase activity.

In conclusion, we have shown that the treatment of 10 cells with AII enhanced the levels of Giα-2 and iα-3 proteins and mRNA and not of Gsα. The enıced levels of Giα proteins were not associated with creased Gi functions. These effects of AII treatment pear to be dependent on the PKC activation and ggest a cross-talk between two signaling pathways. om these results, it can also be suggested that AII, ıose levels are increased in hypertension, may be one of the possible contributing factors responsible for exhibiting an enhanced expression of Giα protein in hypertension.

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