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The Role of the Intracellular Domain and Small Peptide Fragments of NPR-C Receptor in Adenylyl Cyclase Signaling

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The Role of the Intracellular Domain and Small Peptide Fragments of NPR-C Receptor in Adenylyl Cyclase Signaling

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Memoire accepté le: Date_____

To my family, and

To Diane,

who have provided support and love.

To my future,

For which my efforts mean.

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RESUME

Le peptide natriurêtique auriculaire (ANP) régule une variété de paramètres physiologiques incluant la pression sanguine, le volume intravasculaire, la secretion de progésterone ainsi que la libération de rénine, en interagissant avec différents récepteurs présents sur la membrane plasmique. Il existe 3 sous-types des récepteurs de l'ANP : le récepteur NPR-A,-B et le NPR-C. Les récepteurs NPR-A et NPR-B sont des récepteurs couplés à la guanylate cyclase alors que les récepteurs NPR-C sont couplés à l'inhibition de l'adénylate cyclase ou à l'activation de la phopholipase C par l'intermédiare d'une protéine G inhibitrice. Le récepteur NPR-C est un homodimère disulfurique possédant un domaine extracellulaire composé d'environ 440 acides amines (aa), un seul domaine transmembranaire et un petit domaine cytoplasmique constitué de 37 acides amines. Des études effectuées dans notre laboratoire ont demontré que le peptide de 37 acides amines correspondant au domaine cytoplasmique du récepteur NPR-C inhibe l'activité de l'adénylate cyclase par l'entremise d'une protéine Gi sensible à la toxine pertussique. Dans cette présente étude, nous avons utilisé sept fragments peptidiques différents du domaine cytoplasmique du récepteur NPR-C avec une completè, partielle ou aucune séquence activatrice de Gi afin d'examiner leurs effets sur l'activité de l'adénylate cyclase. Les peptides employées sont : KKYRITIERNH (#1), RRNHQEESNIGK (#2), HRELREDSIRSH (#3), RRNHQEESNIGKHRELR (#4), QEESNIGK (X), ITIERRNH (Y), ET ITIYKKRRNHRE (Z). Les peptides #1,#3 et #4 possédent

des sequences complètes activatrices de Gi tandis que les peptides #2 et Y contiennent des sequences partielles activatrices dont les extrémités carboxyle ou amine ont été respectivement tronquées. Le peptide X ne détient aucune specificité structurale alors que le peptide Z est le peptide mêlé du peptide contrôle #1. Les peptides #1,#3 et #4 inhibent l'activité de l'adénylate cyclase en fonction de la concentration avec un Ki situé entre 0.1 et 1 nM. Cependant, le peptide #2 supprime l'activité de l'adénylate cyclase avec un Ki plus enlevé qui est d'environ 10nM tandis que les peptides X,Y,et Z ont été incapables d'inhibiter l'activité de l'adénylate cyclase. Les inhibitions maximales observées étaient entre 30-40%. L'inhibition de l'activité de l'adénylate cyclase par les peptides #1-4 nécessite la présence de nucleotides guanines et elle est complétement attenuée par les traitements à la toxine pertussique. De plus, les effets stimulateurs du isoprotérenol, du glucagon et de la forskoline sur l'activité de l'adénylate cyclase ont été inhibés à différents degrés par ces peptides. Les résultats suggèrent que les peptides formés à partir des fragments peptiques du domaine cytoplasmique du récepteur NPR-C contenant 12 ou 17 acides aminés sont suffisants pour inhiber l'activité du l'adénylate cyclase par l'intermédiarè d'une protéine Gi sensitive à la toxine pertussique. Les peptides ayant une complète séquence spécifique aux séquences activatrices de Gi sur les extremités amine et carboxyle ont été des inhibiteurs de l'activité de l'adénylate cyclase plus puissants comparativement aux peptides ayant une extremité carboxyle tronquée, alors que l'extremité amine tronquée atténue complètement l'inhibition de l'adénylate cyclase.

Mots Clés : : adénylate cyclase, peptide natriurêtique auriculaire (ANP), protéine G inhibitrice, récepteur NPR-C

SUMMARY

Atrial natriuretic peptide (ANP) regulates a variety of physiological parameters, including blood pressure and intravascular volume, progesterone secretion, and renin release by interacting with different receptors present on the plasma membrane. ANP receptors three subtypes are: NPR-A,-B and NPR-C receptors. NPR-A and NPR-B receptors are guanylyl cyclase receptors, whereas NPR-C receptors are coupled to adenylyl cyclase inhibition or phospholipase C activation through inhibitory guanine nucleotide-regulating protein. NPR-C receptor is a disulfide linked homodimer with an approximately 440-amino acid (aa) extracellular domain, a single transmembrane domain and a short cytoplasmic domain of 37 amino acids. Previous studies in our lab demonstrated that a 37-amino acid peptide corresponding to the cytoplasmic domain of the NPR-C receptor inhibited adenylyl cyclase activity via pertussis toxin (PT)-sensitive Gi protein. In the present studies we have used seven different peptide fragments of the cytoplasmic domain of the NPR-C receptor with complete, partial or no Gi-activator sequence to examine their effects on adenylyl cyclase activity. The peptides used were KKYRITIERRNH (#1), RRNHQEESNIGK- (#2), HRELREDSIRSH-(#3), RRNHQEESNIGKHRELR-(#4), QEESNIGK-(X) ITIERRNH-(Y) and ITIYKKRRNHRE (Z). Peptides #1, #3 and #4 have complete Gi-activator sequences, whereas peptides #2 and Y have partial Gi-activator sequences with truncated carboxy or amino terminal respectively. Peptide X has

no structural specificity whereas peptide Z is the scrambled peptide control for peptide #1. The peptides #1, #3 and #4 inhibited adenylyl cyclase activity in a concentration-dependent manner with apparent Ki between 0.1 and 1 nM, However, peptide #2 attenuated adenylyl cyclase activity with a higher Ki of about 10 nM and peptides X, Y and Z were unable to inhibit adenylyl cyclase activity. The maximal inhibitions observed were between 30-40%. The inhibition of adenylyl cyclase activity by peptides #1-4 was absolutely dependent on the presence of guanine nucleotides and was completely attenuated by pertussis toxin (PT) treatment. In addition, the stimulatory effects of isoproterenol, glucagon and forskolin on adenylyl cyclase activity were inhibited to different degrees by these peptides. These results suggest that the small peptide fragments of the cytoplasmic domain of the NPR-C receptor containing 12 or 17 amino acids were sufficient to inhibit adenylyl cyclase activity through a PT-sensitive Gi-protein. The peptides having complete structural specificity of Gi-activator sequences at both amino and carboxyl terminals were more potent to inhibit adenylyl cyclase activity as compared to the peptides having a truncated carboxyl terminal, whereas the truncation of the amino terminal motif completely attenuated adenylyl cyclase inhibition.

KEY WORDS: adenylyl cyclase, Gi-protein, atrial natriuretic peptide (ANP), Natriuretic peptide C-receptor (NPR-C)

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LIST OF ABBREVIATIONS

ADC	Adenylyl Cyclase
ANP	Atrial natriuretic peptides
ANP-A,B,C	Atrial natriuretic peptide type A,B,C
BNP	Brain natriuretic peptides
CNP	C-Type natriuretic peptides
cAMP	Cyclic adenosine monophosphate
C-ANF$_{4,23}$	Ring-deleted analogue of atrial natriuritic
cDNA	Complementary deoxyribonucleic acid
cGMP	Cyclic guanine monophosphate
CT	Cholera Toxin
DAG	diacylglycerol
EDRF	endothelium-derived relaxing factor
FSK	Forskolin
G protein	heterotrimeric guanine nucleotide-binding regulatory protein
GC	Guanlylate Cyclase System
GC-A/B	Alternate name for NPR-A/B receptor class
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'-3-O-(thio)triphosphate
IP3	1,4,5-Inositoltriphosphate
ISO	Isoproternol
L-NAME	N^{w}-nitro-L-arginine
MAP	Mitogen activated protein
MAPK	Mitogen activated protein kinase
MEK	MAPK/ Extracellular signal related kinase
NaF	Sodium Flouride
NECA	5'-N-Ethyl-carboxamido-adenosine
NPR-A,B,C	Natriuretic peptide receptor A,B,C
PI-3	Phosphatidylinositol 3-OH
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PT	Pertussis Toxin
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
VSMC	Vascular smooth muscle cells

CHAPTER 1

INTRODUCTION

Review of Literature

I. Atrial Natriuretic Peptide

A. Introduction

The Atrial Natriuretic Peptide (ANP) was discovered by deBold et al (1981,1982). ANP belongs to the family of natriuretic peptides; BNP and CNP make up the other members of the family. When discovered, ANP, was believed to be the much sought out plasma natriuretic substance, but further research proved it to have many physiological responses. ANP is synthesized primarily as a pre-prohormone in atrial granules (Gardner et al.,1981) (Figure #1). It is then cleaved to a pro-hormone of 126 amino acids by enzymatic cleavage. The carboxy terminal of 28 amino acids represents the principal circulating form of ANP (Thibault et al.,1985; Glembotski et al., 1988). The amino terminal fragment is of 98 amino acids and is also present in circulation. The carboxy terminal is responsible for the physiological responses observed by the ANP. A derivative of this carboxy fragment and the major circulating form is designated as $ANP_{(99,126)}$ (Figure #2). The amino terminal $ANP_{(1-98)}$ fragment is processed into $ANP_{(1-30)}$ and $ANP_{(31-67)}$ fragments, which also posses biological activity, but information regarding their biological significance is limited to the fact that they activate the Guanlylate Cyclase (GC) system , promote hypotension and natruresis, and are vasodilators (Winters et al.,1988)

The other members of the natriuretic peptide family are brain natriuretic peptide (BNP) (Sudoh et al., 1988) and C-type natriuretic peptide (CNP) (Sudoh et al.,1990).

The role played by ANP and BNP as endocrine hormones is apparently to be antagonists to vasopressin, endothelins and the renin-angiotensin-aldosterone system. (Brenner et al., 1990; Ruskoaho, 1992).

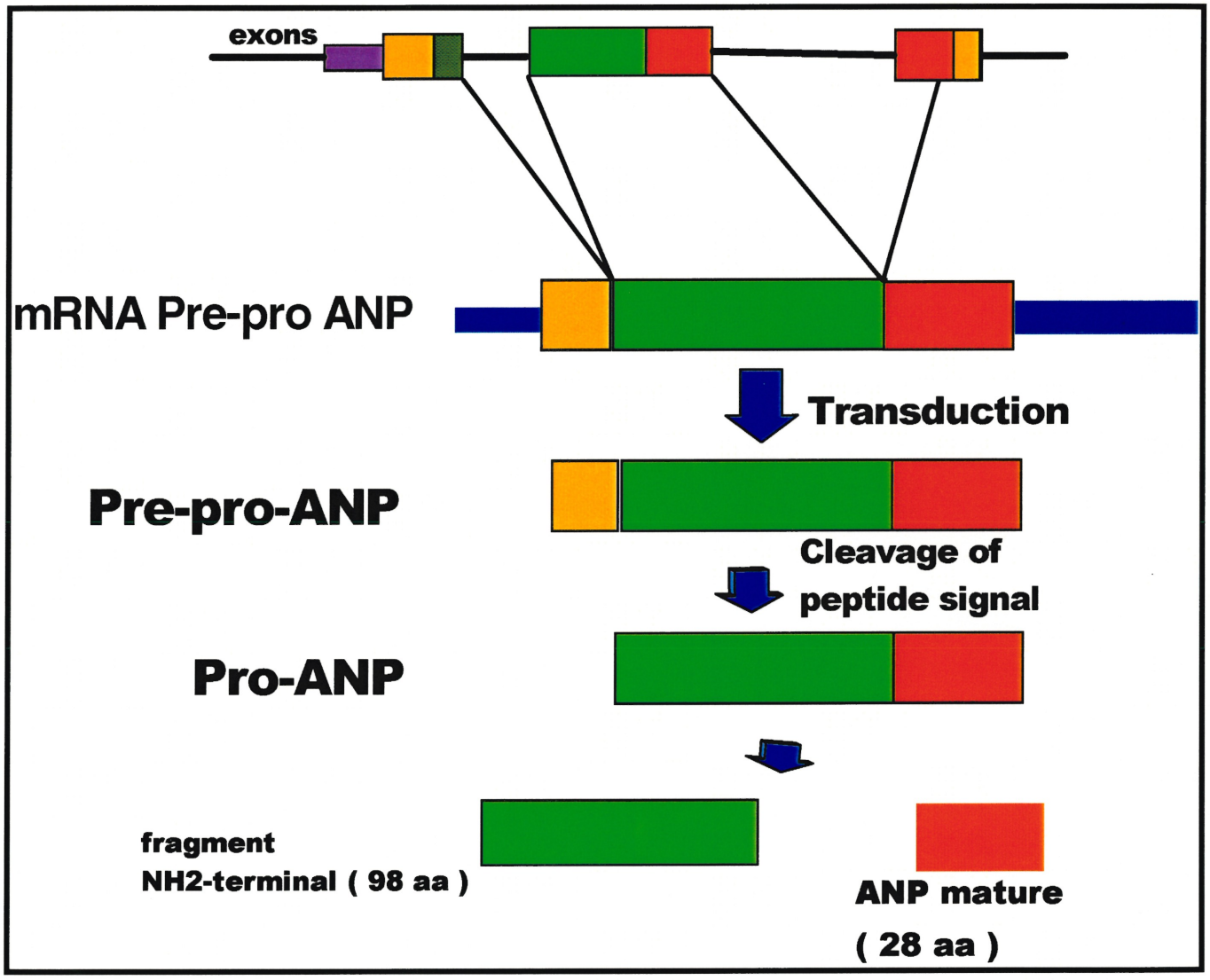
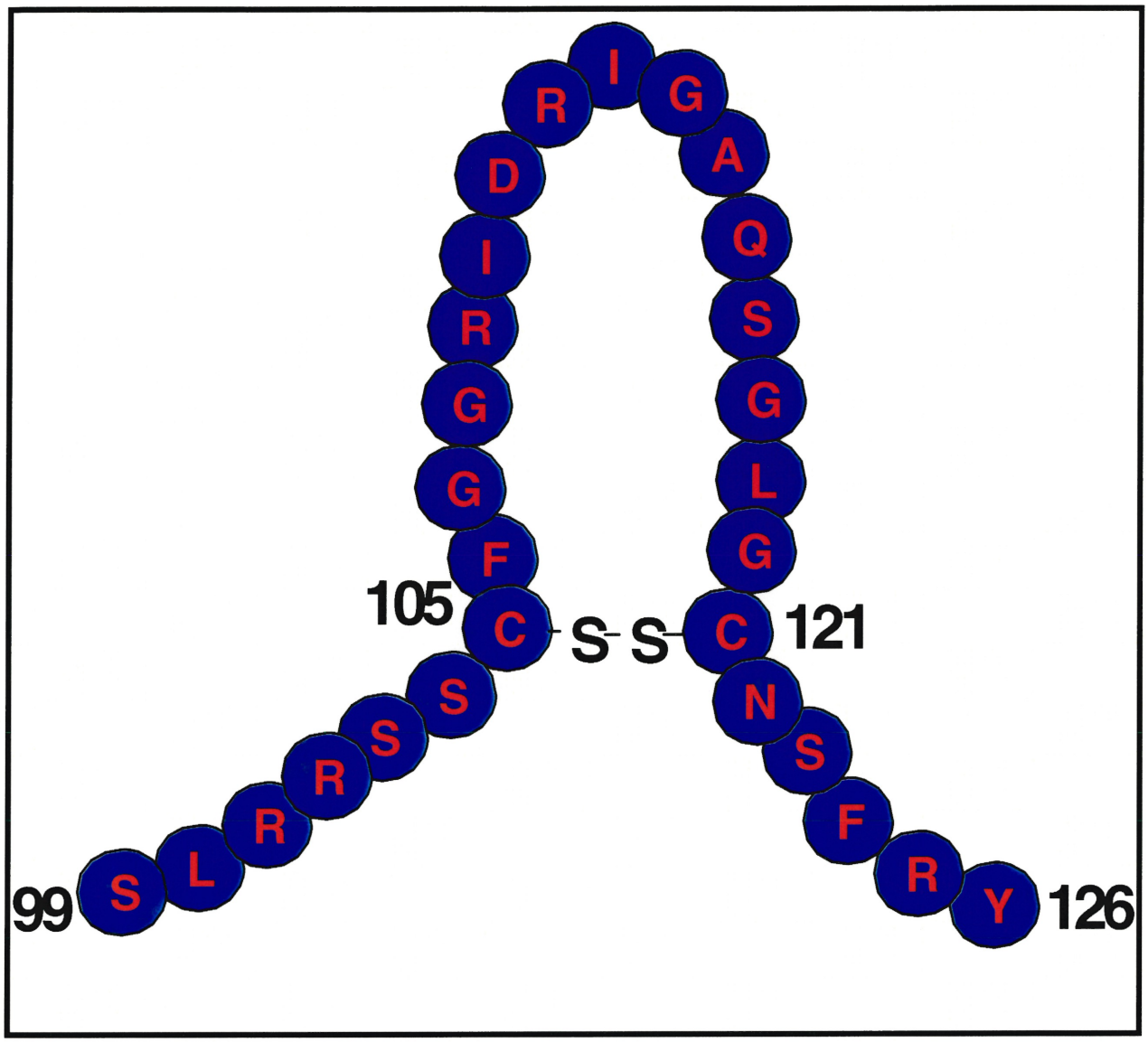


Figure #1: Synthesis of Atrial Natruretic Peptide (ANP)



MAJOR CIRCULATING FORM: $ANP_{(99\text{-}126)}$

Figure #2: Structure of ANP

The role of CNP is less well defined. Although CNP might not be a significant modulator of diuresis and natruresis (Stingo et al., 1992; Clavell et al., 1993), it is a vasodilator expressed by endothlial cells (Sudoh et al., 1990; Suga et al., 1992). Compared with ANP, BNP has an additional six amino acid sequences at its amino terminal end (Kambayashi et al. 1990; Sudoh et al., 1988) whereas CNP lacks the carboxy terminal (Sudoh et al., 1990).

B. Physiological Responses and Signal Transduction Mechanisms

B.1. Introduction

ANP promotes physiological responses by interacting with receptors on the plasma membrane. This interaction results in transmission of signals to effector systems such as adenylyl cyclase, guanylyl cyclase and phospholipase C systems to generate second -messenger molecules such as cyclic AMP (cAMP), cyclic GMP (cGMP) and IP3 respectively. ANP also affects sodium, potassium and calcium channel conductance leading to physiological responses. The primary effects of ANP are perceived to involve actions on: vasculature, kidney, adrenal, heart, lung, endocrine organs, neurons and platelets.

B.2. Overview of Signal Transduction Mechanisms

ANP mediates it's various physiological responses using adenylyl cyclase, guanylyl cyclase , phospholipase C signal transduction systems. ANP is coupled to the guanylate cyclase system via a R1/ANP-A, ANP-B (NPR-A, NPR-B) receptor. ANP

increases the concentration of cGMP, which in turn activates protein kinases, which give varied responses. Also it can suppress the levels of PLC activity, which reduces levels of IP3 and diacylglycerol (DAG) production and alter the permeability to ions.

ANP is coupled the adenylyl cyclase system via an R2/ANP-C (NPR-C) receptors and Gi proteins. ANP decreases the levels of cAMP or activates PLC activity increasing levels of IP3 and DAG. (Figure #3)

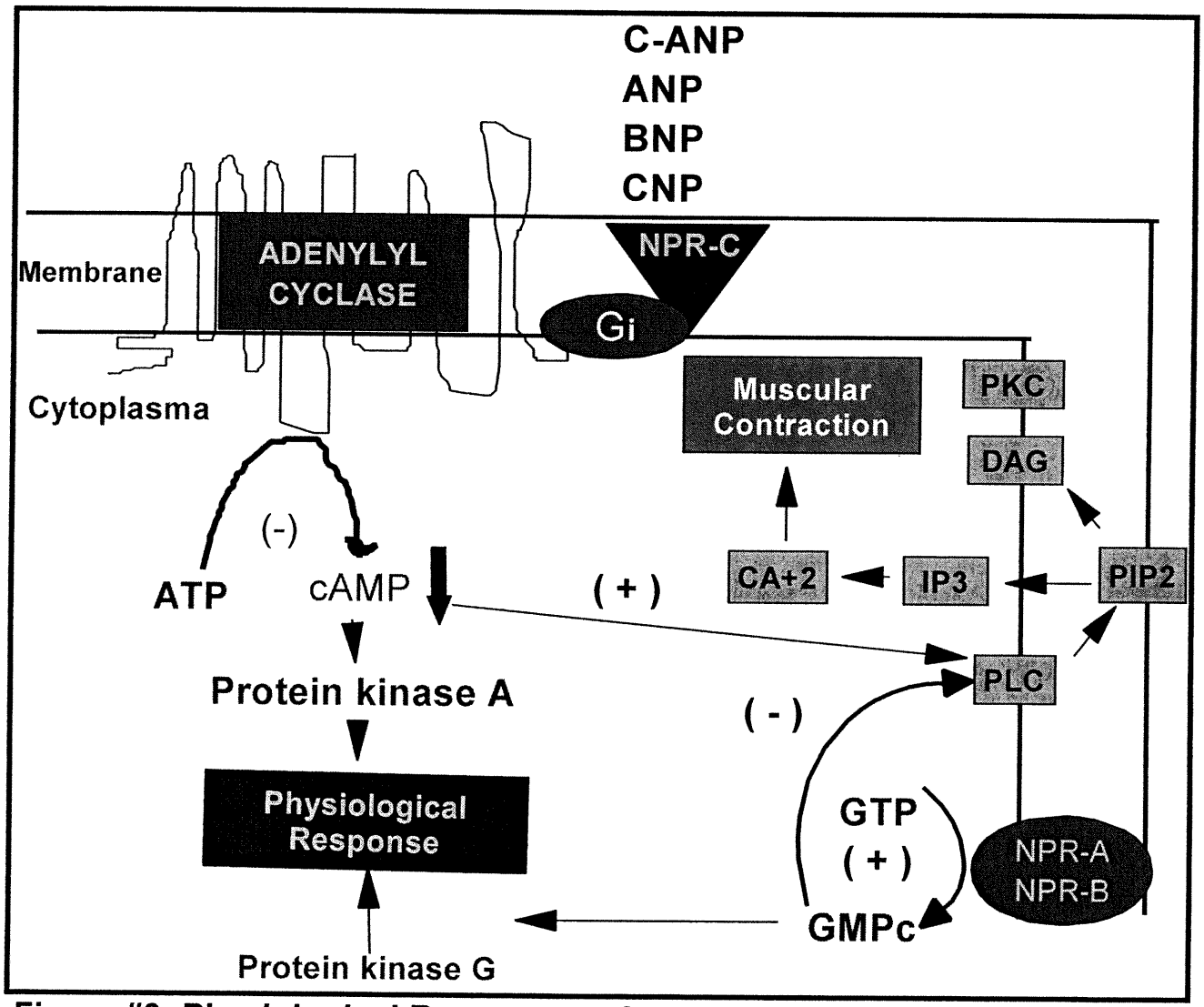


Figure #3: Physiological Responses of the ANP

C.1 Introduction

The ANP-R1 are guanylyl cyclase-coupled receptors and have a relative molecular mass of 130-180 kDA, whereas ANP-R2/ANP-C receptors exist as monomers (66KDa) and dimers (130kDa). Molecular cloning techniques hasrevealed three subtypes of natruretic peptide receptors (NPR), (Figure #4). NPR-A (Chinkers et al., 1989; Lowe et al., 1989), NPR-B (Schulz et al., 1989; Chang et al., 1989) and NPR-C (Fuller et al., 1988; Anand-Srivatava et al., 1987). NPR-A and NPR-B are membrane guanylyl cyclases, whereas NPR-C (clearance receptors) also known as ANP-R2 and ANP-C receptors, are coupled to adenylyl cyclase inhibition through inhibitory guanine nucleotide-regulating protein. (Anand-Srivatava et al., 1989,1990) or to the activation of phospholipase C (Hirata et al., 1989). (Figure # 3)

C.2. NPR-A, NPR-B (ANP-A,ANP-B) Receptors

C2.1. Overview

Natriuretic peptides receptors A and B are linked to the cGMP- dependent signaling cascade and mediate many of the cardiovascular and renal effects of the

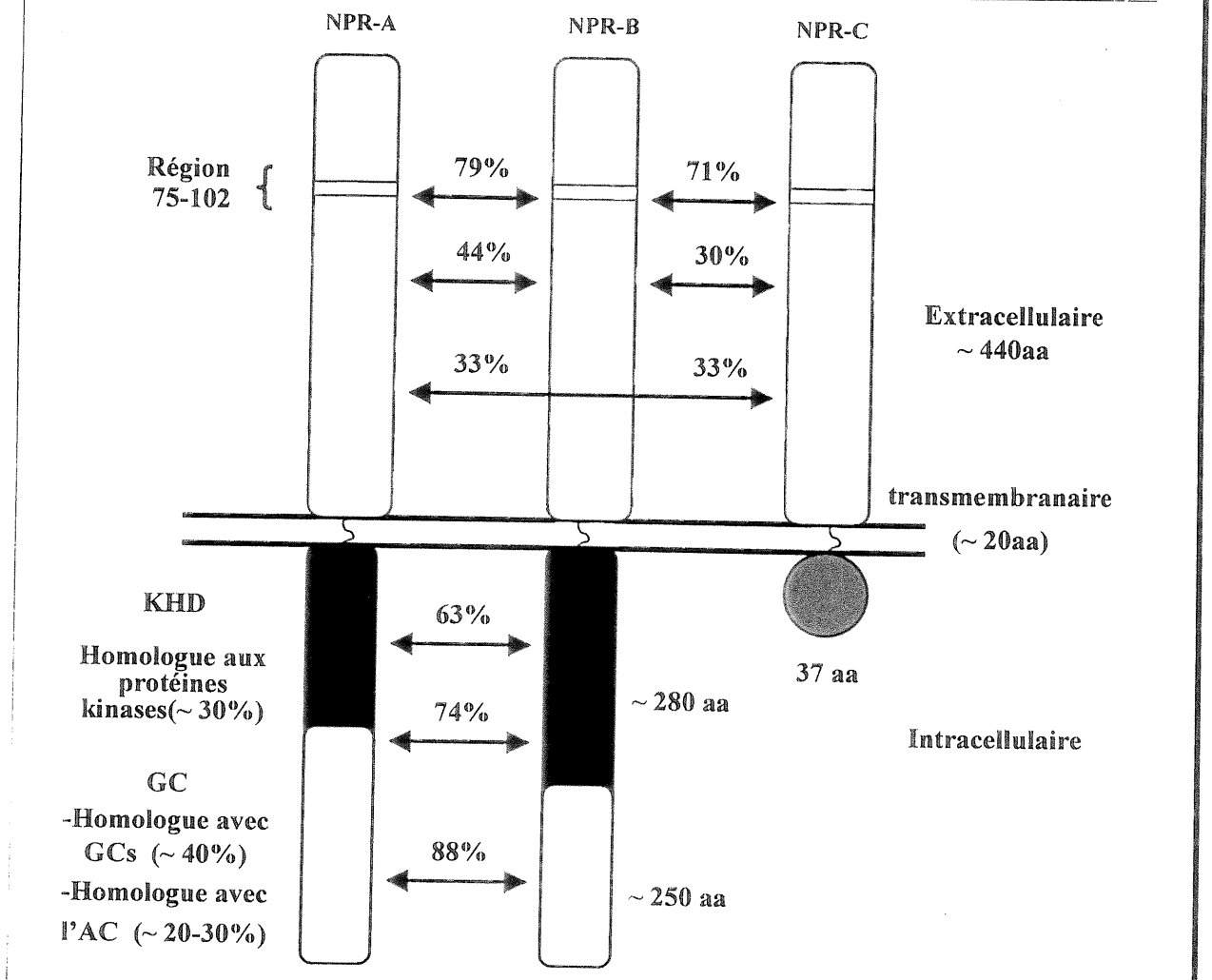


Figure #4: Representation of ANP's three types of receptors and the % homology seen between them.

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natriuretic peptides. Natriuretic peptide receptors A and B are structurally similar, with approximate 44% homology in the ligand-binding extracellular domain. The A receptor binds both atrial and brain natriuretic peptides, with preference for atrial natriuretic peptide. C-type natriuretic peptide is the natural ligand for the B receptor. The A receptor is the most abundant type in large blood vessels, but there are also some B

receptors. The B receptors predominate in the brain. Both receptors are present in the adrenal glands and the kidney. In both A and B receptors, the extracellular portion is linked to the intracellular portion by a single membrane-spanning segment. The intracellular portion contains a kinase-like domain, followed by the guanylyl cyclase catalytic domain. Binding of the natriuretic peptides to their receptors activates guanylyl cyclase, leading to an elevation in intracellular cGMP.

C.2.2. Structure

Initial studies of ANP indicated a homogeneous population of binding sites by Scatchard analysis (Ballerman et al., 1985; Schiffrin et al., 1985). After ANP was recognized as a GC stimulant, comparisons of ANP binding and cGMP production were performed (Hamet et al., 1984; Waldman et al, 1984; Winquist et al., 1984). Truncated analogs of ANP failed to stimulate GC but bound to ANP receptors with the same affinity as native ANP (Scarbourough et al., 1986; Leitman et al., 1988; Leitman and Murad, 1986). Specifically, ANP(103-123) bound to ANP receptors but was a poor stimulator of GC (Leitman et al., 1986), leading to the interpretation that diverse ANP receptors exist in most tissues. The ANP receptor coupled to GC was proposed to be present in low concentrations and to have a low affinity for ANP(103-123). Conversely, the most abundant receptor bound ANP(103-123) with high affinity but did not couple to GC.

Schenk et al. (1985) identified two binding sites for ANP in bovine aortic smooth muscle with molecular masses of 180 000 and 66 000 Da. Leitman et al. (1986) demonstrated that truncated ANP analogs selectively bound to the 66 000 Da site,

indicating that the GC-coupled receptor was of high molecular mass. However, the higher molecular mass ANP receptor was identified as a 130 00 Da receptor in their study and most subsequent studies. Furthermore, ANP binding and GC activity copurified during the course of GC purification, suggesting that ANP bound to a protein containing GC enzyme activity (Kuno et al., 1986). Ultimately, Chinkers et al. (1989) transfected COS-7 cells with brain GC cDNA and demonstrated increased GC activity and ANP binding following transfection. The cDNA encoded a protein with a predicted molecular mass of 115,852 Da, consistent with the electrophoretic results predicting a molecular mass of 120 000 to 180 000 Da. The data of Chinkers et al. (1989) have been recognized as the strongest evidence indicating that ANP binds to a receptor containing a GC moiety. This combination of an ANP receptor binding protein and GC has subsequently been designated GC-A or the NPR-A receptor. The binding of ANP to this receptor is half-maximal at concentrations of 100 to 1000 pM, whereas half-maximal activation of GC usually requires concentrations of 1 to 10 nM ANP. Thus, the binding does not correlate with cGMP production, although, they are presumably mediated by the molecule. The reason for the differing potencies of ANP on these two events, which presumably are mediated by the same molecule, may involve dephosphorylation of the GC, resulting in desensitization to stimuli (Potter and Garbers, 1992).

The gene encoding rat GC-A contains 17.5 kilobases (Yamaguchi et al., 1990) consisting of 22 exons and 21 introns. The first six exons encode the extracellular region of GC-A, accounting for ANP binding. This region contains considerable

homology with the extracellular regions of other ANP receptors. The membrane-spanning domain is encoded by exons 8 to 15, with GC domain encoded by exons 16 to 22.

Another ANP receptor coupling to GC subsequently was identified in human placental tissue and rat brain (Chang et al., 1989; Schulz et al., 1990). This receptor bound ANP but had a higher affinity for either natriuretic peptide (Schulz et al., 1990). This receptor was termed ANP B or GC-B, as opposed to the ANP A receptor described above. The GC-B was cloned and had 63% overall homology with GC-A and 74 to 78% intracellular homology (Chang et al., 1989; Schulz et al., 1990). The predicted molecular mass was 114 952 Da, similar to the GC-A receptor. The poor affinity of the GC-B for either ANP or BNP was inconsistent with a functional role for this receptor in mediating natriuretic effects of either peptide (Schulz et al.,1990). Another ANP-like peptide, CNP (Eguchi et al., 1992), stimulated GC activity of the GC-B receptor more potently than either ANP or BNP, suggesting that CNP is its natrural ligand. The distribution of GC-A and GC-B differed depending on the tissue. The GC-A preponderated in renal tissue, whereas the GC-B was the most abundant form in human fetal brain and porcine atrium (Chang et al., 1989).

C.2.3. Signaling and Physiological Responses

Guanylate Cyclase (GC) is an enzyme that catalyses the conversion of GTP to cGMP, resulting in the phosphorylation of specific proteins through the activation of cGMP dependent protein kinases. These phosphorylated proteins mediate physiological responses to activation of the enzyme. The GC enzyme is located in different cellular compartments and the different number of isoforms is unknown. GC's are divided into two major general categories, soluble and particulate. The soluble GC is composed of two heterodimers with masses of approximately 70 000 and 80 000 Da. (Goy,1991). The soluble GCis activated by nitrovasodilators, nitric oxide, and free radicals (Waldman and Murad, 1987). This enzyme is believed to mediate responses to a variety of vasodilators, such as acetylcholine, after the vasodilators augment the production of an EDRF, probably nitric oxide (Murad, 1986). The soluble GC is not a substrate for ANP and is unrelated to any known biological action of ANP.

Particulate GC's are composed of a single protein with masses of 130 000 and 180 000 Da (Chinkers et al., 1989; Sharma et al., 1989). Five distinct forms of particulate GC's have been identified. NPR-A and NPR-B receptors were characterized as two particulate GC's, GC-A and GC-B (Chinkers and Garbers, 1991; Schulz et al., 1990). ANP stimulates particulate GC activity and elevates cGMP concentrations in most tissues and cell lines. cGMP concentrations are increased in response to ANP concentrations of 0.1 to 100 nM, with an EC_{50} in the range of 1 to 10 nM. The stimulatory action of ANP on GC was observed intially in the kidney (Hamet et

al.,1984); Waldman et al., 1984). Almost all other tissues were found to increase cGMP production in response to ANP, including adrenal (Matsuoka et al., 1985), vascular tissues (Winquist et al., 1984), cardiac tissue (Cramb et al., 1987). Platelets do not contain ANP R1 receptors; therefore, they do not respond to ANP with an elevation of GC activity (Anand-Srivastava et al., 1991; Schiffrin et al., 1991).

The regulation of GC activity is quite distinct from that for adenylyl cyclases. There is no requirement for G-proteins and the catalytic sub-unit is a structural component of the receptor molecule in particulate GC's. The enzyme is activated by ATP (Goraczniak et al., 1992; Marala et al., 1992), and this activation is eliminated by modifications of the protein kinase-like domain of the GC molecule. Such alterations of the protein kinase domain eliminate ATP binding and nearly eliminate the activation of the enzyme by ANP (Marala et al., 1992). Addition of phorbol esters, which activate protein kinase C (Nambi et al., 1987; Jaiswal et al., 1988; Sekiya et al., 1991), attenuate the activation of GC by ANP. This effect of protein kinase C activators is eliminated by PT, suggesting a role for G-proteins in mediating the inhibitory effect (Sekiya et al., 1991). PT alone had no effect on GC activity stimulated with ANP (Sekiya et al., 1991; Drewett et al., 1990; Ljusegren et al., 1990). Agents interacting with sulfhydral groups, such as N-ethylmaleimide, inhibit adenylyl cyclase activity (Ross and Gilman, 1980) but have no effect on GC activity (Sharma et al., 1989). These findings indicate that ANP activates GC independently of G-proteins, but agents acting to inhibit GC activity via protein kinase C may involve a G-protein to mediate their effects.

C3. NPR-C (ANP-C) Receptors

C.3.1. Introduction

NPR-C receptors are homodimers of about 64-66 kDa transmembrane protein (Fuller et al.,1989; Schenk et al., 1985; Leitman et al., 1988) and distributed widely in several tissues and cells (Table 1) including platelets, vascular smooth muscle cells, glomeruli, collecting ducts, pituitary glands, adrenal glands, the zona glomerulosa, the cerebral cortex, the brain striatum, the ciliary process of the eye, Purkinje fibers of the cardiac conduction system and Leydig tumour cells (Schenk et al., 1985). The density of these receptors in most tissues is higher than that of NPR-A/B receptors. For example, in endothelial cells, the NPR-C receptors comprise about 94% of the ANP receptor population (Leitman et al., 1986). (Table 1)

C.3.2 Structure

The NPR-C receptor was characterized initially by Schenk et al. (1985) in bovine aortic smooth muscle. (Figures #4 and #5) The receptor was solubilized with octaethyleneglycol dodecyl ether and purified by passing it over an ANP-agarose column. The receptor migrated with a molecular weight equivalent to 125,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. When reduced with dithiothreitol, the receptor had a electrophoretic mobility consistent with a protein of 60,500 Da. The cDNA for expression of the R2 receptor was isolated from bovine aortic smooth muscle (Fuller et al.,1988) and expressed in Xenopus oocytes. The gene

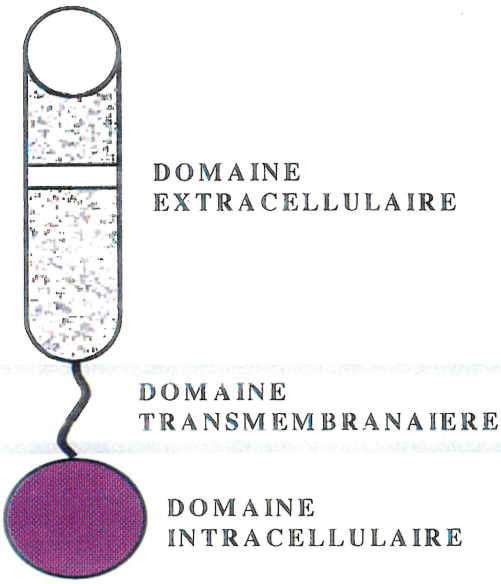

Figure #5: NPR-C's structure, showing its single transmembrane domain and its 37 amino acid cytoplasmic domain.

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encoding this receptor varied in length with bovine forms being 8000 nucleotides in length, whereas human forms were 5600 nucleotides long (Fuller et al., 1988) The protein encoded consisted of 537 amino acids containing a large extracellular region, a single membrane -spanning domain, and a short cytoplasmic tail of 37 amino acids. A similar protein was encoded by human placenta, kidney, and fetal heart with the gene having a nucleotide length of 5400 bases (Porter et al., 1990).

Suzuki et al. (1991) found the gene in bovine tissues to consist of 8500 nucleotides with eight exons and seven massive introns. The extracellular region was coded by exons 1 to 6. Exons 7 and 8 encoded the membrane spanning region and the short cytoplasmic tail, respectively. This pattern is the same as that described for GC-A, except that the gene for GC-A contains an additional 14 exons encoding the intracellular protein kinase and guanylyl cyclase regions
(Saheki et al.,1991; Yamaguchi et al., 1990).

The significance of these differences in gene length is not known, but the products of the human or bovine genes are nearly the same. They all encode a protein of 496 amino acids following removal of a 41-amino acid segment on the amino terminus. Two different cDNAs encoding the NPR-C receptor were also identified in the human umbilical vein. They differed only by the deletion of 123 nucleotides in one of the transcripts (Nunez et al., 1991). The deletion did not alter the open frame. Interestingly, the second exon encoding the R2 receptor contained 123 nucleotides (Saheki et al., 1991). Furthermore, the first exon could combine with the third exon without interrupting the reading frame or altering the amino acid encoded by the third exon. Thus, these different transcripts would encode proteins differing by 41 amino acids, and the deletion would be predicted to occur in the extracellular binding region of the receptor. One report found two vascular R2 receptors of 60,000 and 70,000 Da
(Kato et al., 1991), which is consistent with the presence of distinct NPR-C receptors.

Table #1: Location of ANP Receptors and Properties

LOCATION	RECEPTOR TYPE	%	SIZE (kDA)
VAsCULAR TISSUES	R2: Multiple Binding Sites	94	66
	R1: Endothialium, VSMC	6	130
KIDNEY	R1: Predominant in papillary regions		130
	R2: Predominant in renal cortex, glomeruli, collecting ducts		64
ADRENAL GLAND	R2- Zona Glomerulosa	< 20	67
	R1- Predominant in adrenal glomerulosa	50-80	114
HEART	R2- Cardiac sacrolemma - conduction system of heart, purkinje fibers		65,130
LUNG	R2: Predominates as dimer or monomer	90	66,130
ENDOCRINE ORGANS	R2- thyroid cells, pituitary glands,Leydig Tumour Cells		70
	R1- testis		130-180
NEURONS	R1- Predominant in CNS		180
	R2- Predominant in Astrocytes, cerbral cortex, brian striatium		70
PLATELETS	R2- only no R1, as monomer or Dimer	100	65,125

D.Adenylyl Cyclase/cAMP Signal Transduction System

D.1. Introduction

The adenylyl cyclase/cAMP system is one of the best-characterized signal transduction systems, mediating physiological responses of a variety of hormones and neurotransmitters. Adenylyl cyclase is composed of three components: a receptor, a catalytic subunit (such as an enzyme, ion channel, or transporter) and a stimulatory guanine nucleotide regulatory protein (Gs) or inhibitory guanine nucleotide regulatory protein (Gi). Each component 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 sites. 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 adenylyl cyclase enzyme) The hormonal stimulation and inhibition of adenylyl cyclase are mediated through the Gs and Gi proteins, respectively, (Gilman,1984), resulting in increased and decreased formation of cAMP. In this process, 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. The 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 #6).

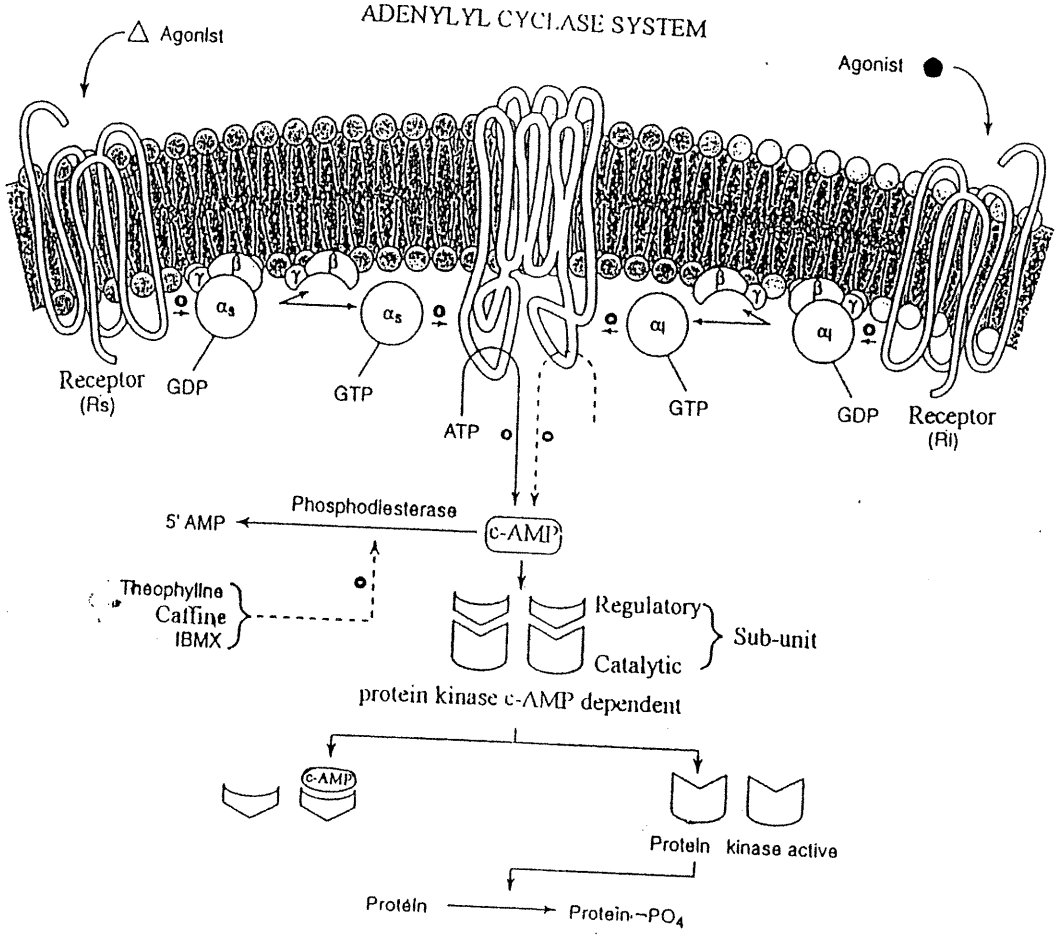


Figure #6: The adenylyl cyclase / cAMP System

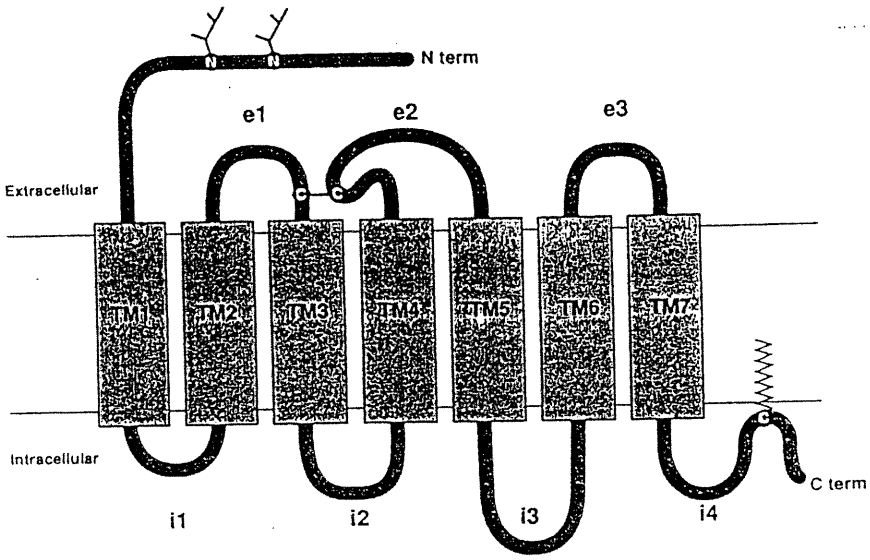
Krupinski J (1991); " The adenylyl cyclase family. " Mol. Cell. Biochem. **104**: 73-79.

D.2. G-protein Linked Cell Surface Receptors

D.2.1. Structure

Specialized cells in living organism communicate with each other through extracellular signalling molecules. These signalling molecules elicit their metabolic responses within the cell by interacting either with one or a combination of cell-surface receptors present on the plasma membrane. The superfamily of G-protein linked receptors make up the majority of cell surface receptors (Gudermann and Nurenberg , 1995). It is generally assumed that 50-60% of all clinically relevant drugs exert their actions via G-protein-coupled receptors. More than 300 G-protein-coupled receptors have been cloned; the total number is assumed to exceed 1000. The primary structures of various G-protein-linked receptors, including the light receptor rhodopsin, seem to be designed according to a common structural principal. 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 transmembane 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 # 7). Most 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.

Examples of G-protein coupled receptors that have the characteristic of seven-membrane-domain receptors include the angiotensin II, beta-adenergic, α2-adenergic, endothelin B and muscarinic 2 and 4 subtypes and also of the dopamine D2 receptor, in which the third



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.

Figure #7

cytoplasmic loop plays a role in determining G protein coupling and specificity (Okamoto et al., 1992; Aramori et al., 1992; Lefkowitz 1988). That being said not all pathways for G protein activation need a seven-transmembrane-domain receptor. The neuronal protein Gap-43 stimulates GTP bindind to Go, suggesting that an intracellular protein can activate a G protein pathway (Strittmatter et al., 1990). Also, mastoparan, a 14-amino-acid wasp-venom peptide, activates G proteins directly (Nigashijima et al, 1988). The single-transmembrane-domain insulin and insulin-like growth factor II/mannose 6-phosphate receptors have also been reported to activate G-protein coupled pathways (Nishimoto et al., 1987,1989).

D.2.2. Function and Desensitization

Receptors such as G-protein-coupled receptors, have specific binding domains (Gudermann and Nurenberg 1995), that interact with specific ligands. However, different binding domains of receptor subtypes can bind the same ligand. There is a wide variety of signalling ligands for those binding domains. These ligands include amines, amino acids and derivatives, peptides and proteins, nucleotides, fatty acid derivatives, phospholipid derivatives, multi-structural 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 recognition is 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 recptor is phosphorylatyed in the cytoplasminc domains of the receptor molecule that interact with Gs by a specific cAMP-dependent β-adrenergic receptor kinase as well as by both the cAMP-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.

E. G-Proteins

E.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, αsubunits readily dissociate from the βγ complex. Each αsubunit 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) Gi α(inhibitory G-protein) respectively (Figure # 8) (Gilman 1984; Stryer and Bourne 1986). Molecular cloning 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, Giα2, and Giα-3 encoded by 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 α subunit of transduction is distinct from other G-protein γ subunits, but the relationship among the γ subunits of G-proteins other than transduction is not clear.

E.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 start another cycle.

In solution, G-protein activation is accompanied by dissociation of α from βγ 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 βγ 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. Hydroylsis of GTP α to GDP returns the α subunit to a state of higher affinity for the βγ complex.

The βγ complex has been regarded as an " inhibitor ", keeping G-protein in its inactive form. Binding of GTP, promoted by activated receptor , is necessary to overcome inhibition by βγ. The βγ complex, however, may be required for association

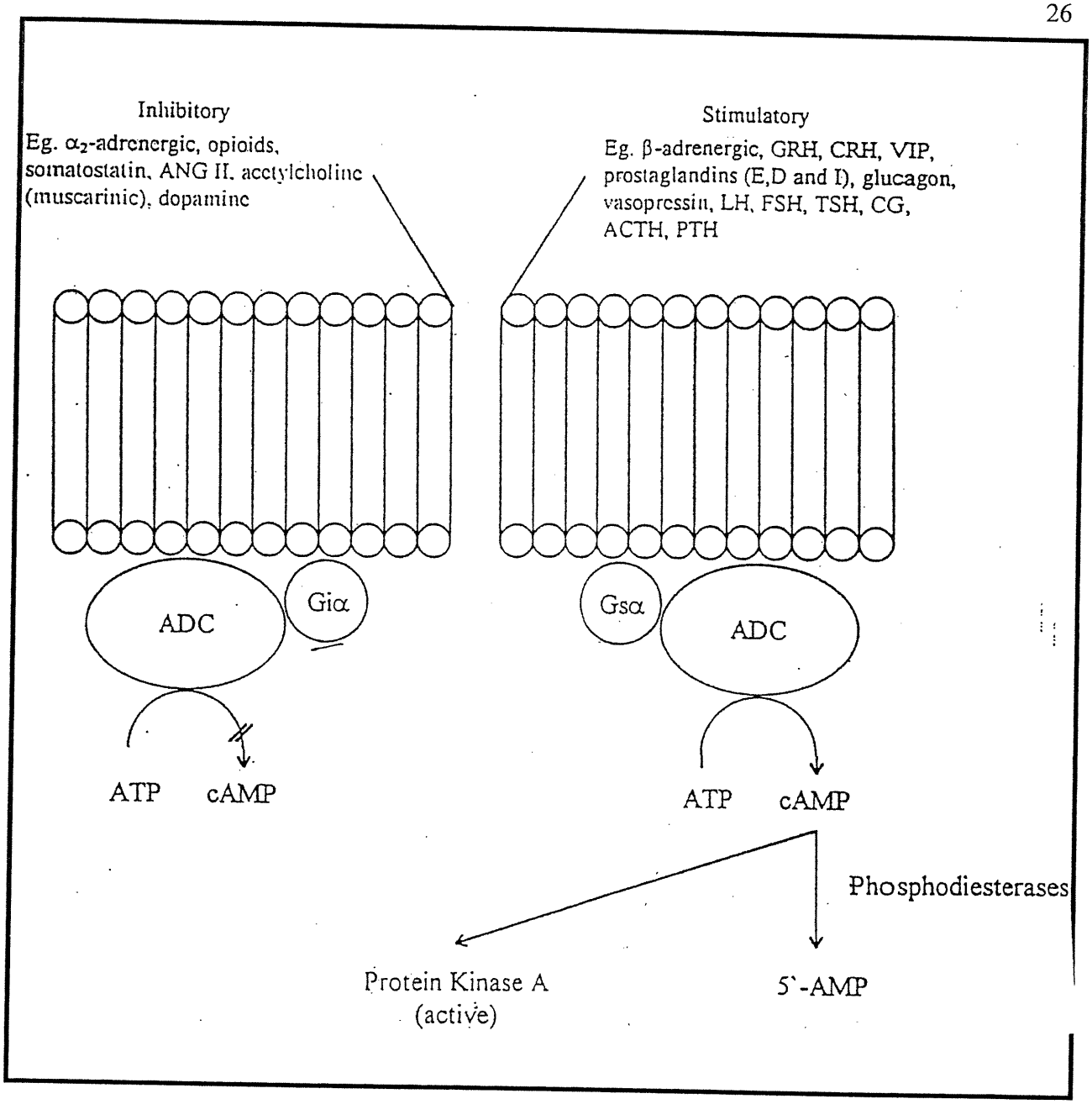


Figure 8: The hormonal stimulation and inhibition of adenylyl cyclase are mediated by two G-proteins: stimulatory (Gs) and inhibitory (Gi),

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

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 βγ 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.

E.3. Mechanism of Activation

The activation/inactivation cycle of G-proteins is illustrated in (Figure # 9). When GTP is bound to the α-subunit, it is depicted to be active; however when GDP is bound to the $\tilde{\alpha}$-subunit, it is shown as being inactive. Conversion between these two active states is governed by the cell surface receptor with which the G-protein is associated with. 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 $\tilde{\alpha}$-subunit is replaced by GTP. The active G-protein dissociates itself from the receptor which favours

the release of α- and βγ-subunits. The α- and βγ 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 hydrolyses GTP to GDP. Once GTP is hydrolyzed and the inorganic phosphate released, the α-subunit, and βγ dimer re-associate to form the inactive G-protein heterodimer. The 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 signalling molecules.

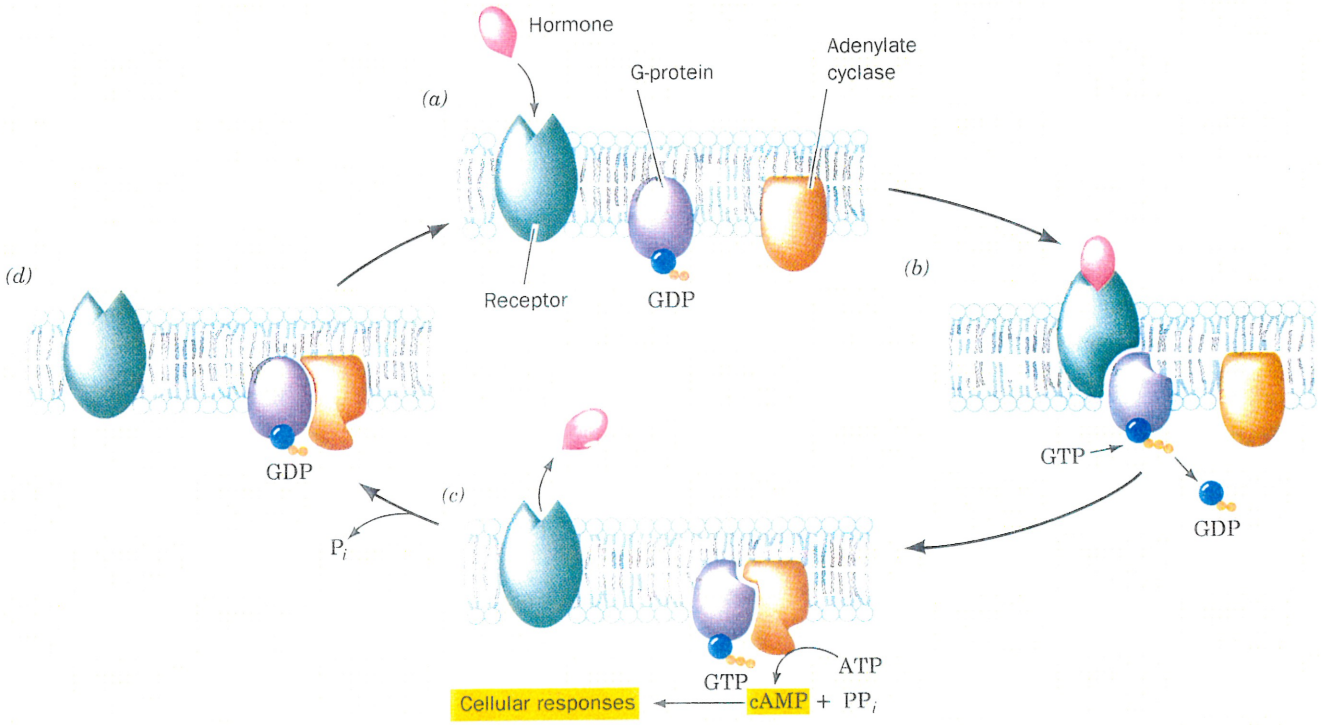
E.4. Interaction of G-protein with Receptors

A single receptor may interact with a single or several G-proteins, and multiple receptors 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 in expression and on the sub-cellular localization of proteins.

Evidence obtained from expression of α2-adrenergic receptors demonstrates that the receptor interacts with Gi-1, Gi-2 and Gi-3 to inhibit adenylyl cyclase / cAMP 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).

E.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 2). The 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 shown that the HT_{1a} receptors preferentially interacted with Giα-3



The activation/deactivation cycle for hormonally stimulated adenylate cyclase. (*a*) In the absence of hormone, G-protein binds GDP and adenylate cyclase is catalytically inactive. (*b*) The hormone–receptor complex stimulates G-protein to exchange its bound GDP for GTP. (*c*) The G-protein–GTP complex, in turn, binds to and thereby activates adenylate cyclase to produce cAMP. (*d*) The eventual G-protein–catalyzed hydrolysis of its bound GTP to GDP causes G-protein to dissociate from and hence deactivate adenylate cyclase.

Figure #9: The activation/deactivation cycle of G-Proteins

and in turn Giα-3 inhibited adenylyl cyclase/ cAMP (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, attention is paid towards the presence of GTPase inhibitors and their role in cellular signalling. 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 been demonstrated 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 βγ subunits. (Hepler and Gilman 1992). The βγ dimers were 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 demonstrated regulation by the βγ-subunit in the presence of stimulated α2 subunits (Tang et al.1991). In addition, Katada et al.(1984) have shown that βγ-subunits stimulate the mouse lymphoma S49 *cyc*$^-$ cell membrane adenylyl cyclase. It also showed that phospholipase C from HL-60 cells and neutrophils can be stimulated by βγ (Camps et al. 1992).

Table 2: Interactions of G-protein with receptors and effectors.

Family and subunits	*Mass (kDa)*	*% AA identity*	*Toxin*	*Receptors*	*Effectors*
Gs					
α s(short S)	44.2	100	C		ADC, Ca+2, Na+channels
α s(long L)	45.7		C	Odorant	K+ channel
α olf	55.7	88	C		ADC
Gi/o/t					
iα1	40.3	100	P		K+ channel
iα2	40.5	88	P	5-HT, C5a,etc	Ca+2 channels,ADC
iα3	40.5	94	P	M_2-AchR ,somatostatin	
α o1	40.0	73	P		PLC, PLA2
α o2	40.1	73	P		
α t1	40.0	68	C + P	Rhodopsin	cGMP-PDE
α t2	40.1	68	C + P	Cone opsin	cGMP-PDE
α g	40.5	67			cAMP-PDE
α z	40.9	60			ADC?
Gq					
α q	42.0	100		m_1-AchR αi	PLC
α 11	42.0	88		AR,TRH, etc.	βs+others?,PLC
α 14	41.5	79		IL-8 and others ?	PLC?
α 15	43.0	57		IL-8 and others /	?
α 16	43.5	58		IL-8,C5a and others?	PLC-β2+others
G12					
α 12	44.0	100		?	?
α 13	44.0	67		?	?

Gilman AG (1987): " G-protein: Transducers of receptor generated signals." Annu. Rev Biochem. **56**: 615-649 C=Cholera toxin; P=Pertussis toxin

E.6. Modification of G-protein by Toxins

It is well known that some toxins can prolong the activation 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 terminal of the α-subunit. The ADP-ribosylation functionally uncouples Gi proteins from receptors (Gilman 1987), and attenuates the GTP-depedent and receptor-mediated inhibitory adenylyl cyclase response.

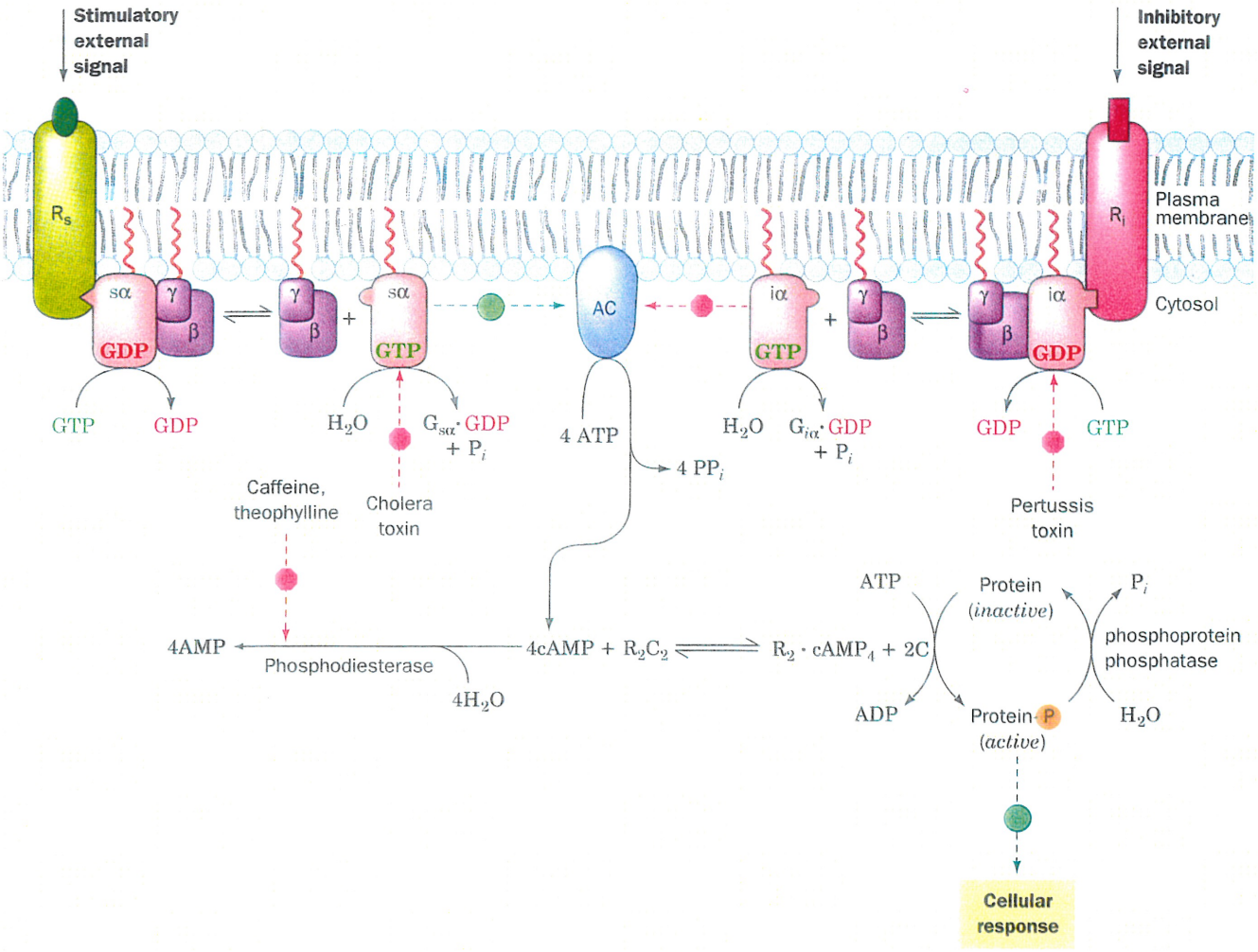
The cholera toxin, a secretory product of the bacterium *Vibrio cholera*, is responsible for the significant fluid and electrolyte loss in cholera (Kelly 1986). Like pertussis toxin, cholera toxin is an A-B type toxin composed of one enzymatically-active A subunit and five B subunits responsible for 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 occuring 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 activation/deactivation are shown in (Figure # 10).

F. Adenylyl Cyclase

F.1 Structure

The first adenylyl cyclase isoform was cloned from the brain. (Krupinski et al, 1989,1992). Krupinski and co-workers 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 polymerase chain reaction has now permitted isolation of seven additional full-length clones: types II-VI and VIII (Bakalyar and Reed; De Vivo and Iyengar 1994).



Figure#10: Effects of Pertussis Toxin and Cholera Toxin on G-proteins

Adenylyl cyclase has an approximate molecular weight of 120 kDA, it contains 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 (C1 and C2) , punctuated by two intensely hydrophobic stretches (M1 and M2); each of the latter is hypothesized to contain six transmembrane helices (Figure # 11) It is this complex and widely distributed group of adenylyl cyclases that is responsive to stimulatory and inhibitory regulation by hormones and neurotransmitters, acting via the intermediary of both receptors and G-proteins (Gilman 1987). Different isoformes of adenylyl cyclase have been identified (Krupinski et al. 1992), and their distribution in different tissues has been determined by immunoblot and cloning studies (Table 3). 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).

F.2. Regulation of Adenylyl Cyclase Activity

All isoforms of adenylyl cyclase are activated by both forskolin and the GTP-bound α-subunit of the stimulatory G-protein Gs. They are all inhibited by certain adenosine analogs termed P-site inhibitors; 2"- deoxy-3"- AMP is particularly potent. However, all isoforms of adenylyl

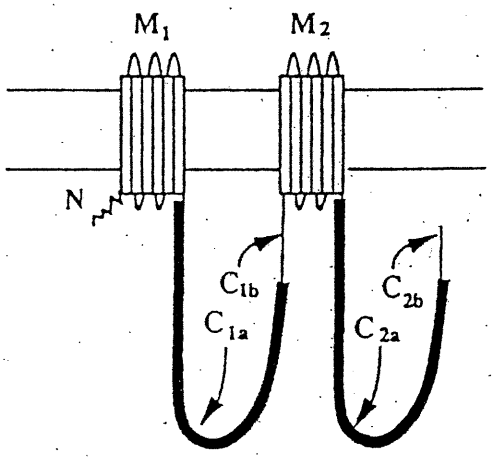


Figure #11: The structures of adenylyl cyclase. Wei-Jen Tang et al.

Cell (1992) 70; 869-872.

N indicates the N-terminal cytoplasmic domain; M1, the first six transmembrane-spanning domain; C1a, the first cytoplasmic catalytic domain; C1b, the first cytoplasmic linker domain; M2, the second six transmembrane spanning domain; C2a, the second cytoplasmic catalytic domain; and C2b, the second cytoplasmic noncatalytic domain.

TABLE # 3 : Different Isoformes and Properties of adenylyl cyclase

Properties of Eukaryotic Adenylyl Cyclases

Effect of G Proteins

Type	Amino Acid Residues	Expression	Gα	βγ	Ca^{+2} Calmodulin	Forskolin
I	1134	Brain	1[b]		1	1
II	1090	Brain, Lung	1	1	0	1
III	1144	Offactory	1	0	1	1
IV	1064	Brain,Others	1	1	0	1
V	1184	Heart,Brain,Others	1	0	0	1
VI	1165	Heart,Brain,Others	1	0	0	1
Rutabaga	2249	Mushroom Body	1	0	1	1
AC-A	1407	During Aggregation	2[c]	2	2	0
AC-G	858	Fruiting Body	2[d]	2[d]	2[d]	0
CYR1	2026	Constitutive	0[e]	0	0	0

[a] Adenylyl cyclases types I-VI are mammalian;rutabaga is from Drosophila. AC-A and AC-G are from Dictysstelium CYR1 is fgrom Saccharomyces cerevisiae. References: I (Krupininski et al.,1989), II (Feinstein et al.,1991) III (Bakaylar and Reed,1990) IV (Gao and Gilman,1991), V (Ishikawa et al.,1992), VI (Premont et al., 1992; Katsuashika 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 G1α 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 adenylyl cyclases." J Biol. Chem. **270**(1): 1-4.

cyclase are further regulated in type-specific patterns by other inputs, 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). Different types of adenylyl cyclase are activated by different regulators; adenylyl cyclase type II and IV are activated by Gβγin 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 # 12 and Table 2)

G. Phospholipase C (PLC), Diacylglycerol (DAG), and Inositol Trisphosphate (IP3)

In this system, the activated receptor stimulates, via a G protein, a plasma-membrane effector enzyme called phospholipase C. This enzyme catalyzes the breakdown of a plasma-membrane phospholipid (Figure # 3) known as phosphatidylinositol bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol trisphosphate (IP_3). Both DAG and IP_3 then function as second messengers, but via different mechanisms. DAG activates a particular protein kinase known as protein kinase C, which then phosphorylates a large number of other proteins leading to the cell's response. In particular, protein kinase C plays an important role, via phosphorylation, in the regulation of proteins associated with the plasma membrane.

IP_3, in contrast to DAG (as well as cAMP and cGMP), does not exert its second-messenger role by directly activating a protein kinase. Rather, IP_3, enteracts with an IP_3 receptor which is a Ca++ channel. The concentration of calcium is much higher in the

endoplasmic reticulm than in the cytosol, and so calcium diffuses out of the organelle into the cytosol, significantly increasing the

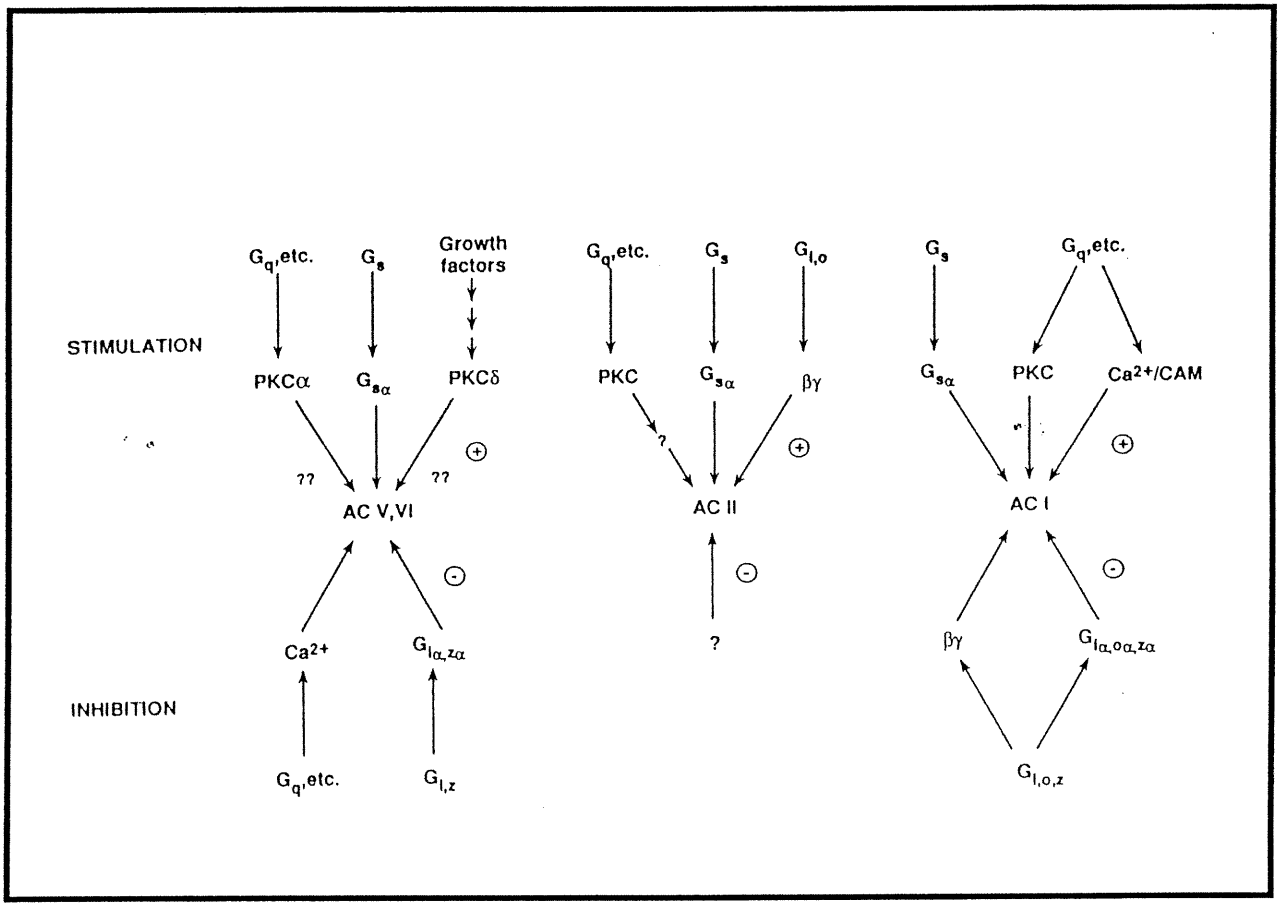


Figure #12: Patterns of regulation of adenylyl cyclase activity.

Ronald and Gilman. J.Biol.Chem. (1995) **270**: No 1: 1-4:

Gi,o,q,s,z = different isoformes of G-proteins

cytosolic calcium concentration. This increased calcium concentration then continues the sequence of events leading to the cell's response to the first messenger.

H. NPR-C Receptor Cell Signaling and Physiological Responses

ANP has been shown to inhibit adenylyl cyclase activity in various vascular tissues including aorta, renal arteries, and mesenteric arteries (Anand-Srivastava et al, 1984). The maximal inhibition observed was 40 to 50% with an apparent K*i* of 0.1 to 1 nM. ANP also inhibited forskolin- and- hormone-stimulated adenylyl cyclase activity. The inhibitory effect of ANP was dependent on the presence of guanine nucleotides, suggesting the involvement of G-proteins in the coupling of ANP receptors to adenylyl cyclase. No decrease in adenylyl cyclase activity was seen in spleen, testis, adrenal medulla, and proximal tubule of the kidney (Anand-Srivastava et al, 1984), suggesting the absence of these receptors in these tissues.

Maack and his colleagues (1987) demonstrated that NPR-C receptors are non-GC coupled receptors and are biologically silent regarding renal actions. They proposed that the primary function of these receptors was the sequestration and metabolic clearence of ANP, and hence, called these receptors clearance or C-receptors. However, using human thyroid cultured cells possessing only NPR-C receptors, Tseng et al. (1990) showed that ANP inhibited cAMP production and thyroglobulin release; furthermore the inhibition of thyoglobulin release paralleled declines in cAMP concentrations, suggesting that ANP acts via a cAMP pathway in thyroid cells. These studies indicated that NPR-C receptors are coupled to adenylyl cyclase/cAMP signal transduction pathways and refuted the hypothesis that they are biologically silent. In addition Anand-Srivastava et al. (1991) reported that rat platelets, devoid of particulate

GC, responded to ANP inhibiting adenylyl cyclase activity and reducing cAMP levels. The inhibition was dependent on the presence of guanine nucleotides and was blocked by Pertussis Toxin treatment or amiloride treatment, suggesting that NPR-C receptors couple to the negative regulation of adenylyl cyclase/cAMP system. These results were confirmed in NIH-3T3 cells possessing pure populations of NPR-C receptors. ANP inhibited adenylyl cyclase activity in a concentration-dependent manner with apparent Ki of about 100 pM (Anand-Srivastava et al., unpublished observations). In addition, ANP reduced cAMP levels in HeLa cells expressing predominantly NPR-C receptors (Koyama et al., 1992), supporting the contention that NPR-C receptors negatively regulate the adenylyl cyclase/cAMP signal transduction system.

Further confirmation regarding the coupling of NPR-C/cANP receptors to the adenylyl cyclase/cAMP system was shown using a ring-deleted analog of ANP, c-ANP (4,23), and other linear truncated analogs, interact directly with NPR-C receptors (Maack et al. , 1987). These analogs inhibited adenylyl cyclase in many tissue in a concentration-dependent manner; dependent on the presence of guanine nucleotides and blocked by PT or amiloride treatment (Anand-Srivastava et al.,1990). The cANP also inhibited the production of cAMP but not cGMP, indicating that cANP/R2 receptors are coupled to the adenylyl cyclase/cAMP signal transduction system. This inhibitory effect was additive to that of ANP(99,126), indicating that cANP receptors are NPR-C receptors (Anand-Srivastava et al., 1990). In addition, cANP inhibited the basal, as well as luteinizing hormone-stimulated, production of progesterone secretion,

suggesting that NPR-C receptors are not biologically silent but have a physiological role.

ANP inhibited adenylyl cyclase activity in various renal structures, such as glomeruli, loops of Henle, and collecting ducts, but not in proximal tubules (Anand-Srivastava et al., 1986). The maximal inhibitory effects were 45% in glomeruli and collecting ducts, with an apparent Ki of of 100 to 1000 pM and 30% in loops of Henle with an apparent K*i* of 10 to 50 pM. The inhibitory effect was dependent on the presence of guanine nucleotides (Anand-Srivastava et al, 1986). Umemura et al. ,(1989) demonstrated that ANP inhibited parathyroid hormone -stimulated increases in cAMP production in human glomeruli in a concentration-dependent manner, to a maximum of 50 %. ANP also significantly reduced arginine vasopressin and forskolin-stimulated cAMP levels in cultured rat renal papillary collecting tubule cells (Ishikawa et al., 1985).

These studies and others (Table #4) indicate that ANP suppresses the adenylyl cyclase activity in the majority of tissues studied. The suppression of adenylyl cyclase activity in broken cell preparations is critically dependent on the presence of GTP or GTP analogs, and was completely attenuated by PT treatment, suggesting the implication of Gi proteins in the coupling of NPR-C receptor to adenylyl cyclase (Anand-Srivastava et al., 1987, 1989, 1991,1996; Mittag et al., 1987; Resink et al., 1988 , Hempel, 1998). These studies also suggest that ANP is an important factor in the signal transduction pathways of vascular tissue, cardiac, endocrine, lung neuronal tissues, and in platelets.

Table #4: Studies showing the Inhibition of Adenylyl Cyclase Activity by the NPR-C Receptor

STUDY	TISSUE	RESULTS
Anand-Srivastava et al.,1984	Aorta,renal,mesenteric arteries	↓ cAMP production, Ki of 0.1to 1nM
Anand-Srivastava et al.,1985	Adrenal cortical membranes	↓ACTH, Ang II, FSK stimulated cAMP production,Ki 50 to 100pM
Matasuoka et al.,1985.	Rat adrenal capsular tissue	↓aldesterone and cAMP production
Ishikawa et al., 1985.	Rat renal papillary collecting tubule cells	↓arginine vasopressin and FSK-stimulated cAMP levels
Obana et al., 1985.	Posterior pituitary glands	↓ cAMP levels and vasopressin release
Pandey et al., 1985.	Leydig tumor cells	↓ cAMP production
Waldman 1985.	Adrenal cortical membranes	↓ACTH, Ang II, FSK stimulated cAMP production
Anand-Srivastava 1986.	Glomeruli, loops of Henle, collecting ducts	↓ cAMP production, Ki of 10 to 50 pM, dependent on guanine nucleotides
Anand-Srivastava and Cantin,1986.	Heart sacrolemma	↓ cAMP production
Maack et al.,1987.	Renal actions	NPR-C receptors are non GC-coupled
Naruse et al.,1987.	Adenoma tissue	↓ACTH-aldesterone secretion,↓ cAMP

		production
Resink et al., 1988.	Rat lung membrane	↓ cAMP production
Anand-Srivastava et al.,1989.	Purkinje fibers	↓ cAMP production
Umemura et al., 1989.	Human glomeruli	↓parathyroid hormone-stimulated increases in cAMP production
Vargas et al., 1989.	Fetal rat bone cultures	↓parathyroid hormone-stimulated increases in cAMP production
Tseng et al., 1990.	Human thyroid cultured cells	↓thyroglobulin release, ↓cAMP
Anand-Srivastava et al., 1990.	Many tissues using $cANP_{(4,23)}$	↓ cAMP production
McCall and Fried, 1990.	Rat myocardial cells	↓ cAMP production,↓ contraction velocity and calcium influx
Anand-Srivastava et al.,1991.	Rat platelets, NIH-3T3 cells	↓ cAMP production, Ki 100pM
Koyama et al.,1992.	HeLa cells	↓ cAMP production
Hempel et al., 1998.	Rat coronary endothelium	↓ cAMP production

Other physiological roles of the NPR-C receptor have been demonstrated in other studies. Levin and Frank (1991) showed that ANP inhibits rat astroglial proliferation through NPR-C receptors. Johnson et al. (1991) also reported the NPR-C receptor-mediated inhibition of electrically induced purinergic and adrenergic contractile force generation in rabbit isolated vasa deferentia. In addition ANP-induced inhibition of endothelial and vascular smooth muscle cell proliferation was also reported to be mediated through NPR-C receptors (Cahill and Hassid;1991 Itoh et al., 1988). Studies by Drewett et al. (1992), demonstrated the inhibition of adenylyl cyclase and neurotransmission by cANP in nerve growth factor-treated pheochromocytoma cells,

further supporting the physiological role and coupling of these receptors to the adenylyl cyclase/cAMP signal transduction system. In another study by Hu et al, (1992), cANP and nanopiperazine ANP(11-15)NH2, agents selective for the NPR-C receptor, inhibited the in vivo translation of the endothelin message and the endothelin secretion from cultured bovine aortic endothelial cells. The cANP-mediated decrease in endothelin secretion was reversed by 8-bromo cAMP or amiloride, (Anand-Srivastava et al., 1990,1991). These data strongly support the hypothesis that NPR-C receptors elicit physiological responses through their interaction with cAMP signal transduction mechanisms.

Hirata et al., (1989) showed that ANP and ANP(103-123) stimulate phosphatidylinositol turnover in the presence of guanine nucleotides in cultured bovine aortic smooth muscle cells. ANP(103-123) was 10-fold more potent than ANP, suggesting that NPR-C receptors coupled to phosphatidylinositol turnover through guanine nucleotide regulatory proteins. This observation suggests that NPR-C receptors couples to two different intracellular messengers, cAMP and phosphatidylinositol turnover. Alternatively there may exist a cross-talk between these two second messengers. ANP could inhibit adenylyl cyclase/cAMP through its interaction with NPR-C receptors, and the decreased cAMP may be the stimulus for increased turnover of phosphatidylinositol by ANP in cultured bovine aortic cells. This suggests that the stimulation of phosphatidylinositol turnover by ANP in cultured bovine aortic cells (Hirata et al., 1989) may be a secondary event mediated through the adenylyl cyclase/cAMP system coupled to NPR-C receptors.

The GTP dependence of ANP effects on adenylyl cyclase and phospholipase C activities suggests the involvement of inhibitory G-proteins in the coupling of NPR-C receptors to adenylyl cyclase inhibition and phospholipase C activation. Receptors coupled to G-proteins typically have seven transmembrane-spanning domains, such as angiotensin II, adrenergic, endothelin B and muscarinic 2 and 4 subtypes, and also of the dopamine D2 receptor, in which the third cytoplasmic loop plays a role in determining G protein coupling and specificity. The NPR-C receptor contains only a single-transmembrane domain and a short intracellular cytoplasmic domain of 37 amino acids. Other findings that oppose to the notion that G-protein coupled receptors are seven transmembrane domains include the observation by Okamoto el al., (1990). This study defined the structural requirement for the insulin-like growth factor II mannose 6-phosphate receptor coupling to G-proteins as a 14-amino acid intracellular segment enriched in basic amino acids. This segment of the insulin-like growth factor II receptor had a specific Gi activator sequence that was able to activate Gi protein directly in the same manner as that of conventional Gi-coupled receptors. This sequence (Gi activator) was characterized by the presence of two basic amino acids at the NH2 terminus and BBXB or BBXXB at the COOH terminus, where B and X denote basic amino acid and nonbasic amino acid respectively. Interestingly, the cytoplasmic domain of the NPR-C receptor contained 14 basic amino acids and several of these Gi activator sequences amongst its 37-amino acid structure. The data of Okamoto et al. (1990) would predict that the NPR-C receptor is capable of interacting with G-proteins, consistent with the functional studies mentioned above.

The cytoplasmic domain of the ANP-C receptor was shown to inhibit adenylyl cyclase activity via pertussis toxin (PT)-sensitive Gi protein (Anand-Srivastava et al., 1996; Hempel et al., 1998). Incubation of polyclonal rabbit antisera raised against a 37-amino-acid synthetic peptide (R37A) corresponing to the cytoplasmic domain of the NPR-C receptor with the rat heart particulate fractions blocked ANP-dependent inhibition of adenylyl cyclase. The inhibition of adenylyl cyclase mediated by cytoplasmic domain peptides R37A and TMC (10 residues of the transmembrane domain appended to R37A) was GTP-dependent and was blocked by pertussis toxin treatment. The inhibition of adenylyl cyclase by these peptides was not due to the net positive charge, since scrambled peptide K37A with the same composition, as R37A did

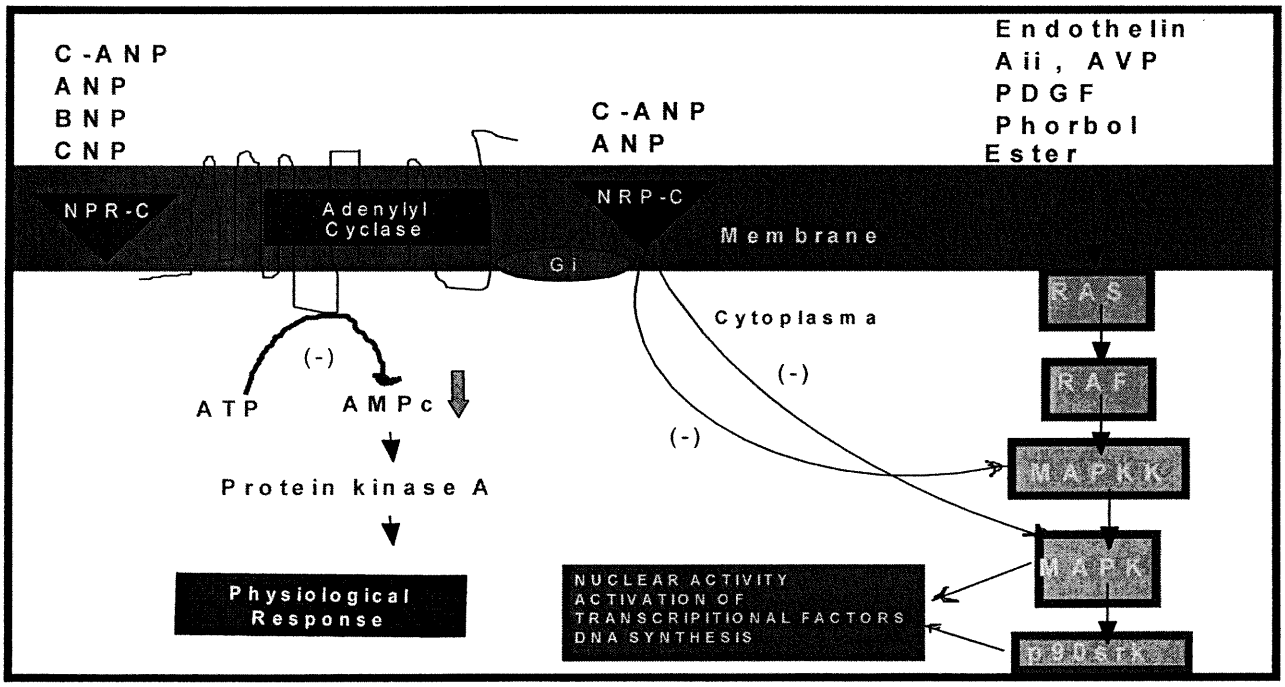


Figure #13 : Cell Proliferation Control by the NPR-C Recptor

not inhibit adenylyl cyclase activity. These data suggest that the cytoplasmic domain of the NPR-C receptor might have structural specificity like those of other single-transmembrane-domain receptors in order to exert its inhibitory effects on adenylyl cyclase.

Prins et al., (1996) showed that both C-ANP$_{4,23}$ and ANP inhibited the mitogen-activated protein kinase (MAPK) activity stimulated by endothelin-3, platelet-derived growth factor and phorbol-12-myristate 13-acetate (Fig # 13). The inhibition of MAPK appeared to be mediated via the inhibition of upstream kinases including MAPK kinase. C-ANP also inhibited mitogen-induced stimulation of DNA synthesis in the astrocytes; however, C-ANP did not inhibit the production of cAMP. These results indicate that the ANP-C receptor can inhibit proliferation via the inhibition of MAPK. This suggests that the ANP-C receptor might also be coupled to other signal transduction mechanisms or that there could be an interaction of the ANP-C receptor with other signalling pathways.

In other recent studies, Kanwal et al. (1999) showed that a 15 amio-acid peptide fragment of NPR-C receptor (Arg^{1}-Gln^{15}) attenuated dopamine efflux in pheochromocytoma cells (PC12). The involvement of the NPR-C receptor was confirmed in further studies (Trachte, 2000) using antisense treatment against the NPR-C receptor. An antisense nucleotide, specific for the ANP-C receptor, caused an inhibition of the action of the NPR-C receptor on dopamine efflux. Murthy and Makhlouf (1999) showed that a 17 amino-acid (Arg^{469}- Arg^{485}) peptide representing the middle part of the cytoplasmic domain of the NPR-C receptor, containing a specific sequence at the amino and carboxy terminal, demonstrated it could activate selectively G_{i1} and G_{i2}

in tenia coli smooth muscle. This activation lead to the stimulation of phosphoinositide hydroylysis by activating PLC-β3 via the βγ subunits of both G-proteins, inhibited adenylyl cyclase activity via the α subunits, and induced muscle contraction. The peptide also increased activation of G_{i1} and G_{i2} by c-$ANP_{(4,23)}$ to activate, as well as stimulating phosphoinositide hydrolysis, inducing contraction, and inhibiting forskolin-stimulated cAMP.

Hempel et al. (1998) demonstrated that the NPR-C receptor participates in modulating endothelial permeability. They used an endothelial monolayer from rat coronary endothelium and measured albumin flux. They applied either ANP or a ring-deleted ANP (c-$ANP_{(4,23)}$), which only stimulated the NPR-C receptor. ANP and c-$ANP_{(4,23)}$ both decreased permeability from 100 pM to 100nM by 60 and 30% respectively. Moreover the action of c-$ANP_{(4,23)}$ was completely blocked by pertussis toxin treatment. They concluded that agonist binding to the NPR-C receptor inhibited cAMP production via a Gi protein-coupled signaling system, and believe this inhibition contributed to the decreased endothelial permeability evoked by ANP.

The studies of Jaubert et al. (1999) have shown that three allelic mutations that cause skeletal overgrowth involve the natriuretic peptide receptor C gene (Npr3). In 1979, a BALB/cJ mouse was identified with an exceptionally long body. This phenotype was found to be caused by a recessive mutation, designated longjohn (lgj), that mapped to the proximal region of chromosome 15. Several years later , a mouse with a similarly elongated body was identified in an outbred stock after chemical mutagenesis with ethylnitrosourea. This phenotype also caused by a recessive

mutation, designated strigosus (stri). The two mutations were found to be allelic. A third allele was identified in a DBA/2J mouse and was designated longjohn-2J (lgj(2J)). In addition a recent study reported that mice overexpressing brain natriuretic peptide, exhibited a skeletal-overgrowth syndrome with endochondral ossification defects. (Jaubert et al.,1999) reported in their study that all three mutations involve the Npr3 gene and provides evidence in vivo that there is a natriuretic-related bone pathway, underscoring the importance of natruretic peptide clearance by NPR-C receptor.

I. Regulation of NPR-C Receptor-Mediated Inhibition of Adenylyl Cyclase

The NPR-C receptor-mediated inhibition of adenylyl cyclase is clearly dependent on the presence of guanine nucleotides (Anand-Srivastava et al., 1987, 1989, 1991,1996; Mittag et al., 1987; Resink et al., 1988; Hempel et al.1998). The optimal concentration of GTP or guanosine 5'-(O-thiotriphosphate) (GTPγS) required to elicit the maximal inhibition is tissue dependent. For example, in the pituitary the maximal ihnibition was observed for 3-5 pmol/l, whereas maximal inhibitory effects in other tissues were induced by 10 pmol/l GTPγS. Above this concentration, the inhibitory effect was decreased and, at higher concentrations, the effect was abolished (Anand-Srivastava and Trachte, 1993).

The inhibition of adenylyl cyclase by ANP is regulated by a variety of agents. Phorbol Ester and calcium phospholipid-dependent protein kinase (C-Kinase) attenuated the inhibitory effect of ANP on adenylyl cyclase (Anand-Srivastava, 1992). The effect of ANP on adenylyl cyclase was also abolished by N-ethylmaleimide, which

uncouples receptors from the catalytic subunit of adenylyl cyclase. The ANP-mediated inhibition of adenylyl cyclase was also attenuated by neurominidase treatment, indicating that the glycoprotein moiety is inviloved in eliciting the inhibitory response of ANP (Anand-Srivastava, 1992). Phospholipids were also shown to be involved in the expression of the inhibitory effect of ANP on adenylyl cyclase (Anand-Srivastava, 1992).

ANP, as reported for other inhibitory hormone receptors, inhibited adenylyl cyclase more effectively at higher sodium concentrations, whereas potassium and lithium suppressed the inhibitory effects of ANP (Anand-Srivastava, 1992). NPR-C receptor levels, have been reported to be very sensitive to NaCl (Katafuchi et al., 1992) in vascular endothelial cells.

These results indicate that ANP-C receptor-mediated inhibition of adenylyl cyclase is regulated by various modulators. The inhibition is absolutely dependent on the presence of guanine nucleotides. The downregulation of the ANP-C receptor byNaCl in vascular endothelial cells could contribute to the development of hypertension in the DOCA-salt model.

Other possible ways of altering NPR-C receptor function include evidence shown in studies by Pedro et al. (1998), that the NPR-C has phosphorylation sites present on it's cytoplasmic domain. NPR-C purification and phosphoamino acid analysis clearly demonstrate that NPR-C exists as a phosphoprotein in RASM cells and that phosphorylation occurs exclusively on serine residues. Transient expression of bovine

NPR-C in Cos-P cells of kidney origin confirmed that phosphorylation occurs within the cytoplasmic domain of the receptor.

J. Hormonal Regulation of NPR-C Receptors

The NPR-C receptor has also been reported to be regulated by various hormones. Angiotensin II (AII) downregulates the ANP-C receptor without affecting the ANP-A and ANP-B receptors (Chabrier et al., 1988), whereas ANP might or might not downregulate the ANP receptor; however, ANP-C receptor-mediated inhibition of adenylyl cyclase is attenuated AII and ANP ($C\text{-}ANP_{4,23)}$ treatments. (Palaparti and Anand-Srivastava, 1996).

Transforming growth factor-β_1 (TGF-β_1) has been reported to regulate gene expression of various ANP receptors differently. TGF-β_1 treatment of a murine thymic stromal cell line (MRL 104.8a) augmented the ANP-C receptor binding sites, which were shown to be at the transcriptional level, whereas ANP-A and ANP-B receptors were downregulated by TGF-β_1 (Agui et al., 1995).

The regulation of ANP receptors by catecholamines in cultured vascular smooth muscle cells has also been examined. That treatment of the cells with noradrenaline decreased the ANP binding was attributed to downregulation of the NPR-C receptor (Kishimoto et al., 1994). The downregulation of the NPR-C receptor was time- and dose-dependent and antagonized by a β_2-selective adrenergic antagonist (ICI 118,551) , (Kishimoto et al., 1994). Forskolin, NaF and 8-bromocyclic AMP (8-Br-cAMP) have also been shown to decrease the density of ANP-C receptors Isoproterenol and 8-Br-

cAMP decreased the ready state level of NPR-C receptor messenger RNA(Kishimoto et al., 1994). By contrast, ANP-A and ANP-B receptor messenger RNA labels were notaffected by isoproterenol and 8-Br-cAMP (Kishimoto et al., 1994).

II. OBJECTIVES

The NPR-C receptor has been found to inhibit adenylyl cyclase activity using specific natriuretic peptide analogs, such as $cANP_{(4,23)}$. Previous work in our lab demonstrated that the 37-amino acid peptide (R37A) corresponding to the cytoplasmic domain of the NPR-C receptor inhibited adenylyl cyclase activity in rat heart particulate fractions, which was completely blocked by the polyclonal rabbit antisera raised against R37A (Anand-Srivastava et al., 1996). The inhibition was dependent on the presence of GTP and was blocked by pertussis toxin treatment. In addition, a scrambled peptide of 37 amino acids K37A, did not inhibit adenylyl cyclase activity, suggesting the inhibition was not due to the positive charges and that a certain structural specificity present in the cytoplasmic domain of the NPR-C receptor may be responsible for exerting inhibitory effects on adenylyl cyclase. Okamoto et al. have shown that a short intracellular region of 14 amino acids (Arg^{2410}-Lys^{2423}) of insulin-like growth factor II receptor (IGF-II R) having a specific Gi activator sequence was able to activate Gi-protein directly in a same manner as that of conventional Gi-coupled receptors (31).This sequence (Gi-activator) was characterized by the presence of two basic amino acids at the amino terminal (N- terminal) and B-B-X-B or B-B-X-X-B at carboxy terminal (C - terminal), where B and X denote basic amino acid and non-basic amino acid respectively. The cytoplasmic domain of NPR-C receptor contains several of these Gi-

activator sequences. In the present studies we have used seven different synthetic peptide fragments of the cytoplasmic domain of NPR-C receptor with complete, partial or no Gi activator sequence in order to examine their effects on adenylyl cyclase activity. The peptides used were KKYRITIERRNH (#1), RRNHQEESNIGK- (#2), HRELREDSIRSH-(#3), RRNHQEESNIGKHRELR-(#4), QEESNIGK-(X), ITIERRNH-(Y) and ITIYKKRRNHRE (Z). Peptides #1, #3 and #4 have complete Gi-activator sequences, whereas peptides #2 and Y have partial Gi-activator sequences with truncated carboxy or amino terminal respectively, peptide X has no structural specificity whereas peptide Z is the scrambled peptide control for peptide #1. Our objective in using these seven different peptides was to isolate and determine, precisely, the amino acid sequence required to activate Gi-proteins. Is the entire cytoplasmic domain of the NPR-C receptor required to interact with Gi-proteins and inhibit adenylyl cyclase activity, or is there a shorter specific amino acid sequence that is responsible for this effect?

CHAPTER 2

Cytoplasmic Domain of Natriuretic Peptide Receptor-C (NPR-C) Constitutes Gi-Activator Sequences that Inhibit Adenylyl Cyclase *

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Running Title: Gi- Activator Sequences of NPR-C Cytoplasmic Domain Inhibit Adenylyl Cyclase

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ABSTRACT

We have recently demonstrated that a 37-amino acid peptide corresponding to the cytoplasmic domain of the NPR-C receptor inhibited adenylyl cyclase activity via pertussis toxin (PT)-sensitive Gi protein In the present studies we have used seven different peptide fragments of the cytoplasmic domain of the NPR-C receptor with complete, partial or no Gi-activator sequence to examine their effects on adenylyl cyclase activity. The peptides used were KKYRITIERRNH (#1), RRNHQEESNIGK-(#2), HRELREDSIRSH-(#3), RRNHQEESNIGKHRELR-(#4), QEESNIGK-(X) ITIERRNH-(Y) and ITIYKKRRNHRE (Z). Peptides #1, #3 and #4 have complete Gi-activator sequences, whereas peptides #2 and Y have partial Gi-activator sequences with truncated carboxy or amino terminal respectively, peptide X has no structural specificity whereas peptide Z is the scrambled peptide control for peptide #1. The peptides #1, #3 and #4 inhibited adenylyl cyclase activity in a concentration-dependent manner with apparent Ki between 0.1 and 1 nM, however, peptide #2 inhibited adenylyl cyclase activity with a higher Ki of about 10 nM and peptides X, Y and Z were unable to inhibit adenylyl cyclase activity. The maximal inhibitions observed were between 30-40%. The inhibition of adenylyl cyclase activity by peptides #1-4 was absolutely dependent on the presence of guanine nucleotides and was completely attenuated by pertussis toxin (PT) treatment. In addition, the stimulatory effects of isoproterenol, glucagon and forskolin on adenylyl cyclase activity were inhibited to different degrees by these peptides. These results suggest that the small peptide fragments of the

cytoplasmic domain of the NPR-C receptor containing 12 or 17 amino acids were sufficient to inhibit adenylyl cyclase activity through a PT-sensitive Gi-protein. The peptides having complete structural specificity of Gi-activator sequences at both amino and carboxyl terminals were more potent to inhibit adenylyl cyclase activity as compared to the peptides having a truncated carboxyl terminal, whereas the truncation of the amino terminal motif completely attenuates adenylyl cyclase inhibition.

INTRODUCTION

Atrial natriuretic peptide (ANP), a member of the family of natriuretic peptides (NP), discovered by deBold et al., (1,2) regulates a variety of physiological parameters including the blood pressure, progesterone secretion, renin release and vasopressin release by interacting with different receptors on the plasma membrane.(3-11) The other members of the natriuretic peptide family are brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) (12-14). The role played by ANP and BNP as endocrine hormones is apparently to be antagonists to vasopressin, endothelins and the renin-angiotensin-aldosterone system (12, 15). The role of CNP in vivo is less well defined. Although CNP might not be a significant modulator of diuresis and natriuresis (16,17), it is a vasodilator expressed by endothelial cells (14,18). Compared with ANP, BNP has an additional six amino acid sequence at its amino terminal end (5,13,19) whereas CNP lacks the carboxy terminal extension. (14).

Molecular cloning techniques revealed three subtypes of natriuretic peptide receptors

(NPR): (NPR)-A (20,21), NPR-B (22, 23) and NPR-C (24, 25). NPR-A and NPR-B are membrane guanylyl cyclases, whereas NPR-C (clearance receptor) lacks guanylyl cyclase activity. NPR-A catalyses the production of cGMP in response to ANP and BNP, whereas NPR-B is the target for CNP. NPR-C receptors on the other hand are coupled to adenylyl cyclase inhibition through inhibitory guanine nucleotide-regulatory protein (25,26) or to activation of phospholipase C (27).

NPR-C receptors are disulfide linked homodimers of 64-66 kDa, having a single transmembrane domain (24,28,29)., an extracellular domain of approximately 440-amino acid, and a short 37 amino acid cytoplasmic domain or tail. We have recently demonstrated that the 37 amino acid peptide (R37A) corresponding to the cytoplasmic domain of the NPR-C receptor inhibited adenylyl cyclase activity in rat heart particulate fractions which was completely blocked by the polyclonal rabbit antisera raised against R37A (30).. The inhibition was dependent on the presence of GTP and was blocked by pertussis toxin (PT) treatment. Furthermore, inhibition of adenylyl cyclase by R37A was not due to the positive charges, because a scrambled peptide K37A with the same composition as that of R37A but a different sequence did not inhibit adenylyl cyclase activity (30). These data suggested that certain structural specificity present in the cytoplasmic domain of the NPR-C receptor may be responsible to exert inhibitory effects on adenylyl cyclase. Okamoto et al. have shown that a short intracellular region of 14 amino acids (Arg^{2410}-Lys^{2423}) of insulin-like growth factor II receptor (IGF-II R) having a specific Gi activator sequence was able to activate Gi-protein directly in a same manner as that of conventional Gi-coupled receptors (31).This sequence (Gi-activator) was characterized by the presence of two basic amino acids at the amino terminal (N- terminal) and B-B-X-B or B-B-X-X-B at carboxy terminal (C -terminal), where B and X denote basic amino acid and non-basic amino acid respectively. The cytoplasmic domain of NPR-C receptor contains several of these Gi-activator sequences. In the present studies we have used seven different synthetic peptide fragments of the cytoplasmic domain of NPR-C receptor with complete, partial or no Gi

activator sequence in order to examine their effects on adenylyl cyclase activity. We have shown that small fragment peptides of the cytoplasmic domain of the NPR-C receptor with complete Gi- activator sequence were more potent inhibitors of adenylyl cyclase activity, compared to the peptides having partial or no consensus Gi-activator sequence.

EXPERIMENTAL PROCEDURES

Materials - ATP, cAMP, and isoproternol were purchased from Sigma. Creatine kinase (EC 2.7.4.3) and GTPγS were purchased from Boehringer Mannheim. [alpha-^{32}P]ATP was purchased from Amersham Corp. Pertussis Toxin was from List Biochemicals (Campbell, CA). Rat ANP, a ring-deleted analog of ANP, c-ANP$_{4-23}$ and Angiotensin II were from Pennisula Laboratories (Belmont,CA). Peptides R37A, #1, #2, #3 , #4, X, Y and Z were synthesized by standard solid phase techniques and highly purified (95%-99%) by high performance liquid chromatography (Pennisula Laboratories and Chiron Technologies).

Preparation of Heart Particulate Fractions- Heart ventricles were dissected from Sprague Dawley rats (200-300 g), quickly frozen in liquid nitrogen, and stored at -80 C until used. Frozen hearts were pulverized to a fine powder with a mortar and pestle pre-cooled in liquid nitrogen. The heart powder was homogenized using a Teflon-glass homogenizer (12 strokes) in a buffer containing 10mM Tris-HCl and 1mM EDTA, pH 7.5, then centrifuged at 1000x g for 10 min. The supernatant was discarded and the pellet was homogenized in the above buffer and centrifuged at 1000 x g for 10 min. The pellet was finally suspended by homogenization in 10mM Tris-HCl and 1 mM EDTA pH 7.5, and used directly for adenylyl cyclase activity determination. Protein was determined essentially as described (32) with crystalline bovine serum albumin as standard.

Cell Culture and Incubation-

The A10 cell line from embryonic thoracic aorta of rat was obtained from American Type Culture Collection Rockville, MA, USA. 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 Dulbeccos modified Eagles medium (DMEM) (with glucose, L-glutamine, and sodium bicarbonate) containing antibiotics and 10% heat-activated 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. The cells were scraped into ice-cold homogenization buffer using a rubber policeman, and collected by centrifugation at 4C for 10 min at 600 x g. The cells were then homogenized in a Dounce homogenizer (10 strokes) and the homogenate was used for adenylyl cyclase assay.

Pertussis Toxin (PT) Treatment- PT treatment was performed as described earlier (25,33,34). Briefly, heart particulate fractions were incubated in 25 mM glycylglycine buffer, pH 7.5, containing 1 mM NAD, 0.4mM ATP, 0.4 mM GTP, 15mM thymidine, 10 mM dithiothretiol , and ovalbumin (0.1 mg/ml) with and without PT (5ug/ml) for 30 min at 30C. The particulate fraction was washed two to three times with 10 mM Tris, 1 mM EDTA buffer, pH 7.5, and finally suspended in the same buffer and used for adenylyl cyclase activity determination. Preincubation of the membranes at 30C for 30 min in the absence or presence of PT resulted in a significant loss of enzyme activity (~ 40%) which was independent of the presence of PT in the incubation medium. However, the percent inhibition of adenylyl cyclase activity by the different peptides used remained unchanged.

Adenylyl Cyclase Activity Determination- Adenylyl Cyclase activity was determined by measuring [^{32}P] cAMP formation from [α-^{32}P]ATP as described previously (26,33). The typical assay medium contained 50 mM glycylglycine, pH 7.5, 0.5 mM MgATP, [α-^{32}P]ATP

(1-1.5 x 10^6 cpm), 5 mM $MgCl_2$, 100mM NaCl, 0.5 mM cAMP, 1mM 3-isobutyl-1-methylxanthine, 0.1 μM EGTA, 10μM GTPγS, and an ATP-regenerating system consisting of 2mM creatine phosphate, 0.1 mg of myokinase per ml in a final volume of 200□l. Incubations were initiated with the addition of reaction mixture to the membranes (30-70 μg) which had been preincubated at 37°C for 10 min. The reactions conducted in triplicate at 37° C for 10min were terminated by the addition of 0.6 ml of 120 mM zinc acetate, cAMP was purified by co-precipation of other nucleotides with $ZnCO_3$ by the addition of 0.5 ml of 144mM Na_2CO_3 and by subsequent chromatography using the double column system (35). Under these assay conditions, adenylyl cyclase activity was linear with respect to protein concentration and time of incubation.

RESULTS

Effect of different fragments of the cytoplasmic domain of NPR- C receptor on adenylyl_cyclase activity-

The 37 amino acid peptide (R37A) corresponding to the cytoplasmic domain the NPR-C receptor has been shown to inhibit adenylyl cyclase activity by interacting directly with Gi-proteins (30). In order to investigate if the small peptides fragments of the cytoplasmic domain that consist of partial or complete Gi- activator sequences could also mimic the effect of R37A or ANP on NPR-C receptor-mediated inhibition of adenylyl cyclase, seven different synthetic peptides were used to examine their effects on adenylyl cyclase activity. The peptide fragments represent different parts of the cytoplasmic domain as shown in Fig. 1. These consist of 12 amino acids (peptide #1, #2, #3 and Z), 17 amino acids (peptide #4) or 8 amino acids (peptides X and Y). Peptides #1, #3 and #4 possess the required Gi-activator sequences; two basic amino acids at the N- terminal and B-B-X-B, B-X-B or B-B-X-X-B at the C- terminal respectively, where B is basic amino acid and X is non-basic amino acid, whereas peptide #2 has two basic amino acids at the N- terminal but does not have the consensus sequence at the C- terminal. On the other hand peptide Y has only the consensus sequence at C-terminal and peptide X lacks Gi-activator sequence, whereas peptide Z is the scrambled peptide and serves as control for peptide #1..

Figure 2A shows that peptides # 1, 2, 3 and 4 of the cytoplasmic domain of the NPR-C receptor at 10^{-7} M, inhibited adenylyl cyclase activity by 40,30,35 and 36 %

respectively in rat heart particulate fractions. In addition ANP_{99-126}, $C\text{-}ANP_{4-23}$, R37A and Angiotensin II (AII) as reported earlier, (3, 26, 30, 36) also inhibited the enzyme activity by about 20-25%. However, peptides X, Y and Z did not inhibit adenylyl cyclase activity. Similar results were also observed in A-10 vascular smooth muscle cells (Fig. 2B), however the inhibitions were greater (55%) in these cells. These results suggest that small peptides of the cytoplasmic domain of the NPR-C receptor with 12 amino acids or more with specific Gi-activator sequence could inhibit adenylyl cyclase activity.

Figure 3 shows the effects of various concentrations of the seven peptides on adenylyl cyclase activity in heart particulate fractions. The peptides #1, #3 and #4 inhibited adenylyl cyclase activity in a concentration-dependent manner with apparent Ki between 0.1 and 1nM, however, peptide #2 inhibited adenylyl cyclase activity with a higher Ki of about 10 nM. The maximal inhibitions of adenylyl cyclase activity by peptides #1, #2, #3 and #4 were about 40, 30, 35 and 36% respectively. The inhibitory effect of these peptides was not due to the positive changes per se, because the scrambled peptide (peptide Z) of peptide #1 that has the same amino acid composition was unable to exert any inhibitory effect on adenylyl cyclase. In addition, peptides X and Y also did not inhibit adenylyl cyclase activity. These data suggest that the peptides possessing the required Gi-activator sequence at the carboxy terminal as well as at the amino terminal are more potent inhibitors of adenylyl cyclase activity than the peptides having partial or no structural specificity.

Dependence of Peptides #1, #2 ,#3 and #4-mediated Inhibition of Adenylyl Cyclase on Guanine Nucleotides- The inhibitory effect of the R37A peptide

corresponding to the cytoplasmic domain of the NPR-C receptor on adenylyl cyclase activity has been reported to be dependent on the presence of guanine nucleotides. (30) In order to investigate if the inhibition of adenylyl cyclase mediated by the small peptides also requires guanine nucleotides, the effect of different peptides on adenylyl cyclase activity in the absence or presence of GTPγS was examined. The results shown in Figure 4 indicate that all four active peptides (#1, #2, #3 and #4) did not exert any inhibitory effect on adenylyl cyclase in the absence of GTPγS however, in the presence of various concentrations of GTPγS, the four peptides inhibited the enzyme activity in a concentration- dependent manner. The maximal inhibitions (30-40%) was observed at 10 μM GTPγS. These results indicate that the inhibition of adenylyl cyclase by all the four peptides of the cytoplasmic domain of NPR-C is also dependent on the presence of guanine nucleotides.

Effect of Pertussis Toxin (PT) on Peptide #1, #2, #3 and #4-mediated Inhibition of Adenylyl Cyclase-The coupling of NPR-C receptors to adenylate cyclase through inhibitory guanine nucleotide regulatory protein (Gi) has been demonstrated (25,30). Furthermore, we have shown that R37A inhibited adenylyl cyclase through a PT-sensitive Gi protein. To examine if the inhibition of adenylyl cyclase by these four peptides is mediated through Gi, the effect of PT treatment on the inhibitory effects of the four peptides on adenylyl cyclase activity was examined. Figure 5 shows that all four peptides inhibited the enzyme activity in a concentration- dependent manner in control heart particulate fractions which was attenuated by PT treatment. These results indicate

that like R37A, the small peptides of the cytoplasmic domain of NPR-C receptor also inhibit adenylyl cyclase through a PT -sensitive Gi-protein.

Inhibition of Agonist -stimulated Adenylate Cyclase activity by small peptide fragments of Cytoplamic domain- Since ANP $_{99\text{-}126}$, C-ANP $_{4\text{-}23}$ as well as R37A peptide have been shown to inhibit the stimulatory effects of various agonists on adenylyl cyclase activity (25, 26, 30), it was of interest to examine if small peptides of cytoplasmic domain of NPR-C receptor are also capable of inhibiting the stimulated adenylyl cyclase activity in heart particulate fractions. As shown in Figure 6, glucagon, isoproterenol and forskolin stimulated adenylyl cyclase activity by about 160, 190 and 1000% respectively which was inhibited by all four peptides (0.1 μM) to various degrees. Glucagon-mediated stimulation was inhibited by about 30%, whereas isoproternol-stimulated enzyme activity was inhibited by about 20-25% and forskolin-stimulated enzyme activity was inhibited by about 15-35%.

DISCUSSION

We have previously shown that 37 amino acid synthetic peptide (R37A) corresponding to the cytoplasmic domain of NPR-C receptor inhibited adenylyl cyclase activity via PT-sensitive Gi protein (30). In the present studies, we demonstrate for the first time that the cytoplasmic domain peptide of NPR-C receptor has several Gi activator sequences that inhibit adenylyl cyclase activity in GTP dependent manner via PT-sensitive Gi proteins.

The small peptide fragments of cytoplasmic domain of NPR-C receptor containing 12 amino acids (Lys^{2}-His^{13}, peptide #1) with consensus sequence for Gi activation at both N-terminal and C terminal, i.e. B-B at N-terminal and B-B-X-B motif at C-terminal inhibited adenylyl cyclase activity in rat heart particulate fractions, cultured vascular smooth muscle cells and aorta. The other peptide fragments containing 17 amino acids (Arg^{10}- Arg^{26}, peptide #4) with consensus sequence of B-B at N-terminal and B-B-X-X-B at C-terminal also inhibited adenylyl cyclase activity in these tissues/cells. Both these peptide fragments (peptides #1 & 4) inhibited the enzyme activity in a concentration dependent manner with an apparent Ki between 0.1-1 nM, the maximal inhibition observed was about 30-35%. The potency of these peptide fragments to inhibit adenylyl cyclase activity was in the same range as that of the entire cytoplasmic domain peptide R37A as reported earlier (30). The inhibitory effect of these peptides on adenylyl cyclase was not due to the net positive charge present (ie. amino acid composition) since the scrambled peptide Z with the same composition as peptide

#1 but lacking Gi activator sequence at N- and C-terminal did not inhibit adenylyl cyclase activity. On the other hand, the presence of partial C-terminal motif B-X-B but intact N-terminal motif (B-B) in the peptide did not change the potency of the peptide to inhibit adenylyl cyclase activity, suggesting that partial C-terminal motif in the peptide may be sufficient to exert inhibitory effect on adenylyl cyclase activity. However, the truncation of C-terminal motif (B-X-B) from peptide #2 (Arg^{10}-Lys^{21}) inhibited adenylyl cyclase activity with lower potency (Ki ~10 nM), suggesting that C-terminal motif may be important to increase the potency of the peptides to elicit adenylyl cyclase inhibition. These results are in agreement with the recent studies of Kanwal et al. (37) who have shown that 15 amino acid peptide fragment of NPR-C receptor (Arg^{1}-Gln^{15}) that lacks C-terminal motif attenuated dopamine efflux in pheochromocytoma cells (PC12). Similarly, R37A peptide corresponding to the cytoplasmic domain of NPR-C receptor that lacks C-terminal motif B-X-B has also been reported to inhibit adenylyl cyclase activity (30) as well as neurotransmission (37). However, our results are in contrast with studies of Murthy and Makhlouf (38) who have shown that cytoplasmic domain peptide fragment of human NPR-C receptor that lacks C-terminal was inactive in stimulating PLC-β activity in gastric and tenia coli smooth muscle. The truncation of N-terminal motif of the peptide #2 results in inactivation of the peptide to inhibit adenylyl cyclase activity suggesting that N-terminal consensus sequence is important to interact with Gi proteins to exert inhibition of adenylyl cyclase. This is further substantiated by our results showing that peptide Y that has consensus sequence at C-terminal but lacks N-terminal sequence was unable to inhibit adenylyl cyclase activity. However, Murthy and

Makhlouf (38) have shown that peptide containing consensus sequence at N-terminal was unable to stimulate PLC-β in tenia coli smooth muscle cell membranes. This apparent discrepancy may be attributed to the difference in cell/ tissue system utilized in the two studies. Moreover, these investigators have shown that 17-amino acid peptide of human NPR-C receptor cytoplasmic domain stimulated PLC-β activity at higher concentrations with EC_{50} of 1.3 μM, whereas we have shown that the potency of the active peptides #1, 3 and 4 was at least 1000-fold higher in inhibiting adenylyl cyclase activity. In addition, the peptide #2 that lacks C-terminal motif sequence although was less active than peptides #1, 3 and 4 but was still able to inhibit adenylyl cyclase activity, whereas Murthy and Makhlouf (38) did not observe any effect of the peptide lacking C-terminal consensus sequence on PLC-β activity. The lack of effect of this peptide in their studies may be due to the possibility that this peptide also did not have the N-terminal consensus sequence and thus is similar to the peptide X in our studies which was also unable to inhibit adenylyl cyclase activity. Taken together, it can be suggested that N-terminal motif of these peptides may be the important site for the interaction with Gi-protein and thereby to activate the effector systems.

Our studies on the dependence on guanine nucleotides of active peptides to adenylyl cyclase inhibition and its attenuation by PT treatment, are consistent with previous reports (25, 26, 30) and suggest that the small active peptide fragments of cytoplasmic domain of NPR-C receptor like entire cytoplasmic domain peptide R37A could also inhibit adenylyl cyclase via PT-sensitive Gi-protein. In addition, the inhibition of glucagon, isoproterenol- and forskolin-mediated stimulation of adenylyl cyclase by

small peptides is also consistent with our previous studies on ANP, C-ANP_{4-23} and R37A and adenylyl cyclase signalling (25, 26, 30).

In conclusion, we have provided the first evidence to demonstrate that the cytoplasmic domain peptides of NPR-C receptor of 12 amino acids possessing complete Gi activator sequence at C-terminal and N-terminal are sufficient to inhibit adenylyl cyclase activity through a PT-sensitive Gi protein with same potency as that of entire cytoplasmic domain peptide, whereas the peptides with truncated C-terminal inhibited the enzyme activity with low potency, however, the truncation of N-terminal motif completely attenuates adenylyl cyclase inhibition.

FOOTNOTES

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[1] Abbreviations: ANP, rat natriuretic peptide (28 amino acids); BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; $CANP_{4-23}$ [des-(Gln^{18},Ser^{19},Gly^{20},Leu^{21},Gly^{22}] rat ANP $_{(4-23)}$; NPR-C natriuretic peptide receptor- C; G-protein, heterotrimeric guanine nucleotide regulatory protein; PT, pertussis toxin; GTPγS, 5-(3-O-thio)-triphosphate; c-GMP, cyclic 3-,5- guanosine monophosphate; PBS, phosphate buffered saline; aa, amino acid.

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FIGURE LEGENDS

Figure #1:**Sequence of the entire cytoplasmic domain of NPR-C and the various synthetic peptides corresponding to different regions of the cytoplasmic domain.** B-basic amino acid, X-non basic amino acid.

Figure #2 : **Effect of different peptides on adenylyl cyclase activity in rat heart (A), and vascular smooth muscle cells (B).** The effect of peptides on adenylyl cyclase activity was determined as described under "Experimental Procedures " in the presence of 10 uM GTPγS. The basal adenylyl cyclase activity was 225 +/- 9 pmol cAMP /mg of protein /10min in heart and 180 6 pmol cAMP/mg proein/10 min in vascular smooth muscle cells. Values are the means of +/- S.E. of three to four separate experiments.

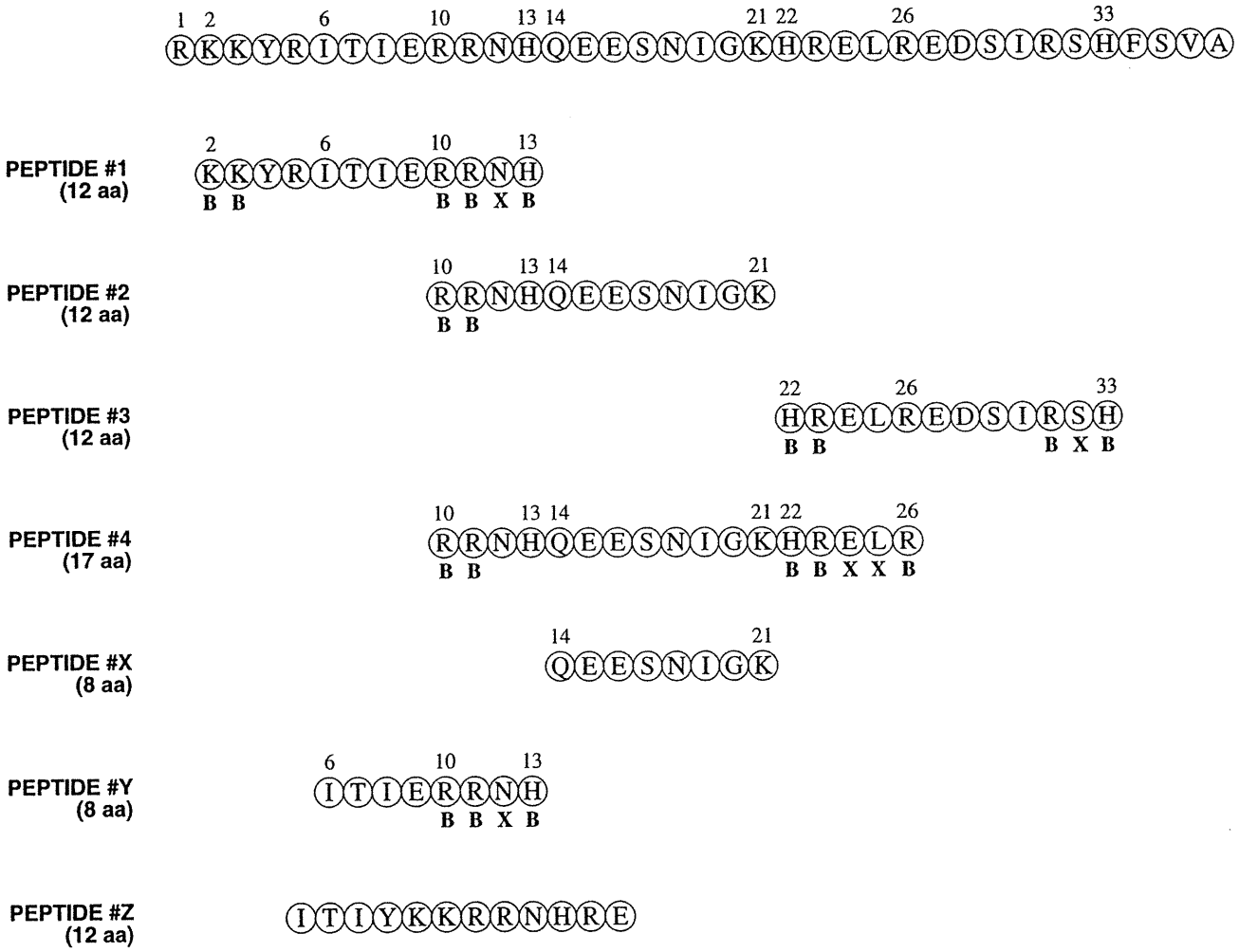
Figure #3 : **Inhibition of rat heart adenylyl cyclase activity**. The effect of different concentrations of peptides #1,#2,#3, #4,X, Y and Z on adenylyl cyclase activity was determined as described under " Experimental Procedures " in the presence of 10 *uM* GTPγS. The basal adenylyl cyclase activity was 225 +/- 9 pmol of cAMP/mg of protein /10min. Values are the means of +/- S.E. of four separate experiments.

Figure #4: **Guanine nucleotide dependence of adenylyl cyclase inhibition by peptides #1,#2,#3 and #4.** Adenylyl cyclase activity was determined in the presence of various concentrations of GTPγS alone (basal) or in combination with 0.1 *uM* of the four peptides. Values are the means of +/- S.E. of four separate experiments.

Figure #5: **Effect of pertussis toxin on different peptide- mediated inhibition of rat heart adenylyl cyclase.** Heart particulate fractions were treated without (-PT) or with (+PT) pertussis toxin. Adenylyl cyclase activity was determined in rat heart particulate fractions treated without (-PT) or with (+PT) pertussis toxin in the absence or presence of various concentrations of four peptides(A-D) as described under " Experimental Procedures ". Values are the means +/- S.E. of three separate experiments. The basal enzyme activities in control and PT treated heart particulate fractions were 285 +/- 10 and 201 +/- 8 pmol of cAMP/mg protein/10min, respectively.

Figure #6: **Inhibition of agonist - stimulated rat heart adenylate cyclase activity by different peptides fragments** - Adenylyl cyclase activity was determined in the presence of 1 uM glucagon (A), 10 uM isoproterenol (B) and 50 uM forskolin

(C) alone or in combination with 0.1 uM peptides as described in " Experimental Procedures " Values are the means +/- S.E. of three separate experiments. The basal enzyme activity was 180 +/- 9 pmol of cAMP/mg of protein/10 min.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PEPTIDE #1 (12 aa)
2 6 10 13
K K Y R I T I E R R N H
B B B B X B
PEPTIDE #2 (12 aa)
10 13 14 21
R R N H Q E E S N I G K
B B
PEPTIDE #3 (12 aa)
22 26 33
H R E L R E D S I R S H
B B B X B
PEPTIDE #4 (17 aa)
10 13 14 21 22 26
R R N H Q E E S N I G K H R E L R
B B B B X X B
PEPTIDE #X (8 aa)
14 21
Q E E S N I G K
PEPTIDE #Y (8 aa)
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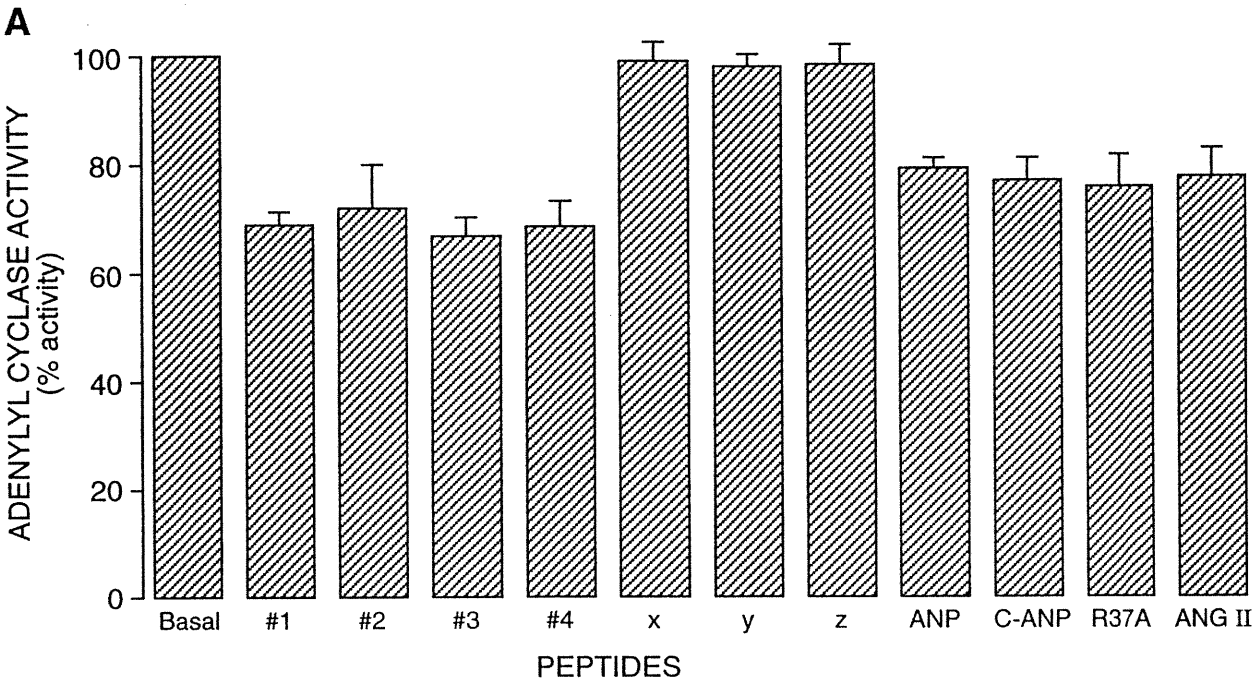
I T I E R R N H
B B X B
PEPTIDE #Z (12 aa)
I T I Y K K R R N H R E

Fig 1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80
60
40
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#2
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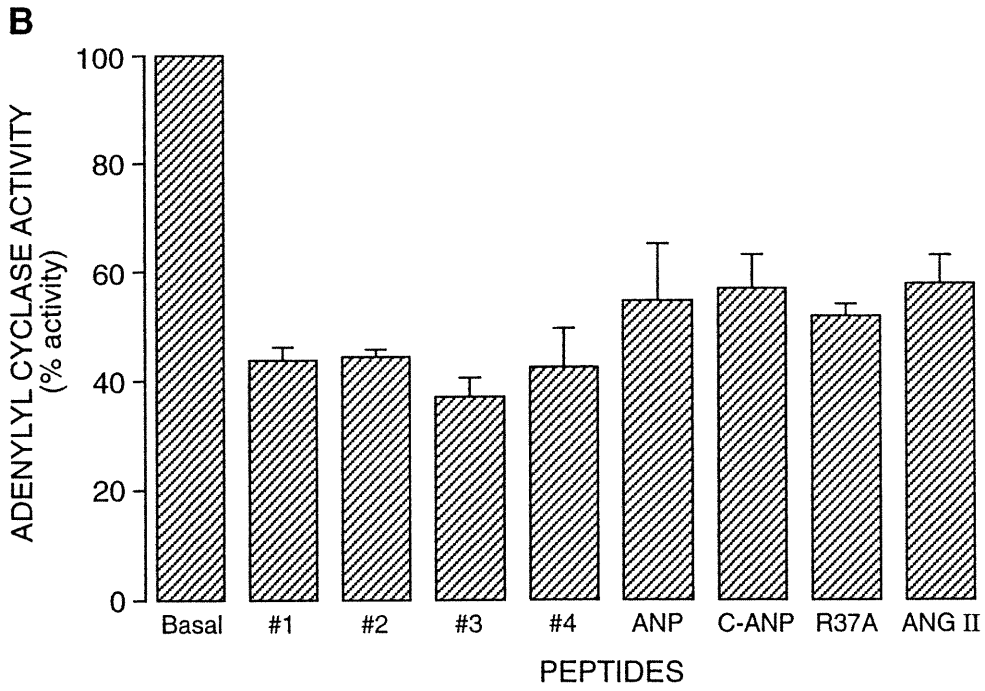
ANG II
PEPTIDES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ADENYLYL CYCLASE ACTIVITY
(% activity)
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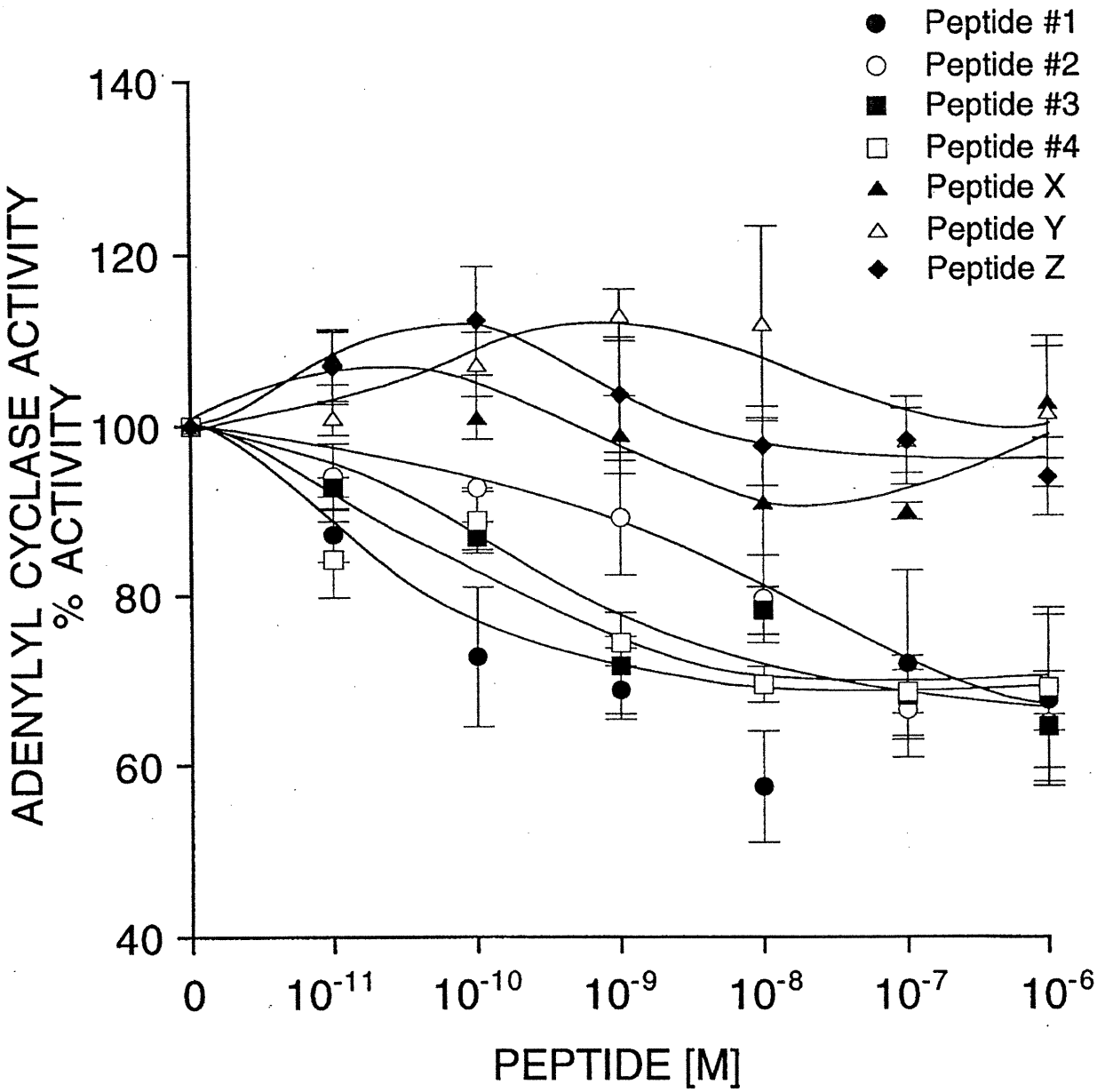
ANG II
PEPTIDES

Fig. 2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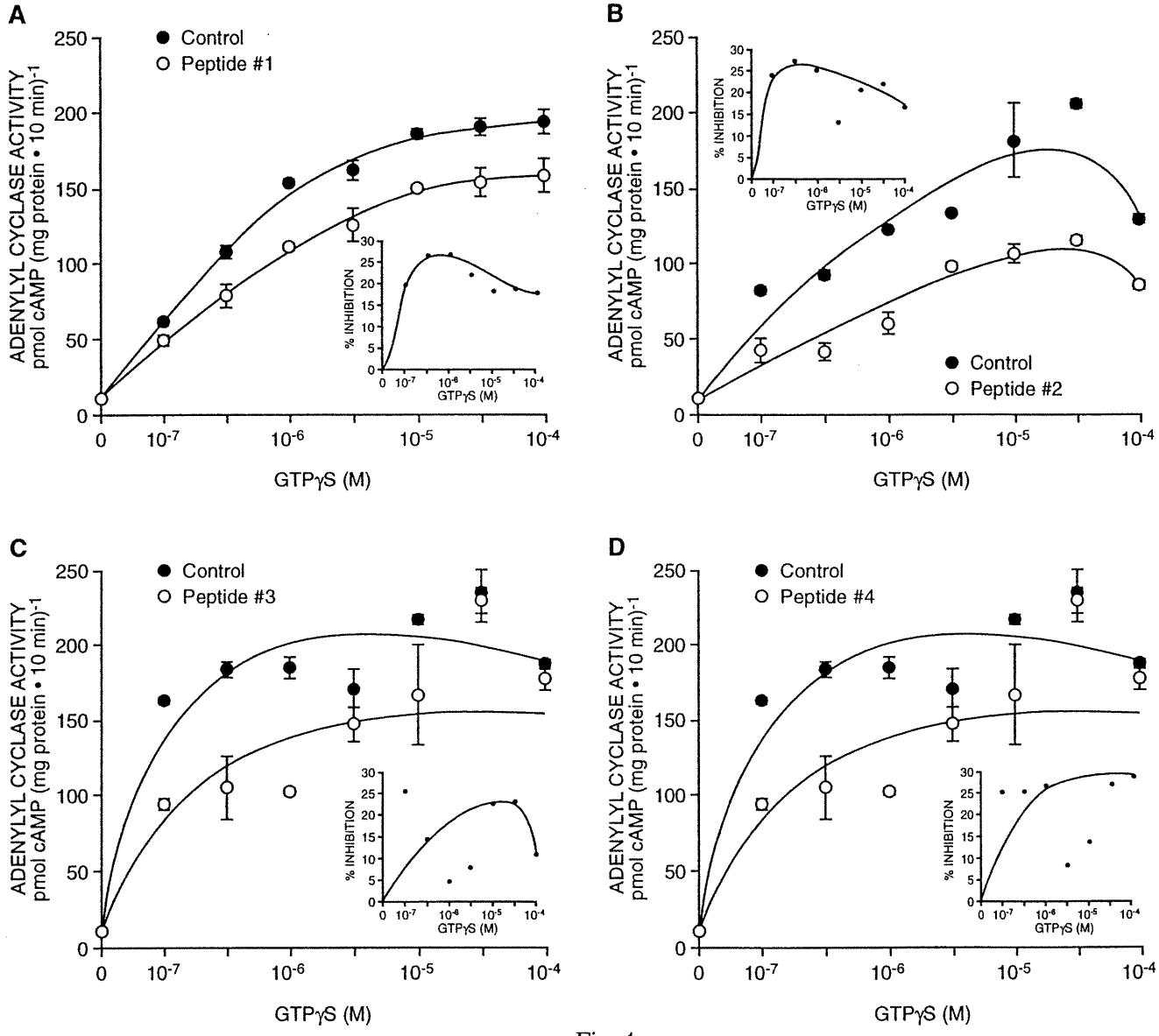
PEPTIDE [M]

Fig. 3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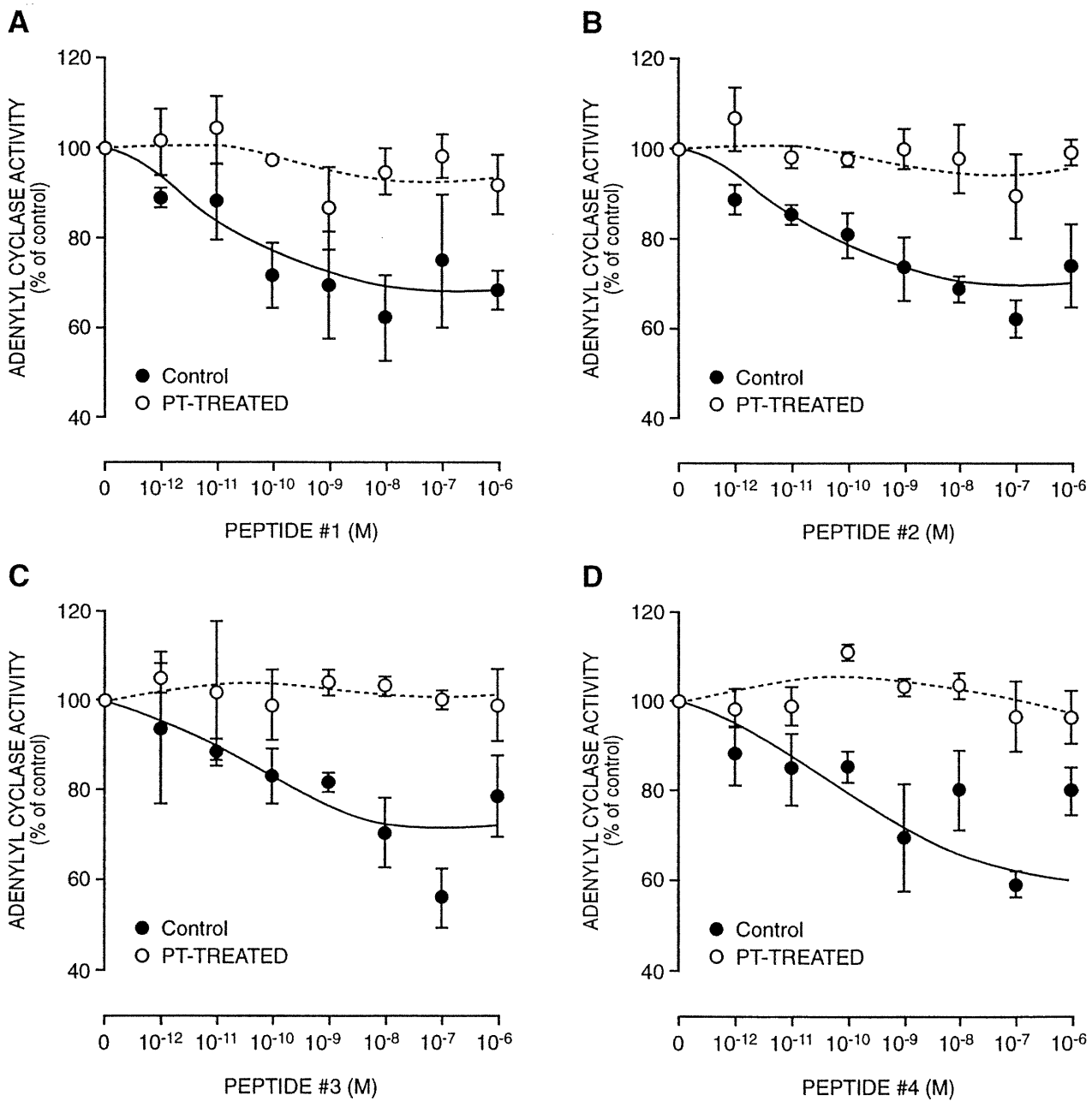
Peptide #2
C
Control
Peptide #3
D
Control
Peptide #4

Fig. 4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B
C
D
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100
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Control
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10-11
10-10
10-9
10-8
10-7
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PEPTIDE #1 (M)
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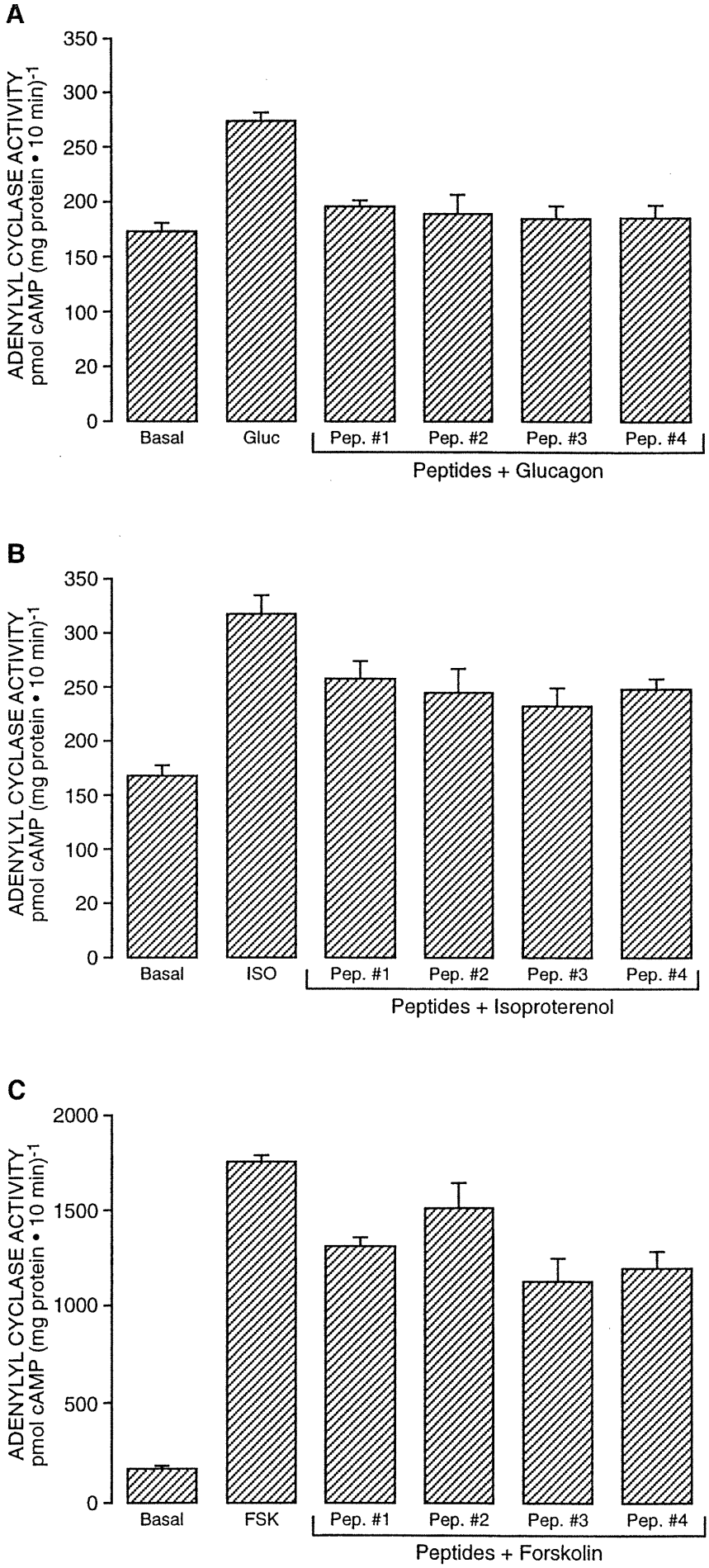
PEPTIDE #3 (M)
PEPTIDE #4 (M)

Fig. 5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300
250
200
150
100
20
0
Basal
Gluc
Pep. #1
Pep. #2
Pep. #3
Pep. #4
Peptides + Glucagon
B
ADENYLYL CYCLASE ACTIVITY
pmol cAMP (mg protein • 10 min)-1
350
300
250
200
150
100
20
0
Basal
ISO
Pep. #1
Pep. #2
Pep. #3
Pep. #4
Peptides + Isoproterenol
C
ADENYLYL CYCLASE ACTIVITY
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2000
1500
1000
500
0
Basal
FSK
Pep. #1
Pep. #2
Pep. #3
Pep. #4
Peptides + Forskolin

Fig. 6

CHAPTER 3

DISCUSSION AND CONCLUSIONS

DISCUSSION

It has been over 20 years since the Atrial Natriuretic Peptide was discovered in 1981 by deBold et al. The much-sought out plasma natriuretic substance was not biologically silent as first thought to be. ANP can regulate several physiological parameters such as blood pressure, progesterone secretion, renin release, and vasopressin release by interacting with different receptors on the plasma membrane. Molecular cloning revealed two major categories of ANP receptors; NPR-A/B receptors, which are coupled to guanylyl cyclase, and NPR-C receptors coupled to adenylyl cyclase. The NPR-C has demonstrated involvement in various physiological responses. Levin and Frank (1991) showed the NPR-C receptor inhibits rat astroglial proliferation; Johnson et al.1991 discovered that it inhibited the electrically induced purinergic and adrenergic contractile force in rabbit isolated vas deferentia and recently using knockout mice, Jaubert et al,1999, showed the NPR-C receptor to be important in skeletal development. In addition, several studies demonstrated that NPR-C receptors had the ability to inhibit adenylyl cyclase activity in many different tissues, in the presence of guanine nucleotides, via a pertussis toxin Gi protein. These examples and others prove that the NPR-C receptor is biologically active and isn't just a clearance receptor as first postulated.

Schenk et al., (1987), characterized the NPR-C receptor, their work uncovered that the protein consisted of 537 amino acids containing a large extracellular region, a single membrane-spanning domain and a short cytoplasmic

tail of 37 amino acids. Previous studies in our lab showed that a 37-amino acid synthetic peptide (R37A) corresponding to the cytoplasmic domain of the NPR-C receptor inhibited adenylyl cyclase activity via a pertussis toxin Gi protein. This data suggested that the cytoplasmic domain of the NPR-C receptor might have a structural specificity like those of other single-transmembrane-domain receptors (Neuronal protein GAP-43, mastoparan, insulin and insulin-like growth factor II/mannose 6-phosphate) in order to exert its inhibitory effects on adenylyl cyclase.

In the present studies, we further explored the possible presence of structural specificity of the NPR-C receptor. We took our previous results with R37A and the work by Okamoto et al., that postulated that the specific Gi-activator sequence consisted of two basic amino acids at the NH_2 terminal and a carboxy terminal ending in B-B-X-B or B-B-X-X-B, where B=basic amino acid and X=non-basic amino acid. The cytoplasmic domain contains many of these Gi-activator sequences. We used seven different peptide fragments of 8,12 and 17 amino acids, containing complete, partial or no Gi-activator sequence requirements. The results demonstrated that that the cytoplasmic domain peptides of NPR-C receptor of 12 amino acids possessing complete Gi activator sequence at C-terminal and N-terminal are sufficient to inhibit adenylyl cyclase activity through a PT-sensitive Gi protein with same potency as that of entire cytoplasmic domain peptide. These results are in agreement with the studies of Murthy and Makhlouf (1999), who showed the inhibition of adenylyl cyclase activity in gastric and tenia coli smooth muscle, using a similar 17 aa peptide with Gi- activator sequence structural specficity at both the amino and carboxy terminal. Furthermore our studies showed

that the peptides with truncated C-terminal inhibited the enzyme activity with low potency, whereas, the truncation of N-terminal motif completely attenuated adenylyl cyclase inhibition. These results are in agreement with the work of Kanwal et al. (1999) who have shown that a 15 amino acid peptide fragment of NPR-C receptor (Arg^{1}-Gln^{15}) that possed the N-terminal motif but lacks the C-terminal motif attenuated dopamine efflux in pheochromocytoma cells (PC12). These results differ from the results of Murthy and Makhlouf (1999), who have shown that cytoplasmic domain peptide fragment of human NPR-C receptor that lacks C-terminal was inactive in stimulating PLC-β activity in gastric and tenia coli smooth muscle. This apparent discrepancy may be attributed to the difference in cell/ tissue system utilized in the two studies. Moreover, these investigators have shown that 17-amino acid peptide of human NPR-C receptor cytoplasmic domain stimulated PLC-β activity at higher concentrations with EC_{50} of 1.3 μM, whereas we have shown that the potency of the active peptides #1, 3 and 4 was at least 1000-fold higher in inhibiting adenylyl cyclase activity. In addition, the peptide #2 that lacks C-terminal motif sequence although was less active than peptides #1, 3 and 4 but was still able to inhibit adenylyl cyclase activity, whereas Murthy and Makhlouf (1999) did not observe any effect of the peptide lacking C-terminal consensus sequence on PLC-β activity. The lack of effect of this peptide in their studies may be due to the possibility that this peptide also did not have the N-terminal consensus sequence and thus is similar to the peptide X in our studies which was also unable to inhibit adenylyl cyclase activity. Taken together, it can be

suggested that N-terminal motif of these peptides may be the important site for the interaction with Gi-protein and thereby to activate the effector systems.

Our next studies were to explore if these same small peptide fragments, that inhibited adenylyl cyclase activity, would be able to show physiological responses. Prin et al., (1996), showed that C-ANP$_{(4,23)}$ and ANP inhibited the mitogen-activated protein kinase (MAPK) activity stimulated by endothelin-3, platelet derived growth factor and phorbol-12-myristrate 13-acetate. These results indicate that the NPR-C receptor plays a role in cell proliferation through inhibition of MAPK. In our lab we tested the effects of the peptide fragments #1,2,3 and 4 on cell proliferation. The results showed that the peptides were able to inhibit mitogen-activated protein kinase (MAPK) activity stimulated by Angiotensin II, endothelin and vasopressin, (Unpublished results). All four peptides yielded similar results by decreasing both the levels of DNA synthesis and transcription. The precise mechanism in which these peptides effectuate this inhibition is not known, and further exploration is required. As shown in figure #13, the possible mechanism of inhibition of nuclear activity, could be the inhibition of MAPKK and MAPK via the four peptides and their Gi-activator sequence. In future experiments the effect of these peptide fragments on other physiological functions such as progesterone secretion; vasopressin release, catecholamine release and other neuromodulatory functions will be explored.

In summary, we have shown that small peptide fragments from the cytoplasmic domain of the NPR-C receptor to be very active. Our results, as discussed above, are supported by the studies of Kanwal et al. (1999), and Murthy

and Makhlouf (1999). Kanwal et al., (1999) showed that a 15 aa small peptide, with N-terminal specficity, could attenuate the dopamine efflux in PC12 cells and Murthy and Makhlouf (1999) were able to stimulate phospholipase Cβ in tenia coli smooth muscle cell membranes, using a 17 aa peptide having both N and carboxy terminal structural speficity. Taken together with the fact that these small peptide fragments have been shown to inhibit adenylyl cyclase activity, and that the adenylyl cyclase transduction system is responsible in mediating many physiological parameters, one can suggest that there is a possibility that these small peptides can be used as therapeutic agents in controlling and remedying certain illnesses and pathologies such as hypertension. The use of these peptides as pharmacological agents would require that the peptides be modified in order to pass through the plasma membrane. Possible modifications could be meristylation and specific drug delivery techniques, which isolate tissue and timing of delivery.

CONCLUSIONS

We have provided the first evidence to demonstrate that the cytoplasmic domain peptides of NPR-C receptor of 12 amino acids possessing complete Gi activator sequence at C-terminal and N-terminal are sufficient to inhibit adenylyl cyclase activity through a PT-sensitive Gi protein with same potency as that of entire cytoplasmic domain peptide. The peptides with truncated C-terminal inhibited the enzyme activity with low potency, whereas, the truncation of N-

terminal motif completely attenuates adenylyl cyclase inhibition. We've also shown that these peptides were capable of inhibiting mitogen-activated protein kinase (MAPK) activity stimulated by Angiotensin II, endothelin and vasopressin.

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Cytoplasmic Domain of Natriuretic Peptide Receptor C Constitutes G_i Activator Sequences That Inhibit Adenylyl Cyclase Activity*

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We have recently demonstrated that a 37-amino acid peptide corresponding to the cytoplasmic domain of the natriuretic peptide receptor C (NPR-C) inhibited adenylyl cyclase activity via pertussis toxin (PT)-sensitive G_i protein. In the present studies, we have used seven different peptide fragments of the cytoplasmic domain of the NPR-C receptor with complete, partial, or no G_i activator sequence to examine their effects on adenylyl cyclase activity. The peptides used were KKYRITIERRNH (peptide 1), RRNHQEESNIGK (peptide 2), HRELREDSIRSH (peptide 3), RRNHQEESNIGKHRELR (peptide 4), QEESNIGK (peptide X), ITIERRNH (peptide Y), and ITIYKKRRNHRE (peptide Z). Peptides 1, 3, and 4 have complete G_i activator sequences, whereas peptides 2 and Y have partial G_i activator sequences with truncated carboxyl or amino terminus, respectively. Peptide X has no structural specificity, whereas peptide Z is the scrambled peptide control for peptide 1. Peptides 1, 3, and 4 inhibited adenylyl cyclase activity in a concentration-dependent manner with apparent K_i between 0.1 and 1 nM; however, peptide 2 inhibited adenylyl cyclase activity with a higher K_i of about 10 nM, and peptides X, Y, and Z were unable to inhibit adenylyl cyclase activity. The maximal inhibitions observed were between 30 and 40%. The inhibition of adenylyl cyclase activity by peptides 1–4 was absolutely dependent on the presence of guanine nucleotides and was completely attenuated by PT treatment. In addition, the stimulatory effects of isoproterenol, glucagon, and forskolin on adenylyl cyclase activity were inhibited to different degrees by these peptides. These results suggest that the small peptide fragments of the cytoplasmic domain of the NPR-C receptor containing 12 or 17 amino acids were sufficient to inhibit adenylyl cyclase activity through a PT-sensitive G_i protein. The peptides having complete structural specificity of G_i activator sequences at both amino and carboxyl termini were more potent to inhibit adenylyl cyclase activity as compared with the peptides having a truncated carboxyl terminus, whereas the truncation of the amino-terminal motif completely attenuates adenylyl cyclase inhibition.

Atrial natriuretic peptide (ANP),[1] a member of the family of natriuretic peptides (NP), discovered by de Bold *et al.* (1, 2), regulates a variety of physiological parameters including the blood pressure, progesterone secretion, renin release, and vasopressin release by interacting with different receptors on the plasma membrane (3–11). The other members of the natriuretic peptide family are brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) (12–14). The role played by ANP and BNP as endocrine hormones is apparently to be antagonists to vasopressin, endothelins, and the renin-angiotensin-aldosterone system (12, 15). The role of CNP *in vivo* is less well defined. Although CNP might not be a significant modulator of diuresis and natriuresis (16, 17), it is a vasodilator expressed by endothelial cells (14, 18). Compared with ANP, BNP has an additional six-amino acid sequence at its amino-terminal end (5, 13, 19), whereas CNP lacks the carboxyl-terminal extension. (14).

Molecular cloning techniques revealed three subtypes of natriuretic peptide receptors (NPR): NPR-A (20, 21), NPR-B (22, 23), and NPR-C (24, 25). NPR-A and NPR-B are membrane guanylyl cyclases, whereas NPR-C (clearance receptor) lacks guanylyl cyclase activity. NPR-A catalyzes the production of cGMP in response to ANP and BNP, whereas NPR-B is the target for CNP. NPR-C receptors on the other hand are coupled to adenylyl cyclase inhibition through inhibitory guanine nucleotide-regulatory protein (25, 26) or to activation of phospholipase C (27).

NPR-C receptors are disulfide-linked homodimers of 64–66 kDa, having a single transmembrane domain (24, 28, 29), an extracellular domain of ~440 amino acids, and a short 37-amino acid cytoplasmic domain or tail. We have recently demonstrated that the 37-amino acid peptide (R37A) corresponding to the cytoplasmic domain of the NPR-C receptor inhibited adenylyl cyclase activity in rat heart particulate fractions, which was completely blocked by the polyclonal rabbit antisera raised against R37A (30). The inhibition was dependent on the presence of GTP and was blocked by pertussis toxin (PT) treatment. Furthermore, inhibition of adenylyl cyclase by R37A was not due to the positive charges, because a scrambled peptide K37A with the same composition as that of R37A but a different sequence did not inhibit adenylyl cyclase activity (30). These data suggested that certain structural specificity present in the cytoplasmic domain of the NPR-C receptor may be responsible for exerting inhibitory effects on adenylyl cyclase. Okamoto *et al.* (31) have shown that a short intracellular region of 14 amino acids (Arg^{2410}–Lys^{2423}) of insulin-like growth factor II receptor having a specific G_i activator sequence was able to activate G_i protein directly in the same manner as that of conventional G_i-coupled receptors. This sequence (G_i activator) was characterized by the presence of two basic amino acids

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[1] The abbreviations used are: ANP, rat atrial natriuretic peptide (28 amino acids); BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; C-ANP-(4–23), [des-Gln^{18},Ser^{19},Gly^{20},Leu^{21},Gly^{22}]rat ANP-(4–23); NPR, natriuretic peptide receptor; PT, pertussis toxin; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate.

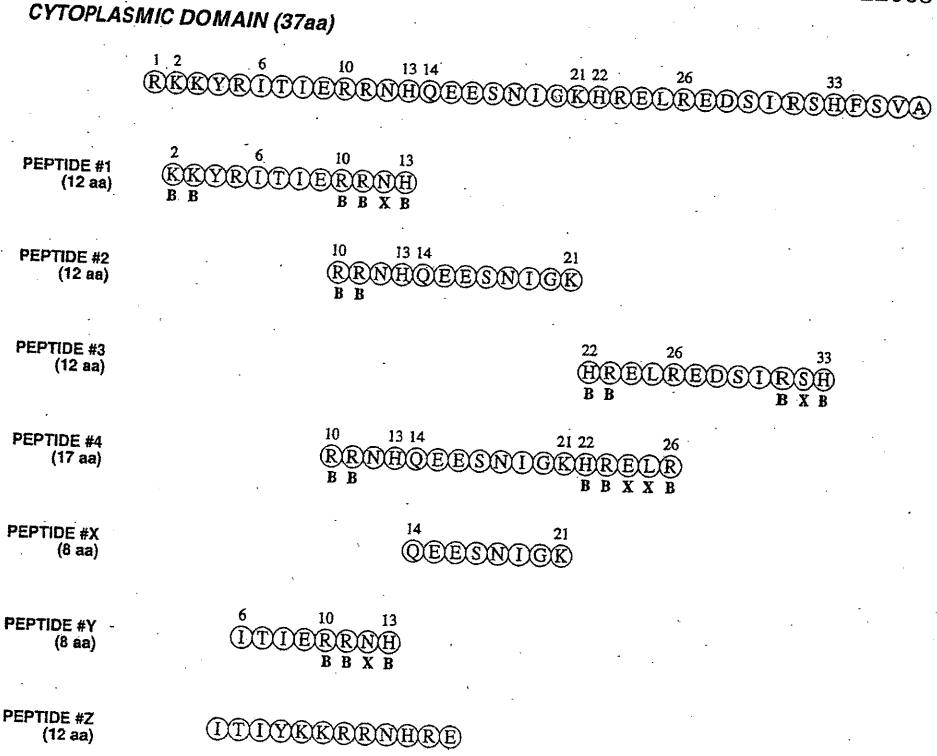

FIG. 1. **Sequence of the entire cytoplasmic domain of NPR-C and the various synthetic peptides corresponding to different regions of the cytoplasmic domain.** *B*, basic amino acid; *X*, nonbasic amino acid.

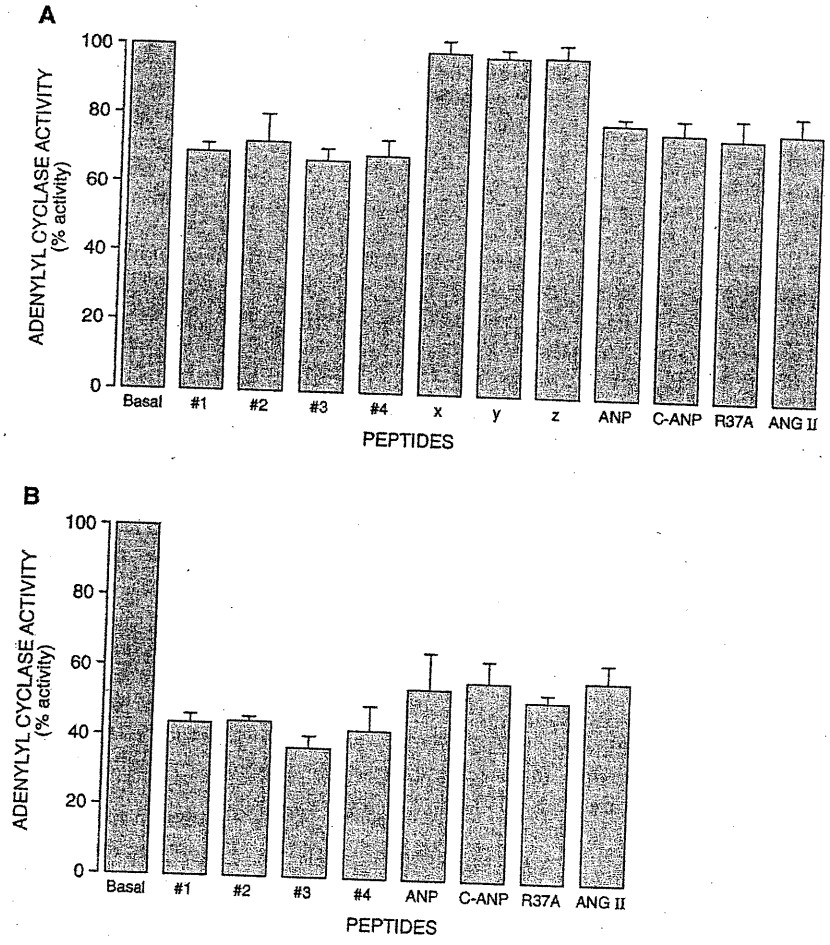

FIG. 2. **Effect of different peptides on adenylyl cyclase activity in rat heart (*A*) and vascular smooth muscle cells (*B*).** The effect of peptides on adenylyl cyclase activity was determined as described under "Experimental Procedures" in the presence of 10 μM GTPγS. The basal adenylyl cyclase activity was 225 ± 9 pmol of cAMP/mg of protein/10 min in heart and 180 ± 6 pmol of cAMP/mg of protein/10 min in vascular smooth muscle cells. Values are the means ± S.E. of three to four separate experiments.

the NH_2 terminus and BB*X*B or BB*XX*B at the COOH terminus, where B and *X* denote basic amino acid and nonbasic amino acid, respectively. The cytoplasmic domain of NPR-C receptor contains several of these G_i activator sequences. In the present studies, we have used seven different synthetic peptide fragments of the cytoplasmic domain of NPR-C receptor with complete, partial, or no G_i activator sequence in order to examine their effects on adenylyl cyclase activity. We have shown that small fragment peptides of the cytoplasmic domain of the NPR-C receptor with complete G_i activator sequence were more

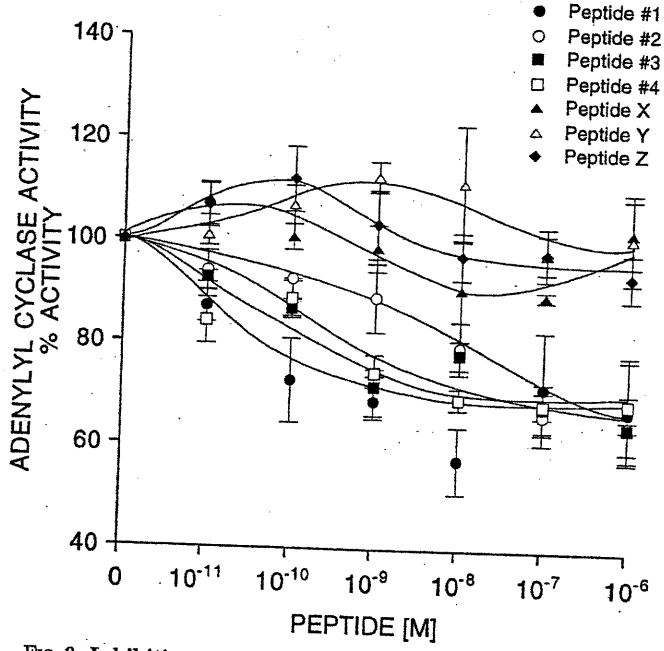

FIG. 3. **Inhibition of rat heart adenylyl cyclase activity.** The effect of different concentrations of peptides 1, 2, 3, 4, X, Y, and Z on adenylyl cyclase activity was determined as described under "Experimental Procedures" in the presence of 10 μM GTPγS. The basal adenylyl cyclase activity was 225 ± 9 pmol of cAMP/mg of protein/10 min. Values are the means ± S.E. of four separate experiments.

potent inhibitors of adenylyl cyclase activity, compared with the peptides having partial or no consensus G_i activator sequence.

EXPERIMENTAL PROCEDURES

Materials—ATP, cAMP, and isoproterenol were purchased from Sigma. Creatine kinase (EC 2.7.4.3) and GTPγS were purchased from Roche Molecular Biochemicals. [α-^{32}P]ATP was purchased from Amersham Pharmacia Biotech. Pertussis toxin was from List Biochemicals (Campbell, CA). Rat ANP, a ring-deleted analog of ANP, c-ANP-(4–23), and angiotensin II were from Peninsula Laboratories (Belmont, CA). Peptides R37A, 1, 2, 3, 4, X, Y, and Z were synthesized by standard solid phase techniques and highly purified (95–99%) by high performance liquid chromatography (Peninsula Laboratories and Chiron Technologies).

Preparation of Heart Particulate Fractions—Heart ventricles were dissected from Harlan Sprague-Dawley rats (200–300 g), quickly frozen in liquid nitrogen, and stored at −80 °C until used. Frozen hearts were pulverized to a fine powder with a mortar and pestle precooled in liquid nitrogen. The heart powder was homogenized using a Teflon-glass homogenizer (12 strokes) in a buffer containing 10 mM Tris-HCl and 1 mM EDTA, pH 7.5, and then centrifuged at 1000 × *g* for 10 min. The supernatant was discarded, and the pellet was homogenized in the above buffer and centrifuged at 1000 × *g* for 10 min. The pellet was finally suspended by homogenization in 10 mM Tris-HCl and 1 mM EDTA, pH 7.5, and used directly for adenylyl cyclase activity determination. Protein was determined essentially as described (32) with crystalline bovine serum albumin as a standard.

Cell Culture and Incubation—The A10 cell line from embryonic thoracic aorta of rat was obtained from the American Type Culture Collection (Manassas, VA). The cells were plated in 7.5-cm^2 flasks and incubated at 37 °C in a 95% air and 5% CO_2 humidified atmosphere in Dulbecco's modified Eagle's medium (with glucose, L-glutamine, and sodium bicarbonate) containing antibiotics and 10% heat-activated calf serum. The cells were passaged upon reaching confluence with 0.5% trypsin containing 0.2% EDTA and utilized between passages 5 and 15. The 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 an adenylyl cyclase assay.

PT Treatment—PT treatment was performed as described earlier (25, 33, 34). Briefly, heart particulate fractions were incubated in 25 mM glycylglycine buffer, pH 7.5, containing 1 mM NAD, 0.4 mM ATP, 0.4 mM GTP, 15 mM thymidine, 10 mM dithiothreitol, and ovalbumin (0.1 mg/ml) with and without PT (5 μg/ml) for 30 min at 30 °C. The particulate fraction was washed two to three times with 10 mM Tris, 1 mM EDTA buffer, pH 7.5, and finally suspended in the same buffer and used for adenylyl cyclase activity determination. Preincubation of the membranes at 30 °C for 30 min in the absence or presence of PT resulted in a significant loss of enzyme activity (~40%), which was independent of the presence of PT in the incubation medium. However, the percentage inhibition of adenylyl cyclase activity by the different peptides used remained unchanged.

Adenylyl Cyclase Activity Determination—Adenylyl cyclase activity was determined by measuring [^{32}P]cAMP formation from [α-^{32}P]ATP as described previously (26, 33). The typical assay medium contained 50 mM glycylglycine, pH 7.5, 0.5 mM MgATP, [α-^{32}P]ATP (1–1.5 × 10^6 cpm), 5 mM $MgCl_2$, 100 mM NaCl, 0.5 mM cAMP, 1 mM 3-isobutyl-1-methylxanthine, 0.1 μM EGTA, 10 μM GTPγS, and an ATP-regenerating system consisting of 2 mM creatine phosphate, 0.1 mg of myokinase per ml in a final volume of 200 μl. Incubations were initiated with the addition of reaction mixture to the membranes (30–70 μg), which had been preincubated at 37 °C for 10 min. The reactions conducted in triplicate at 37 °C for 10 min were terminated by the addition of 0.6 ml of 120 mM zinc acetate, and cAMP was purified by co-precipitation of other nucleotides with $ZnCO_3$ by the addition of 0.5 ml of 144 mM Na_2CO_3 and by subsequent chromatography using the double column system (35). Under these assay conditions, adenylyl cyclase activity was linear with respect to protein concentration and time of incubation.

RESULTS

Effect of Different Fragments of the Cytoplasmic Domain of NPR-C Receptor on Adenylyl Cyclase Activity—The 37-amino acid peptide (R37A) corresponding to the cytoplasmic domain of the NPR-C receptor has been shown to inhibit adenylyl cyclase activity by interacting directly with G_i proteins (30). In order to investigate if the small peptide fragments of the cytoplasmic domain that consist of partial or complete G_i activator sequences could also mimic the effect of R37A or ANP on NPR-C receptor-mediated inhibition of adenylyl cyclase, seven different synthetic peptides were used to examine their effects on adenylyl cyclase activity. The peptide fragments represent different parts of the cytoplasmic domain as shown in Fig. 1. These consist of 12 amino acids (peptides 1, 2, 3, and Z), 17 amino acids (peptide 4), or 8 amino acids (peptides X and Y). Peptides 1, 3, and 4 possess the required G_i activator sequences: two basic amino acids at the NH_2 terminus and BBXB, BXB, or BBXXB at the COOH terminus, respectively (where B represents a basic amino acid and *X* represents a nonbasic amino acid), whereas peptide 2 has two basic amino acids at the NH_2 terminus but does not have the consensus sequence at the COOH terminus. On the other hand, peptide Y has only the consensus sequence at the COOH terminus, and peptide X lacks G_i activator sequence, whereas peptide Z is the scrambled peptide and serves as control for peptide 1.

Fig. 2*A* shows that peptides 1, 2, 3, and 4 of the cytoplasmic domain of the NPR-C receptor at 10^{-7} M inhibited adenylyl cyclase activity by 40, 30, 35, and 36%, respectively, in rat heart particulate fractions. In addition, ANP-(99–126), C-ANP-(4–23), R37A, and angiotensin II, as reported earlier (3, 26, 30, 36), also inhibited the enzyme activity by about 20–25%. However, peptides X, Y, and Z did not inhibit adenylyl cyclase activity. Similar results were also observed in A-10 vascular smooth muscle cells (Fig. 2*B*); however, the inhibitions were greater (55%) in these cells. These results suggest that small peptides of the cytoplasmic domain of the NPR-C receptor with 12 amino acids or more with specific G_i activator sequence could inhibit adenylyl cyclase activity.

Fig. 3 shows the effects of various concentrations of the seven peptides on adenylyl cyclase activity in heart particulate fractions. Peptides 1, 3, and 4 inhibited adenylyl cyclase activity in a concentration-dependent manner with apparent K_i between 0.1 and 1 nM; however, peptide 2 inhibited adenylyl cyclase activity with a higher K_i of about 10 nM. The maximal inhibi-

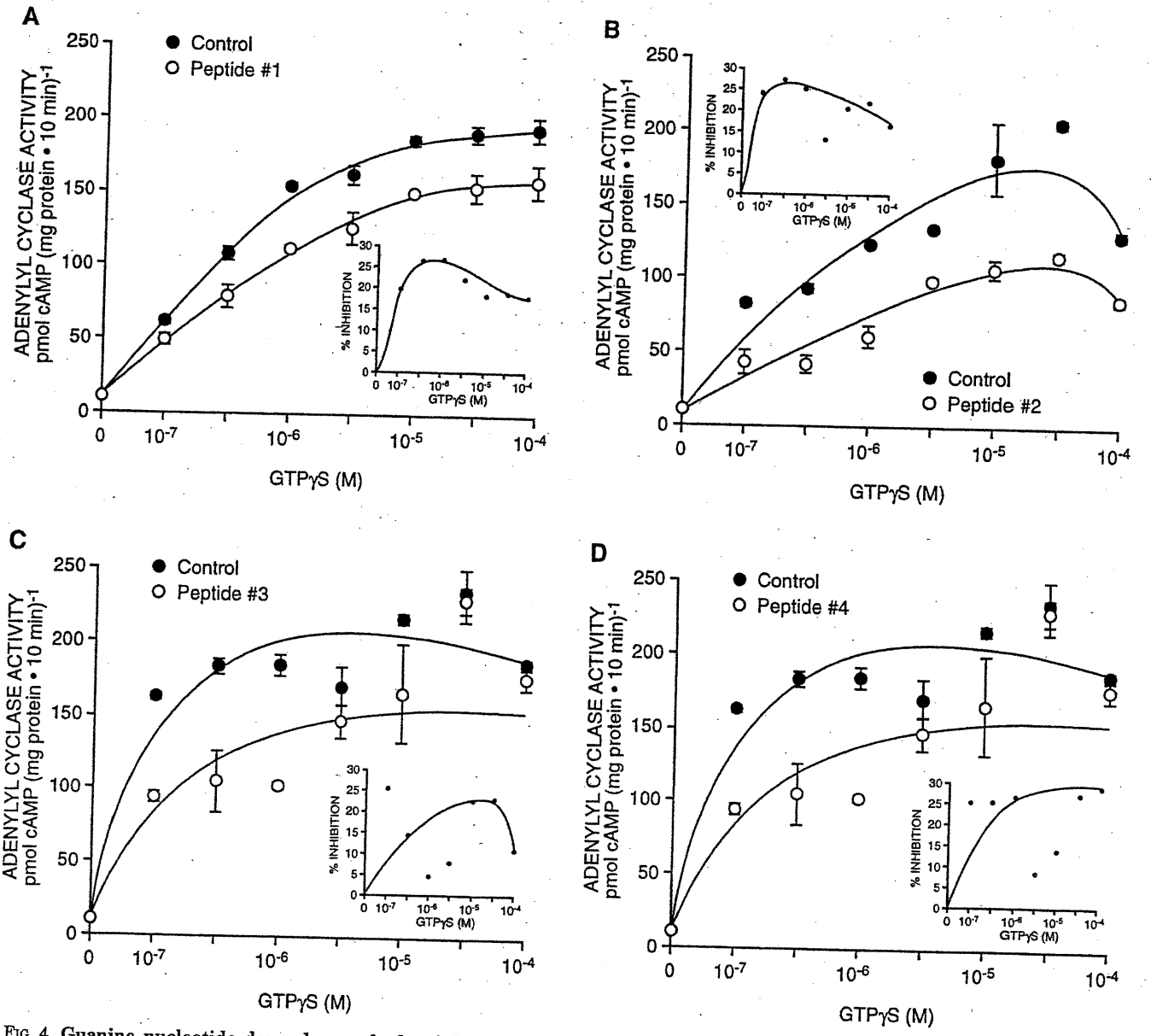

FIG. 4. **Guanine nucleotide dependence of adenylyl cyclase inhibition by peptides 1, 2, 3, and 4.** Adenylyl cyclase activity was determined in the presence of various concentrations of GTPγS alone (basal) or in combination with 0.1 μM concentrations of the four peptides. Values are the means ± S.E. of four separate experiments.

tions of adenylyl cyclase activity by peptides 1, 2, 3, and 4 were about 40, 30, 35, and 36% respectively. The inhibitory effect of these peptides was not due to the positive charges *per se*, because the scrambled peptide (peptide Z) of peptide 1 that has the same amino acid composition was unable to exert any inhibitory effect on adenylyl cyclase. In addition, peptides X and Y also did not inhibit adenylyl cyclase activity. These data suggest that the peptides possessing the required G_i activator sequence at the carboxyl terminus as well as at the amino terminus are more potent inhibitors of adenylyl cyclase activity than the peptides having partial or no structural specificity.

Dependence of Peptide 1-, 2-, 3-, and 4-mediated Inhibition of Adenylyl Cyclase on Guanine Nucleotides—The inhibitory effect of the R37A peptide corresponding to the cytoplasmic domain of the NPR-C receptor on adenylyl cyclase activity has been reported to be dependent on the presence of guanine nucleotides (30). In order to investigate if the inhibition of adenylyl cyclase mediated by the small peptides also requires guanine nucleotides, the effect of different peptides on adenylyl cyclase activity in the absence or presence of GTPγS was examined. The results shown in Fig. 4 indicate that all four active peptides (peptides 1–4) did not exert any inhibitory effect on adenylyl cyclase in the absence of GTPγS; however, in the presence of various concentrations of GTPγS, the four peptides inhibited the enzyme activity in a concentration-dependent manner. The maximal inhibitions (~30–40%) was observed at 10 μM GTPγS. These results indicate that the inhibition of adenylyl cyclase by all four peptides of the cytoplasmic domain of NPR-C is also dependent on the presence of guanine nucleotides.

Effect of PT on Peptide 1-, 2-, 3-, and 4-mediated Inhibition of Adenylyl Cyclase—The coupling of NPR-C receptors to adenylate cyclase through G_i has been demonstrated (25, 30). Furthermore, we have shown that R37A inhibited adenylyl cyclase through a PT-sensitive G_i protein. To examine if the inhibition of adenylyl cyclase by these four peptides is mediated through G_i, the effect of PT treatment on the inhibitory effects of the four peptides on adenylyl cyclase activity was examined. Fig. 5 shows that all four peptides inhibited the enzyme activity in a concentration-dependent manner in control heart particulate

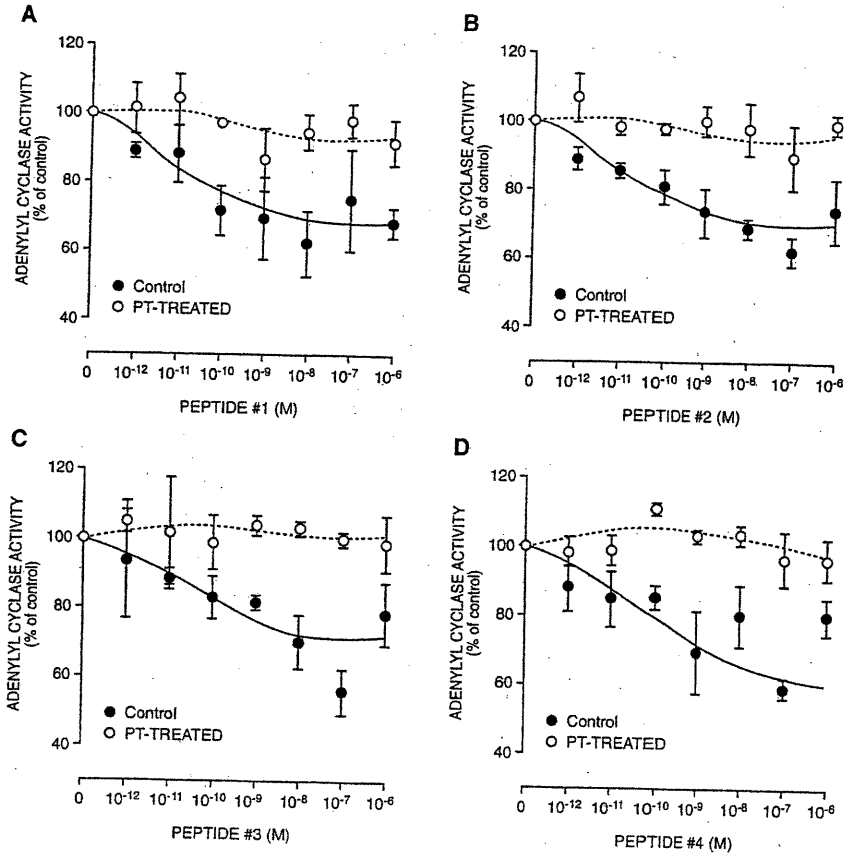

FIG. 5. **Effect of pertussis toxin on different peptide-mediated inhibition of rat heart adenylyl cyclase.** Heart particulate fractions were treated without (*Control*) or with pertussis toxin (*PT-treated*) as described under "Experimental Procedures." Adenylyl cyclase activity was determined in control and PT-treated heart particulate fractions in the absence or presence of various concentrations of four peptides (*A–D*) as described under "Experimental Procedures." Values are the means ± S.E. of three separate experiments. The basal enzyme activities in control and PT-treated heart particulate fractions were 285 ± 10 and 201 ± 8 pmol of cAMP/mg of protein/10 min, respectively.

fractions, which was attenuated by PT treatment. These results indicate that, like R37A, the small peptides of the cytoplasmic domain of NPR-C receptor also inhibit adenylyl cyclase through a PT-sensitive G_i protein.

Inhibition of Agonist-stimulated Adenylyl Cyclase Activity by Small Peptide Fragments of Cytoplasmic Domain—Since ANP-(99–126) and C-ANP-(4–23) as well as R37A peptide have been shown to inhibit the stimulatory effects of various agonists on adenylyl cyclase activity (25, 26, 30), it was of interest to examine if small peptides of the cytoplasmic domain of the NPR-C receptor are also capable of inhibiting the stimulated adenylyl cyclase activity in heart particulate fractions. As shown in Fig. 6, glucagon, isoproterenol, and forskolin stimulated adenylyl cyclase activity by about 160, 190, and 1000%, respectively, which was inhibited by all four peptides (0.1 μM) to various degrees. Glucagon-mediated stimulation was inhibited by about 30%, whereas isoproterenol-stimulated enzyme activity was inhibited by about 20–25%, and forskolin-stimulated enzyme activity was inhibited by about 15–35%.

DISCUSSION

We have previously shown that 37-amino acid synthetic peptide (R37A) corresponding to the cytoplasmic domain of NPR-C receptor inhibited adenylyl cyclase activity via PT-sensitive G_i protein (30). In the present studies, we demonstrate for the first time that the cytoplasmic domain peptide of NPR-C receptor has several G_i activator sequences that inhibit adenylyl cyclase activity in a GTP-dependent manner via PT-sensitive G_i proteins.

The small peptide fragments of the cytoplasmic domain of the NPR-C receptor containing 12 amino acids (Lys^2–His^{13}, peptide 1) with consensus sequence for G_i activation at both NH_2 and COOH terminus (*i.e.* BB at the NH_2 terminus and the BBXB motif at the COOH terminus) inhibited adenylyl cyclase activity in rat heart particulate fractions, cultured vascular smooth muscle cells, and aorta. The other peptide fragments containing 17 amino acids (Arg^{10}–Arg^{26}, peptide 4) with a consensus sequence of BB at the NH_2 terminus and BBXXB at the COOH terminus also inhibited adenylyl cyclase activity in these tissues/cells. Both of these peptide fragments (peptides 1 and 4) inhibited the enzyme activity in a concentration-dependent manner with an apparent K_i between 0.1 and 1 nM, and the maximal inhibition observed was about 30–35%. The potency of these peptide fragments to inhibit adenylyl cyclase activity was in the same range as that of the entire cytoplasmic domain peptide R37A as reported earlier (30). The inhibitory effect of these peptides on adenylyl cyclase was not due to the net positive charge present (*i.e.* amino acid composition), since the scrambled peptide Z with the same composition as peptide 1 but lacking the G_i activator sequence at the NH_2 and COOH terminus did not inhibit adenylyl cyclase activity. On the other hand, the presence of partial COOH-terminal motif (BXB) but intact N-terminal motif (BB) in the peptide did not change the potency of the peptide to inhibit adenylyl cyclase activity, suggesting that a partial COOH-terminal motif in the peptide may be sufficient to exert inhibitory effect on adenylyl cyclase activity. However, the truncation of COOH-terminal motif (BXB) from peptide 2 (Arg^{10}–Lys^{21}) inhibited adenylyl cyclase activity with lower potency (K_i ~10 nM), suggesting that the COOH-terminal motif may be important to increase the potency of the peptides to elicit adenylyl cyclase inhibition. These results are in agreement with the recent studies of Kanwal *et al.* (37), who have shown that the 15-amino acid peptide fragment of NPR-C receptor (Arg^1–Gln^{15}) that lacks the COOH-terminal motif attenuated dopamine efflux in pheochromocytoma cells (PC12). Similarly, R37A peptide corresponding to the cytoplasmic domain of the NPR-C receptor that lacks the COOH-terminal motif BXB has also been reported to inhibit adenylyl cyclase activity (30) as well as neurotransmission (37). However, our results are in contrast with studies of Murthy and Makhlouf (38), who have shown that the cytoplasmic domain peptide

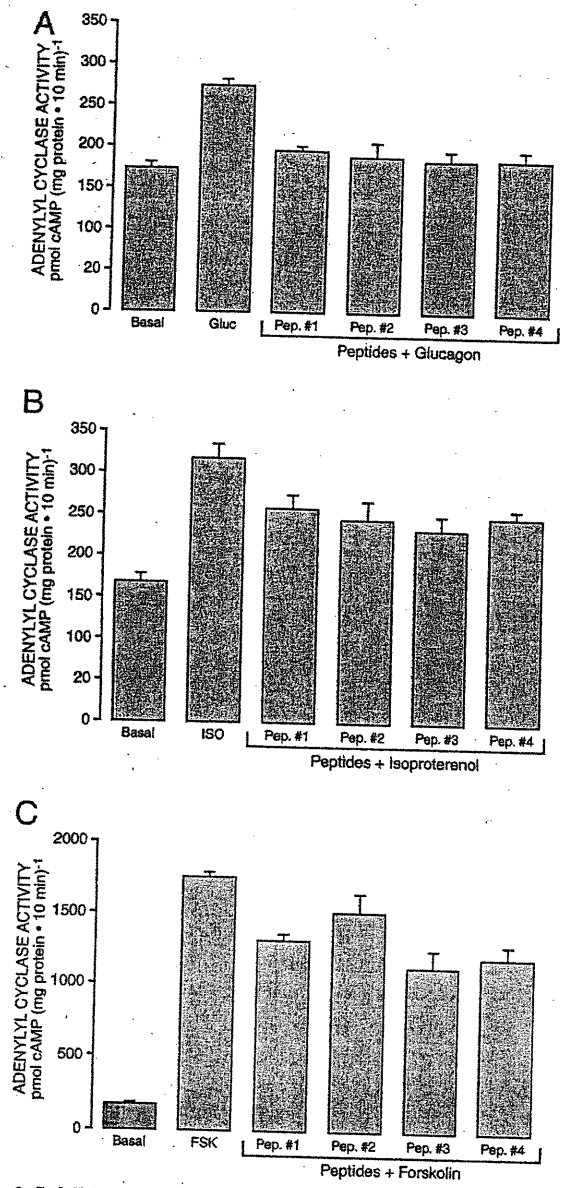

FIG. 6. **Inhibition of agonist-stimulated rat heart adenylyl cyclase activity by different peptide fragments.** Adenylyl cyclase activity was determined in the presence of 1 μM glucagon (*A*), 10 μM isoproterenol (*B*), and 50 μM forskolin (*C*) alone or in combination with 0.1 μM peptides as described under "Experimental Procedures." Values are the means ± S.E. of three separate experiments. The basal enzyme activity was 180 ± 9 pmol of cAMP/mg of protein/10 min.

fragment of the human NPR-C receptor that lacks the COOH terminus was inactive in stimulating phospholipase Cβ activity in gastric and tenia coli smooth muscle. The truncation of the NH_2-terminal motif of peptide 2 results in inactivation of the peptide to inhibit adenylyl cyclase activity, suggesting that NH_2-terminal consensus sequence is important to interact with G_i proteins to exert inhibition of adenylyl cyclase. This is further substantiated by our results showing that peptide Y that has the consensus sequence at the COOH terminus but lacks the NH_2-terminal sequence was unable to inhibit adenylyl cyclase activity. However, Murthy and Makhlouf (38) have shown that peptide containing the consensus sequence at the NH_2 terminus was unable to stimulate phospholipase Cβ in tenia coli smooth muscle cell membranes. This apparent discrepancy may be attributed to the difference in the cell/tissue system utilized in the two studies. Moreover, these investigators have shown that the 17-amino acid peptide of the human NPR-C receptor cytoplasmic domain stimulated phospholipase Cβ activity at higher concentrations with an EC_{50} value of 1.3 μM, whereas we have shown that the potency of the active peptides 1, 3, and 4 was at least 1000-fold higher in inhibiting adenylyl cyclase activity. In addition, peptide 2 that lacks the COOH-terminal motif sequence was less active than peptides 1, 3, and 4 but was still able to inhibit adenylyl cyclase activity, whereas Murthy and Makhlouf (38) did not observe any effect of the peptide lacking the COOH-terminal consensus sequence on phospholipase Cβ activity. The lack of effect of this peptide in their studies may be due to the possibility that this peptide also did not have the NH_2-terminal consensus sequence and thus is similar to peptide X in our studies, which was also unable to inhibit adenylyl cyclase activity. Taken together, it can be suggested that the NH_2-terminal motif of these peptides may be the important site for the interaction with G_i protein and thereby to activate the effector systems.

Our studies on the dependence on guanine nucleotides of active peptides-mediated adenylyl cyclase inhibition and its attenuation by PT treatment are consistent with previous reports (25, 26, 30) and suggest that the small active peptide fragments of the cytoplasmic domain of NPR-C receptor, like the entire cytoplasmic domain peptide R37A, could also inhibit adenylyl cyclase via PT-sensitive G_i protein. In addition, the inhibition of glucagon-, isoproterenol-, and forskolin-mediated stimulation of adenylyl cyclase by small peptides is also consistent with our previous studies on ANP, C-ANP-(4–23), and R37A and adenylyl cyclase signaling (25, 26, 30).

In conclusion, we have provided the first evidence to demonstrate that the cytoplasmic domain peptides of the NPR-C receptor of 12 amino acids possessing complete G_i activator sequence at the COOH and NH_2 termini are sufficient to inhibit adenylyl cyclase activity through a PT-sensitive G_i protein with the same potency as that of the entire cytoplasmic domain peptide, whereas the peptides with a truncated COOH terminus inhibited the enzyme activity with low potency; however, the truncation of the NH_2-terminal motif completely attenuates adenylyl cyclase inhibition.

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