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C-type natriuretic peptide and brain natriuretic peptide inhibit adenylyl cyclase activity: interaction with ANF-R2/ANP-C receptors

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Abstract C-type natriuretic peptide (CNP) and brain natriuretic peptide (BNP) are members of the natriuretic peptide family, which have been shown to interact with ANP-C/ANF-R2 receptors in addition to ANP-B receptor subtypes. The present study was undertaken to investigate if the interaction of CNP and BNP with ANP-C receptors results in the inhibition of adenylyl cyclase activity. CNP and BNP inhibited adenylyl cyclase activity in heart and brain striatal membranes in a concentration dependent manner with an apparent K_i between 0.1 and 1.0 nM. Maximal inhibition observed in heart membranes were about 25% and 35% for BNP and CNP respectively, however the inhibitions in brain striatal membranes were smaller (~20%). The inhibition was dependent on the presence of guanine nucleotides and was attenuated by pertussis toxin treatment. In addition, CNP inhibited the stimulatory effect of isoproterenol on adenylyl cyclase, whereas CNP as well as BNP showed an additive effect with the inhibitory response of angiotensin II on adenylyl cyclase activity. When the combined effect of C-ANF_{4-23}/BNP, C-ANF_{4-23}/CNP and BNP/CNP at optimal concentrations was studied together on adenylyl cyclase activity, the percent inhibition remained the same for C-ANF_{4-23} and BNP or C-ANF_{4-23} and CNP, however, an additive inhibitory effect was observed for BNP and CNP. These results suggest that CNP and BNP like C-ANF_{4-23} interact with ANP-C receptors and result in the inhibition of adenylyl cyclase activity. On the other hand, CNP and BNP interact with the ANP-C receptor, however, the interaction may be at different sites or there may be two subpopulations of ANP-C receptors specific for each of the peptides. These results indicate that BNP and CNP, like ANP and C-ANF_{4-23}, inhibit the adenylyl cyclase/cAMP signal transduction system through an inhibitory guanine nucleotide regulatory protein, by interacting with ANP-C receptor subtypes.

Key words: CNP; BNP; Adenylyl cyclase; G_i protein

1. Introduction

Atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) are a family of structurally related peptides containing a seventeen amino acid ring formed by a disulfide bridge. As compared to ANP, BNP has an additional six amino acid sequence in its amino terminal extend [1,3,4] while CNP lacks the carboxy-terminal extend [2].

ANP has been reported to regulate a variety of physiological processes affecting cardiovascular homeostasis [5]. BNP also shares the same characteristics [2,3,6,7] but is less potent than ANP and has long lasting effects [8,9]. CNP, on the other hand is produced in the brain, anterior pituitary and cerebellum [6,10–12] and acts as a neurotransmitter to inhibit the same effects as those of ANP and BNP [13]. The concentration of CNP in brain is higher than the two other peptides [10]. CNP has also been shown to be secreted from cultured vascular smooth muscle cells [9], where it may function as growth inhibitor [14]. CNP influences muscular tone but has no natriuretic effect in mammals [15,16]. The diuresis and hypotension provoked by CNP are weaker than those of ANP and BNP [2].

ANP has been reported to stimulate guanylyl cyclase/cGMP and to inhibit the adenylyl cyclase/cAMP system in several tissues by interacting with their receptors [5]. By molecular cloning techniques, three subtypes of natriuretic peptide receptor (NPR) have been identified. These are NPR-A [17,18], NPR-B [19,20] and NPR-C [21]; NPR-A and NPR-B are membrane guanylyl cyclase, whereas NPR-C (clearance receptors) also known as ANF-R2 or ANP-C receptors have been shown to be coupled to adenylyl cyclase inhibition through the G_i regulatory protein [22,23] or to activation of phospholipase C [24]. NPR-A responds to stimulation by both ANP and BNP, however, ANP is more potent than BNP in stimulating guanylyl cyclase [17], however NPR-B is more responsive to BNP than ANP [19,20]. CNP on the other hand interacts with NPR-B with higher affinity and is probably its natural ligand while BNP could interact with a NPR-B subtype or with another receptor [7,13,25].

The interaction of BNP and CNP with NPR-C has also been reported by receptor binding studies [14,21,26], however, it is not known whether this interaction results in the functional coupling to adenylyl cyclase/cAMP transduction system to which ANP-C receptors are coupled. We have therefore undertaken the present studies to examine if BNP and CNP, like ANP, could also inhibit adenylyl cyclase/cAMP system through G_i regulatory protein.

2. Experimental

2.1. Preparation of heart membranes

The rat heart membranes were prepared in a similar manner as that of aorta-washed particles as described previously [22,23]. The heart ventricles were dissected out, and quickly frozen in liquid nitrogen and pulverized to a fine powder by using a mortar cooled in liquid nitrogen. The powder was homogenized by Teflon/Glass homogenizer in a buffer containing 10 mM Tris-HCl and 1 mM EDTA, pH 7.5, the homogenate was centrifuged at 1000 × g for 15 min at 4°C. The supernatant was

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Abbreviations: ANP, atrial natriuretic peptide (99–126); C-ANF_{4-23}, a ring-deleted analog of atrial natriuretic factor; C-ANF_{4-23} (des[Gln^{18}, Ser^{19}, Gln^{20}, Leu^{21}, Gly^{22}]ANF_{4-23}-NH_2); CNP, C-type natriuretic peptide; BNP, brain natriuretic peptide; PT, pertussis toxin; G_i, an inhibitory guanine nucleotide regulatory protein; G_s, a stimulatory guanine nucleotide regulatory protein; GTPγS; guanosine 5′-0-(3-thiotriphosphate); AII, angiotensin II.

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discarded and the pellet was suspended in 10 mM Tris-HCl/1 mM EDTA. The membranes were washed two times and suspended finally in the 10 mM Tris-HCl and 1 mM EDTA pH 7.5 for adenylyl cyclase activity determination and ADP-ribosylation studies.

2.2. Preparation of brain striatal membranes and aorta washed particles

The brain striatal membranes and aorta washed particles were prepared as described previously [23].

2.3. Pertussis toxin-catalysed ADP-ribosylation

The pertussis toxin (PT) treatment was performed as described previously [22]. The heart membranes were incubated in a buffer containing 25 mM glycylglycine (pH 7.5), 1 mM NAD^+, 0,4 mM ATP, 0.4 mM GTP, 15 mM thymidine, 10 mM dithiothreitol, and ovalbumin (0.1 mg/ml) without or with PT (5 μg/ml) for 30 min at 30°C. The membranes were washed two to three times with 10mM Tris, 1 mM EDTA buffer, pH 7.5 and finally suspended in the same buffer and used for adenylyl cyclase activity determination. Pre-incubation of heart 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 percent inhibition of adenylyl cyclase by CNP or BNP remained unchanged (data not shown).

2.4. Adenylyl cyclase activity determination

Adenylyl cyclase activity was determined by measuring cyclic [α-^{32}P]AMP formation from [α-^{32}P]ATP as described previously [22,23]. The typical assay medium contained 50 mM glycylglycine (pH 7.5), 0.5 mM MgATP, [α-^{32}P]PATP (1–1.5 × 10^6 CPM) 5 mM $MgCl_2$ (in excess of ATP concentration), 100 mM NaCl, 0.5 mM cAMP, 1 mM 3-isobutyl-1-methylxanthine, 0.1 mM EGTA, 10 μM GTPγS and an ATP-regenerating system consisting of 2 mM creatine phosphate, 0.1 mg of creatine kinase/ml and 0.1 mg of myokinase/ml in a final volume of 200 μl. Incubations were initiated with the addition of a reaction mixture to the membranes (30–70 μg), which had been thermically equilibrated for 2 min at 37°C. The reactions were conducted in triplicate at 37°C for 10 min and were terminated by the addition of 0.6 ml of 120 mM zinc acetate. 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 subsequent chromatography using double column system described by Salomon et al. [27]. Under these assay conditions, adenylyl cyclase activity was linear with respect to protein concentration and time of incubation. The protein concentration was determined essentially as described by Lowry et al. [28] with crystalline bovine serum albumin as standard.

2.5. Materials

Male Sprague–Dawley rats (weight, 250 g) were provided by Charles River Canada inc. (St.-Constant, Quebec, Canada). ATP, cAMP, pertussis toxin and isoproterenol were purchased from Sigma (St.-Louis, MO, USA). Creatine kinase (EC 2.7.3.2), myokinase (EC 2.7.4.3), GTP and guanosine 5′-[γ-thio]triphosphate (GTPγS) were purchased from

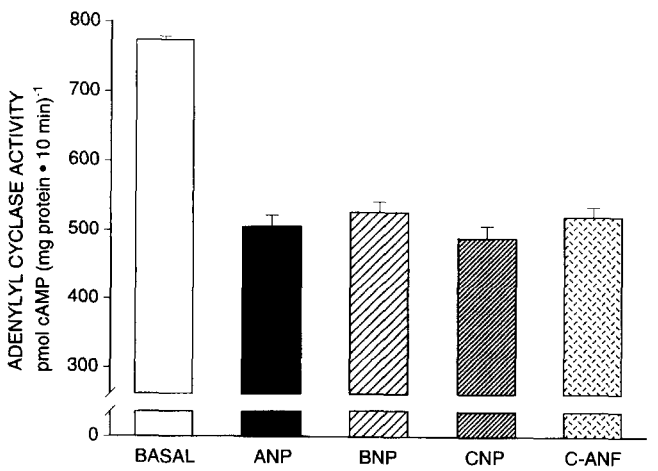

Fig. 1. Effect of various natriuretic peptides on adenylyl cyclase activity in rat heart membranes. Adenylyl cyclase activity was determined in the absence (basal, empty column) or presence of 0.1 μM ANP_{99-126} (black column), 0.1 μM BNP (hatched column), 0.1 μM CNP (finely hatched column) or 0.1 μM C-ANF_{4-23} (cross-hatched column), as described in section 2. Values are the means ±S.E.M. of three separate experiments.

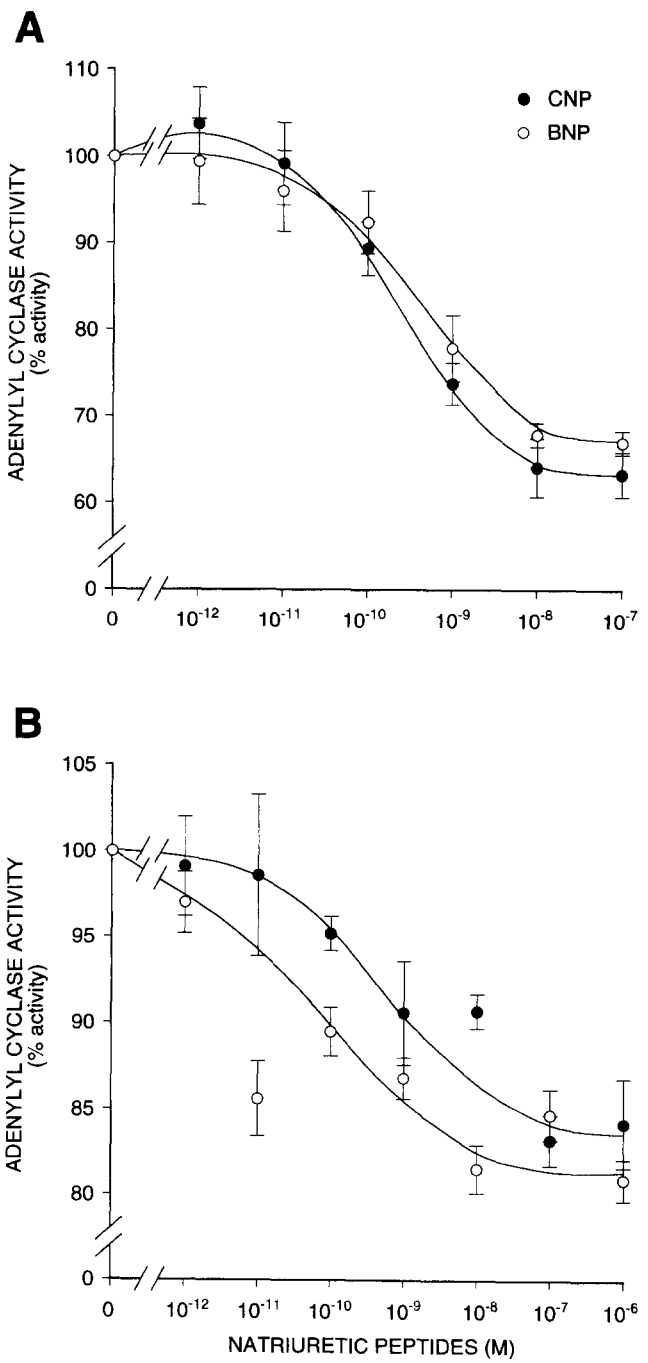

Fig. 2. Effect of CNP and BNP on adenylyl cyclase activity in heart membranes (A) and brain striatal membranes (B). Adenylyl cyclase activity was determined as described in section 2 in the presence of various concentration of CNP (closed circles) or BNP (open circles). Values are the means ± S.E.M. of three separate experiments. The absolute enzyme activity in the presence of 10 μM GTPγS in heart membranes was 677.3 ± 12.9 pmol cAMP (mg protein × 10 min)$^{-1}$ and brain striatal membranes was 839.7 ± 70.8 pmol cAMP (mg protein × 5 min)$^{-1}$, respectively.

Boehringer Mannheim (Montreal, Quebec, Canada). [α-^{32}P]ATP was from Amersham Corp. (Oakville, Ontario, Canada). BNP (32 amino acids) and CNP (22 amino acids) were obtained from Peninsula Lab. and Bachem (CA, USA), respectively.

3. Results

3.1. Effect of BNP and CNP on adenylyl cyclase activity

In order to investigate if CNP and BNP interact with ANP-C receptors and result in the inhibition of adenylyl cyclase, the effect of these peptides were studied on adenylyl cyclase activity and were compared with the inihibitions elicited by ANP and C-ANF$_{4-23}$. The results shown in Fig. 1 indicate that ANP, C-ANF$_{4-23}$, CNP and BNP at (0.1 μM) inhibited adenylyl cyclase activity to almost similar extent (between 30–40%) in heart membranes, indicating that BNP and CNP like ANP and C-ANF$_{4-23}$ may also interact with adenylyl cyclase signal transduction system. Fig. 2 shows adenylyl cyclase inhibition by BNP and CNP at various concentrations in heart and brain striatal membranes. BNP and CNP both inhibited adenylyl cyclase activity in a concentration dependent manner in heart (A) and brain striatal (B) membranes with an apparent K_i between 0.1 and 1 nM. The maximal inhibition observed in heart were about 35%, whereas about 20% inhibition was observed in brain striatum. Similar results were also observed in aorta (data not shown).

3.2. Dependence of CNP inhibition of adenylyl cyclase on guanine nucleotides

The inhibitory effect of ANP and C-ANF$_{4-23}$ on adenylyl cyclase has been reported to be dependent on the presence of guanine nucleotides [22,23]. To examine if the inhibition elicited by the BNP and CNP is also dependent on the presence of guanine nucleotides, the effect of GTPγS was studied on the peptide-mediated inhibition of adenylyl cyclase activity. Table 1 illustrates the effect of CNP on adenylyl cyclase from heart membranes in the absence and presence of various concentrations of GTPγS. CNP did not exert any effect on adenylyl cyclase activity in the absence of GTPγS; however, in the presence GTPγS, CNP was able to inhibit the enzyme activity in a concentration-dependent manner. The maximal inhibition elicited by CNP (~25%) was at 1 μM GTPγS. Similar results were also observed with BNP (data not shown). These results indicate that the receptors for BNP and CNP may also be coupled to adenylyl cyclase system through guanine nucleotide regulatory protein.

Table 1
Dependence on guanine nucleotides of inhibition of adenylyl cylcase by CNP in heart membranes

	Adenylyl cyclase activity pmol cAMP (mg protein × 10 min)$^{-1}$)		
GTPγS (M)	−CNP	+CNP	Inhibition (%)
None	109.0 ± 2.0	103.0 ± 0.8	5.5 ± 2.5
10^{-9}	143.9 ± 2.7	123.8 ± 0.1	14.0 ± 1.7
10^{-8}	389.0 ± 2.9	330.8 ± 7.3	15.0 ± 2.5
10^{-7}	761.8 ± 4.2	618.8 ± 13.8	18.8 ± 2.3
10^{-6}	953.2 ± 13.9	732.6 ± 1.5	23.1 ± 1.3
10^{-5}	1042.1 ± 29.5	824.4 ± 18.7	20.9 ± 4.0

Adenylyl cyclase activity was determined in the presence of various concentrations of GTPγS alone or in combination with 0.1 μM CNP as described in section 2. Values are the means ± S.E.M. of three separate experiments done in triplicate.

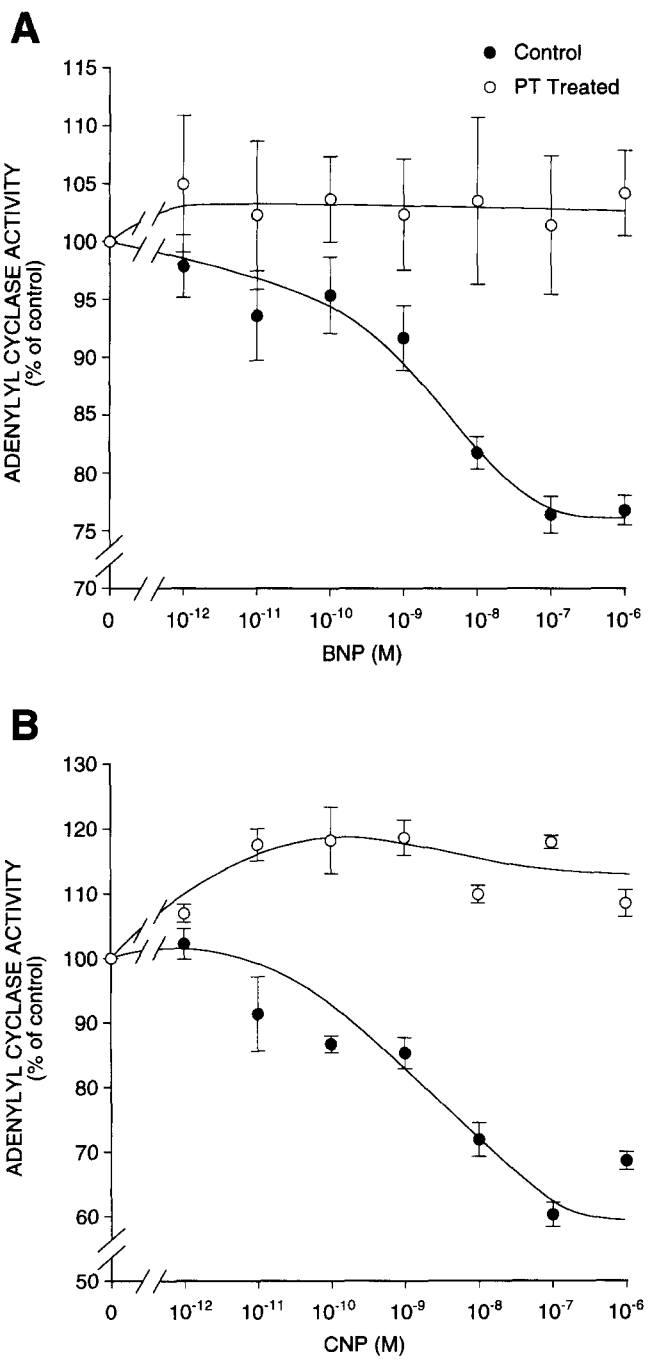

Fig. 3. Effect of PT on BNP and CNP-mediated inhibition of adenylyl cyclase in rat heart membranes. Heart membranes were treated without (control) or with PT as described in section 2. Adenylyl cyclase activity was determined in the absence and presence of various concentrations of BNP (A) or CNP (B) in control (closed circle) and PT treated (open circle) membranes as described in section 2 in the presence of 10 μM GTPγS. Values are the means ± S.E.M. of three separate experiments. The basal enzyme activities in control and PT-treated membranes were 528.2 ± 6.6 and 506.9 ± 4.9 pmol cAMP (mg protein × 10 min)$^{-1}$, respectively.

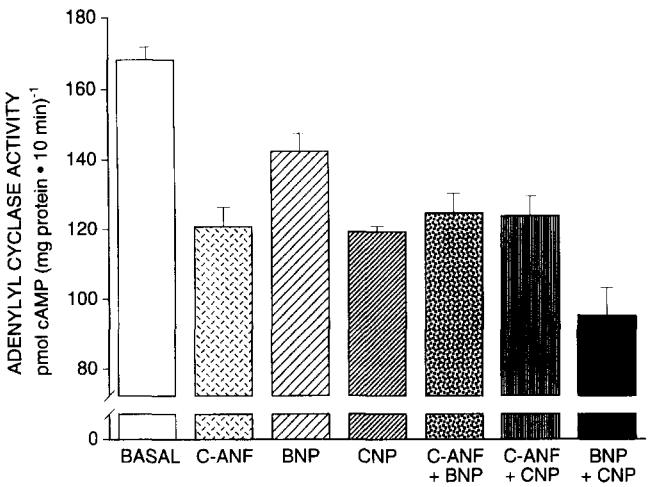

Fig. 4. Interaction of BNP, CNP and C-ANF$_{4-23}$ on adenylyl cyclase activity in rat aorta membranes. Adenylyl cyclase activity was determined in the absence (Basal, open column) or presence of 0.1 μM C-ANF$_{4-23}$ (C-ANF, cross-hatched column), 0.1 μM BNP (hatched column), 0.1 μM CNP (finely hatched column), alone or in combination with C-ANF$_{4-23}$ + BNP (dotted column), C-ANF$_{4-23}$ + CNP (vertically hatched column) or BNP + CNP (black column) as described in section 2. Values are the means ± S.E.M. of three separate experiments.

3.3. *Effect of pertussis toxin on BNP- and CNP-mediated inhibition of adenylyl cyclase*

Fig. 3 shows the effect of PT treatment on BNP- and CNP-mediated inhibition of adenylyl cyclase. BNP and CNP inhibited the enzyme activity in a concentration-dependent manner in control membranes, however, the inhibitions were completely abolished by PT treatment. These data indicate that like ANP [22,23], the inhibitory effects of CNP and BNP on adenylyl cyclase are also mediated through G_i regulatory protein.

3.4. *Interaction of BNP, CNP and C-ANF$_{4-23}$ on adenylyl cyclase activity*

Since BNP and CNP, like ANP and C-ANF$_{4-23}$, inhibited adenylyl cyclase activity through G_i regulatory protein, it was of interest to examine if these peptides interact with the same ANP-C receptor population or interact with two distinctly different receptor populations. Fig. 4 shows that C-ANF$_{4-23}$, BNP and CNP inhibit adenylyl cyclase by 28, 15 and 29%, respectively, in aorta-washed particles, and when the effect of an optimal concentration of C-ANF$_{4-23}$ and BNP or C-ANF$_{4-23}$ and CNP was studied together on adenylyl cyclase, the percent inhibition remained the same. These data indicate that the BNP and CNP like C-ANF$_{4-23}$ elicit inhibition of adenylyl cyclase by interacting with the ANP-C receptor population. However, when the effect of an optimal concentration of CNP and BNP was studied together on adenylyl cyclase, almost additive inhibitory effect was observed. These results indicate that CNP and BNP may be interacting with ANP-C receptors at distinctly different sites or there may be two subpopulations of ANP-C receptors specific for each of the peptides and coupled to adenylyl cyclase/cAMP signal transduction system.

3.5. *Effect of CNP and BNP on stimulatory and inhibitory effects of hormones on adenylyl cyclase activity*

The interaction of ANP with various stimulatory or inhibitory hormones on adenylyl cyclase has been reported in various tissues [23,29,30]. Since CNP also inhibits adenylyl cyclase activity through G_i protein, it was of interest to examine if CNP could also be able to interact with various agonists which stimulate or inhibit adenylyl cyclase through G_s and G_i proteins respectively. The results shown in Table 2 indicate that β-adrenergic agonist isoproterenol stimulated adenylyl cyclase activity in heart membranes and CNP inhibited these stimulations. On the other hand, AII as reported previously [31] inhibited adenylyl cyclase activity by about 30% and CNP as well as BNP showed an additive effect with the inhibitory response of AII. These data suggest that CNP or BNP and AII inhibit adenylyl cyclase by distinctly different mechanisms. A similar additive effect of ANP and AII has been reported in glomeruli and aorta [29,32].

4. Discussion

In the present studies, we demonstrate that BNP and CNP, which have been reported to bind to the ANF-R2/ANP-C receptor [14,21,26] inhibited adenylyl cyclase activity in a concentration dependent manner in heart as well as in brain striatal membranes, suggesting that the other signalling pathway through which these peptides mediate their physiological responses may be adenylyl cyclase/cAMP signal transduction pathway to which ANP-C receptors are coupled [23,30]. A decrease in cAMP levels by BNP has recently been shown in rat vascular smooth muscle cells [33], however, these studies did not provide any mechanistic details of this effect.

The inhibition of adenylyl cyclase by CNP and BNP like ANP or C-ANF$_{4-23}$ was also dependent on the presence of guanine nucleotides and was attenuated by PT treatment and suggest that CNP- and BNP-mediated inhibition of adenylyl cyclase requires the G_i regulatory protein as has been reported for the inhibition elicited by ANP or C-ANF$_{4-23}$.

ANP or C-ANF$_{4-23}$ have been reported to interact with other hormone receptors which are coupled to adenylyl cyclase signal transduction system G_s or G_i proteins [29–31]. In the present studies, we have shown that CNP and BNP (data not shown) like ANP or C-ANF$_{4-23}$ [23,29,30] were also able to inhibit the

Table 2
Effect of natriuretic peptides on stimulatory and inhibitory responses of hormones on adenylyl cyclase activity

Additions	Adenylyl cyclase activity pmol (cAMP mg protein · 10 min)$^{-1}$
Experiment 1	
None	145.3 ± 24
CNP (0.1 μM)	95.3 ± 5
Isoproterenol (50 μM)	425.9 ± 1.5
Isoproterenol + CNP	339.0 ± 34.8
Experiment 2	
None	251.05 ± 8.2
BNP (0.1 μM)	181.75 ± 8.6
CNP (0.1 μM)	195.38 ± 14
AII (10 μM)	176.94 ± 10.65
BNP + AII	150.38 ± 11.9
CNP + AII	159.0 ± 16.6

Adenylyl cyclase activity was determined in heart (Experiment 1) and aorta membranes (Experiment 2) in the presence of 10 μM GTP or 10 μM GTPγS respectively as described in section 2. Values are means ± S.E.M. of three separate experiments conducted in triplicate.

hormone-stimulated enzyme activity and showed additive effect with inhibitory responses of AII. These data suggest that all the three peptides mediate adenylyl cyclase inhibition by the same or similar mechanism. This notion is further substantiated by our results on the combined effect of C-ANF$_{4-23}$ and BNP or C-ANF$_{4-23}$ and CNP, which resulted in the same extent of inhibition of adenylyl cyclase as that exerted by either of these peptides. These results strongly suggest that C-ANF$_{4-23}$, BNP and CNP use the same receptor ANP-C for inhibiting adenylyl cyclase/cAMP transduction system. The involvement of a common neuromodulatory receptor ANP-C for C-ANF$_{4-23}$, CNP and ANP in mediating the inhibition of purinergic and adrenergic neurotransmission in isolated rabbit vas deference has recently been suggested [34].

Our results showing an additive inhibitory effect of CNP and BNP on adenylyl cyclase activity are intriguing and suggest that both these peptides may interact with ANP-C receptors at two different sites and inhibit adenylyl cyclase by distinctly different mechanism or alternatively, there may be two different subpopulations of ANP-C receptors for the interaction of CNP and BNP. The presence of ANP-C receptor subtype has also been shown in vascular tissue [35]. In addition, two different cDNAs encoding ANP-C receptor were identified in human umbical vein which differed only by deletion of 123 nucleotides in one of the transcripts [36]. A recent study by Koyama et al. [33] has shown that C-ANF$_{4-23}$ and BNP but not CNP decreased cAMP levels in vascular smooth muscle cells which may suggest that these cells may have a single population of ANP-C receptor subtype responsive to BNP and C-ANF$_{4-23}$ and not to CNP. In addition Trachte et al. [37] by receptor binding studies also suggested the existence of ANP-C receptor subtypes in PC12 cells.

In conclusion, our results provide the first evidence that CNP and BNP interact with ANP-C receptors and elicit adenylyl cyclase inhibition through G_i regulatory protein. It can be suggested that adenylyl cyclase/cAMP signal transduction system may be the other possible mechanism by which these natriuretic peptides elicit their physiological responses.

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