Ring-deleted Analogs of Atrial Natriuretic Factor Inhibit Adenylate Cyclase/cAMP System

POSSIBLE COUPLING OF CLEARANCE ATRIAL NATRIURETIC FACTOR RECEPTORS TO ADENYLATE CYCLASE/cAMP SIGNAL TRANSDUCTION SYSTEM*

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We have recently shown that atrial natriuretic factor (ANF) inhibits adenylate cyclase activity in rat platelets where only one population of ANF receptors (ANF-R2) is present, indicating that ANF-R2 receptors may be coupled to the adenylate cyclase/cAMP system. In the present study, we have used ring-deleted peptides which have been reported to interact with ANF-R2 receptors also called clearance receptors (C-ANF) without affecting the guanylate cyclase/cGMP system, to examine if these peptides can also inhibit the adenylate cyclase/cAMP system. Ring-deleted analog C-ANF₄₋₂₃ like ANF₉₉₋₁₃₆ inhibited the adenylate cyclase activity in a concentration-dependent manner in rat aorta, brain striatum, anterior pituitary, and adrenal cortical membranes. The maximal inhibition was about 50–60% with an apparent Kᵢ between 0.1 and 1 nM. In addition, C-ANF₄₋₂₃ also decreased the cAMP levels in vascular smooth muscle cells in a concentration-dependent manner without affecting the cGMP levels. The maximal decrease observed was about 60% with an apparent Kᵢ of about 1 nM. Furthermore, C-ANF₄₋₂₃ was also able to inhibit cAMP levels and progesterone secretion stimulated by luteinizing hormone in MA-10 cell line. Other smaller fragments of ANF with ring deletions were also able to inhibit the adenylate cyclase activity as well as cAMP levels. Furthermore, the stimulatory effects of various agonists such as 5'-(N-ethylcarboxamido)dadenosine, dopamine, and forskolin on adenylate cyclase activity and cAMP levels were also significantly inhibited by C-ANF₄₋₂₃. The inhibitory effect of C-ANF₄₋₂₃ on adenylate cyclase was dependent on the presence of GTP and was attenuated by pertussis toxin treatment. These results indicate that ANF-R2 receptors or so-called C-ANF receptors are coupled to the adenylate cyclase/cAMP signal transduction system through inhibitory guanine nucleotide regulatory protein.

Atrial natriuretic factor (ANF) has been reported to regulate a variety of physiological processes affecting cardiovascular homeostasis. ANF inhibits adenylate cyclase and cAMP levels and stimulates guanylate cyclase/cGMP accumulation in several tissues suggesting that these two second messenger systems may be responsible in mediating the physiological responses of ANF. The inhibition of adenylate cyclase by ANF has been reported to be dependent on the presence of guanine nucleotides (7, 8) indicating that the inhibition is a receptor-coupled phenomenon. Recently, the involvement of G regulatory protein in the coupling of ANF receptors to the adenylate cyclase system has also been demonstrated (12, 13). Cross-linking studies have identified at least two classes of ANF receptors with an apparent molecular weight of 130,000 and 66,000 designated as ANF-R1 and ANF-R2 (14-16). ANF-R1 receptors are coupled to the guanylate cyclase system (14), whereas the coupling of ANF-R2 receptors to other signal transduction systems is not clearly demonstrated.

Recently, Maack and his colleagues (17) by using a ring-deleted analog of ANF, C-ANF₄₋₂₃ (des[Gln₁⁸, Ser²⁰, Gin²⁰, Leu²¹, Gly²²]ANF₁₋₃₆-NH₂), have reported that this analog binds with high affinity to ANF-R2 receptors without affecting the cGMP levels as well as any of the renal vascular, hemodynamic, and excretory effects of ANF (17). However, the ring-deleted analog of ANF was able to lower the blood pressure and increase sodium excretion in intact anesthetized rats. From these studies, these investigators postulated that the majority of the renal and vascular receptors are biologically silent receptors and the primary function of this receptor is sequestration and metabolic clearance of ANF and have therefore designated them as clearance receptors (C-ANF receptors). However, the effects of ring-deleted analogs on the other signal transduction systems have not been studied. Hirata et al. (18) have recently shown that ANF and atriopeptin I stimulated phosphatidylinositol (PI) turnover in the presence of guanine nucleotide in cultured bovine aortic smooth muscle cells, where atriopeptin I was 10 times more potent than ANF and suggested that ANF-R2 receptors are coupled to the PI turnover system through guanine nucleotide regulatory protein. On the other hand, by using platelets which are devoid of particulate guanylate cyclase (19), we...
have demonstrated that ANF-R2 receptors may be coupled to the adenylate cyclase/cAMP system (20). In the present studies, we have used C-ANF-4-23 and other ring-deleted analogs of ANF which interact with only ANF-R2 or so-called "C-ANF" receptors to investigate if these analogs were also able to inhibit the adenylate cyclase/cAMP system.

**EXPERIMENTAL PROCEDURES**

**Preparation of Anterior Pituitary Homogenates**—Anterior pituitary homogenates were processed as described previously (7). After decapsulation, the anterior pituitaries of female Sprague-Dawley rats (200-300 g, Path) were homogenized in 10 mM Tris-HCl and 1 mM EDTA (pH 7.5) and homogenized manually with a Teflon-glass homogenizer. The homogenates thus obtained were used for adenylate cyclase determination.

**Preparation of Aorta-washed Particles**—Aorta-washed particles were prepared as described elsewhere (2). The aortae were dissected out, quickly frozen in liquid nitrogen, and pulverized to a fine powder with a percussion mortar cooled in liquid nitrogen. They were stored at -70 °C until used. Homogenization was performed in a motor-driven Teflon-glass homogenizer with a buffer containing 10 mM Tris-HCl and 1 mM EDTA (pH 7.5). The homogenate was then centrifuged at 16,000 × g for 10 min. The supernatant was discarded, and the pellets were finally suspended in 10 mM Tris-HCl, 1 mM EDTA buffer (pH 7.5) and used for adenylate cyclase determination.

**Culture of Vascular Smooth Muscle Cells**—Vascular smooth muscle cells were cultured from explants of rat aorta as described previously (21). Colonies of vascular smooth muscle cells grew out from some of the explants within 7-14 days. When sufficiently confluent, the colonies were trypsinized with 0.05% trypsin in phosphate-buffered saline (Ca++- and Mg++-free, containing 0.02% EDTA). The resulting suspension of smooth muscle cells was centrifuged and the cell pellet was dispersed in a small volume of Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 1 mM Hepes, and antibiotics and plated at a cell density of 1 × 10^3-1 × 10^4 cells in 9-cm glass Petri dishes. The medium was changed after attachment of the cells and twice weekly thereafter. The cells were again passaged when they reached confluence.

**Preparation of Adrenal Cortical Membranes**—Adrenal cortical membranes were prepared as described elsewhere (8). Adrenal glands were dissected out from Sprague-Dawley rats (200-300 g). The capsular zone (zona glomerulosa) was separated from subcapsular area (zona fasciculata) and was homogenized using a Teflon-glass homogenizer in a buffer containing 10 mM Tris-HCl and 1 mM EDTA (pH 7.5). The homogenate was centrifuged at 10,000 × g for 10 min. The supernatant was discarded, and the pellets were homogenized in the above buffer with the aid of a glass-Teflon homogenizer. This preparation was used for hormone determination.

**Preparation of Brain Striatal Membranes**—The brain striatal membranes were prepared as described previously (22). Sprague-Dawley rats weighing about 250-300 g were killed by decapitation. Brains were removed, and the striata were dissected out and placed in ice-cold buffer containing 10 mM imidazole and 1 mM EDTA (pH 7.5). Striata (two striata per 3 ml) were homogenized with the aid of a glass-Teflon homogenizer. The homogenate was centrifuged at 1,000 × g for 10 min. The pellet was suspended in 1 mM NaHCO_3 (pH 7.5) and centrifuged at 16,000 × g for 10 min. The supernatant fraction was discarded, and the pellet was finally suspended in a buffer containing 10 mM imidazole and 1 mM EDTA (pH 7.5) and used for adenylate cyclase determination.

**Pertussis Toxin (PT) Treatment**—PT treatment was performed as described earlier (12, 23). The brain striatal membranes were incubated in 95 mM glycolglycine buffer (pH 7.5) containing 1 mM NAD, 9.4 mM ATP, 0.4 mM GTP, 15 mM thymidine, 10 mM diothiothreitol, and ovalbumin (0.1 mg/ml) with and without PT (5 μg/ml) for 30 min at 30 °C. The striatal membranes were 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 adenylate cyclase activity determination. Preincubation of striatal 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 adenylate cyclase by ANF remained unchanged (data not shown).

**Adenylate Cyclase Determination**—Adenylate cyclase activity was determined by measuring [γ-32P]cAMP formation from [α-32P]ATP as described previously (5, 12). The typical assay medium contained 50 mM glycolglycine (pH 7.5), 0.5 mM MgATP, [α-32P]ATP (1-1.5 x 10^6 c.p.m.), 5 mM MgCl_2, GTP (excess of the ATP concentration), 100 mM NaCl, 0.5 mM cAMP, 1 mM 3-isobutyl-1-methylxanthine, 0.1 mM EGTA, 10 μM GTP, and an adenylate-regenerating system consisting of 2 mM creatine phosphate, 0.1 mg of creatine kinase/mi, and 0.1 mg of myokinase/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. cAMP was purified by co-precipitation of other nucleotides with ZnCO_3 by the addition of 0.5 ml of 144 mM NaCO_3 and by subsequent chromatography, using the double column system described by Salomon et al. (24). Under these chromatographic conditions, adenylate cyclase activity was linear with respect to protein concentration and time of incubation.

**Determination of cAMP and cGMP Levels**—cAMP and cGMP levels in vascular smooth muscle cells were determined by radioimmunoassay as described previously (25) by using a radioimmunoassay kit from Du Pont (Mississauga, Ontario, Canada) and Amer- sham Corp. (Ontario, Canada).

**Progesterone Synthesis in Mouse Leydig Tumor Cells**—The MA-10 clone of mouse Leydig tumor cells was propagated and maintained in culture by slight modifications of the procedure of Ascoli (26). The cells were plated in 24 multwell plates in the presence of 10% horse serum in OPTIMEM medium (GIBCO) for 72 h. The cells were washed with serum-free medium and incubated with ovine luteinizing hormone (LH) in the absence or presence of various concentrations of ANF-R1 or C-ANF-4-23 in a total volume of 0.5 ml under 95% CO_2, 5% air at 37 °C for 60 min. At the end of the incubation, the amount of progesterone secreted into the medium was estimated by a specific radioimmunoassay (27).

**Results**

**Effect of C-ANF-4-23 on Adenylate Cyclase Activity**—To investigate if the C-ANF receptors are coupled to the adenylate cyclase system, the effect of ring-deleted analog C-ANF-4-23 on adenylate cyclase activity from several tissues was studied and was compared with the effects observed with ANF. Fig. 1 shows that C-ANF-4-23 like ANF inhibited the adenylate cyclase activity in a concentration-dependent manner in anterior pituitary homogenates, aorta-washed particles, brain striatal and adrenal cortical membranes. Both the peptides were equally effective in inhibiting the enzyme activity. The maximal inhibitions elicited by both these peptides were between 50 and 60% in anterior pituitary, aorta, and brain striatum, whereas about 30% inhibition was observed in adrenal cortical membranes. Both peptides were almost equipotent (apparent K_i between 0.1 and 1 nM) in eliciting the inhibitory effects on the adenylate cyclase activity. C-ANF-4-23 was also able to inhibit adenylate cyclase activity by about 40% in Leydig tumor cells (data not shown).

**Effect of C-ANF-4-23 on cAMP and cGMP Levels**—Fig. 2 shows the effect of C-ANF-4-23 and ANF on cAMP and cGMP levels in cultured vascular smooth muscle cells from rat aorta. Both peptides decreased cAMP levels in a concentration-dependent manner with an apparent K_i of about 1 nM. The maximal inhibition observed was about 60-70%. On the other hand, C-ANF-4-23 as reported earlier (17) was ineffective in stimulating cGMP levels, whereas cGMP levels were dose dependently increased by ANF. These results indicate that C-ANF-4-23 does not interact with ANF-R1 receptors associated with the guanylate cyclase/cGMP system but interacts with ANF-R2 or C-ANF receptors and results in an
**FIG. 1.** Effect of C-ANF_{4-23} and ANF_{99-116} on adenylate cyclase activity in anterior pituitary homogenates (A), aorta-washed particles (B), brain striatal membranes (C), and adrenal cortical membranes (D). Adenylate cyclase activity was determined as described under "Experimental Procedures" in the presence of various concentrations of C-ANF_{4-23} ( △ — △ ) or ANF_{99-116} ( ○ — ○ ). Values are means ± S.E. of three different experiments.

**Fig. 2.** Effect of C-ANF_{4-23} and ANF_{99-116} on cAMP (upper panel) and cGMP levels (lower panel) in cultured vascular smooth muscle cells from rat aorta. The cultured vascular smooth muscle cells were preincubated with 1 mM isobutylmethylxanthine for 5 min and were further incubated in the absence or presence of various concentrations of C-ANF_{4-23} ( △ — △ ) or ANF_{99-116} ( ○ — ○ ) for 10 min at 37 °C. The reaction was stopped by the addition of trichloroacetic acid (6%). The cAMP and cGMP levels in the supernatants were determined by radioimmunoassay kits from Du Pont. Values are means ± S.E. of three separate experiments.

**Fig. 3.** Dependence of guanine nucleotide of inhibition of adenylate cyclase by C-ANF_{4-23} in the rat brain striatal membranes. Adenylate cyclase activity was determined in the presence of various concentrations of GTP_{γS} alone (○ — ○ ) or in combination with 0.1 μM C-ANF_{4-23} ( △ — △ ) as described under "Experimental Procedures." Values are the means ± S.E. of three separate experiments.

inhibition of adenylate cyclase/cAMP system.

Dependence of C-ANF_{4-23} Inhibition of Adenylate Cyclase on Guanine Nucleotides—The inhibitory effects of ANF on adenylate cyclase have been reported to be dependent on the presence of guanine nucleotides (5, 6). If C-ANF_{4-23} interacts with the same receptor populations associated with adenylate cyclase, the inhibitory effect of C-ANF_{4-23} should also require the presence of guanine nucleotides. Fig. 3 illustrates the effect of C-ANF_{4-23} on adenylate cyclase from brain striatum in the absence and presence of various concentrations of GTP_{γS}. C-ANF_{4-23} did not exert any effect on adenylate cyclase activity in the absence of GTP_{γS}; however, in the presence of various concentrations of GTP_{γS}, C-ANF_{4-23} inhibited the enzyme activity in a concentration-dependent manner. The maximal inhibition (~60%) was observed at 30 μM GTP_{γS}. These results indicate that C-ANF receptors are coupled to adenylate cyclase through guanine nucleotide regulatory protein.
Effect of PT on C-ANF4-23-mediated Inhibition of Adenylate Cyclase—The coupling of ANF receptors to adenylate cyclase through inhibitory guanine nucleotide regulatory protein (Gi) has been demonstrated recently (12, 13). To examine if C-ANF receptors are also coupled to adenylate cyclase through Gi, the effect of PT treatment on C-ANF-mediated inhibition was studied. As shown in Fig. 4, C-ANF4-23 inhibited the enzyme activity in a concentration-dependent manner, and this inhibition was attenuated by PT treatment indicating the involvement of Gi regulatory protein in the coupling of C-ANF receptors to adenylate cyclase.

Interaction of C-ANF and ANF99-126 on Adenylate Cyclase Activity—Since both ANF99-126 and C-ANF4-23 were equipotent in inhibiting the adenylate cyclase activity, it was of interest to investigate if both peptides interact with the same receptor population to elicit inhibition or interact with two distinctly different receptor populations. Fig. 5 shows that C-ANF4-23 and ANF99-126 inhibit adenylate cyclase activity by 28 and 32%, respectively, and when the effect of an optimal concentration of both these peptides was studied together on adenylate cyclase, the percent inhibition remained the same. These data indicate that both these peptides interact with the same population of ANF receptors.

Effect of Some Linear Fragments of ANF on Adenylate Cyclase and cAMP Levels—Table I shows that NECA, dopamine, and forskolin stimulated adenylate cyclase activity to various degrees in brain striatum and C-ANF4-23 as shown before inhibited basal activity as well as the stimulatory effects of all these agonists on enzyme activity. For example, NECA, stimulated the enzyme activity by about 300% which was inhibited to about 130% (~50% inhibition) in the presence of 0.1 μM C-ANF4-23. Similarly, the stimulation of adenylate cyclase by forskolin (~50%) was inhibited to about 200% in the presence of 0.1 μM C-ANF4-23. Furthermore, C-ANF4-23 was also able to decrease the basal as well as dopamine- or forskolin-stimulated cAMP levels in cultured vascular smooth muscle cells from rat aorta. The similar inhibitory effects of ANF101-126 or ANF99-126 on hormone-responsive adenylate cyclase activities and cAMP levels have also been reported previously in several other tissues (2-9).

Effect of C-ANF4-23 on Agonist-stimulated Adenylate Cyclase and cAMP Levels—Table I shows that NECA, dopamine, and forskolin stimulated adenylate cyclase activity to various degrees in brain striatum and C-ANF4-23 as shown before inhibited basal activity as well as the stimulatory effects of all these agonists on enzyme activity. For example, NECA, stimulated the enzyme activity by about 300% which was inhibited to about 130% (~50% inhibition) in the presence of 0.1 μM C-ANF4-23. Similarly, the stimulation of adenylate cyclase by forskolin (~50%) was inhibited to about 200% in the presence of 0.1 μM C-ANF4-23. Furthermore, C-ANF4-23 was also able to decrease the basal as well as dopamine- or forskolin-stimulated cAMP levels in cultured vascular smooth muscle cells from rat aorta. The similar inhibitory effects of ANF101-126 or ANF99-126 on hormone-responsive adenylate cyclase activities and cAMP levels have also been reported previously in several other tissues (2-9).

DISCUSSION

The data presented in the current studies demonstrate that ANF-R2 or C-ANF receptors are coupled to the adenylate cyclase/cAMP signal transduction system.

ANF elicits its physiological responses by interacting with its receptors. Two subclasses of ANF receptors, ANF-R1 and ANF-R2 have been reported to be present in most of the tissues (14-16). The ANF-R1 receptor has a molecular mass of 130 kDa and co-purifies with particulate guanylate cyclase (14). The interaction of ANF with ANF-R1 receptors results reported that a linear truncated analog of ANF: des-Cys105, Cys121-ANF (104-126) specifically labeled a 65,000-dalton protein referred to ANF-R2 receptors and did not activate guanylate cyclase in rabbit lung membranes. To investigate if the linear analog of ANF can also interact with the adenylate cyclase/cAMP system, the effects of various truncated linear analogs of ANF on cAMP levels and adenylate cyclase activity in cultured aorta cells were studied and compared to the effects elicited by ANF99-126 and C-ANF4-23. As shown in Table II, all the truncated linear fragments of ANF were able to inhibit/decrease adenylate cyclase activity and cAMP levels, respectively, to various degrees. The inhibitions were quite comparable with those exerted by ANF99-126 or C-ANF4-23. These results indicate that truncated linear analogs of ANF like C-ANF4-23 may also interact, with ANF-R2 receptors.

Effect of C-ANF4-23 on cAMP Levels and Progesterone Secretion in Leydig Tumor Cells—ANF has been reported to inhibit cAMP levels and progesterone secretion in Leydig tumor cells (30). In order to examine if the observed decrease in progesterone secretion by ANF was due to its interaction with ANF-R2 receptors, we studied the effect of C-ANF4-23 on progesterone secretion using the Leydig tumor cell line (MA-10 cells). As shown in Table III, C-ANF4-23 and ANF99-126 did not show any significant effect on basal progesterone secretion up to 10⁻⁹ M; however, luteinizing hormone-stimulated progesterone secretion was significantly inhibited (~25-35%) by both C-ANF4-23 and ANF99-126. In addition, C-ANF4-23 as well as ANF99-126 were also able to decrease cAMP levels (~40%) in these cells stimulated by luteinizing hormone (data not shown).

Fig. 4. Effect of PT on C-ANF4-23-mediated inhibition of adenylate cyclase in rat brain striatal membranes. Brain striatal membranes were treated without (control) or with PT as described under "Experimental Procedures." Adenylate cyclase activity was determined in the absence (basal, B) or presence of 0.1 μM ANF4-23 (A), or combination of both peptides (■) as described under "Experimental Procedures." Values are the means ± S.E. of three separate experiments.
C-ANF Receptors Coupled to Adenylate Cyclase/cAMP Signal Transduction System

Effect of C-ANF<sub>99-126</sub> on basal and agonist-stimulated adenylate cyclase activity and cAMP levels

Adenylate cyclase activity was determined in brain striatal membranes as described under "Experimental Procedures." Values are the means ± S.E. of three separate experiments, each done in triplicate. cAMP levels in cultured vascular smooth muscle cells were determined as described under "Experimental Procedures." Vascular smooth muscle cells were preincubated with 1 mM isobutylmethylxanthine for 5 min at 37°C. Agonists were added to the cells and were further incubated for 10 min. The cells were washed, and the reaction was stopped by the addition of 6% trichloroacetic acid and cAMP levels in the supernatants were determined by the radioimmunoassay kit from Du Pont. Values are means ± S.E. of three separate experiments.

| Addition | Adenylate cyclase activity | cAMP levels |
|----------|---------------------------|-------------|
|          | Control | C-ANF<sub>4-23</sub> (10<sup>-7</sup> M) | Control | C-ANF<sub>4-23</sub> (10<sup>-7</sup> M) |
|          | pmol cAMP (mg protein 5 min)<sup>-1</sup> | pmol/mg protein |
| None     | 410 ± 30 | 287 ± 40 | 1605 ± 36 | 512 ± 48 |
| NECA (10 μM) | 1700 ± 52 | 670 ± 80 | — | — |
| Dopamine (100 μM) | 880 ± 80 | 490 ± 42 | 2880 ± 140 | 1180 ± 77 |
| Forskolin (50 μM) | 2505 ± 57 | 849 ± 20 | 4030 ± 151 | 2400 ± 151 |

Effect of various linear truncated analogs of ANF on adenylate cyclase and cAMP levels in vascular smooth muscle cells from rat aorta

Adenylate cyclase activity was determined as described under "Experimental Procedures." Values are the means ± S.E. of three experiments, each done in triplicate. cAMP levels in cultured vascular smooth muscle cells were determined as described under "Experimental Procedures." Vascular smooth muscle cells were preincubated with 1 mM isobutylmethylxanthine for 5 min at 37°C. Agonists were added to the cells and were further incubated for 10 min. The cells were washed, and the reaction was stopped by the addition of 6% trichloroacetic acid and cAMP levels in the supernatants were determined by the radioimmunoassay kit from Du Pont. Values are means ± S.E. of three separate experiments.

| ANF analogs | Adenylate cyclase activity | cAMP levels |
|-------------|---------------------------|-------------|
| None        | 67 ± 10                  | 4125 ± 71   |
| ANF<sub>99-126</sub> | 19 ± 7             | 4565 ± 77   |
| C-ANF<sub>4-23</sub> | 81 ± 19           | 4150 ± 69   |
| 2-Naphthylacetyl<sup>106</sup>-r-ANF<sub>106-113</sub>[NH<sub>2</sub>]<sup>3</sup> | 75 ± 19         | 3087 ± 515  |
| r-ANF<sub>106-120</sub>[OH] | 69 ± 8             | 5200 ± 42   |
| [p-F-Phe<sup>3</sup>-d-Ala<sup>6</sup>] r-ANF<sub>8-15</sub>-NH<sub>2</sub> | 76 ± 20         | 3250 ± 143  |
| (d-Ala)<sup>6</sup>r-ANF<sub>8-15</sub>-NH<sub>2</sub> | 75 ± 10          | 3087 ± 515  |
| (p-F-Phe)<sup>3</sup>r-ANF<sub>8-15</sub>-NH<sub>2</sub> | 76 ± 1           | 4000 ± 130  |
| r-ANF<sub>8-15</sub>-NH<sub>2</sub> | 80 ± 5            | 3850 ± 299  |

Effect of ANF<sub>90-126</sub> and C-ANF<sub>4-23</sub> on progesterone secretion from mouse Leydig tumor cells

Mouse Leydig tumor cells (MA-10) were incubated for 1 h in a total volume of 0.5 ml including the various additives as shown. The experiment was terminated and medium separated from the cell mat for determination of progesterone content by radioimmunoassay (n = 3) per incubation.

| Additions | Concentration | Progesterone secretion |
|-----------|---------------|------------------------|
|           | ng/well/0.5 ml |                        |
| None      | 0.131 ± 0.069 |                        |
| ANF<sub>90-126</sub> | 10<sup>-9</sup> | 0.124 ± 0.018         |
| C-ANF<sub>4-23</sub> | 10<sup>-9</sup> | 0.097 ± 0.004         |
| LH (3 ng) | 158.30 ± 14.97 |                        |
| LH + ANF<sub>99-126</sub> | 10<sup>-10</sup> | 119.15 ± 4.014       |
| LH + C-ANF<sub>4-23</sub> | 10<sup>-10</sup> | 119.90 ± 2.244       |
| LH + C-ANF<sub>4-23</sub> | 10<sup>-10</sup> | 108.29 ± 3.099       |

* Indicates statistically significant difference (p < 0.05) when compared to 1H alone group.

in the activation of particulate guanylate cyclase and thereby increases cGMP levels suggesting that the ANF-R1 receptors are coupled to guanylate cyclase system. On the other hand, the ANF R2 receptor has a molecular mass of 66 kDa and is generally the more abundant form of ANF receptor in most tissues. The functions of this receptor are not known, and it has been reported that this receptor is a non-guanylate cyclase-coupled receptor. Since ANF inhibits adenylate cyclase activity in a variety of tissues (2-9) in a GTP-dependent manner, it is clear that the ANF-mediated inhibition of adenylate cyclase is a receptor-coupled phenomenon, and it is highly likely that one of the ANF receptors is coupled to this signal transduction system. We have recently shown that platelets which are devoid of particulate guanylate cyclase activity have ANF-R2 receptors and since ANF inhibits adenylate cyclase activity in platelets in a GTP-dependent manner, we suggested that ANF-R2 receptors are coupled to the adenylate cyclase/cAMP system (20).

Recently, Maack and colleagues (17) by using the ring-deleted analog of ANF (C-ANF<sub>4-23</sub>) have suggested that ANF-R2 receptors are biologically inactive because of the fact that the interaction of C-ANF<sub>4-23</sub> with ANF-R2 receptors did not result in an enhancement of cGMP levels. However, in the present studies we have shown that C-ANF<sub>4-23</sub> like ANF<sub>99-126</sub> also inhibits adenylate cyclase activity in anterior pituitary, aorta, brain striatum, and adrenal cortex in a concentration-dependent manner. The maximal inhibition and the potency of C-ANF<sub>4-23</sub> to inhibit enzyme activity was in the same range as was observed with ANF<sub>99-126</sub>. In addition, C-ANF<sub>4-23</sub>...
was also able to decrease cAMP levels in cultured vascular smooth muscle cells without affecting cGMP levels, whereas ANF99-126 was able to decrease cAMP levels and increase cGMP levels. These data as reported earlier (17) indicate that C-ANF4-23 does not interact with ANF-R1 receptors associated with the guanylate cyclase/cGMP system but interacts with ANF-R2 or C-ANF receptors and results in an inhibition of the cAMP/adenylate cyclase system. Thus, these results contradict the earlier hypothesis postulated by Maack et al. (17) and suggest that the C-ANF receptors are not biologically inactive but are coupled to the adenylate cyclase/cAMP signal transduction system.

The inhibition of adenylate cyclase by C-ANF4-23 was also dependent on the presence of guanine nucleotides and was attenuated by PT treatment. Similar attenuation of ANF-mediated inhibition of adenylate cyclase by PT was shown previously (12, 13). These results indicate that C-ANF and/or ANF-R2 receptors represent the same receptor population and are coupled to adenylate cyclase system through the Gi regulatory protein. This notion is further substantiated by our results showing that the inhibition exerted by optimal concentration of ANF99-126 or C-ANF4-23 was not additive when the combined effect of both the ANF99-126 and C-ANF4-23 was studied on adenylate cyclase activity.

As shown earlier for ANF99-126 or ANF101-126 (2-9), the C-ANF4-23 was also able to inhibit the stimulatory effects of guanine nucleotides and hormones, which are mediated through Gi (stimulatory guanine nucleotide regulatory protein) on adenylate cyclase and cAMP levels. Similarly, the stimulation of adenylate cyclase caused by forskolin, which interacts directly with catalytic subunit or a component associated with it (31), was also inhibited by C-ANF4-23. However, C-ANF4-23 did not inhibit the basal enzyme activity (inactive form), which may be due to the fact that the inhibition of adenylate cyclase by ANF is a receptor-coupled phenomenon and requires GTP-binding protein (Gi) and GTP to elicit the inhibition of adenylate cyclase as has been reported previously (12). These data further support that ANF receptors are coupled to the adenylate cyclase system and may not be biologically silent (17) and mediate some of the physiological responses of ANF. This fact is further supported by our present studies showing that C-ANF4-23 like ANF99-126 inhibited the progesterone secretion stimulated by LH in the Leydig tumor cells, the phenomenon which is known to be mediated through the adenylate cyclase/cAMP system (30).

Recently, Hirata et al. (18) have shown that ANF-R2 receptor activation results in the stimulation of phosphoinositide (PI) hydrolysis in cultured bovine aortic smooth muscle cells. These results were based on the assumption that atriopeptin I was more potent than ANF in exerting the stimulatory effects on PI hydrolysis. However, it should be noted that API has been shown to bind to both ANF-R1 and ANF-R2 receptors (32), hence these studies cannot conclusively demonstrate that ANF-R2 receptors are coupled to PI turnover. On the other hand, the present studies strongly suggest that ANF-R2 or C-ANF receptors are coupled to the adenylate cyclase/cAMP system through Gi-guanine nucleotide regulatory protein. The involvement of cAMP in mediating some of the physiological effects of ANF such as inhibition of aldosterone secretion, inhibition of renin release, inhibition of vasopressin release, etc. has been demonstrated (1). It is also known that not all of the physiological effects of ANF could be explained by the increases in cGMP levels (17). Taken together, it may be possible that these physiological effects of ANF are mediated through its interaction with ANF-R2 receptors which are coupled to the adenylate cyclase/cAMP signal transduction system. Furthermore, it has been reported that the infusion of C-ANF4-23 in the rat resulted in an increase in the plasma levels of endogenous immunoreactive ANF and natriuresis (17). It has also been shown that agents such as forskolin, isoproterenol, etc. which increase cAMP levels by the stimulation of adenylate cyclase activity can decrease the secretion of ANF (33), whereas vasopressin and angiotensin II, which inhibit the adenylate cyclase activity and cAMP levels are the stimulators of ANF secretion (34). Taken together, it is possible that the increase in the plasma levels of immunoreactive ANF by C-ANF4-23 may also be the result of such an inhibition of the adenylate cyclase/cAMP system.

In conclusion, we have demonstrated that C-ANF4-23 and other linear truncated analogs of ANF that specifically interact with ANF-R2 or C-ANF receptors only inhibit adenylate cyclase activity and cAMP levels in various tissues and suggest that C-ANF receptors are coupled to the adenylate cyclase/cAMP signal transduction system through inhibitory guanine nucleotide regulatory protein.

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