Downregulation of Atrial Natriuretic Peptide ANP-C Receptor Is Associated with Alterations in G-Protein Expression in A10 Smooth Muscle Cells†

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ABSTRACT: Atrial natriuretic peptide (ANP) receptors A and B are guanylyl cyclase receptors, whereas ANP-C receptors are coupled to adenylyl cyclase through inhibitory guanine nucleotide (Gi) protein. ANP has been shown to downregulate ANP-A and -B receptors and cGMP response in various tissues. In the present studies, we have examined the regulation of ANP-C receptor−adenylyl cyclase signal transduction by ANP and [des(Gln^{18},Ser^{19},Gln^{20},Leu^{21},Gly^{22})ANP$_{4-23}$-NH$_2$](C-ANP$_{4-23}$) that interacts specifically with ANP-C receptor in A10 smooth muscle cells (SMC). Treatment of the cells with C-ANP$_{4-23}$ for 24 h resulted in a reduction in ANP receptor binding activity. [^{125}I]ANP$_{99-126}$ bound to control and C-ANP$_{4-23}$-treated cell membranes at a single site with dissociation constants of 33.7 ± 6 and 35.0 ± 4.5 pM and B_{max} of 74.0 ± 5.0 and 57.6 ± 4.0 fmol/mg of protein, respectively. C-ANP$_{4-23}$ inhibited adenylyl cyclase activity in a concentration-dependent manner in control cells. A maximal inhibition observed was about 30−40% with an apparent K_i of about 1 nM; however, this inhibition was completely attenuated in cells pretreated with ANP$_{99-126}$ or C-ANP$_{4-23}$ (10^{-7} M). However, the inhibition of adenylyl cyclase by 17-amino acid peptide (RRNHQEESNIGKHRELR) (R17A) of cytoplasmic domain of ANP-C receptor was attenuated by about 50% but was not completely abolished by C-ANP$_{4-23}$ treatment. The attenuation of C-ANP$_{4-23}$-mediated inhibition of adenylyl cyclase was dependent on the concentration and time of pretreatment of the cells with C-ANP$_{4-23}$. In addition, angiotensin II- (Ang II-) mediated inhibition of adenylyl cyclase (∼30%) was also abolished by C-ANP$_{4-23}$ treatment, indicating that the desensitization elicited by ANP was heterologous. In addition, C-ANP$_{4-23}$ treatment decreased the expression of Giα-2 and Giα-3 proteins by about 40 and 60%, respectively, and their mRNA by 40%. However, the levels of Gi proteins were not altered when the cells were treated for shorter period of time (2−4 h) or with lower concentrations of C-ANP$_{4-23}$ (10^{-10} M). On the other hand, the levels of Gsα but not of Gβ were increased by about 35% by C-ANP$_{4-23}$ treatment. Furthermore, the stimulations exerted by GTPγS, isoproterenol, FSK, and NaF on adenylyl cyclase were also augmented in cells treated with C-ANP$_{4-23}$. These results indicate that C-ANP$_{4-23}$ treatment of A10 cells desensitizes ANP-C receptor-mediated inhibition of adenylyl cyclase which may be due to the downregulation of ANP-C receptor and decreased expression of Giα proteins to which these receptors are coupled.

Atrial natriuretic peptide (ANP),[1] a member of the family of natriuretic peptides, was discovered by de Bold et al. (*1*, *2*). ANP regulates a variety of physiological parameters, including the blood pressure, progesterone secretion, renin release, and vasopressin release (*3*, *4*), by interacting with receptors on the plasma membrane either to generate second messengers such as cyclic AMP (cAMP) (*5*−*9*) and cyclic GMP (cGMP) (*10*−*12*) or to affect ion channels (*4*).

ANP receptors are divided into two major categories, those that activate guanylyl cyclase (referred to as ANP-R$_1$) and those that do not (referred to as ANP-R$_2$ or ANP-C). The ANP-R$_1$ are guanylyl cyclase-coupled receptors and have a relative molecular mass of 130−180 kDa, whereas ANP-R$_2$/ANP-C receptors exist as monomers (66 kDa) and dimers (130 kDa). Molecular cloning techniques revealed three subtypes of natriuretic peptide receptor (NPR). These are NPR-A (*13*, *14*), NPR-B (*15*, *16*), and NPR-C (*17*, *18*). NPR-A and NPR-B are membrane guanylyl cyclases, whereas NPR-C (clearance receptors), also known as ANP-R$_2$ and ANP-C receptors, are coupled to adenylyl cyclase inhibition through inhibitory guanine nucleotide regulatory protein (*17*, *19*) or to activation of phospholipase C (*20*) and have been shown to mediate some of the physiological effects of ANP. The physiological role of ANP-C receptor and cAMP signal transduction includes inhibition of progesterone secretion from Leydig tumor cells, inhibition of thyroglobulin release from human thyroid cultured cells, inhibition of endothelial and vascular smooth muscle proliferation, inhibition of adrenergic and purinergic neurotransmission and in

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[1] Abbreviations: ANP, atrial natriuretic peptide; C-ANP$_{4-23}$, [des(Glu^{18},Ser^{19},Glu^{20},Leu^{21},Gly^{22})ANP$_{4-23}$-NH$_2$]; R17A, 17 amino acid peptide of cytoplasmic domain of ANP-C receptor (10−26AA, RRNHQEESNGKHRELR); Gi, inhibitory guanine nucleotide regulatory protein; Gs, stimulatory guanine nucleotide regulatory protein; GTPγS, guanosine 5′-0-(3-thiotriphosphate); FSK, forskolin; Ang II, angiotensin II.

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vivo translation of the endothelin message and the secretion of endothelin from cultured bovine endothelial cells (see review *4*).

The ANP-C receptors have been reported to be regulated by various agents. Phorbol esters, calcium phospholipid-dependent protein kinase (PKC), and *N*-ethylmaleimide (NEM) attenuated the inhibitory effect of ANP on adenylyl cyclase (*21*) which may be due to the uncoupling of the ANP-C receptors from the catalytic subunit of the adenylyl cyclase. ANP receptors have also been shown to be regulated (down- or upregulated) in various pathophysiological conditions which are associated with increased levels of plasma ANP (*4*). ANP and angiotensin II (Ang II) appear to act as physiological antagonists in the regulation of blood pressure and fluid homeostasis through receptor-mediated actions in various target tissues (*3*, *22*−*24*). We have recently shown that Ang II attenuated the ANP-C receptor-mediated inhibition of adenylyl cyclase without affecting the ANP receptor binding sites in A10 vascular smooth muscle cells (VSMC) (*25*). In the present studies, we have examined the regulation of ANP-C receptor by ANP and C-ANP$_{4-23}$ in A10 VSMC. We have shown that both these peptides desensitize ANP-C receptor-mediated inhibition of adenylyl cyclase which may be attributed to the downregulation of ANP-C receptor and inhibition of Gi protein which couples the ANP-C receptor to adenylyl cyclase signal transduction system.

EXPERIMENTAL PROCEDURES

Materials. Adenosine triphosphate, cyclic AMP, and other chemicals necessary for total RNA extraction and Northern blot analysis were obtained from Sigma Chemical Co. (St Louis, MO). Creatine kinase (EC 2.7.3.2), myokinase (EC 2.7.4.3), GTP, and GTPγS were purchased from Boehringer-Mannheim, Montreal, Quebec, Canada. 3-Isobutyl-l-methylxanthine (IBMX) was purchased from Aldrich Chemical Corporation (Milwaukee, WI). [α-^{32}P]ATP, [α-^{32}]dCTP, and carrier-free [^{32}P]orthophosphate were purchased from Amersham Corp. (Oakville, Ontario, Canada). Angiotensin II and C-ANP$_{4-23}$ were from Peninsula Laboratories Inc. (Belmont, CA). AS/7, EC/2, RM/1, and SW/1 antibodies directed against specific C-terminus sequences of Giα2, Giα3, Gsα, and Gβ, respectively, were purchased from Dupont (Mississauga, Ontario).

Cell Culture and Incubation. The A10 cell line from embryonic thoracic aorta of rat was obtained from American Type Culture Collection, Rockville, MA. The cells were plated in 7.5 cm^2 flasks and incubated at 37 °C in 95% air and 5% CO_2 humidified atmosphere in Dulbecco's modified Eagle's medium (DMEM) (with glucose, L-glutamine, and sodium bicarbonate) containing antibiotics and 10% heat-inactivated calf serum (FCS), as described previously (*25*). The cells were passaged upon reaching confluence with 0.5% trypsin containing 0.2% EDTA and utilized between passages 5 and 15. Confluent cell cultures were starved by incubation for 3 h in DMEM without FCS at 37 °C. These cells were then incubated with C-ANP$_{4-23}$ or ANP$_{99-126}$ (10^{-7} M) or as otherwise indicated for 24 h at 37 °C. After incubation, cells were washed twice with ice-cold homogenization buffer (10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA). 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 adenylyl cyclase assay and immunoblotting.

Adenylyl Cyclase Activity Determination. Adenylyl cyclase activity was determined by measuring [^{32}P]cAMP formation from [α-^{32}P]ATP, as described previously (*17*, *19*).

Immunoblotting. Immunoblotting of G-proteins was performed as described previously (*26*, *27*).

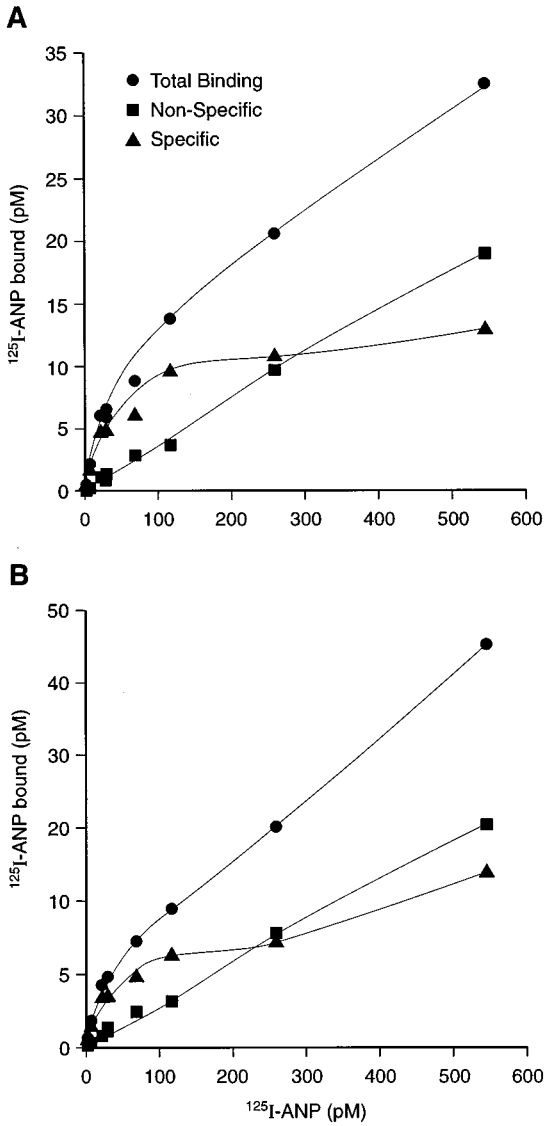
Total RNA Extraction and Northern Blot Analysis. Total RNA was extracted from A10 cells by the method of Sambrook et al. (*28*) as described earlier (*31*). Northern blot analysis was performed as described earlier (*29*).

ANP-C Receptor Binding Determination. ANP receptor binding was determined as described previously (*25*, *30*). Briefly, [^{125}I]ANP binding was determined at 25 °C by incubating 20 μL of membranes ($\sim$100 μg) for 60 min with 10 pM ^{125}I-ANP$_{99-126}$ peptide or as otherwise indicated (specific radioactivity 1000 Ci/mmol) in 200 μL of a reaction mixture consisting of 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM aprotinin, bacitracin (1 mg/mL), BSA (4 mg/mL), 0.5 mM phenylmethylsulfonyl fluoride, and various concentrations of the C-ANP$_{4-23}$ or as otherwise indicated. Binding reactions were initiated by the addition of membrane protein. The receptor−[^{125}I]ANP complex was separated from free ^{125}I-ANP by filtration through GP/C filters pretreated with polyethyleneamine. The filters were washed three times with 4 mL of ice-cold Tris-HCl buffer (pH 7.5), and the associated radioactivity was determined in a LKB-Wallac 1277 Gamma Master counter.

RESULTS

Effect of C-ANP$_{4-23}$ Treatment on ANP Receptors. ANP has been shown to downregulate ANP receptors and cGMP response (*31*, *32*) in vascular smooth muscle cells; however, the studies on ANP-C receptor downregulation have not been conducted. Since C-ANP$_{4-23}$ interacts specifically with ANP-C receptors, we investigated the effect of C-ANP$_{4-23}$ treatment on receptor binding activity using [^{125}I]ANP$_{99-126}$. As illustrated in Figure 1, [^{125}I]ANP binds to control and C-ANP$_{4-23}$-treated A10 smooth muscle cell membranes in a saturable manner. Computer analysis of the saturation binding of labeled ANP over the concentration range studied in control and C-ANP$_{4-23}$-treated cells revealed a single class of saturable high-affinity binding site with a dissociation constant (K_d) of 33.7 $\pm$ 6.0 and 35.0 $\pm$ 4.5 pM, respectively, and a receptor density (B_{max}) of 74.0 $\pm$ 5.0 and 57.0 $\pm$ 4.0 fmol/mg of protein (n = 3), respectively. In addition, the specificity of [^{125}I]ANP binding to VSMC membranes was also studied by competitive displacement of radiolabeled 10 pM [^{125}I]ANP by unlabeled C-ANP$_{4-23}$ peptide. As shown in Figure 2, the total number of binding sites was significantly decreased in membranes prepared from C-ANP$_{4-23}$-treated cells as compared to control cells (3.7 $\pm$ 1.8 versus 5.6 $\pm$ 2.0 pmol/mg of protein, n = 3) without a significant change in the affinity (9.4 $\pm$ 1.0 versus 11.8 $\pm$ 1.5 nM, n = 3).

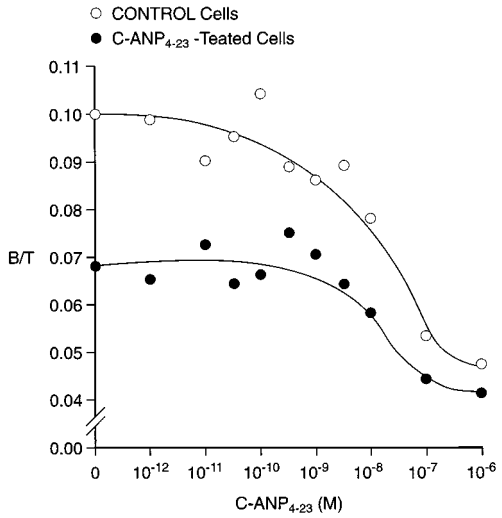
ANP-C Receptor-Mediated Inhibition of Adenylyl Cyclase. To investigate if the downregulation of ANP receptor by C-ANP$_{4-23}$ treatment is reflected in ANP-C receptor-mediated inhibition of adenylyl cyclase, the effect of C-ANP$_{4-23}$ treatment on C-ANP$_{4-23}$-mediated inhibition of adenylyl



FIGUREE 1: Saturation binding of [^{125}I]ANP$_{99-126}$ to control and C-ANP$_{4-23}$-treated A10 smooth muscle cell membranes. A10 cells were incubated in the absence (control, A) or presence of 10^{-7} M C-ANP$_{4-23}$ (treated, B) for 24 h as described in Experimental Procedures. Membranes were prepared as described in Experimental Procedures. A10 cell membranes were incubated with increasing amounts of labeled ANP$_{99-126}$ peptide at 25 °C for 60 min in the absence (total binding) or presence (nonspecific binding) of 1 μM unlabeled ANP$_{99-126}$ as described in Experimental Procedures. Specific binding is the difference between two curves. The curves were analyzed by the ALLFIT program (*47*). Each point represents the mean of triplicate determinations from a single experiment: (A) K_d 30.0 pM, B_{max}, 70.0 fmol/mg of protein; (B) K_d 23.0 pM, B_{max} 44.0 fmol/mg of protein. Data from three separate independent experiments are given in the Results.

cyclase was examined in A10 smooth muscle cells, and the results are shown in Figure 3A. C-ANP$_{4-23}$ inhibited adenylyl cyclase activity in a concentration-dependent manner in control cells with an apparent K_i of about 1 nM. The maximal inhibition observed was about 30−40%. However, this inhibition was completely abolished in cells treated with C-ANP$_{4-23}$. In addition, ANP$_{99-126}$ treatment of the cells also resulted in a complete attenuation of C-ANP$_{4-23}$-mediated inhibition of adenylyl cyclase (Figure 3B).

The desensitization of the response was concentration (Figure 4A) and time dependent (Figure 4B). Pretreatment of the cells with increasing concentrations of C-ANP$_{4-23}$



FIGUREE 2: Effect of c-ANP$_{4-23}$ treatment on competition binding of [^{125}I]ANP in control and C-ANP$_{4-23}$-treated A10 smooth muscle cells. A10 cells were incubated in the absence (control) or presence (treated) of 10^{-7} M C-ANP$_{4-23}$ for 24 h as described in Experimental Procedures. Membranes were prepared as described in Experimental Procedures. A10 cell membranes were incubated at 25 °C for 60 min with 10 pM [^{125}I]ANP and increasing concentrations of unlabeled peptide as described in Experimental Procedures. Each point represents the mean of tripilicate determination from a single experiment. The binding curves are derived from the specific binding data analyzed by the ALLFTT program (multiple regression): control cells, K_d 12.4 nM, B_{max} 4.2 pmol/mg of protein; C-ANP$_{4-23}$-treated cells, K_d 12.8 nM, B_{max} 2.8 pmol/mg of protein. Data from three separate experiemnts are given in the Results.

(10^{-12}, 10^{-10}, 10^{-9}, 10^{-8}, and 10^{-7} M) attenuated the C-ANP$_{4-23}$-mediated inhibition by 23%, 46%, 76%, 77%, and 88%, respectively (n = 3). In addition, about 50% attenuation of C-ANP$_{4-23}$-mediated inhibition was observed after 2−4 h of pretreatment of the cells with C-ANP$_{4-23}$ which was almost completely abolished after 8 h of treatment and persisted up to 24 h.

Effect of C-ANP$_{4-23}$ Pretreatment on Ang II-Mediated Inhibition of Adenylyl Cyclase. Ang II receptor (AT_1 subtype) activation results in the inhibition of adenylyl cyclase activity (*33*) and activation of PLC signaling pathway (*34*). Ang II has been shown to regulate ANP receptors in vascular smooth muscle cells (*24*). We have recently shown that Ang II pretreatment of A10 smooth muscle cells for 24 h resulted in the heterologous desensitization of Ang II and ANP-C receptor-mediated adenylyl cyclase inhibition (*25*). To investigate if C-ANP$_{4-23}$ pretreatment of A10 cells also results in an attenuation of Ang II-mediated inhibition of adenylyl cyclase, the effect of C-ANP$_{4-23}$ pretreatment on Ang II-mediated inhibition of adenylyl cyclase was examined, and the results are shown in Figure 5. Ang II as reported previously (*25*) inhibited adenylyl cyclase activity in a concentration-dependent manner in control cells with an apparent K_i of about 5 nM. The maximal inhibition observed was about 35%. However, the inhibition elicited by various concentrations of Ang II was almost completely abolished in the cells pretreated with C-ANP$_{4-23}$.

Effect of C-ANP$_{4-23}$ Pretreatment on G-Protein Levels. A partial downregulation of ANP receptor and a complete attenuation of C-ANP$_{4-23}$-mediated inhibition of adenylyl cyclase by C-ANP$_{4-23}$ treatment for 24 h may suggest the involvement of postreceptor components in the desensitized

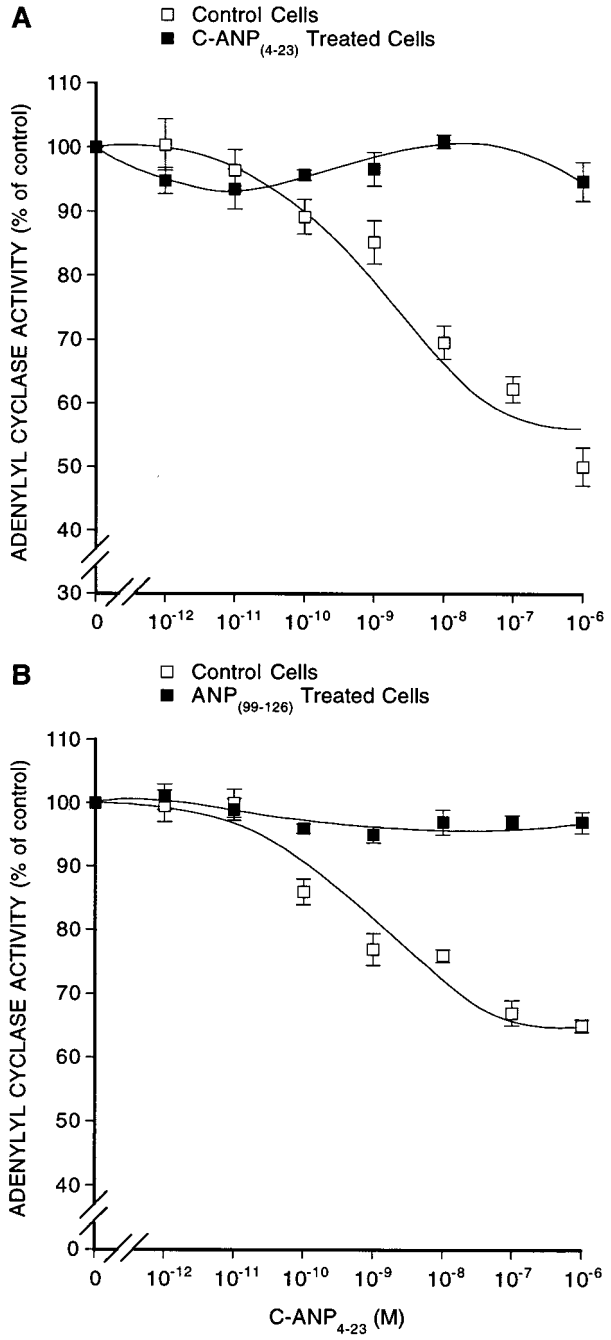


FIGURE 3: Effect of C-ANP$_{4-23}$ and ANP pretreatment on C-ANP$_{4-23}$-mediated inhibition of adenylyl cyclase activity in A10 smooth muscle cells. (A) A10 cells were incubated in the absence (control) or presence of 10^{-7} M C-ANP$_{4-23}$ (treated) for 24 h as described in Experimental Procedures. Membranes were prepared as described in Experimental Procedures. Adenylyl cyclase activity was determined in the membranes in the absence or presence of various concentrations of C-ANP$_{4-23}$ as described in Experimental Procedures. Values are SEM of three separate experiments performed in triplicates. Basal enzyme activities in the presence of 10 μM GTPγS in control and C-ANP$_{4-23}$-treated membranes were 185.3 $\pm$ 9.0 and 161.5 $\pm$ 5 pmol (mg protein·10 min)$^{-1}$, respectively. (B) A10 cells were incubated in the absence (control) or presence of 10^{-7} M ANP$_{99-126}$ (treated) for 24 h as described in Experimental Procedures. Adenylyl cyclase activity was determined in the membranes in the absence or presence of various concentrations of C-ANP$_{4-23}$ as described in Experimental Procedures. Values are SEM of three separate experiments performed in triplicates. Basal enzyme activities in the presence of 10 μM GTPγS in control and C-ANP$_{4-23}$-treated membranes were 167.0 $\pm$ 8.0 and 170.0 $\pm$ 5.4 pmol (mg of protein·10 min)$^{-1}$, respectively.

response. To investigate this possibility, the levels of G-proteins that couple ANP-C receptor to adenylyl cyclase (*17*, *19*) were determined by immunoblotting technique using specific antibodies against Giα-2 and Giα-3 in control and C-ANP$_{4-23}$-treated cells. As reported previously, AS/7 antibodies that react with both Giα-1 and Giα-2 (*35*) and EC/2 antibodies against Giα-3 recognized a single protein of 40 kDa (Giα-2) and 41 kDa (Giα-3) in control and C-ANP$_{4-23}$-treated cells (Figure 6A); however, the relative amounts of immunodetectable Giα2 and Giα-3 were significantly decreased in a concentration-dependent manner in C-ANP$_{4-23}$-treated cells. At 10^{-7} M, the levels of Giα-2 and Giα-3 were decreased by about 35.0 $\pm$ 3.5% (n = 3) and 40.0 $\pm$ 4.4% (n = 3), respectively, as compared to control cells as determined by densitometric scanning (Figure 6B); however, at lower concentrations of C-ANP$_{4-23}$ (10^{-10} M), the levels of Giα-2 and Giα-3 proteins were not significantly altered, suggesting that Gi proteins may not be involved in the desensitization of ANP-C receptor-mediated inhibition of adenylyl cyclase observed at lower concentration of C-ANP$_{4-23}$ treatment. The decreased levels of Gi proteins at higher concentration of C-ANP$_{4-23}$ treatment may be responsible for the attenuation of C-ANP$_{4-23}$-mediated inhibition of of adenylyl cyclase.

In addition, the levels of Gsα and Gβ were also determined and the results are shown in Figure 6A. RM/1 antibodies against Gsα and SW/1 antibodies against Gβ recognized a single protein of 45 and 35 kDa, respectively, in control and C-ANP$_{4-23}$-treated cells; however, in contrast to Giα, the relative amounts of immunodetectable Gsα were increased by 35.6 $\pm$ 4.0% (n = 3) in C-ANP$_{4-23}$ treated cells as compared to control cells and the levels of Gβ were not significantly different in control and C-ANP$_{4-23}$-treated cells as determined by densitometric scanning (Gβ, in arbitrary units, 1.72 $\pm$ 0.05; C-ANP$_{4-23}$-treated cells, 1.67 $\pm$ 0.07).

We then extended our studies further to investigate if mRNA levels of Giα2 and Giα-3 change concomitantly with protein levels by C-ANP$_{4-23}$ treatment and determined mRNA levels by Northern blot analysis using specific cDNA probes encoding for Giα-2 and Giα-3 (Figure 7A). The Giα-2 cDNA probe detected a message of 2.3 kb in control and C-ANP$_{4-23}$-treated cells, but the amount of Giα2 mRNA was decreased significantly by about 60 $\pm$ 5% (n = 3) in C-ANP$_{4-23}$-treated cells as compared to control cells as determined by densitometric scanning (Figure 7B). Similarly, the Giα-3 cDNA probe detected a message of 3.5 Kb in both control and C-ANP$_{4-23}$ treated cells (Figure 7A), yet the levels of Giα-3 mRNA were significantly decreased by about 40 $\pm$ 3% (n = 3) in C-ANP$_{4-23}$ treated cells (Figure 7B) as determined by densitometric scanning.

Time-Course of C-ANP$_{4-23}$ Pretreatment and Gi-Protein Levels. To further investigate a correlation between the decreases in the levels of Gi proteins and C-ANP$_{4-23}$-mediated inhibition of adenylyl cyclase, the levels of Gi proteins were determined at different times of pretreatment of cells with C-ANP$_{4-23}$. Results shown in Figure 8 indicate that the levels of Giα-2 (A) and Giα-3 (B) were not altered at 2 and 4 h of pretreatment with C-ANP$_{4-23}$; however, pretreatment for 8 h inhibited the levels by about 30−40% as determined by densitometric scanning. These results suggest that the attenuation of C-ANP$_{4-23}$-mediated inhibition of adenylyl cyclase by 50% as observed after 2−4 h of

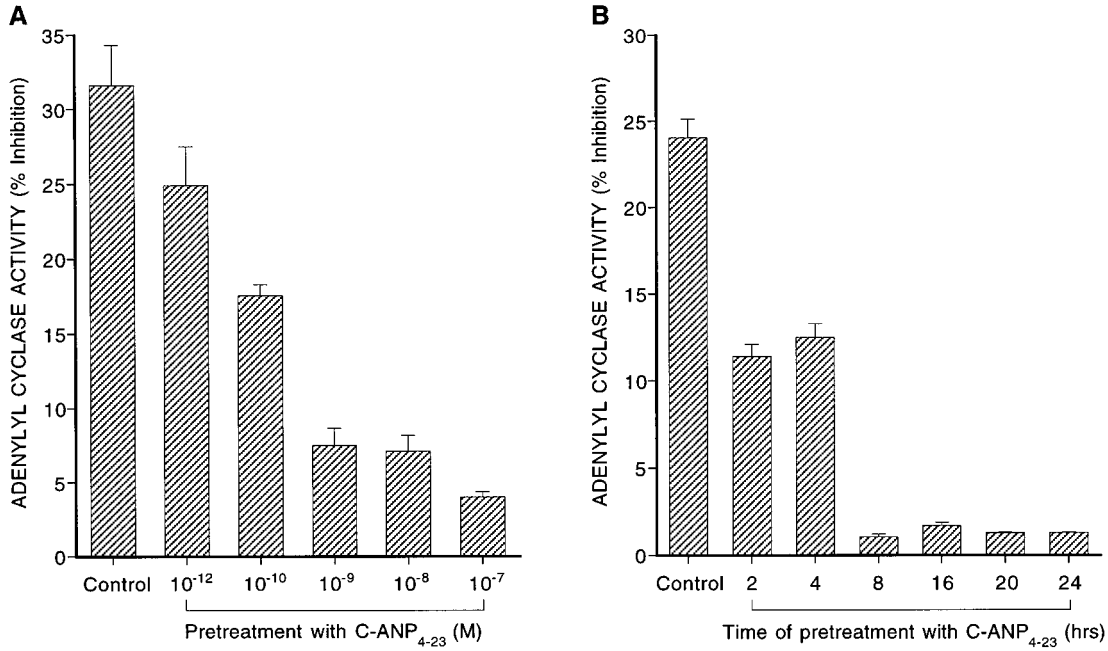


FIGURE 4: (A) Effect of various concentration of C-ANP$_{4-23}$ pretreatment on C-ANP$_{4-23}$-mediated inhibition of adenylyl cyclase activity in A10 smooth muscle cells. A10 cells were incubated in the absence (control) or presence of various concentration of C-ANP$_{4-23}$ (10^{-12}–10^{-7} M) for 24 h as described in Experimental Procedures. Membranes were prepared as described in Experimental Procedures. Adenylyl cyclase activity was determined in the membranes in the absence (basal) or presence of 10^{-7} M C-ANP$_{4-23}$ as described in Experimental Procedures. Values are means ± SEM of three separate experiments performed in triplicates. Basal adenylyl cyclase activity in the presence of 10 μM GTPγS in control membranes was 196.8 ± 13.4 pmol of cAMP (mg of protein·10 min)$^{-1}$. (B) Time course of C-ANP$_{4-23}$ pretreatment on C-ANP$_{4-23}$-mediated inhibition of adenylyl cyclase activity in A10 smooth muscle cells. A10 cells were incubated in the presence of 10^{-7} M C-ANP$_{4-23}$ for 0, 2, 4, 8, 12, 16, 20, and 24 h as described in Experimental Procedures. Membranes were prepared as described in Experimental Procedures. Adenylyl cyclase activity was determined in the membranes in the absence (basal) or presence of 10^{-7} M C-ANP$_{4-23}$ as described in Experimental Procedures. Values are means ± SEM of three separate experiments performed in triplicates. Adenylyl cyclase activities in the presence of 10 μM GTPγS alone or in combination with 10^{-7} C-ANP$_{4-23}$ in control membranes were 203.4 ± 11.1 and 154.5 ± 7 pmol of cAMP (mg of protein·10 min)$^{-1}$, respectively.

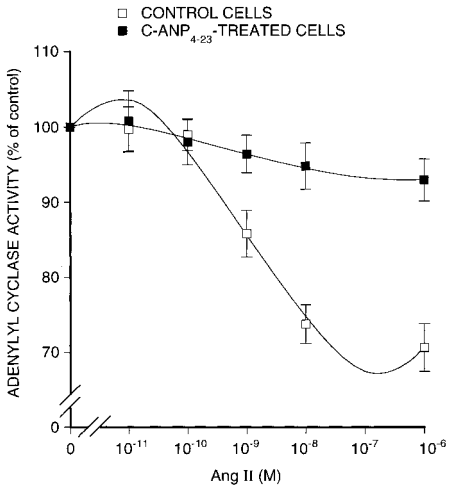


FIGURE 5: Effect of C-ANP$_{4-23}$ pretreatment on Ang II-mediated inhibition of adenylyl cyclase activity in A10 smooth muscle cells. A10 cells were incubated in the absence (control) or presence of 10^{-7} M C-ANP$_{4-23}$ (treated) for 24 h as described in Experimental Procedures. Membranes were prepared as described in Experimental Procedures. Adenylyl cyclase activity was determined in the membranes in the absence or presence of various concentrations of Ang II as described in Experimental Procedures. Values are means ± SEM of three separate experiments performed in triplicates. Basal adenylyl cyclase activity in the presence of 10 μM GTPγS in control and C-ANP$_{4-23}$-treated A10 membranes were 243.6 ± 5.8 and 210.4 ± 7.9 pmol of cAMP (mg of protein·10 min)$^{-1}$, respectively.

pretreatment (Figure 4B) may not be attributed to the decreases in the levels of Gi proteins and may be due to the reduction in ANP-C receptor. But when the levels of Gi proteins were also inhibited (~40%), after 8 h or longer period of treatment with C-ANP$_{4-23}$ (Figure 6A,B), a complete attenuation of C-ANP$_{4-23}$-mediated inhibition of adenylyl cyclase was observed.

Effect of Inhibition of Gi Protein on Adenylyl Cyclase Inhibition. We have recently shown that 37-amino acid peptide (R37A) corresponding to cytoplasmic domain of ANP-C receptor directly inhibits adenylyl cyclase activity through its interaction with Giα protein (*36*). To investigate if the inhibition of Gi proteins (~40%) by C-ANP$_{4-23}$ treatment results in complete or partial attenuation of adenylyl cyclase inhibition by cytoplasmic domain peptide of ANP-C receptor, the effect of the 17-amino acid peptide of cytoplasmic domain (RRNHQEESNIGKHRELR) (R17A) of ANP-C receptor on adenylyl cyclase was examined. Results shown in Figure 9 indicate that R17A inhibited adenylyl cyclase activity in a concentration-dependent manner in control cells. However, inhibition of the levels of Gi proteins (~40%) by C-ANP$_{4-23}$ treatment resulted in partial attenuation (~50%) of R17A-mediated inhibition of adenylyl cyclase activity.

Effect of C-ANP$_{4-23}$ Treatment on Gi Functions. To investigate if the decreased levels of Giα were also reflected in Gi functions, the effect of low concentrations of GTPγS on forskolin- (FSK-) stimulated adenylyl cyclase activity was examined in control and C-ANP$_{4-23}$-treated cells. Figure 10 illustrates that GTPγS inhibited FSK-stimulated adenylyl cyclase activity in control and C-ANP$_{4-23}$-treated cells in a

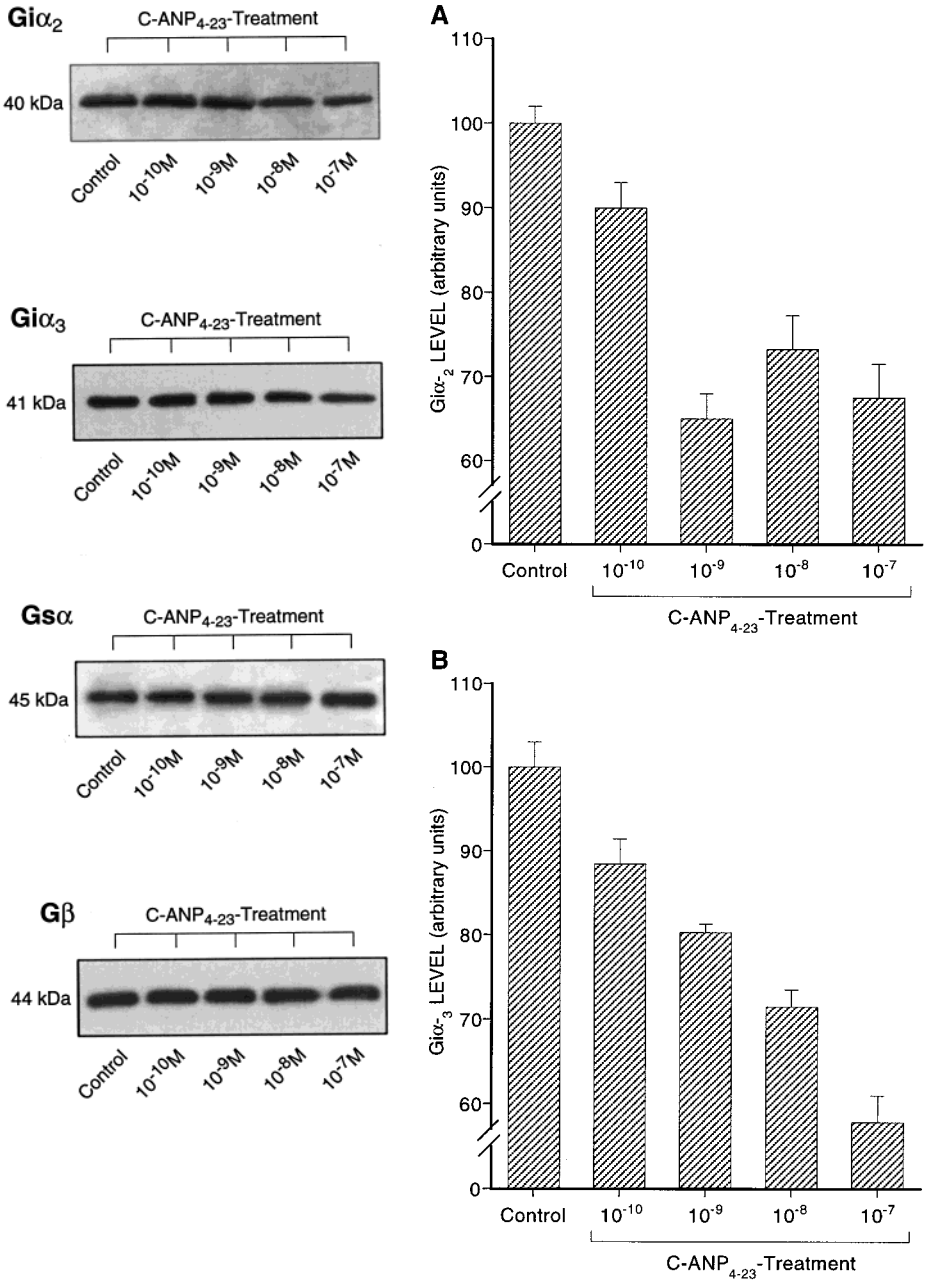


FIGURE 6: Left: Determination of G-protein levels (Giα-2, Giα-3, Gsα, Gβ) in membranes from control and C-ANP$_{4-23}$-treated A10 smooth muscle cells by immunoblotting. A10 cells were incubated in the absence (control) or presence of various concentrations (10^{-10}, 10^{-9}, 10^{-8}, and 10^{-7} M) of C-ANP$_{4-23}$ for 24 h as described in Experimental Procedures. Membranes were prepared as described in Experimental Procedures and used for immunoblotting. The membrane proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose which was then immunoblotted using AS/7 antibody for Giα-1 and Giα-2, EC/1 antibody for Giα-3, or RM/1 antibody for Gsα or SW/1 antibody for Gβ and were detected by using the ECL Western blotting technique as described in Experimental Procedures. The immunoblots are representatives of three separate experiments. Right: Densitometric scanning of Giα-2 (A) and Giα-3 (B) proteins from control and C-ANP$_{4-23}$-treated A10 smooth muscle cells. The results are expressed as arbitrary units, and control values were taken as 100. Values are means ± SEM of 3 separate experiments.

concentration-dependent manner; however, the inhibition was significantly attenuated in C-ANP$_{4-23}$-treated cells.

Effect of C-ANP$_{4-23}$ Treatment on Gs Functions. The effect of cholera toxin (CT) treatment on GTP-sensitive adenylyl cyclase activity was also examined to investigate if the increased expression of Gsα by C-ANP$_{4-23}$ treatment was reflected in Gs functions. Results shown in Table 1 indicate that CT stimulated GTPγS-sensitive adenylyl cyclase activity in both control and C-ANP$_{4-23}$-treated cells; however, the fold stimulation was significantly increased in C-ANP$_{4-23}$-treated cells as compared to control cells. These results indicate that Gs function was also augmented by C-ANP$_{4-23}$ treatment.

Effect of C-ANP$_{4-23}$ Treatment on Agonist-Stimulated Adenylyl Cyclase Activity. Since C-ANP$_{4-23}$ treatment increased the levels and functions of Gsα, it was of interest to also examine if the altered levels/functions of Gsα could affect the responsiveness of adenylyl cyclase to guanine nucleotides, stimulatory hormones, and other agonists which activate adenylyl cyclase by receptor-independent mechanisms. Figure 11 shows the effect of C-ANP$_{4-23}$ treatment on GTPγS-stimulated adenylyl cyclase activity in control and

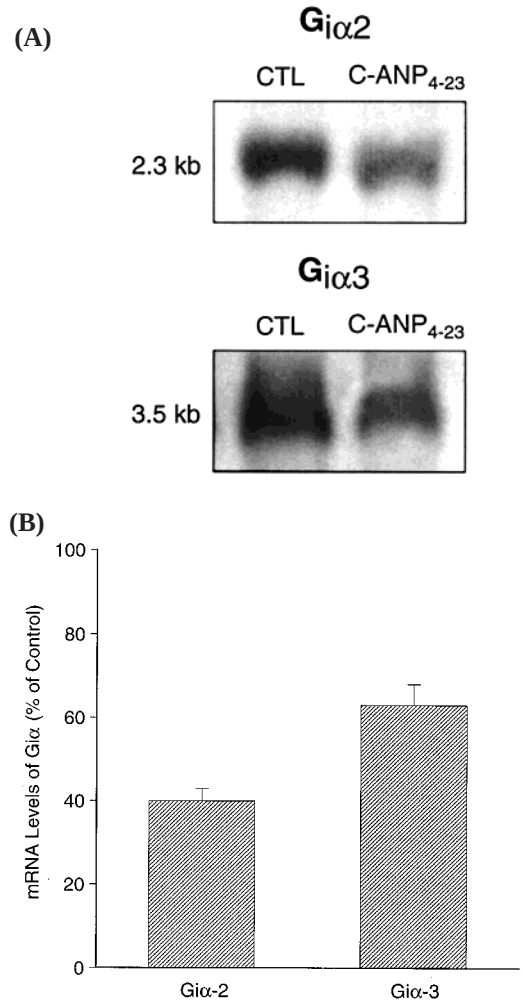


FIGURE 7: (A) Expression of Giα-2 and Giα-3 mRNA in control and C-ANP$_{4-23}$-treated A10 smooth muscle cells. Total RNAs (10 μg) isolated from control and C-ANP$_{4-23}$-treated A10 cells were separated on 1% agarose and transferred to nylon membranes, which was then hybridized with a full length cDNA probe encoding for Giα-2 and Giα-3 as described in Experimental Procedures. The autoradiogram is representative of 3 separate experiments. (B) Densitometric scanning of Giα and Giα-3 mRNA from control and C-ANP$_{4-23}$-treated A10 smooth muscle cells. The results are expressed as percent of control, which has been taken as 100%. Values are means ± SEM of 3 separate experiments.

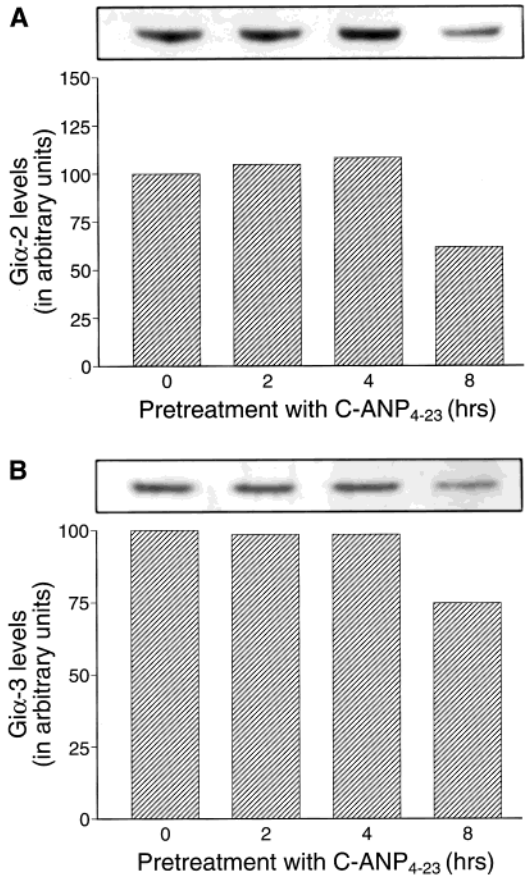


FIGURE 8: Time course of C-ANP$_{4-23}$ pretreatment and Gi protein levels in A10 smooth muscle cells. A10 cells were incubated in the absence (control) or presence of 10^{-7} M C-ANP$_{4-23}$ for 2, 4, and 8 h as described in Experimental Procedures. Membranes were prepared as described in Experimental Procedures and used for immunoblotting. The membrane proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose which was then immunoblotted using AS/7 antibody for Giα-1 and Giα-2 (A, upper panel) and EC/1 antibody for Giα-3 (B, upper panel) and were detected by using the ECL Western blotting technique as described in Experimental Procedures. The immunoblots are representative of three separate experiments. Lower panel: Densitometric scanning of the autoradiograms shown in upper panels. The results are expressed as percent Giα-2 or Giα-3 protein levels over control which has been taken as 100%.

C-ANP$_{4-23}$-treated cells. GTPγS stimulated adenylyl cyclase activity in a concentration-dependent manner in control and C-ANP$_{4-23}$-treated cells, but the stimulation was significantly enhanced in C-ANP$_{4-23}$-treated cells. For example, at 10 μM, GTPγS stimulated adenylyl cyclase activity by 100% in control and by 700% in C-ANP$_{4-23}$-treated cells. In addition, forskolin (FSK) (A), isoproterenol (ISO), and sodium fluoride (NaF) (B) stimulated adenylyl cyclase activity to various degrees in both control and C-ANP$_{4-23}$-treated cells; however, the extent of stimulation by these agonists was significantly augmented by about 2.5-, 2.3-, and 2.8-fold, respectively, in C-ANP$_{4-23}$-treated cells as compared to control cells (Figure 12).

DISCUSSION

The present studies demonstrate that pretreatment of A-10 smooth muscle cells by C-ANP$_{4-23}$ or ANP$_{99-126}$ for 24 h results in a downregulation of ANP-C receptors which is reflected in an attenuation of ANP-C receptor-mediated inhibition of adenylyl cyclase. Our results are in agreement with the studies of other investigators (*31*, *32*, *37*) who have reported a significant decrease in ANP sites in vascular smooth muscle cells (VSMC) and A10 cells after 18 h exposure with ANP. In addition, a decreased responsiveness of guanylyl cyclase/cGMP system to ANP stimulation in VSMC exposed to ANP has also been demonstrated suggesting a downregulation of GC-coupled ANP receptor subtype by ANP treatment (*31*, *32*). However, these investigators have not performed any studies to examine the relationship between the changes in ANP sites and ANP receptor−adenylyl cyclase signal transduction but provided evidence suggesting that G-cyclase uncoupled sites are largely susceptible to a downregulation mechanism (*31*). On the other hand, our results are inconsistent with the previous studies (*38*), where 18 h exposure of VSMC to ANP did not result in the downregulation of GC-coupled and uncoupled (ANP-C) ANP receptors. These apparent discrepancies may be due to the difference in the cell type (A10 cells versus VSMC) or to the method of treatment. We also demonstrate

Table 1: Effect of Cholera Toxin (CT) Treatment on Adenylyl Cyclase Activity in Control and C-ANP$_{4-23}$-Treated A10 Smooth Muscle Cells[a]

	adenylyl cyclase activity, pmol of cAMP (mg of protein·10 min)$^{-1}$					
	control cells			C-ANP$_{4-23}$-treated cells		
additions	−CT	+CT	% stimulation	−CT	+CT	% stimulation
GTPγS (10 μM)	174.4 ± 4.6	290.4 ±26	66.6	145.4 ± 15.3	412.4 ± 25.8	183.6

[a] A10 smooth muscle cells were treated in the absence (control cells) or presence of 10^{-7} M ANP$_{4-23}$ (C-ANP$_{4-23}$-treated cells) for 24 h as described in Experimental Procedures. The membranes were prepared and were incubated for 30 min in the absence or presence of cholera toxin (CT) as described in Experimental Procedures. Adenylyl cyclase activity was determined in the membranes in the presence of 10 μM GTP as described in Experimental Procedures. Values are means ± SEM of four separate experiments.

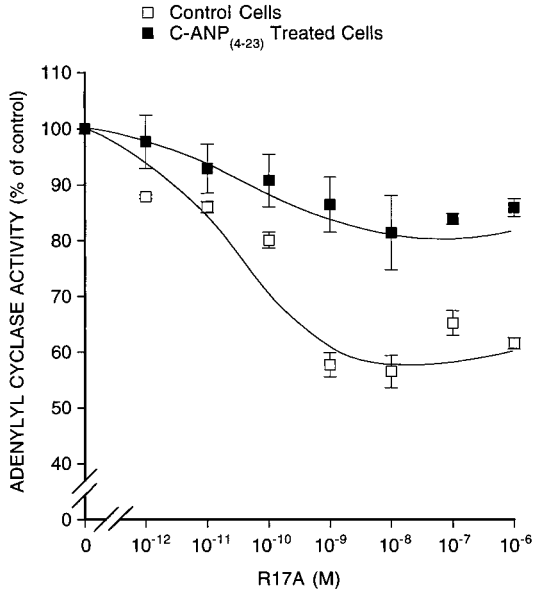


FIGURE 9: Effect of C-ANP$_{4-23}$ pretreatment on R17A-mediated inhibition of adenylyl cyclase activity in A10 smooth muscle cells. A10 cells were incubated in the absence (control) or presence of 10^{-7} M C-ANP$_{4-23}$ (treated) for 24 h as described in Experimental Procedures. Membranes were prepared as described in Experimental Procedures. Adenylyl cyclase activity was determined in the membranes in the absence or presence of various concentrations of R17A as described in Experimental Procedures. Values are mean ± SEM of three separate experiments performed in triplicates. Basal enzyme activities in the presence of 10 μM GTPγS in control and C-ANP$_{4-23}$-treated membranes were 197.0 ± 10.5 and 175.7 ± 5.5 pmol (mg of protein·10 min)$^{-1}$, respectively.

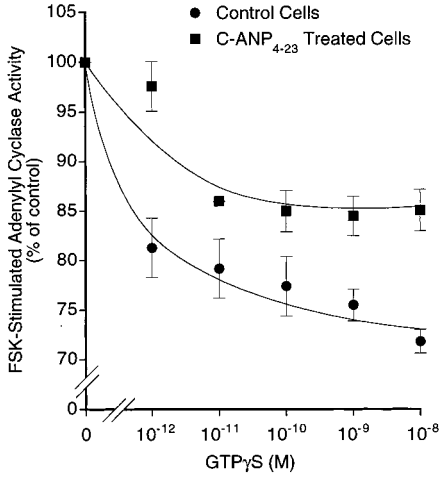


FIGURE 10: Inhibition of Gi functions by C-ANP$_{4-23}$ treatment in A10 smooth muscle cells. A10 cells were incubated in the absence (control) or presence (treated) of 10^{-7} M C-ANP$_{4-23}$ for 24 h as described in Experimental Procedures. A10 cell membranes were prepared as described in Experimental Procedures. Adenylyl cyclase activity was determined in the membranes in the presence of 100 μM forskolin (FSK) alone or in combination with various concentrations of GTPγS as described in Experimental Procedures. Values are means ± SEM of three separate experiments performed in triplicates. Enzyme activities in the absence or presence of FSK were 67.2 ± 4.5 and 1190 ± 8.9 pmol of cAMP (mg of protein·10 min)$^{-1}$ in control cells, respectively, and 37.3 ± 2.0 and 1398.4 ± 59.4 pmol cAMP (mg of protein·10 min)$^{-1}$, respectively in C-ANP$_{4-23}$-treated cells. V_{FSK}/V_{basal} was taken as 100%.

that C-ANP$_{4-23}$ treatment resulted in an attenuation of adenylyl cyclase inhibition in response to Ang II, which acts via a separate membrane-bound receptor, suggesting a heterologous desensitization. Our results are in agreement with previous studies showing a heterologous desensitization of inhibitory adenylyl cyclase-coupled receptors by adenosine and Ang II treatments (*25*, *39*).

A complete attenuation of ANP-C receptor-mediated inhibition of adenylyl cyclase associated with partial down-regulation (~35%) of ANP-C receptors after 24 h of C-ANP$_{4-23}$ treatment is in agreement with our recent studies showing that the treatment of VSMC with ANP-C receptor antisense that inhibited the expression of ANP-C receptor protein by about 50% attenuated completely the ANP-C receptor-induced inhibition of adenylyl cyclase (*40*) and suggests that postreceptor modification such as an alteration in the levels of Giα proteins that couple ANP-C receptor to adenylyl cyclase system may also be responsible for the observed desensitization of adenylyl cyclase inhibition. In this regard, a relationship between the levels of Gi proteins and functions has been reported by several investigators (*28*, *41*−*43*). In the present studies, we have shown that the treatment of A-10 cells with C-ANP$_{4-23}$ for 24 h decreased the levels of Giα proteins by about 40%, which may partially contribute to the attenuation of C-ANP$_{4-23}$-mediated inhibition of adenylyl cyclase. This is further supported by the observation that the reduction in the levels of Gi proteins by about 40% by C-ANP$_{4-23}$ treatment inhibited partially (~50%) but not completely the R17A-mediated inhibition of adenylyl cyclase. On the other hand, treatment of the cells for shorter period of time (2−4 h) or with lower concentrations of C-ANP$_{4-23}$ (10^{-10} M) that attenuated adenylyl cyclase inhibition by about 50% without altering the levels of Gi proteins suggests that Gi proteins may not be implicated in the desensitization of ANP-C receptor-mediated inhibition of adenylyl cyclase observed after 2−4 h of C-ANP$_{4-23}$ treatment. However, when the levels of Gi proteins were also inhibited upon longer exposure (8 or 24 h) or with higher concentration of C-ANP$_{4-23}$, a complete attenuation of C-ANP$_{4-23}$-mediated inhibition of adenylyl cyclase was observed. These data strongly suggest that a complete attenuation of C-ANP$_{4-23}$-mediated inhibition of adenylyl cyclase after 24 h of C-ANP$_{4-23}$ treatment may not solely be attributed to the decreased levels of Giα proteins that

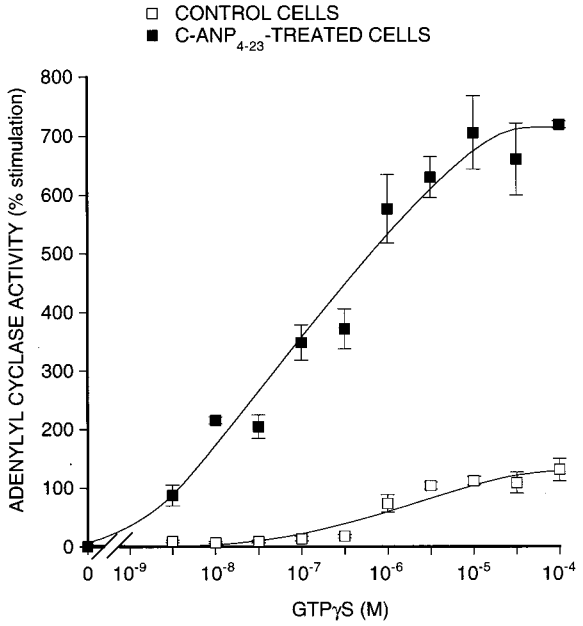


FIGURE 11: Effect of C-ANP$_{4-23}$ treatment on GTPγS-stimulated adenylyl cyclase activity in A10 smooth muscle cells. A10 cells were incubated in the absence (control) or presence (treated) of 10^{-7}M C-ANP$_{4-23}$ for 24 h as described in Experimental Procedures. A10 cell membranes were prepared as described in Experimental Procedures. Adenylyl cyclase activity was determined on the membranes in the absence or presence of various concentration of GTPγS as described in Experimental Procedures. Values are means $\pm$ SEM of three separate experiments performed in triplicate. Basal enzyme activities in control and C-ANP$_{4-23}$-treated cells in the absence of GTPγS were 62.3 $\pm$ 2.7 and 47.1 $\pm$ 4.5 pmol of cAMP (mg of protein·10 min)$^{-1}$, respectively.

couple ANP-C receptor to adenylyl cyclase or to the decreased receptor density but most likely reflects a cumulative effect of both the components.

We have also shown that the levels of Giα-2 and Giα-3 mRNA were also decreased by C-ANP$_{4-23}$ treatment suggesting that decreased transcription of the genes for Giα-2 and Giα-3 may be responsible for the observed decrease in protein levels. The mechanism by which C-ANP$_{4-23}$ decreased the expression of Giα proteins is not known; however, it may be possible that ANP-C receptor-mediated inhibition of adenylyl cyclase and cAMP levels may be responsible for the observed decreases in Giα protein expression in A10 smooth muscle cells. In this regard, a chronic exposure of adipocytes with adenosine agonist N^6-phenylisopropyladenosine that interacts with inhibitory adenosine receptor (A1) and inhibits adenylyl cyclase activity and cAMP levels has also been reported to decrease the levels of Giα protein (*39*). In addition, isoprenaline that stimulates adenylyl cyclase activity and cAMP levels has been shown to augment the levels of Giα (*44*). However, in contrast, Ang II decreases cAMP levels and increases the levels of Gi proteins (*25*), suggesting that other mechanisms or factors may also be responsible for the regulation of Giα protein expression.

We have also shown for the first time that C-ANP$_{4-23}$ treatment of A10 cells augmented the levels of Gsα proteins which was also reflected in increased functions of Gsα as was demonstrated by increased stimulation of GTPγS-sensitive adenylyl cyclase activity by cholera toxin. In addition, an enhanced stimulation of adenylyl cyclase by

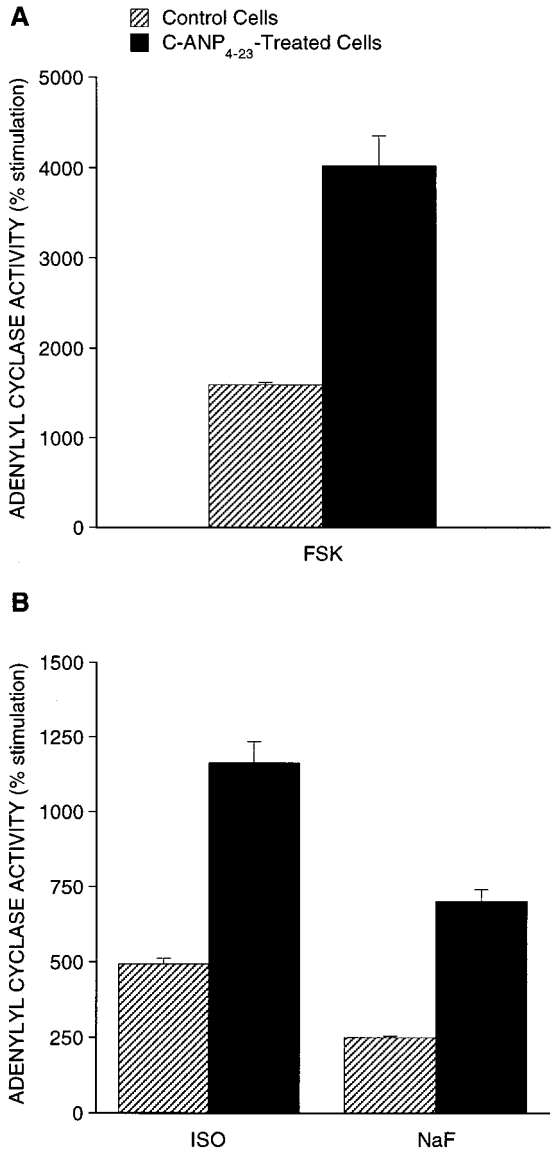


FIGURE 12: Effect of C-ANP$_{4-23}$ treatment on agonist-stimulated adenylyl cyclase activity in A10 smooth muscle cells. A10 cells were incubated in the absence (control) or presence (treated) of 10^{-7} M C-ANP$_{4-23}$ for 24 h as described in Experimental Procedures. Membranes were prepared as described in Experimental Procedures. Adenylyl cyclase activity in the membranes was determined in the absence (basal) or presence of 50 μM forskolin (FSK), 50 μM isoproterenol (ISO) + 10 μM GTP, or 10 mM sodium fluoride (NaF) as described in Experimental Procedures. Values are mean $\pm$ SEM of three separate experiments performed in triplicate. Basal enzyme activities in the absence or presence of 10 μM GTP were 63.6 $\pm$ 2.9 and 93.9 $\pm$ 3.3 pmol of cAMP (mg of protein·10 min)$^{-1}$, respectively, in control and 33.9 $\pm$ 1.6 and 37.2 $\pm$ 1.6 pM cAMP (mg of protein·10 min)$^{-1}$, respectively, in C-ANP$_{4-23}$-treated cells.

GTPγS in C-ANP$_{4-23}$-treated cells as compared to control cells may be attributed to the increased levels and functions of Gsα, whereas decreased levels and functions of Giα, upregulation of β-adrenergic receptors, and increased levels and functions of Gsα may contribute to an increased responsiveness of adenylyl cyclase to isoproterenol stimulation. In this regard, a relationship between decreased levels of Gi and augmented responsiveness of adenylyl cyclase to stimulatory hormones has been shown by previous studies, where pertussis toxin (PT) and amiloride treatments which inactivate Giα proteins resulted in an augmentation of

stimulatory responses of hormones on adenylyl cyclase (*17*, *45*). Furthermore, platelets from spontaneously hypertensive rats (*43*) and hypertensive patients (*44*) that exhibit decreased levels of Giα proteins elicited enhanced stimulation of adenylyl cyclase by *N*-ethylcarboxamide adenosine (NECA) and prostaglandins (PGE1). An augmented stimulation of adenylyl cyclase by FSK in C-ANP$_{4-23}$-treated cells may be due to hypersensitivity of the catalytic subunit of adenylyl cyclase system *per se* or to the decreased expression of Giα or to the enhanced expression of Gsα or to the alterations in all the components of adenylyl cyclase system. Our results are in agreement with the previous studies showing an increased stimulation of adenylyl cyclase by FSK in adipocytes exposed to adenosine inhibitory receptor agonist (*39*). The Gi-mediated regulation of FSK-stimulated enzyme activity can be further supported by the results of various studies showing an augmentation of FSK-stimulated adenylyl cyclase activity by PT treatment. In addition, the platelets from SHR (*43*) and hypertensive patients (*44*) that exhibited decreased levels of Giα showed an increased stimulation of adenylyl cyclase by FSK. Similarly, the overexpression of Giα has been shown to result in an attenuation of FSK-stimulated adenylyl cyclase activity (*26*). On the other hand, the requirement of Gsα and guanine nucleotides for the FSK activation of adenylyl cyclase has also been shown (*46*), which may suggest that C-ANP$_{4-23}$-induced enhanced levels of Gsα in A10 cells contribute to the enhanced sensitivity of adenylyl cyclase to FSK stimulation.

In conclusion, we have shown that exposure of A10 smooth muscle cells to C-ANP$_{4-23}$ resulted in the down-regulation of ANP-C receptor−adenylyl cyclase signal transduction system involving Giα proteins to which these receptors are coupled. In addition, Gsα protein and Gsα-mediated functions were also augmented by such treatment. From these results, it may be suggested that ANP, by regulating the levels of G-proteins and thereby cAMP, may play an important role in the regulation of various physiological responses mediated by ANP-C receptor. The desensitization of ANP-C receptors in vivo may be one of the possible mechanisms responsible for the pathophysiology of hypertension.

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