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Atrial Natriuretic Peptide-C Receptor-Induced Attenuation of Adenylyl Cyclase Signaling Activates Phosphatidylinositol Turnover in A10 Vascular Smooth Muscle Cells

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ABSTRACT

Atrial natriuretic peptide (ANP)-C receptor activation has been shown to inhibit adenylyl cyclase (AC) activity as well as to stimulate phospholipase C (PLC) signaling pathways. The present studies were undertaken to investigate whether ANP-C receptor-mediated decreased cAMP levels contribute to the activation of PLC signaling. C-ANP$_{4-23}$ [des(Gln^{18},Ser^{19},Glu^{20},Leu^{21},Gly^{22})ANP$_{4-23}$-NH$_2$], a ring-deleted peptide of ANP that interacts specifically with ANP-C receptor, stimulated inositol 1,4,5-tris-phosphate (IP$_3$) production (PLC activity) in A10 vascular smooth muscle cells in a concentration- and time-dependent manner. The maximal stimulation observed was about 75% at 2 h of treatment, with an apparent EC$_{50}$ of about 20 to 30 nM. Pertussis toxin treatment of the cells completely abolished the C-ANP$_{4-23}$–mediated stimulation of IP$_3$ production. Forskolin (FSK), a stimulator of adenylyl cyclase, dibutyryl cAMP (db cAMP), and isoproterenol (ISO), a β-adrenergic agonist that stimulates adenylyl cyclase activity and cAMP levels, inhibited IP$_3$ production by about 35, 30, and 50%, respectively, whereas dideoxyadenosine (DDA), an inhibitor of adenylyl cyclase activity, and oxotremorine stimulated IP$_3$ production by about 90 and 80%, respectively, in these cells, suggesting a functional interaction between these two signaling pathways. Treatment of the cells with antisense oligonucleotide of ANP-C receptor that attenuated ANP-C receptor-mediated inhibition of adenylyl cyclase resulted in a complete attenuation of C-ANP$_{4-23}$–induced stimulation of IP$_3$ formation, whereas FSK, db cAMP, and ISO-mediated decrease and oxotremorine and endothelin-1 (ET-1)-induced increase in IP$_3$ production was not affected by this treatment. Furthermore, C-ANP$_{4-23}$–induced increase in IP$_3$ formation was significantly potentiated by DDA and inhibited by FSK and db cAMP, whereas ET-1–induced increase in IP$_3$ production was not affected by FSK. In addition, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline (H-89), an inhibitor of protein kinase A, completely abolished C-ANP$_{4-23}$ and not ET-1–induced stimulation of IP$_3$ production. These results indicate that ANP-C receptor activation by C-ANP$_{4-23}$ and resulting decrease in cAMP levels may be responsible for the activation of phosphatidylinositol (PI) turnover signaling, suggesting a cross-talk between ANP-C receptor-mediated adenylyl cyclase and PLC signaling pathways.

Atrial natriuretic peptide (ANP), a member of the family of natriuretic peptides, was discovered by de Bold et al. (de Bold et al., 1981; de Bold, 1982). ANP regulates a variety of physiological parameters, including blood pressure, progesterone secretion, renin release, and vasopressin release, by interacting with receptors on the plasma membrane, either to generate second messengers such as cAMP (Anand-Srivastava et al., 1985a,b, 1986; Anand-Srivastava and Cantin, 1986; Bianchi et al., 1986) and cGMP (Hamet et al., 1984; Waldman et al., 1984) or to affect ion channels (Anand-Srivastava and Trachte, 1993). The other members of the natriuretic peptide family are brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) (Brenner et al., 1990; Sudoh et al., 1990). ANP and BNP, endocrine hormones, are apparently antagonists to vasopressin, endothelins, and the renin-angiotensin-aldosterone system (Brenner et al., 1990; Ruskoaho, 1992). The role of CNP in vivo is less well defined. Although CNP might not be a significant modulator of diuresis and natriuresis (Stingo et al., 1992; Clavell et al., 1993), it is a vasodilator expressed by endothelial cells (Sudoh et al., 1990; Suga et al., 1992).

Molecular cloning techniques revealed three subtypes of natriuretic peptide receptor (NPR): NPR-A (Chinkers et al., 1989; Lowe et al., 1989), NPR-B (Chang et al., 1989; Schulz et al., 1989) and NPR-C (clearance receptor) (Anand-Srivastava

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ABBREVIATIONS: ANP, rat natriuretic peptide (28 amino acids); BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; NPR, natriuretic peptide receptor; C-ANP$_{4-23}$ [des(Gln^{18},Ser^{19},Glu^{20},Leu^{21},Gly^{22})ANP$_{4-23}$-NH$_2$]; PLC, phospholipase C; VSMC, vascular smooth muscle cell; PI, phosphatidylinositol; FSK, forskolin; DDA, dideoxyadenosine; db, dibutyryl; ET-1, endothelin-1; ISO, isoproterenol; PT, pertussis toxin; H-89, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; PKA, protein kinase A; IP$_3$, inositol 1,4,5-trisphosphate; SMC, smooth muscle cells.

et al., 1987; Fuller et al., 1988). NPR-A and NPR-B are membrane guanylyl cyclases, whereas NPR-C (or ANP-C receptor) lacks guanylyl cyclase activity. NPR-A catalyzes the production of cGMP in response to ANP and BNP, whereas NPR-B is the target for CNP. The stimulation of cGMP by NPR-A or NPR-B receptor activation has also been reported to inhibit phospholipase C (PLC) signaling (Abdel-Latif, 2001). On the other hand, ANP-C receptors are coupled to adenylyl cyclase inhibition through inhibitory guanine nucleotide-regulatory protein (Anand-Srivastava et al., 1987, 1990).

ANP-C receptors are disulfide-linked homodimers of 64 to 66 kDa that have a single transmembrane domain (Schenk et al., 1985; Fuller et al., 1988; Leitman et al., 1988), an extracellular domain of ~440 amino acids, and a short 37-amino acid cytoplasmic domain or tail. We have recently demonstrated that small fragment peptides of the cytoplasmic domain of the ANP-C receptor with complete G_i activator sequence were sufficient to inhibit adenylyl cyclase activity through a pertussis toxin-sensitive G_i protein with the same potency as that of the entire cytoplasmic domain peptide (Anand-Srivastava et al., 1996; Pagano and Anand-Srivastava, 2001). In addition, we have also shown that the elimination of the ANP-C receptor by antisense oligonucleotide treatment resulted in the attenuation of C-ANP_{4-23}–induced inhibition of adenylyl cyclase in vascular smooth muscle cells (VSMCs), Leydig tumor cells, and pheochromocytoma cells.

On the other hand, Hirata et al. (1989) have reported that ANP_{99-126} and $ANP_{103-123}$ (atriopeptin) stimulate phosphatidylinositol (PI) turnover in the presence of guanine nucleotides in cultured bovine aortic smooth muscle cell homogenates or particulate fractions. $ANP_{103-123}$, an atrial peptide analog truncated at the carboxyl terminus that binds selectively to the ANP-C receptor (Leitman and Murad, 1987), was about 10-fold more potent than ANP_{99-126} with regard to the formation of inositol phosphates. These results suggested that ANP-C receptors may be coupled to PI turnover signaling through guanine nucleotide-regulatory proteins. Recently, Murthy and Makhlouf (1999) reported the stimulation of PLC-β activity by C-ANP_{4-23} and small peptide fragments of cytoplasmic domain of ANP-C receptor in guinea pig tenia coli smooth muscle cells.

Because cAMP and cGMP have been reported to regulate PI turnover (Abdel-Latif, 2001), it may be possible that the stimulation of PI turnover by atrial natriuretic peptides may be mediated through the inhibition of adenylyl cyclase/cAMP system to which ANP-C receptors are coupled. The present studies were undertaken to investigate this possibility. We have used VSMCs (A10), which have been reported to have both NPR-A/B and ANP-C receptors (Palaparti et al., 2000). The majority of the receptors in VSMCs are of the ANP-C subtype (Anand-Srivastava and Trachte, 1993). We have provided the first evidence that the ANP-C receptor-mediated decrease in cAMP levels contributes to the activation of PLC signaling and suggests a cross-talk between ANP-C receptor-mediated adenylyl cyclase and PLC signaling pathways.

Materials and Methods

Materials. Forskolin (FSK), dideoxyadenosine (DDA), db cAMP, endothelin-1 (ET-1), isoproterenol (ISO), and pertussis toxin (PT) were purchased from Sigma. H-89, a protein kinase A (PKA) inhibitor, was purchased from Calbiochem (San Diego, CA). [*myo*-2-^{3}H]Inositol was purchased from Amersham Biosciences (Piscataway, NJ). A ring-deleted analog of ANP, C-ANP_{4-23}, was purchased from Peninsula Laboratories (Belmont, CA). Antisense (5′-CAG CAG CAG CGA CCG CAT-3′) and sense (5′-ATG CGG TCC CTG CTG CTG-3′) oligodeoxynucleotides were purchased from Institut Armand Frappier (Laval, QC, Canada). The sequence of the phosphorothioate oligodeoxynucleotide (antisense) is complementary to positions 17 to 34 on ANP-C mRNA (sense). This sequence is not related to NPR-A or NPR-B mRNA.

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

Antisense and Sense Oligonucleotide Treatment. Antisense treatment of the cells was performed as described previously (Palaparti et al., 2000). Confluent cell cultures were washed with 5 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum and incubated in 2 ml of medium in the absence or presence of 2.5 μM (or as otherwise indicated) antisense or sense oligonucleotide for 48 h at 37°C. After 24-h incubation with antisense or sense oligonucleotide, 5 μCi/ml [*myo*-2-^{3}H]inositol was added for 24 h. The cells were further treated with C-ANP_{4-23}, FSK, db cAMP, or ISO for 2 h, and the reaction was terminated by adding 0.9 ml of methanol/chloroform/HCl (40:20:1). Cells were then homogenized and the homogenates were used for determination of IP_3 levels.

PT Treatment. The confluent VSMCs were incubated for 24 h in a serum-free Dulbecco's modified Eagle's medium with 1 μg/ml PT and 5 μCi/ml [*myo*-2-^{3}H]inositol. The cells were further treated with ET-1 or C-ANP_{4-23} for 2 h, and the reaction was terminated by adding 0.9 ml of methanol/chloroform/HCl (40:20:1). Cells were then homogenized and the homogenates were used for determination of IP_3 levels.

H-89 (Protein Kinase A Inhibitor) Treatment. The confluent VSMCs were incubated for 24 h in a serum-free Dulbecco's modified Eagle's medium with 5 μCi/ml [*myo*-2-^{3}H]inositol. The cells were further treated with H-89 (10^{-5} M) for 30 min and then with C-ANP_{4-23} for 2 h, and the reaction was terminated by adding 0.9 ml of methanol/chloroform/HCl (40:20:1). Cells were then homogenized and the homogenates were used for determination of IP_3 levels.

Determination of IP_3 Levels. The confluent VSMCs were incubated for 24 h in a serum-free Dulbecco's modified Eagle's medium with 5 μCi/ml [*myo*-2-^{3}H]inositol.Cells were washed three times with warm (37°C) Earle's balanced salt solution obtained from Invitrogen (Carlsbad, CA) after removing the cultured medium containing unincorporated isotope. The cells were further incubated for 30 min in the same buffer containing 20 mM lithium chloride to inhibit the conversion of the inositol phosphates to inositol so that the radiolabeled inositol phosphates could accumulate within the cell (Eid and de Champlain, 1988; Dean and Beaven, 1989). Agonists were applied for various periods and the reaction was terminated by adding 0.9 ml of methanol/chloroform/HCl (40:20:1) (Gunther et al., 1982). The cells were scraped using a rubber policeman and were then homogenized. Chloroform (0.5 ml) and 0.5 ml distilled water were added to the homogenates, and the samples were then centrifuged to separate lipid and aqueous phases. The aqueous phase was transferred to a column containing 0.8 ml of AG1-X8 resin (200–400 mesh, formate form; obtained from Bio-Rad, Hercules, CA) from which inositol phosphates were eluted sequentially with ammonium formate buffers of increasing molarity (Dean and Beaven, 1989). The radioactivity was measured in a liquid scintillation counter. The lipid phase was counted to measure the phosphatidylinositol pool. The accumulation of inositol phosphate was expressed as the ratio of inositol triphosphate to phosphatidylinositol pool $\times 10^3$ to correct for the variation in the labeling of the lipid pool.

Statistical Analysis. Data are presented as mean ± S.E.M. Comparison between groups were made using student's *t* test or analysis of variance where appropriate. The results were considered significantly different at $P < 0.05$.

Results

Effect of C-ANP$_{4-23}$ on PLC Activity. Atriopeptin I and ANP, peptides that interact with both NPR-A/B and ANP-C receptor, have been reported to stimulate PI turnover in bovine aortic SMC (Hirata et al., 1989) and RIMCT cells (Berl et al., 1991) through Gi regulatory protein. However, the effect of C-ANP$_{4-23}$, a peptide that interacts specifically with ANP-C receptor and inhibits adenylyl cyclase activity, has not been examined on PI turnover in VSMCs. Figure 1 shows the effect of C-ANP$_{4-23}$, ET-1 (used as a positive control), and oxotremorine, another G-protein-coupled receptor agonist linked to Gi on PI turnover in A10 SMCs. C-ANP$_{4-23}$ (10^{-7} M), ET-1 (10^{-7} M), and oxotremorine (5×10^{-5}M) stimulated IP$_3$ production by about 75, 690, and 80% respectively, in these cells, suggesting a coupling of ANP-C receptor to PLC signaling pathway. However, when the effect of ET-1 and C-ANP$_{4-23}$ or ET-1 and oxotremorine on PI turnover was examined together, an additive effect was observed.

Figure 2A shows the effect of various concentrations of C-ANP$_{4-23}$ on IP$_3$ production. C-ANP$_{4-23}$ stimulated IP$_3$ production in a concentration-dependent manner, with an apparent EC$_{50}$ of about 20 to 30 nM. The maximal stimulation observed was about 70%. The stimulation of IP$_3$ production by C-ANP$_{4-23}$ (10^{-7} M) was also time-dependent and peaked at 2 h (Fig. 2B).

Effect of db cAMP, FSK, and DDA on IP$_3$ Formation. To investigate the implication of cAMP in PI turnover signaling, we examined the effects of db cAMP and FSK, agents that increase cAMP and DDA, an adenosine analog that inhibits adenylyl cyclase, on IP$_3$ formation. Figure 3 shows that FSK and db cAMP inhibited IP$_3$ formation in A10 SMCs in a concentration-dependent manner. The maximal inhibition of about 35% was observed at 50 μM FSK (Fig. 3A) and 0.1 μM db cAMP (Fig. 3B). In addition, ISO, a β-adrenergic agonist, inhibited IP$_3$ production by about 50% in these cells

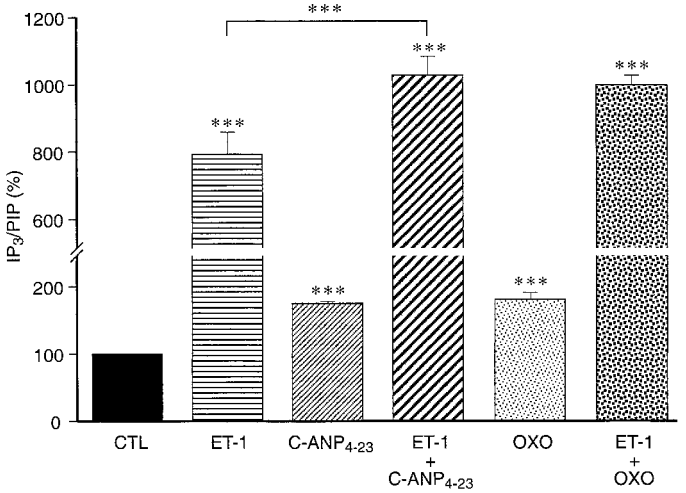

Fig. 1. Effect of ET-1 and C-ANP$_{4-23}$ on IP$_3$ formation in A10 VSMCs. A10 VSMCs were incubated in the presence of [*myo*-2-^{3}H]inositol for 24 h as described under *Materials and Methods*. The cells were further incubated in the absence (CTL) or presence of C-ANP$_{4-23}$ (10^{-7} M), oxotremorine (5×10^{-5} M) or ET-1 (10^{-7} M), alone or in combination with C-ANP$_{4-23}$ plus ET-1 or oxotremorine + ET-1 for 2 h at 37°C, and IP$_3$ formation was determined as described under *Materials and Methods*. Values are the means ± S.E.M. of three to four separate experiments performed in triplicate. Control is taken as 100%. ***, $P < 0.001$.

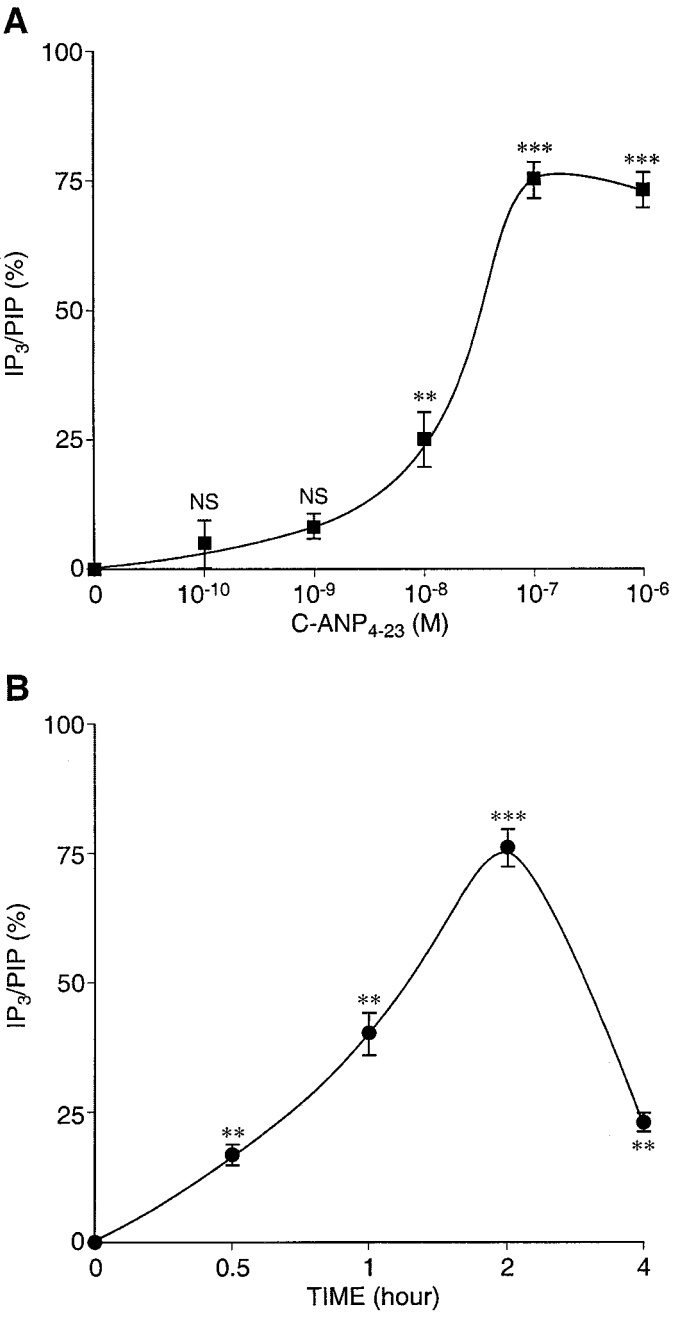

Fig. 2. A, effect of various concentrations of C-ANP$_{4-23}$ on IP$_3$ formation in VSMCs. A10 VSMCs were incubated in the presence of [*myo*-2-^{3}H]inositol for 24 h as described under *Materials and Methods*. The cells were further incubated in the presence of various concentrations of C-ANP$_{4-23}$ (10^{-10}-10^{-6}M) for 2 h. IP$_3$ formation was determined as described under *Materials and Methods*. Values are the means ± S.E.M. of three separate experiments performed in triplicate. **, $P < 0.01$; ***, $P < 0.001$; NS; not significant. B, time dependence of IP$_3$ formation in the presence of C-ANP$_{4-23}$. A10 VSMCs were incubated in the presence of [*myo*-2-^{3}H]inositol for 24 h as described under *Materials and Methods*. The cells were further incubated in the presence of C-ANP$_{4-23}$ (10^{-7} M) for different time periods at 37°C. IP$_3$ formation was determined as described under *Materials and Methods*. Values are means ± S.E.M. of three to four separate experiments performed in triplicate. **, $P < 0.01$; ***, $P < 0.001$; NS, not significant.

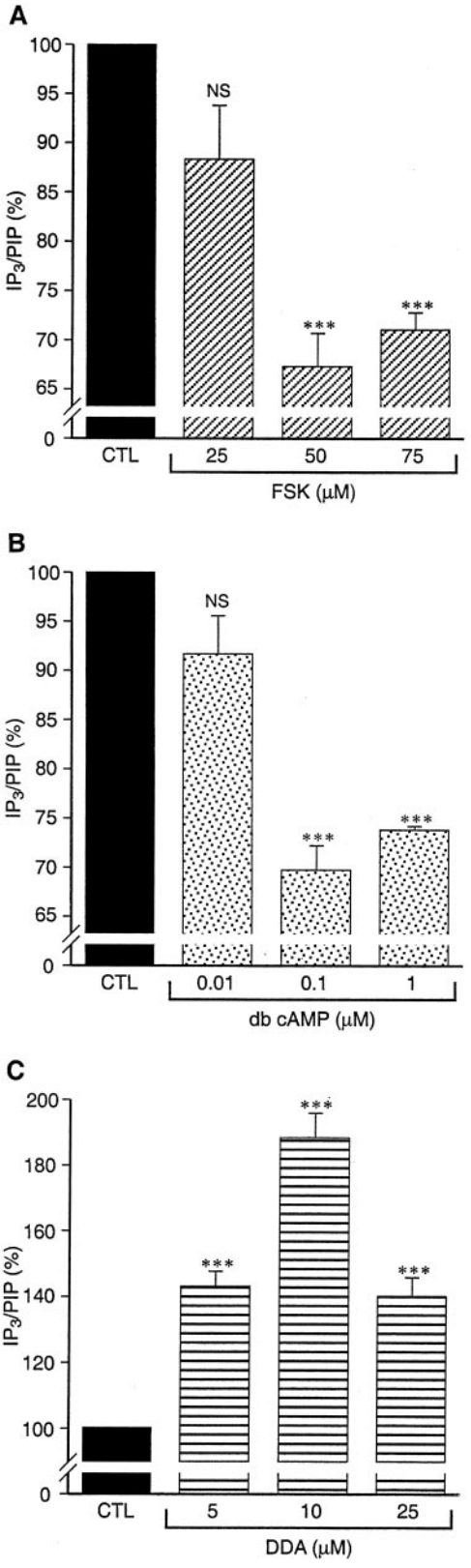

Fig. 3. Effect of FSK, db cAMP, and DDA on IP_3 formation in A10 VSMCs. A10 VSMCs were incubated in the presence of [*myo*-2-^{3}H]inositol for 24 h as described under *Materials and Methods*. The cells were further incubated in the presence of various concentrations of FSK (A), db cAMP (B), and DDA (C) for 2 h at 37°C. IP_3 formation was determined as described under *Materials and Methods*. Values are means ± S.E.M. of three separate experiments performed in triplicate. ***, $P < 0.001$; NS, not significant.

(Fig. 5). On the other hand, DDA stimulated IP_3 production, and maximal stimulation of about 90% was observed at 10 μM DDA (Fig. 3C). These results suggest that cAMP can modulate the production of IP_3 in A10 VSMCs; the elevated level of intracellular cAMP inhibits and the reduced level of intracellular cAMP stimulates IP_3 formation.

Effect of ANP-C Receptor Antisense on ANP-C Receptor-Mediated IP_3 Production in A10 VSMCs. We have recently shown that ablation of ANP-C receptor by antisense oligodeoxynucleotide treatment of A10 VSMCs resulted in a complete attenuation of ANP-C receptor-mediated inhibition of adenylyl cyclase (Palaparti et al., 2000). To investigate whether the ablation of ANP-C receptor by antisense treatment also resulted in the attenuation of ANP-C receptor-mediated PI turnover, the effect of sense and antisense treatment on C-ANP_{4-23}–mediated IP_3 production was examined in A10 VSMCs. Figure 4 shows that C-ANP-$_{4-23}$–induced IP_3 stimulation was inhibited by antisense treatment. About 65% inhibition was observed when the cells were treated with 1 μM antisense, whereas a complete abolition of C-ANP_{4-23}–stimulated IP_3 production was observed at 2.5 μM. However, sense oligomer that has been shown to be ineffective in attenuating ANP-C receptor-mediated inhibition of adenylyl cyclase (Palaparti et al., 2000) was also unable to attenuate C-ANP_{4-23}–induced stimulation of IP_3 production in A10 VSMCs. Furthermore, basal PLC activity

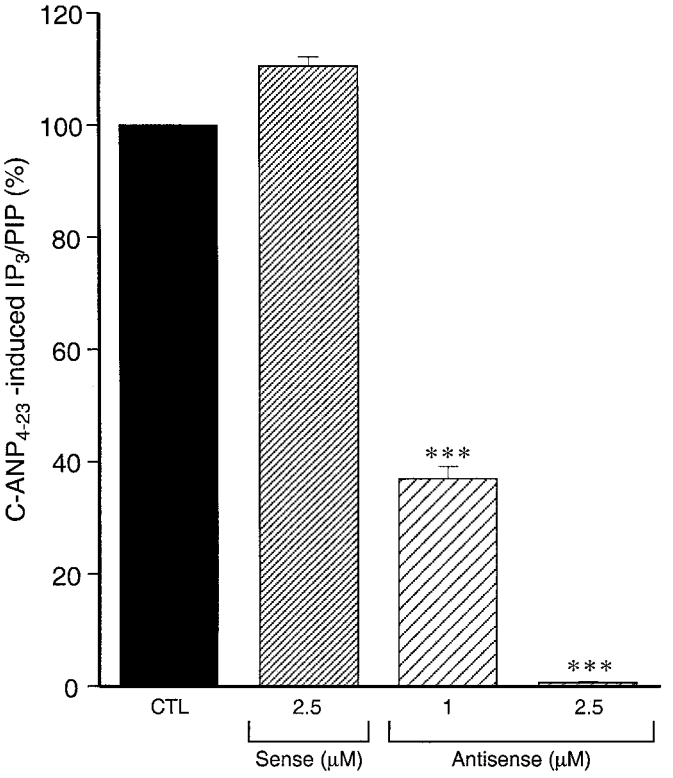

Fig. 4. Effect of ANP-C receptor sense and antisense oligodeoxynucleotide treatment on C-ANP_{4-23}–mediated stimulation of IP_3 formation in A10 VSMCs. A10 VSMCs were pretreated without (CTL) or with sense and various concentrations of antisense deoxynucleotide for 48 h as described under *Materials and Methods*. The cells were then incubated with [*myo*-2-^{3}H]inositol for 24 h as described under *Materials and Methods* and were further incubated in the absence or presence of C-ANP_{4-23} (0.1 μM) for 2 h at 37°C. IP_3 formation was determined as described under *Materials and Methods*. Values are means ± S.E.M. of three to four separate experiments performed in triplicate. ***, $P < 0.001$

was not affected by antisense-oligonucleotide treatment (data not shown).

To investigate whether ANP-C receptor antisense also results in the attenuation of other agonist-induced IP_3 production, the effect of ANP-C receptor antisense treatment on oxotremorine-, endothelin-, Iso-, FSK- and db cAMP-induced stimulation/inhibition of IP_3 formation was examined. The results shown in Fig. 5 indicate that FSK-, db cAMP- and Iso-mediated inhibition or oxotremorine- and ET-induced stimulation of IP_3 production was not different in control and antisense-treated cells, whereas C-$ANP_{4–23}$–mediated stimulation was abolished by antisense oligonucleotide treatment.

Cross-Talk between ANP-C Receptor-Mediated Adenylyl Cyclase and PI Turnover Signaling. To investigate whether ANP-C receptor-mediated decreased cAMP levels contribute to the activation of PI turnover signaling, the effect of cAMP stimulatory and inhibitory agonists on C-$ANP_{4–23}$–induced stimulation of IP_3 production was examined and the results are shown in Fig. 6. db cAMP and FSK inhibited IP_3 production significantly (Fig. 6), whereas C-$ANP_{4–23}$ stimulated IP_3 production (Fig. 6). However, when the C-$ANP_{4–23}$–mediated decrease in cAMP levels was elevated by the addition of db cAMP or FSK, the C-$ANP_{4–23}$–mediated stimulation of IP_3 formation was significantly decreased to control level. For example, C-$ANP_{4–23}$–mediated IP_3 formation that was stimulated by about 75% was almost completely abolished by FSK and db cAMP. However, ET-1–induced increased production of IP_3 was not affected by FSK [IP_3/phosphatidyl inositol phosphate (%), control, 100%; ET-1, 802.0 ± 15.6; FSK, 60.0 ± 3.0; ET-1 + FSK, 804.0 ± 20]. On the other hand, DDA, which decreased cAMP levels, potentiated the stimulatory effect of C-$ANP_{4–23}$ on IP_3 formation. These results suggest that the ANP-C receptor-mediated decrease in cAMP levels may be responsible for the ANP-C receptor-mediated activation of PI turnover.

Effect of Protein Kinase A Inhibitor (H-89) on ANP-C Receptor-Mediated IP_3 Production. To further investigate the implication of cAMP/protein kinase A in C-$ANP_{4–23}$–evoked stimulation of IP_3 production, the effect of H-89, a PKA inhibitor, was examined. Figure 7 shows that C-$ANP_{4–23}$–induced stimulation of IP_3 production was completely abolished by pretreatment of the cells with H-89; however, ET-1–induced stimulation of IP_3 production was not affected by H-89 treatment.

Effect of PT Treatment on ANP-C Receptor-Mediated IP_3 Production. ANP-C receptors are coupled to adenylyl cyclase through Gi regulatory protein (Anand-Srivastava et al., 1987, 1990). The inactivation of Gi proteins by PT has been reported to uncouple inhibitory hormone receptor from adenylyl cyclase and results in the attenuation of Gi-mediated adenylyl cyclase signaling (Anand-Srivastava et al., 1987, 1990). To investigate whether the ANP-C receptor-mediated reduction in cAMP levels is responsible for the C-$ANP_{4–23}$–induced stimulation of IP_3 production, the effect of PT treatment was examined on C-$ANP_{4–23}$–stimulated IP_3

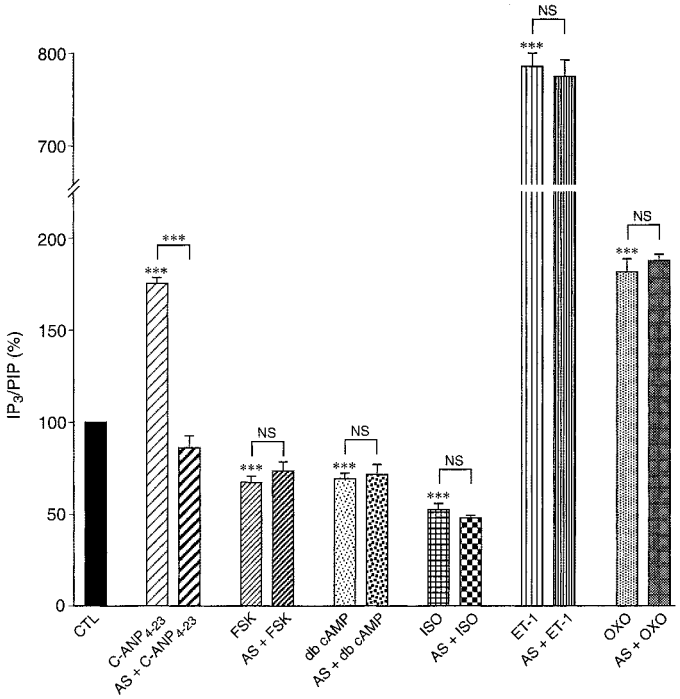

Fig. 5. Effect of antisense-oligodeoxynucleotide treatment on agonist-mediated stimulation and inhibition of IP_3 formation in A10 VSMCs. A10 VSMCs were pretreated without (CTL) or with 2.5 μM of antisense deoxynucleotide (AS) for 48 h as described under *Materials and Methods*. The cells were then incubated with [*myo*-2-^{3}H]inositol for 24 h as described under *Materials and Methods* and were further incubated in the presence of C-$ANP_{4–23}$ (0.1 μM), FSK (50 μM), db cAMP (0.1 μM), oxotremorine (50 μM), ET-1 (0.1 μM), or ISO (50 μM) for 2 h at 37°C. IP_3 levels were determined as described under *Materials and Methods*. Values are the means ± S.E.M. of four separate experiments performed in triplicate. ***, $P < 0.001$; NS, not significant.

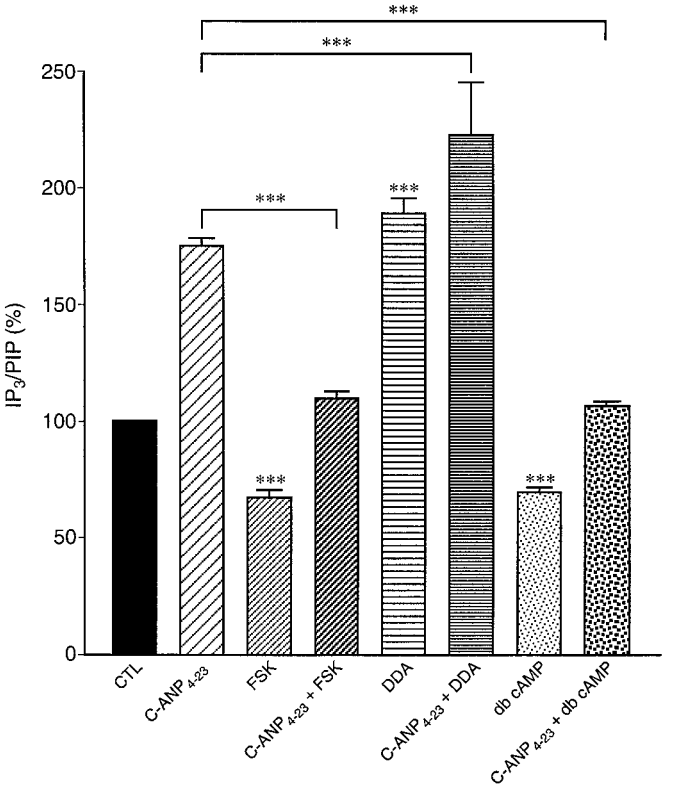

Fig. 6. Effect of DDA, FSK, and db cAMP on C-$ANP_{4–23}$–mediated stimulation IP_3 formation in A10 VSMCs. A10 VSMCs were incubated in the presence of [*myo*-2-^{3}H]inositol for 24 h as described under *Materials and Methods*. The cells were further incubated in the absence (CTL) or presence of C-$ANP_{4–23}$ (0.1 μM), DDA (10 μM), FSK (50 μM), db cAMP (0.1 μM), alone or in combination with C-$ANP_{4–23}$ for 2 h at 37°C. IP_3 formation was determined as described under *Materials and Methods*. Values are the means ± S.E.M. of four separate experiments performed in triplicate. ***, $P < 0.001$; NS, not significant.

production and the results are shown in Fig. 8. C-ANP$_{4-23}$–stimulated IP_3 production by about 80%, which was completely abolished by PT treatment; however, basal IP_3 production was unaffected by PT treatment. On the other hand, increased production of IP_3 induced by ET-1 was also not affected by PT treatment.

Discussion

Previous studies from our group and others have shown the coupling of ANP-C receptors to adenylyl cyclase through Gi-regulatory protein (Anand-Srivastava and Trachte, 1993). In the present studies, we demonstrate that C-ANP$_{4-23}$, which interacts specifically with ANP-C receptors, stimulated PLC activity (IP_3 formation) in A10 VSMCs in a concentration-dependent manner with an apparent EC_{50} between 20 and 30 nM. The maximal stimulation observed was about 75%. Our results are in agreement with the studies of Hirata et al. (1989), who have reported the stimulation of PI turnover by ANP and atriopeptin I in bovine aortic smooth muscle cell homogenates. The C-ANP$_{4-23}$–mediated stimulation of PI turnover may not be attributed to the activation of NPR-A/B receptors and resultant increased levels of cGMP, because C-ANP$_{4-23}$ was shown to be ineffective in altering cGMP levels in A10 VSMCs (Palaparti et al., 2000). The stimulation was dependent on the presence of guanine nucleotides. In addition, Berl et al. (1991) have also demonstrated that low concentrations of ANP stimulated PI turnover via a pertussis toxin-sensitive G-protein, and high concentrations of ANP inhibited PI turnover in cultured rat inner medullary collecting tubule. Murthy and Makhlouf (1999) reported the stimulation of PLC-β activity by C-ANP$_{4-23}$ and small peptide fragments of cytoplasmic domain of ANP-C receptor in guinea pig tenia coli smooth muscle cells. These results suggest that ANP-C receptors may also be coupled to PLC signaling pathway. This notion is further substantiated by our studies showing that ablation of ANP-C receptor by ANP-C receptor antisense attenuates C-ANP$_{4-23}$–mediated stimulation of PI turnover. The inability of ANP-C receptor antisense oligodeoxynucleotides to block FSK-, db cAMP- and ISO-mediated inhibition or ET-1- and oxotremorine-mediated stimulation of IP_3 formation suggests that ANP-C receptor antisense oligonucleotide was specific for ANP-C receptor only and did not affect the other receptor (β-adrenergic, muscarinic, or endothelin) or postre-

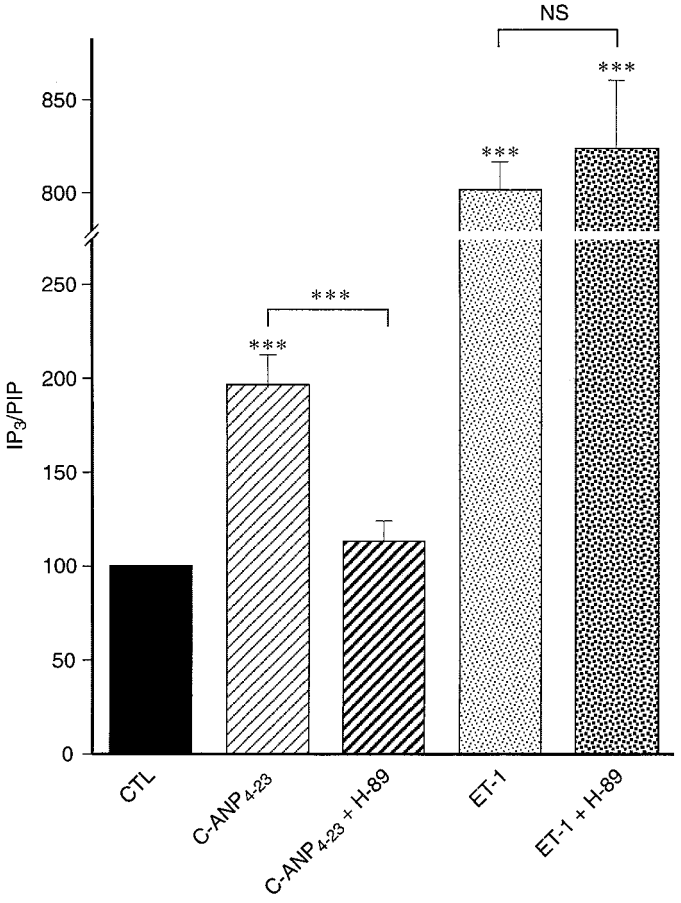

Fig. 7. Effect of protein kinase A inhibitor (H-89) on C-ANP$_{4-23}$–mediated stimulation of IP_3 formation in A10 VSMCs. A10 VSMCs were incubated in the presence of [*myo*-2-^{3}H]inositol for 24 h as described under *Materials and Methods*. The cells were further incubated in the presence of H-89 (10^{-5} M) for 30 min at 37°C and then treated with C-ANP$_{4-23}$ (10^{-7} M) or ET-1 (10^{-7} M) for 2 h at 37°C. IP_3 formation was determined as described under *Materials and Methods*. Values are the means ± S.E.M. of four separate experiments performed in triplicate. ***, $P < 0.001$; NS, not significant.

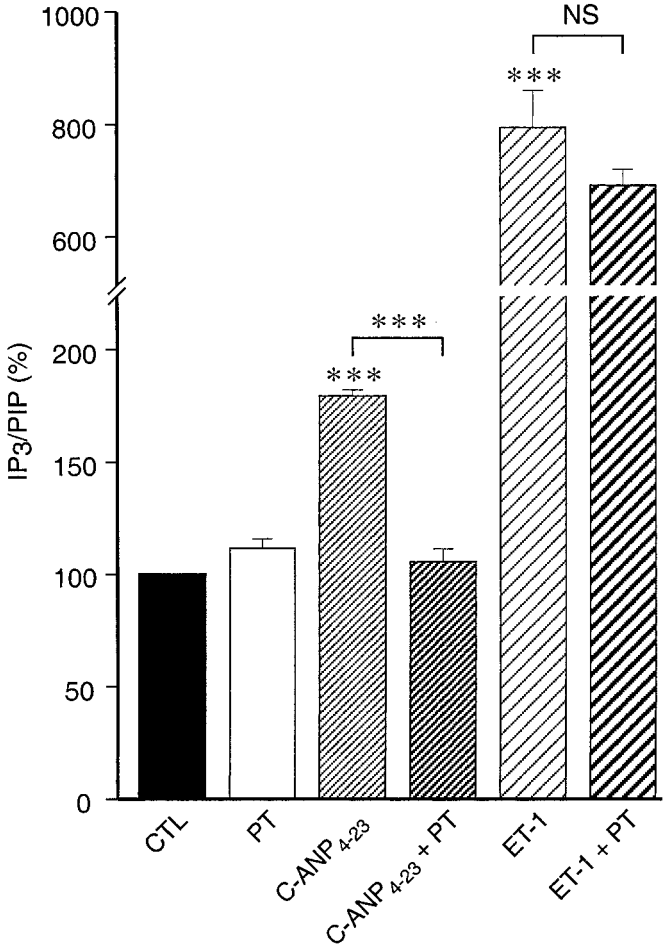

Fig. 8. Effect of PT treatment on C-ANP$_{4-23}$–mediated IP_3 production in A10 VSMCs. A10 VSMCs were incubated in the presence of [*myo*-2-^{3}H]inositol and PT (1 μ g/ml) for 24 h as described under *Materials and Methods*. The cells were further incubated in the presence of C-ANP$_{4-23}$ (10^{-7} M) or ET-1 (10^{-7} M) for 2 h at 37°C. IP_3 formation was determined as described under *Materials and Methods*. Values are the means ± S.E.M. of four separate experiments performed in triplicate. ***, $P <$ 0.001; NS, not significant.

ceptor components such as the catalytic subunit of adenylyl cyclase. The ablation of ANP-C receptor by antisense has also been shown to attenuate ANP-C receptor-mediated inhibition of adenylyl cyclase without affecting β-adrenergic receptor-mediated stimulation of adenylyl cyclase and the other components of adenylyl cyclase system (Palaparti et al., 2000). Taken together, these data suggest that ANP-C receptor-like angiotensin AT_1 receptors may also be coupled to two signaling pathways: adenylyl cyclase and PI turnover (Anand-Srivastava, 1983; Griendling et al., 1986).

We have also demonstrated a role of cAMP in the modulation of PI turnover signaling in A10 VSMCs in the present studies. FSK (a cAMP-inducing agent), db cAMP, and isoproterenol, inhibited IP_3 production, whereas DDA, an inhibitor of adenylyl cyclase and oxotremorine that inhibits adenylyl cyclase through Gi protein, stimulated IP_3 formation in A10 VSMCs. Our results are in accordance with other studies showing that cAMP or FSK treatment inhibited basal and α_1-adrenergic receptor-induced stimulation of IP_3 formation in cultured SMCs from both spontaneously hypertensive and Wistar-Kyoto rats (Wu and de Champlain, 1996). In addition, a cross-talk between these two signaling pathways, cAMP and PI turnover, has been shown by various studies (Abdel-Latif, 2001). The activation of cAMP-dependent protein kinase A has been reported to attenuate PLC signaling in a variety of cells (Abdel-Latif, 2001). G-protein-coupled receptor activation that stimulates adenylyl cyclase and increases intracellular cyclic AMP levels has been shown to decrease IP_3 formation (Abdel-Latif, 2001). In addition, the activation of cGMP-dependent protein kinase by cAMP that results in membrane hyperpolarization has also been reported to inhibit formation of IP_3 (Quast, 1993). However, our studies demonstrate for the first time the interaction between ANP-C receptor-mediated inhibition of adenylyl cyclase activity and increased PI turnover in A10 VSMCs. C-ANP_{4-23} that inhibited adenylyl cyclase activity and decreased intracellular cAMP levels augmented IP_3 formation in A10 VSMCs. However, PT treatment, which inactivates Gi protein and results in the uncoupling of ANP-C receptor from adenylyl cyclase, attenuated ANP-C receptor-mediated stimulation of IP_3 production. In addition, protein kinase A inhibitor also attenuated ANP-C receptor-induced augmentation of IP_3 production. On the other hand, when the levels of cAMP were increased by FSK (a stimulator of adenylyl cyclase) or by db cAMP, the ANP-C receptor-stimulated IP_3 formation was significantly inhibited. On the other hand, FSK was unable to inhibit ET-1–induced increased production of IP_3. Furthermore, DDA (an inhibitor of adenylyl cyclase that decreased cAMP levels) potentiated C-ANP_{4-23}–induced increased formation of IP_3. These results strongly suggest that ANP-C receptor-mediated inhibition of adenylyl cyclase/cAMP may be responsible for the C-ANP_{4-23}–mediated stimulation of PI turnover; in other words, the stimulation of PI turnover by C-ANP_{4-23} in A10 VSMCs may be a secondary event mediated through the inhibition of the adenylyl cyclase/cAMP signaling system to which ANP-C receptors are coupled. On the other hand, ET-1-induced increase in production of IP_3 was not affected by PT or H-89 treatments, suggesting that ET-1–evoked stimulation of PI turnover is not mediated through Gi protein and protein kinase A activation and may involve Gq protein, a PT-insensitive G protein. Thus, the two peptides stimulate PI turnover by two different mechanisms involving Gi or Gq. This notion was further supported by our results showing an additive effect of the peptides C-ANP_{4-23} and ET-1 on IP_3 production.

In conclusion, we have provided the first evidence to demonstrate that ANP-C receptor activation by C-ANP_{4-23} inhibits adenylyl cyclase activity and the resulting decreased levels of cAMP contribute to the increased PI turnover in A10 VSMCs (Fig. 9) and suggest a cross-talk between ANP-C receptor-mediated adenylyl cyclase and PI turnover signaling pathways.

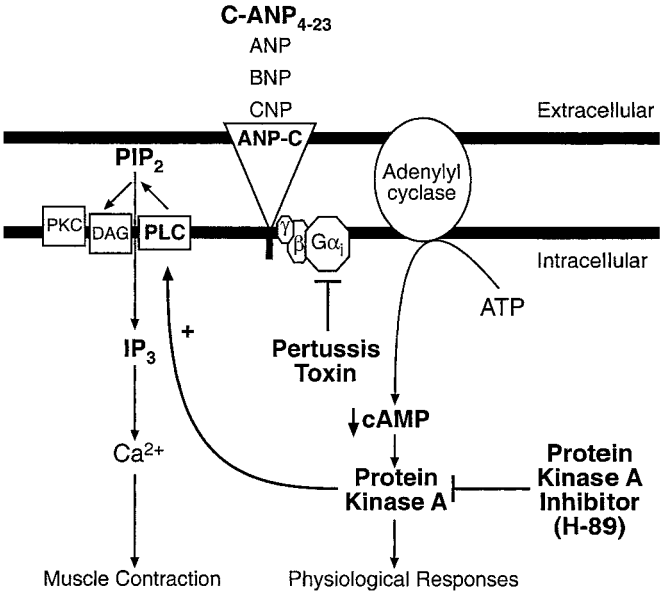

Fig. 9. Summary of the cross-talk between ANP-C receptor-mediated inhibition of adenylyl cyclase and activation of PI turnover. ANP-C receptor activation by C-ANP_{4-23} inhibits adenylyl cyclase activity through PT-sensitive Gi protein and the resultant decreased levels of cAMP stimulates PI turnover in A10 VSMCs.

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