Inhibition of atrial natriuretic peptide (ANP) C receptor expression by antisense oligodeoxynucleotides in A10 vascular smooth-muscle cells is associated with attenuation of ANP-C-receptor-mediated inhibition of adenylyl cyclase

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INTRODUCTION

Atrial natriuretic peptide (ANP), a member of the family of natriuretic peptides, regulates a variety of physiological parameters including blood pressure, progesterone secretion, renin release and vasopressin release by interacting with receptors on the plasma membrane [1,2]. Molecular-cloning techniques have revealed three subtypes of natriuretic peptide receptor; NPR-A [3,4], NPR-B [5,6] and NPR-C [7,8]. NPR-A and NPR-B are membrane guanylyl cyclases, whereas NPR-C (clearance receptor), also known as NPR-C [7,8]. NPR-A and NPR-B are membrane guanylyl cyclase receptors, whereas NPR-C receptors are coupled to phospholipase signalling has also been reported [13,14]. In addition, the coupling of ANP-C receptors to phospholipase signalling has also been reported [15]. The physiological role of the ANP-C receptor and cAMP signal transduction includes inhibition of thyroglobulin release in human thyroid cultured cells possessing only ANP-C receptors [16], inhibition of rat astroglial proliferation [17], electrically induced purinergic and adrenergic contractile force generation in rabbit isolated vasa deferentia [18] and inhibition of endothelial and vascular smooth-muscle cell (VSMC) proliferation [19,20]. In addition, the inhibition of adenylyl cyclase and neurotransmission by C-ANP4–23 [des(Gln18, Ser19, Gln20, Leu21, Gly22)ANP4–23-NH2]mediated inhibition of adenylyl cyclase in vascular smooth-muscle cells (A10). Treatment of the cells with antisense oligonucleotide resulted in the complete attenuation of C-ANP19 to 23mediated inhibition of adenylyl cyclase by antisense oligonucleotide treatment. The attenuation of C-ANP19 to 23mediated inhibition of adenylyl cyclase by antisense oligonucleotide was dose- and time-dependent. A complete attenuation of ANP-C-receptor-mediated inhibition of adenylyl cyclase was observed at 2.5 nM. In addition, treatment of the cells with antisense oligonucleotide and not with sense or missense oligomers resulted in the inhibition of the levels of ANP-C-receptor protein and mRNA as determined by immunoblotting and Northern blotting using antisera against the ANP-C receptor and a cDNA probe of the ANP-C receptor respectively. On the other hand, ANP-A/B-receptor-mediated increases in cGMP levels were not inhibited by antisense-oligonucleotide treatment. Our results demonstrate conclusively that the elimination of ANP-C receptor by antisense oligonucleotide attenuates ANP-induced inhibition of adenylyl cyclase and provide evidence that antisense oligonucleotide of the ANP-C receptor may serve as a useful pharmacological tool to elucidate the physiological functions of the ANP-C receptor. Key words: antisense, cGMP.
oligodeoxynucleotides, which are used mostly as antisense agents, are phosphorothioate oligodeoxynucleotides, in which one of the non-bridging oxygens in the DNA backbone is replaced by sulphur [24]. The inhibition of protein kinase C-α mRNA expression in vitro (C127 mammary epithelial cells) and in vivo (intraperitoneal administration) by antisense oligonucleotides has recently been reported [26]. In the present studies, we employed antisense technique to ablate the ANP-C receptor and assess a functional consequence of this ablation by examining the ANP-C-receptor-mediated adenyl cyclase/CAMP signal-transduction system. We have demonstrated that elimination of the ANP-C receptor resulted in the attenuation of C-ANP-mediated inhibition of adenyl cyclase in VSMCs, Leydig tumour cells and pheochromocytoma cells (PC-12). These results are important and will be helpful in studying the role of the ANP-C receptor and adenyl cyclase/CAMP signalling in mediating physiological functions.

EXPERIMENTAL PROCEDURES

Materials

ATP, cAMP and other chemicals necessary for total RNA extraction and Northern-blot analysis were obtained from Sigma (St. Louis, MO, U.S.A.). Creatine kinase (EC 2.7.3.2), myokinase (EC 2.7.4.3), GTP and guanosine 5'-[γ-thio]-triphosphate (GTP[S]) were purchased from Boehringer Mannheim. 3-Isobutyl-1-methyl-xanthine (IBMX) was purchased from Aldrich (Milwaukee, WI, U.S.A.). [α-32P]ATP and [α-32P]dCTP were purchased from Amersham (Oakville, Ontario, Canada). C-ANP1–33 was purchased from Peninsula Laboratories (Belmont, CA, U.S.A.).

Antisense of oligodeoxynucleotide sequences

Antisense (5'-CAG CAG CAG GCA CCG CAT-3'), sense (5'-ATG CGG TCC CTG CTG CTG-3') and missense (5'-CAG CAG CAG GCA CGC TAC-3') oligodeoxynucleotides were purchased from Oligos Etc. (Wilsonville, OR, U.S.A.). The sequence of the phosphorothioate oligodeoxynucleotide (antisense) is complementary to positions 17–34 on ANP-C sequence of the phosphorothioate oligodeoxynucleotide purchased from Oligos Etc. (Wilsonville, OR, U.S.A.). The CAG CAG GCA GCG TAC-3' phosphorothioate oligodeoxynucleotide was purchased from Amersham (Oakville, Ontario, Canada). C-ANP1–33 was purchased from Peninsula Laboratories (Belmont, CA, U.S.A.).

Cell culture and incubation

A pure VSMC line (A10) from embryonic thoracic aorta of rat was obtained from American Type Culture Collection, Rockville, MA, U.S.A. The cells were plated in 7.5-cm² flasks and incubated at 37 °C in a 95% air/5% CO₂ humidified atmosphere in Dulbecco’s modified Eagle’s medium (with glucose, 1-glutamine and sodium bicarbonate) containing antibiotics and 10% heat-inactivated fetal calf serum as described previously [27]. 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 washed with 5 ml of Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and incubated in 1.5 ml of medium in the absence or presence of 5 μM (or as otherwise indicated) sense, antisense or missense oligonucleotides for 48 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 VSMCs were scraped into ice-cold homogenization buffer using a rubber policeman, and collected by centrifugation at 40 °C for 10 min at 600 g. The cells were then homogenized in a Dounce homogenizer (10 strokes) and the homogenates were used for determination of adenyl cyclase activity and immunoblotting. Preincubation of cells for 48 h in the absence or presence of antisense oligonucleotide resulted in a significant loss of enzyme activity (~40%), which was independent of the presence of the antisense oligonucleotide in the incubation medium.

Determination of adenyl cyclase activity

Adenyl cyclase activity was determined by measuring [32P]cAMP formation from [α-32P]ATP, as described previously [13]. Briefly, the assay medium contained 50 mM glycylglycine, pH 7.5, 0.5 mM MgATP, [α-32P]ATP (5.5 × 10⁶ c.p.m.), 5 mM MgCl₂ (in excess of the ATP concentration), 100 mM NaCl, 0.5 mM cAMP, 1 mM IBMX, 0.1 mM EGTA, 10 μM GTP[S] and an ATP-regenerating system consisting of 2 mM phosphocreatine, 0.1 mg of creatine kinase/ml and 0.1 mg of myokinase/ml in a final volume of 200 μl. Incubations were initiated by the addition of the membrane preparations (20–30 μg) to the reaction mixture, which had been thermally equilibrated for 2 min at 37 °C. The reactions conducted in triplicate for 10 min at 37 °C were terminated by the addition of 0.6 ml of 120 mM zinc acetate. cAMP was purified by co-precipitation of other nucleotides with ZnCO₃, by addition of 0.5 ml of 144 mM Na₂CO₃ and subsequent chromatography by the double-column system, as described by Salomon et al. [28]. Under the assay conditions used, adenyl cyclase activity was linear with respect to protein concentrations and time of incubation. Protein was determined essentially as described by Lowry et al. [29] with BSA as standard.

Determination of intracellular cGMP levels

Intracellular cGMP levels were determined as described previously [13]. After preincubation with antisense oligonucleotide, the cells were washed twice with PBS. The cells were further incubated with 1 mM IBMX for 5 min at 37 °C and stimulated with or without ANP99-130 (28 amino acids, positions 99–126, of ANP) at various concentrations for 10 min. The reaction was terminated by rapid aspiration and addition of ice-cold 1 M HCl. cGMP levels were determined by radioimmunoassay using a radioimmunoassay (RIA) kit from Biomedical Technologies (Stoughton, MA, U.S.A.).

Immunoblotting

Immunoblotting was performed as described previously [30]. After SDS/PAGE, the separated proteins were transferred electrophoretically on to nitrocellulose paper (Schleicher and Schuell) with a semi-dry transblot apparatus (Bio-Rad) at 15 V for 45 min. After transfer, the membranes were washed twice in PBS and were incubated in PBS containing 8% dehydrated milk at room temperature for 2 h. The blots were then incubated with anti-R37A antibody against the ANP-C receptor and β-actin antibody (1:1000) in PBS (containing 3% dehydrated milk and 0.1% Tween-20) at room temperature for 2 h. The antibody-antigen complexes were detected by incubating the blots with goat anti-rabbit IgG (Bio-Rad) conjugated with horseradish peroxidase for 2 h at room temperature. The blots were washed three times with PBS before reaction with enhanced chemiluminescence (ECL®) Western-blotting detection reagents from Amersham. Quantitative analysis of the G-proteins was performed by densitometric scanning of the autoradiographs employing the enhanced laser densitometer, LKB Uitsoncan XL.
and quantified using the gel-scan XL evaluation software (version 2.1) from Pharmacia (Baie d’Urfé, Quebec, Canada).

**Total RNA extraction**

Total RNA was extracted from VSMCs by the method of Sambrook et al. [31] as described in [32].

**Radiolabelling of the probes**

A cDNA insert encoding the ANP-C receptor was radiolabelled with [$\gamma^32P$]ATP by random priming essentially as described by Feinberg and Vogelstein [33]. Specific activities of the labelled probe ranged from $1 \times 10^{6}$ to $3 \times 10^{6}$ c.p.m./$\mu$g of DNA. The 32-mer oligonucleotide recognizing the 28 S rRNA was end-labelled with [$\gamma^32P$]ATP using T4 polynucleotide kinase as described by Sambrook et al. ([31], pp. 1059–1061).

**Northern-blot analysis**

DMSO/glyoxal-treated total RNA was resolved on 1 % agarose gels and transferred on to nylon membrane as described previously [32]. Filters, after prehybridization at 65 °C for 6 h in hybridization solution (600 mM NaCl, 8 mM EDTA, 120 mM Tris, pH 7.4, 0.1 % sodium pyrophosphate, 0.2 % SDS and heparin 500 units/ml), were then hybridized overnight in hybridization solution containing dextran sulphate (10 %, w/v) and the cDNA probe at $1 \times 10^3$–$3 \times 10^3$ c.p.m./ml as described previously [32]. Filters were then rinsed at 65 °C twice for 30 min each in 300 mM NaCl, 4 mM EDTA, 60 mM Tris, pH 7.4, and 0.2 % SDS, and once for 30 min in 150 mM NaCl, 2 mM EDTA, 30 mM Tris, pH 7.4, and 0.1 % SDS. Autoradiography was performed with X-ray films at $-70$ °C. In order to assess the possibility of any variations in the amounts of total RNA in individual samples applied to the gel, each filter was hybridized with the $32P$-end-labelled oligonucleotide, which recognizes a highly conserved region of 28 S ribosomal RNA. The blots which had been probed with the G-protein cDNA were de-hybridized by washing for 1 h at 65 °C in 50 % formamide, 300 mM NaCl, 4 mM EDTA and 60 mM Tris, pH 7.4, and rehybridized overnight at room temperature with the oligonucleotide. Quantitative analysis of the hybridization of probes bound was performed by densitometric scanning of the autoradiograms employing the enhanced laser densitometer, LKB Ultrascan XL, and quantified using the gel-scan XL evaluation software (version 2.1) from Pharmacia.

**RESULTS**

**Effect of antisense oligonucleotide on ANP-C-receptor protein expression**

To investigate if antisense oligodeoxynucleotide treatment of A10 VSMCs resulted in the inhibition of ANP-C-receptor expression, the levels of the ANP-C-receptor protein were determined by Western blotting using ANP-C-receptor-specific anti-R37A antibodies. These R37A polyclonal antisera were raised against the 37-amino acid sequence corresponding to the cytoplasmic domain of bovine ANP-C receptor. The purified protein-derivative-coupled R37A peptide was very immunogenic, with serum titres of 1:500 000 when tested by ELISA against unconjugated R37A [34]. As shown in Figure 1(A), anti-R37A antibodies, which have been reported to react specifically with ANP-C receptors [23], recognized a single protein of approx. 66 kDa on immunoblots from control and sense-, missense- and antisense-treated A10 VSMCs. However, the relative amount of immunodetectable ANP-C receptor was much reduced in cells treated with antisense oligonucleotide (by about 87±2 % compared with control cells, $n = 3$) but not with sense- or missense-oligonucleotide treatments (Figure 1B). Indeed, the levels of ANP-C-receptor protein were increased by 18±2 and 85±3.5 % ($n = 3$ for both) by sense and missense treatments, respectively. In addition, the level of inhibition of ANP-C-receptor protein by antisense oligonucleotide was concentration-dependent (Figure 2A). At low concentrations (0.25 and 0.5 $\mu$M), antisense oligonucleotide was unable to inhibit the levels of ANP-C-receptor protein. However, at 1 and 2.5 $\mu$M, the inhibition was about 50 and 40 %, respectively, as determined by densitometric scanning. Treatment of the cells with 5 $\mu$M antisense oligonucleotide resulted in almost complete loss of the receptor protein (90±5 %, $n = 3$; Figure 2B).
A. Palaparti, Y. Li and M. B. Anand-Srivastava

**Figure 2** Concentration-dependent inhibition of ANP-C-receptor protein in A10 VSMC membrane by antisense oligodeoxynucleotide

(A) A10 VSMCs were incubated in the absence (control) or presence of 0.25, 0.5, 1.0, 2.5 or 5 μM antisense oligodeoxynucleotide for 48 h as described in the Experimental Procedures section. Membranes were prepared as described in the Experimental Procedures section, which were used for immunoblotting. The membrane proteins were resolved by SDS/PAGE and transferred on to nitrocellulose, which was then immunoblotted using antibody anti-R37A against the ANP-C receptor and β-actin antibody as a control, and were detected by ECL Western blotting. The autoradiograms are representative of three separate experiments. (B) Densitometric scanning of the ANP-C-receptor protein in control and antisense-treated A10 VSMCs. The results are expressed as a percentage of the control, taken as 100%. Values are means ± S.E.M. of three separate experiments.

**Figure 3** mRNA expression of the ANP-C receptor in control and antisense-oligodeoxynucleotide-treated A10 VSMCs

(A) Total RNAs from A10 VSMCs of control cells, and cells treated with 0.25, 0.5, 1, 2.5 and 5 μM antisense deoxynucleotides were subjected to 1% agarose gel electrophoresis and transferred on to nylon membrane. The blots were then probed with full-length radiolabelled ANP-C-receptor cDNA probe as described in the Experimental Procedures section. The autoradiograms are representative of three separate experiments. (B) Densitometric scanning of the ANP-C-receptor mRNA in control and antisense-treated A10 VSMCs. The results are expressed as a percentage of the control, taken as 100%. Values are means ± S.E.M. of three separate experiments.

**Effect of antisense oligonucleotide on ANP-C-receptor gene expression**

In order to investigate if antisense oligonucleotide treatment was also able to attenuate the gene expression of ANP-C receptor, we measured the ANP-C-receptor mRNA levels by Northern blotting. As shown in Figure 3(A), the cDNA probe of the ANP-C receptor recognized a message of 1.7 kb in control and antisense-treated cells. However, ANP-C-receptor mRNA was inhibited in a concentration-dependent manner in antisense-treated cells as determined by densitometric scanning (Figure 3B). At 0.5 μM, antisense oligonucleotide was able to inhibit the mRNA levels by about 70%, and at 5.0 μM almost complete attenuation (90 ± 2%, n = 3) of the expression of ANP-C-receptor mRNA was observed. The precise reason for the lack of correlation between mRNA levels and protein levels of ANP-C receptor after treatment with 0.5 μM antisense oligonucleotide is not clear at present. However, it may be possible that residual mRNA (about 30%) remaining after 0.5 μM antisense-oligonucleotide treatment is sufficient to account for most of the ANP-C-receptor protein detected at this dose of antisense oligonucleotide. Further decrease in ANP-C-receptor mRNA levels (by 80% and more) by higher concentrations of antisense oligonucleotide was reflected in decreased ANP-C-receptor protein levels. It is thus possible that a more than 70% decrease in mRNA levels affects ANP-C-receptor protein synthesis.

**Effect of antisense oligonucleotide on ANP-C-mediated inhibition of adenylyl cyclase**

To investigate if the attenuation of the ANP-C-receptor expression is reflected in ANP-C-receptor-mediated functions, the effect of antisense-oligodeoxynucleotide treatment on adenylyl cyclase activity was studied in A10 VSMCs. Figure 4 shows that treatment of VSMCs with antisense oligonucleotide (5 μM)
Atrial natriuretic peptide C receptor antisense attenuates adenylyl cyclase inhibition

Figure 4 Effect of antisense-, missense- and sense-oligomer treatments on C-ANP<sub>4-23</sub>-mediated inhibition of adenylyl cyclase activity in A10 VSMCs

A10 VSMCs were incubated in the absence (control) or presence of 5 μM sense, missense or antisense deoxynucleotide for 48 h, and membranes were prepared, as described in the Experimental Procedures section. Adenylyl cyclase activity was determined in the membranes in the absence (basal) or presence of 1 μM C-ANP<sub>4-23</sub>. Values are means ± S.E.M. of three separate experiments performed in triplicate. Adenylyl cyclase activities in the presence of 10 μM GTP[S] in control and sense-, missense- and antisense-treated membranes were 243.6 ± 7.5, 215.4 ± 5, 221.3 ± 4 and 249.6 ± 15.5 pmol of cAMP/10 min per mg of protein respectively.

resulted in the abolition of C-ANP<sub>4-23</sub>-mediated inhibition of adenylyl cyclase, whereas sense and missense oligomers were ineffective in attenuating the C-ANP<sub>4-23</sub>-mediated inhibition of adenylyl cyclase. Figure 5 shows the effect of various concentrations of C-ANP<sub>4-23</sub> on adenylyl cyclase activity in A10 VSMCs treated with 5 μM antisense, sense or missense oligonucleotides. C-ANP<sub>4-23</sub> inhibited adenylyl cyclase activity in a concentration-dependent manner in control and sense- and missense-treated A10 cells. However, the inhibitory effect of C-ANP<sub>4-23</sub> was abolished in cells treated with antisense oligonucleotide, but not in cells treated with sense or missense oligonucleotides. However, missense-treated cells exhibited greater inhibition of adenylyl cyclase by C-ANP<sub>4-23</sub> compared with control or sense-treated cells.

The attenuation of C-ANP<sub>4-23</sub>-mediated inhibition of adenylyl cyclase by antisense pretreatment was dose-dependent (Figure 6A). At 2.5 μM, antisense-oligonucleotide treatment resulted in the abolition of C-ANP<sub>4-23</sub>-mediated inhibition of adenylyl cyclase. In addition, the attenuation of ANP-C-receptor-mediated inhibition of adenylyl cyclase by antisense oligonucleotide (2.5 μM) was also time-dependent (Figure 6B). Complete abolition of C-ANP<sub>4-23</sub>-mediated inhibition of adenylyl cyclase was observed after 48 h of treatment with antisense oligonucleotide. In addition, antisense treatment was also able to abolish C-ANP<sub>4-23</sub>-mediated inhibition of adenylyl cyclase in Leydig tumour cells and PC-12 cells (results not shown).

Figure 5 Concentration-dependent inhibition of adenylyl cyclase activity in A10 VSMCs treated with oligodeoxynucleotides

A10 VSMCs were incubated in the absence (control, ○) or presence of 5 μM sense (●), missense (□) or antisense (■) oligodeoxynucleotides for 48 h, and membranes were prepared, as described in the Experimental Procedures section. Adenylyl cyclase activity was determined in the membranes in the absence or presence of various concentrations of C-ANP<sub>4-23</sub>. Values are means ± S.E.M. of three separate experiments performed in triplicate. Adenylyl cyclase activities in the presence of 10 μM GTP[S] in control and sense-, missense- and antisense-treated membranes were 243.6 ± 7.5, 215.4 ± 5, 221.3 ± 4 and 249.6 ± 15.5 pmol of cAMP/10 min per mg of protein respectively.

Effect of antisense oligonucleotide on stimulatory and inhibitory responses of agonists on adenylyl cyclase activity

In order to investigate if antisense treatment also results in the attenuation of other receptor-mediated adenylyl cyclase signalling, the effect of antisense treatment on isoproterenol-mediated stimulation and angiotensin II-mediated inhibition of adenylyl cyclase was examined. The results shown in Table 1 indicate that the respective stimulatory and inhibitory effects of isoproterenol and angiotensin II on adenylyl cyclase activity were not different in control and antisense-treated cells, whereas C-ANP<sub>4-23</sub>-mediated inhibition was abolished by antisense-oligonucleotide treatment. In addition, the stimulatory effects of GTP, GTP[S], NaF and forskolin, which stimulate adenylyl cyclase through a receptor-independent mechanism, on adenylyl cyclase activity were not different in control and antisense-treated cells, whereas C-ANP<sub>4-23</sub>-mediated inhibition was abolished by antisense-oligonucleotide treatment.

Effect of antisense-oligonucleotide treatment on cGMP levels

A10 smooth-muscle cells have also been shown to have ANP-A/B receptors [35], which are guanylyl cyclase receptors. In
order to examine the specificity of the ANP-C-receptor antisense oligonucleotide, its effect on ANP$_{1–23}$-mediated increases in cGMP levels was examined in these cells. Results shown in Figure 7 indicate that ANP stimulated cGMP levels in control as well as in antisense-treated cells in a concentration-dependent manner and that the level of stimulation was significantly higher in antisense-treated cells compared with control cells. On the other hand, as expected, C-ANP$_{4–23}$ was unable to increase the levels of cGMP in these cells (basal, 146.2±9 pmol of cGMP/mg of protein; 10$^{-9}$ M C-ANP$_{1–23}$, 154.3±10; 10$^{-8}$ M C-ANP$_{4–23}$, 148.2±5; 10$^{-6}$ M C-ANP$_{4–23}$, 145.6±9). These results suggest that ANP-C-receptor antisense oligonucleotide is specific for the ANP-C receptor and that it does not inhibit ANP-A/B-receptor-mediated signalling.

**DISCUSSION**

Previous studies from our group and others have shown the coupling of ANP-C receptor to inhibition of adenylyl cyclase [2]. However, Hirata et al. [15] have shown that the ANP-C receptor may be coupled to the phospholipase C signalling pathway, whereas others have reported this receptor as a clearance receptor [11,12]. In the present study, we have demonstrated for the first time that ablation of the ANP-C receptor by antisense technique attenuates C-ANP$_{4–23}$-mediated inhibition of adenylyl cyclase and provides strong evidence that ANP-C receptors are functional and may play an important role in the regulation of a variety of physiological functions [2]. We have used the antisense 18-mer phosphorothioate oligodeoxynucleotide (OH-2) against a sequence of nucleotides specific to the ANP-C receptor. The high antisense potency of OH-2 was aided by the fact that it has a GC-rich oligonucleotide with a high melting temperature ($T_m$). In addition, the use of a nuclease-stable phosphorothioate oligonucleotide analogue combined with the use of heat-treated serum to inactivate nucleases enhanced the potency of OH-2.

The inhibition of ANP-C-receptor protein as well as gene expression by antisense oligonucleotide in a concentration-dependent manner suggests that phosphorothioate oligodeoxynucleotides may be used as a useful pharmacological inhibitor of gene expression *in vitro*. The inhibition of ANP-C-receptor expression by antisense oligonucleotide was also reflected in the attenuation of ANP-C-receptor-mediated inhibition of adenylyl cyclase by C-ANP$_{3–23}$. The concentration-dependent attenuation of ANP-C-receptor-mediated inhibition of adenylyl cyclase and the depletion of ANP-C-receptor protein further support a coupling of the ANP-C receptor to the adenylyl cyclase system. Furthermore, the inhibition of ANP-C-receptor protein expression and C-ANP$_{4–23}$-mediated adenylyl cyclase inhibition was specific for antisense oligonucleotide and was not exhibited by sense or missense oligomers. In addition, a slightly increased inhibition of adenylyl cyclase by C-ANP$_{4–23}$ in missense-treated cells may be attributed to an increase in the expression of ANP-C receptor.
C-receptor protein observed in these cells. However, the reason for the increased expression of the ANP-C receptor and resultant increased inhibition of adenyl cyclase by missense oligomer is not clear at present and needs to be investigated. One of the possibilities may be that missense treatment enhanced the transcription or stabilization of ANP-C-receptor mRNA or decreased the degradation of the receptor protein. ANP has been shown earlier to inhibit adenyl cyclase in Leydig tumour cells and PC-12 cells [13,21]. Thus the attenuation of ANP-C-receptor-mediated inhibition and ANP-induced cGMP formation. For example, the predicted increase in cAMP could inhibit cGMP hydrolysis (competitive substrate for this enzyme), thereby potentiating ANP-stimulated cGMP formation. Our results are in agreement with the studies performed in PC-12 cells, where ANP-A-receptor-mediated stimulation of guanylyl cyclase and cGMP levels were not inhibited by ANP-C-receptor antisense treatment [36].

### Table 1: Effect of ANP-C-receptor antisense-oligonucleotide treatment on agonist-mediated stimulation and inhibition of adenyl cyclase in VSMCs

VSMCs were incubated in the absence (control cells) or presence of 5 μM antisense oligodeoxynucleotide for 48 h. Membranes were prepared as described in the Experimental Procedures section. Adenylyl cyclase activity was determined in the membranes in the absence (basal) or presence of 10 μM GTP alone or with 50 μM isoproterenol (ISO), 10 μM GTP[S] alone or in combination with 0.1 μM C-ANP99-126 or 10 μM angiotensin II (AII), 10 mM NaF or 50 μM forskolin (FSK) as described in the Experimental Procedures section. Values are means ± S.E.M. of three separate experiments performed in triplicate. Arrows (↑, ↓) and values in parentheses indicate the percentage of stimulation (↑) or inhibition (↓) over respective basal adenylyl cyclase activity. –, Complete attenuation.

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<tr>
<td>None (basal)</td>
<td>59.0 ± 11</td>
<td>53.3 ± 9.0</td>
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<tr>
<td>GTP (10 μM)</td>
<td>125.2 ± 6.1 (112.2% ↑)</td>
<td>121.3 ± 2.3 (127.6% ↑)</td>
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<tr>
<td>GTP+ISO (50 μM)</td>
<td>659.9 ± 56.2 (427.1% ↑)</td>
<td>622.0 ± 15.0 (412.8% ↑)</td>
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<tr>
<td>GTP[S] (10 μM)</td>
<td>243.6 ± 7.5 (312.9% ↑)</td>
<td>249.6 ± 15.5 (368.3% ↑)</td>
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<tr>
<td>GTP[S]+C-ANP99-126 (0.1 μM)</td>
<td>180.8 ± 5.6 (26% ↓)</td>
<td>247.8 ± 10.2 (–)</td>
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<tr>
<td>GTP[S]+AII (10 μM)</td>
<td>195.3 ± 4.2 (20% ↓)</td>
<td>187.2 ± 5.0 (25% ↓)</td>
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<td>NaF (10 mM)</td>
<td>382.2 ± 37.2 (247.8% ↑)</td>
<td>371.2 ± 6.0 (396.4% ↑)</td>
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<td>FSK (50 μM)</td>
<td>1758.1 ± 63.2 (2879.8% ‡)</td>
<td>1474.5 ± 51.1 (2666.4% ‡)</td>
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In conclusion, we demonstrate for the first time that the elimination of the ANP-C receptor by antisense-oligonucleotide treatment attenuates ANP-induced inhibition of adenyl cyclase and provides evidence that the antisense oligonucleotide of the ANP-C receptor may be used as an important pharmacological tool to investigate the physiological functions of the ANP-C receptor.

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