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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Atrial natriuretic peptide (ANP) mediates a variety of physiological effects through its interaction with ANP-A, ANP-B or ANP-C receptors. However, controversies exist regarding the involvement of ANP-C receptor and adenylyl cyclase/cAMP signal-transduction systems to which these receptors are coupled in mediating these responses. In the present studies, we have employed an antisense approach to eliminate the ANP-C receptor and to examine the effect of this elimination on adenylyl cyclase inhibition. An 18-mer antisense phosphorothioate oligodeoxynucleotide (OH-2) targeted at the initiation codon of the ANP-C receptor was used to examine its effects on the expression of the ANP-C receptor and ANP-C-receptor-mediated inhibition of adenylyl cyclase in vascular smooth-muscle cells (A10). Treatment of the cells with antisense oligonucleotide resulted in complete attenuation of C-ANP_{4–23} [des(Gln¹⁸, Ser¹⁹, Gln²⁰, Leu²¹, Gly²²)ANP_{4–23}-NH₂]-mediated inhibition of adenylyl cyclase, whereas sense and missense oligomers did not affect the inhibition of adenylyl cyclase by C-ANP_{4–23}. In addition, the stimulatory effects of guanine nucleotides, isoproterenol, sodium fluoride and forskolin as well as the inhibitory effects of angiotensin II on adenylyl cyclase were not affected by antisense-

oligonucleotide treatment. The attenuation of C-ANP_{4–23}-mediated 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 μ M. 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.

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 ANP-R_s and ANP-C receptors, exists as a disulphide-linked homodimer, and has a 37-amino acid cytoplasmic domain [9,10]. The role of NPR-C has originally been considered in terms of the clearance of bound ligand by internalization and degradation [11,12]. However, ANP-C receptors have been shown to mediate various physiological effects of ANP [2] through the adenylyl cyclase/cAMP signal-transduction system to which these receptors are coupled [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-ANP_{4–23} [des(Gln¹⁸, Ser¹⁹, Gln²⁰, Leu²¹, Gly²²)ANP_{4–23}-NH₂] in nerve-growth-factor-treated pheochromocytoma cells [21] and ANP-C-receptor-mediated translation *in vivo* of the endothelin message and the secretion of endothelin from cultured bovine aortic endothelial cells [22] has also been reported and further supports the hypothesis of a physiological role for these receptors. Recently, using antibodies specific for the cytoplasmic domain of ANP-C receptors, we have demonstrated the involvement of the cytoplasmic domain of the ANP-C receptor in mediating adenylyl cyclase inhibition [23].

The inhibition of the expression of several viral and mammalian genes *in vitro* by oligonucleotides designed to hybridize to specific pre-mRNA or mRNA sequences (antisense oligonucleotides) has been reported [24,25]. One of the best-characterized classes of

Abbreviations used: ANP, atrial natriuretic peptide; C-ANP_{4–23}, des(Gln¹⁸, Ser¹⁹, Gln²⁰, Leu²¹, Gly²²)ANP_{4–23}-NH₂; ANP_{99–126}, 28 amino acids, positions 99–126, of ANP; GTP[S], guanosine 5'-[γ-thio]-triphosphate; VSMC, vascular smooth-muscle cell; IBMX, 3-isobutyl-1-methyl-xanthine.

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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 adenylyl cyclase/cAMP signal-transduction system. We have demonstrated that elimination of the ANP-C receptor resulted in the attenuation of C-ANP₄₋₂₃-mediated inhibition of adenylyl 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 adenylyl 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-ANP₄₋₂₃ 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 GCG 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 mRNA (sense). This sequence is not related to ANP-A or ANP-B mRNA. The missense is a scrambled peptide of the antisense peptide.

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-cm³ flasks and incubated at 37 °C in a 95% air/5% CO₂ humidified atmosphere in Dulbecco's modified Eagle's medium (with glucose, L-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 μ M (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 adenylyl 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 adenylyl cyclase activity

Adenylyl cyclase activity was determined by measuring [³²P]cAMP formation from [α -³²P]ATP, as described previously [8,13]. Briefly, the assay medium contained 50 mM glycylglycine, pH 7.5, 0.5 mM MgATP, [α -³²P]ATP (1.5×10^6 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 μ M 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 μ l. Incubations were initiated by the addition of the membrane preparations (20–30 μ g) 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, adenylyl 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 ANP₉₉₋₁₂₆ (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 Ultrosan 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 [α - 32 P]dCTP by random priming essentially as described by Feinberg and Vogelstein [33]. Specific activities of the labelled probe ranged from 1×10^8 to 3×10^8 c.p.m./ μ g of DNA. The 32-mer oligonucleotide recognizing the 28 S rRNA was end-labelled with [γ - 32 P]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 1×10^6 – 3×10^6 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 32 P-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 autoradiographs 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 1:500000 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.

Figure 1 Effect of antisense, sense and missense oligodeoxynucleotides on the levels of ANP-C-receptor protein in A10 VSMC membranes

(A) A10 VSMCs were incubated in the absence (control) or presence of 5 μ M sense, missense and antisense oligodeoxynucleotides for 48 h. Membranes prepared as described in the Experimental Procedures section 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 ANP-C receptor and were detected by ECL Western-blotting. The autoradiograms are representative of three separate experiments. For further details see the Experimental Procedures section. (B) Densitometric scanning of the ANP-C-receptor protein in control and sense-, missense- and antisense-oligonucleotide-treated A10 VSMCs. The results are expressed as a percentage of the control, taken as 100%. Values are means $\pm$ S.E.M. of three separate experiments.

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 $87 \pm 2\%$ 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 $85 \pm 3.5\%$ ($n = 3$ 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 μ M), antisense oligonucleotide was unable to inhibit the levels of ANP-C-receptor protein. However, at 1 and 2.5 μ M, the inhibition was about 50 and 40% respectively, as determined by densitometric scanning. Treatment of the cells with 5 μ M antisense oligonucleotide resulted in almost complete loss of the receptor protein ($90 \pm 5\%$, $n = 3$; Figure 2B).

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 μ M 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 $\pm$ 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 μ M, antisense oligonucleotide was able to inhibit the mRNA levels by about 70%, and at 5.0 μ M almost complete attenuation ($90 \pm 2\%$, $n = 3$) 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 μ M antisense oligonucleotide is not clear at present. However, it may be possible that residual mRNA

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 μ M 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 $\pm$ S.E.M. of three separate experiments.

(about 30%) remaining after 0.5 μ M 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 μ M)

Figure 4 Effect of antisense-, missense- and sense-oligomer treatments on C-ANP₄₋₂₃-mediated inhibition of adenylyl cyclase activity in A10 VSMCs

A10 VSMCs were incubated in the absence (control) or presence of 5 μ M 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 μ M C-ANP₄₋₂₃. Values are means $\pm$ S.E.M. of three separate experiments performed in triplicate. Adenylyl cyclase activities in the presence of 10 μ M 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-ANP₄₋₂₃-mediated inhibition of adenylyl cyclase, whereas sense and missense oligomers were ineffective in attenuating the C-ANP₄₋₂₃-mediated inhibition of adenylyl cyclase. Figure 5 shows the effect of various concentrations of C-ANP₄₋₂₃ on adenylyl cyclase activity in A10 VSMCs treated with 5 μ M antisense, sense or missense oligonucleotides. C-ANP₄₋₂₃ inhibited adenylyl cyclase activity in a concentration-dependent manner in control and sense- and missense-treated A10 cells. However, the inhibitory effect of C-ANP₄₋₂₃ 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-ANP₄₋₂₃ compared with control or sense-treated cells.

The attenuation of C-ANP₄₋₂₃-mediated inhibition of adenylyl cyclase by antisense pretreatment was dose-dependent (Figure 6A). At 2.5 μ M, antisense-oligonucleotide treatment resulted in the abolition of C-ANP₄₋₂₃-mediated inhibition of adenylyl cyclase. In addition, the attenuation of ANP-C-receptor-mediated inhibition of adenylyl cyclase by antisense oligonucleotide (2.5 μ M) was also time-dependent (Figure 6B). Complete abolition of C-ANP₄₋₂₃-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-ANP₄₋₂₃-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, $\circ$) or presence of 5 μ M sense ($\bullet$), missense ($\square$) or antisense ($\blacksquare$) 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-ANP₄₋₂₃. Values are means $\pm$ S.E.M. of three separate experiments performed in triplicate. Adenylyl cyclase activities in the presence of 10 μ M 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-ANP₄₋₂₃-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. Furthermore, basal adenylyl cyclase activity was also not affected 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

Figure 6 (A) Effect of various concentrations of antisense oligodeoxynucleotide and (B) time course of antisense-oligodeoxynucleotide treatment on C-ANP₄₋₂₃-mediated inhibition of adenylyl cyclase activity in A10 VSMCs

(A) A10 VSMCs were incubated in the absence (control) or presence of various concentrations of antisense deoxynucleotide (0.25–5 μM) 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, taken as 100%) or presence of 1 μM C-ANP₄₋₂₃. Values are means $\pm$ S.E.M. of three separate experiments performed in triplicate. Adenylyl cyclase activity in the presence of 10 μM GTP[S] in control membranes was 215.5 ± 6.1 pmol of cAMP/min per mg protein. (B) A10 VSMCs were incubated in the presence of 2.5 μM antisense oligodeoxynucleotide for 0, 12, 24, 36 and 48 h. Membranes were prepared as described in the Experimental Procedures section. Adenylyl cyclase activity was determined in the membranes in the presence of GTP[S] alone (basal, taken as 100%) or in combination with 1 μM C-ANP₄₋₂₃ as described in the Experimental Procedures section. Values are means $\pm$ S.E.M. of three separate experiments performed in triplicate. Basal adenylyl cyclase activities in the presence of 10 μM GTP[S] in membranes prepared from cells treated with antisense deoxynucleotide were 697.5 ± 28.4 (0 h), 654.5 ± 6.0 (12 h), 778.3 ± 3.3 (24 h), 721 ± 21.7 (36 h) and 358.5 ± 12.6 (48 h) pmol of cAMP/10 min per mg of protein.

order to examine the specificity of the ANP-C-receptor antisense oligonucleotide, its effect on ANP₉₉₋₁₂₆-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-ANP₄₋₂₃ 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-ANP₄₋₂₃, 154.3 ± 10 ; 10^{-8} M C-ANP₄₋₂₃, 148.2 ± 5 ; 10^{-6} M C-ANP₄₋₂₃, 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-ANP₄₋₂₃-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-ANP₄₋₂₃. 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-ANP₄₋₂₃-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-ANP₄₋₂₃ in missense-treated cells may be attributed to an increase in the expression of ANP-

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

VSMCs were incubated in the absence (control cells) or presence of 5 μ M 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 μ M GTP alone or with 50 μ M isoproterenol (ISO), 10 μ M GTP[S] alone or in combination with 0.1 μ M C-ANP₄₋₂₃ or 10 μ M angiotensin II (All), 10 mM NaF or 50 μ M forskolin (FSK) as described in the Experimental Procedures section. Values are means $\pm$ S.E.M. of three separate experiments performed in triplicate. Arrows ($\uparrow$) and values in parentheses indicate the percentage of stimulation ($\uparrow$) or inhibition ($\downarrow$) over respective basal adenylyl cyclase activity. —, Complete attenuation.

Additions	Adenylyl cyclase activity (pmol of cAMP/10 min per mg of protein)	
	Control cells	Antisense-treated cells
None (basal)	59.0 $\pm$ 11	53.3 $\pm$ 9.0
GTP (10 μ M)	125.2 $\pm$ 6.1 (112.2% $\uparrow$)	121.3 $\pm$ 2.3 (127.6% $\uparrow$)
GTP + ISO (50 μ M)	659.9 $\pm$ 56.2 (427.1% $\uparrow$)	622.0 $\pm$ 15.0 (412.8% $\uparrow$)
GTP[S] (10 μ M)	243.6 $\pm$ 7.5 (312.9% $\uparrow$)	249.6 $\pm$ 15.5 (368.3% $\uparrow$)
GTP[S] + C-ANP ₄₋₂₃ (0.1 μ M)	180.8 $\pm$ 5.6 (26% $\downarrow$)	247.8 $\pm$ 10.2 (—)
GTP[S] + All (10 μ M)	195.3 $\pm$ 4.2 (20% $\downarrow$)	187.2 $\pm$ 8.5 (25% $\downarrow$)
NaF (10 mM)	382.2 $\pm$ 37.2 (547.8% $\uparrow$)	371.2 $\pm$ 8.0 (596.4% $\uparrow$)
FSK (50 μ M)	1758.1 $\pm$ 63.2 (2879.8% $\uparrow$)	1474.5 $\pm$ 51.1 (2666.4% $\uparrow$)

C-receptor protein observed in these cells. However, the reason for the increased expression of the ANP-C receptor and resultant increased inhibition of adenylyl 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 adenylyl cyclase in Leydig tumour cells and PC-12 cells [13,21]. Thus the attenuation of ANP-C-receptor-mediated inhibition of adenylyl cyclase by antisense oligonucleotide in these cells also supports strongly the coupling of the ANP-C receptor to the adenylyl cyclase system in these cells. The blocking of ANP-C-receptor-mediated inhibition by antisense oligonucleotide was also specific, because angiotensin II receptor subtype (AT₁ receptor)-mediated inhibition and β -adrenergic-mediated stimulation of adenylyl cyclase were not affected by such treatment. In addition, the inability of ANP-C-receptor antisense oligonucleotide to block guanine nucleotide-, NaF- and forskolin-stimulated enzyme activity suggests further that the antisense oligonucleotide was specific for ANP-C receptor only and did not affect the post-receptor components such as the G-protein or the catalytic subunit of adenylyl cyclase. Furthermore, ANP-A/B-receptor-mediated stimulation of cGMP levels was also not blocked by such treatment. On the other hand, the enhanced stimulation of cGMP levels by ANP-C-receptor antisense treatment is intriguing and may suggest a possible interaction between the two receptors. It may be possible that antisense oligonucleotide, in preventing ANP-mediated reduction in cAMP, might remove negative constraints exerted by cAMP on 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].

Figure 7 Effect of antisense-oligodeoxynucleotide treatment on ANP₉₉₋₁₂₆-mediated increases in cGMP levels in A10 VSMCs

A10 VSMCs were incubated in the absence (control, $\circ$) or presence of 5 μ M antisense oligodeoxynucleotide ($\bullet$) for 48 h as described in the Experimental Procedures section. The cells were preincubated with 1 mM IBMX for 5 min and were incubated further in the absence or presence of various concentrations of ANP₉₉₋₁₂₆ for 10 min at 37 $^{\circ}$ C. cGMP levels were determined by radioimmunoassay using an RIA kit from Biomedical Technologies. Values are means $\pm$ S.E.M. of the separate experiments.

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 adenylyl 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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