Natriuretic peptide receptor C (NPR-C) is a disulfide-linked homodimer with an approximately 440-amino acid extracellular domain and a 37-amino acid cytoplasmic domain; it functions in the internalization and degradation of bound ligand. The use of NPR-C-specific natriuretic peptide analogs has implicated this receptor in mediating the inhibition of adenyly cyclase or activation of phospholipase C. In the present studies we have investigated the role of the cytoplasmic domain of NPR-C in signaling the inhibition of adenyly cyclase. Polyclonal rabbit antisera were raised against a 37-amino acid synthetic peptide (R37A) corresponding to the cytoplasmic domain of NPR-C. Incubation of anti-R37A with rat heart particulate fractions blocked atrial natriuretic peptide-dependent inhibition of adenyly cyclase. The cytoplasmic domain peptides R37A and TMC (10 residues of transmembrane domain appended on R37A) were equipotent in inhibiting adenyly cyclase (K_1 = 1 nM) in a GTP-dependent manner, whereas K37E (a scrambled peptide control for R37A) did not inhibit adenyly cyclase activity. Prior incubation of membranes with pertussis toxin blocked R37A or TMC inhibition of cAMP production. Detergent solubilization of the rat heart particulate fraction destroyed natriuretic peptide inhibition of adenyly cyclase, but TMC was able to inhibit cAMP production in a dose-dependent manner. Our results provide evidence that the 37-amino acid cytoplasmic domain of NPR-C is sufficient for signaling inhibition of adenyly cyclase through a pertussis toxin-sensitive G protein.

The natriuretic peptides are a family of three polypeptide hormones termed atrial natriuretic peptide (ANP), β brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) (1–3). The role of ANP and BNP as endocrine hormones is apparently to be antagonists to vasopressin and endothelins and to the renin/angiotensin/aldosterone system (1, 4). The role of CNP in vivo is less well defined. Although CNP may not be a significant modulator of diuresis or natriuresis (5, 6), it is a vasodilator expressed by endothelial cells (3, 7).

Two classes of natriuretic peptide receptor (NPR) have been defined by molecular cloning. The first class includes the membrane guanylyl cyclases, NPR-A (8, 9) and NPR-B (10, 11). For this class of receptor ligand binding activates synthesis of the intracellular second messenger cGMP by the cytoplasmic catalytic domain. The second class of natriuretic peptide binding site is a non-guanylyl cyclase receptor (12) termed clearance, NPR-C or ANP-R_2. This receptor is homologous in its extracellular domain to NPR-A and NPR-B, exists as a disulfide-linked homodimer, and has a 37-amino acid cytoplasmic domain (13, 14). The role of NPR-C has originally been considered in terms of the clearance of bound ligand by internalization and degradation (15, 16). However, work from a number of laboratories has implicated NPR-C in mediating signal transduction, either inhibition of adenyly cyclase or activation of phospholipase C (for review, see Refs. 17 and 18).

The ligand specificity of the three NPRs has been well defined, with ANP and BNP as agonists for NPR-A, and CNP for NPR-B (19–21). NPR-C has much broader specificity, binding all three natriuretic peptides as well as smaller peptide analogs that do not activate guanylyl cyclase (15). In vivo many of the responses to natriuretic peptides are thought to be mediated by cGMP, including vasorelaxation (22, 23), increased glomerular filtration, and inhibition of tubular sodium reabsorption (24–26). The involvement of NPR-C in mediating physiological responses to ANP has been inferred by pharmacology; ANP variants that are ineffective in binding and activating receptor guanylyl cyclases will bind to NPR-C and are efficacious in the inhibition of adenyly cyclase or activation of phospholipase C. These responses are dependent on the presence of guanine nucleotides (27, 28), suggesting a receptor-coupled phenomenon. Blocking of these ANP signal transduction pathways by pertussis toxin treatment is similar to the classical G protein-coupled adenyly cyclase inhibitory pathways (29–31) and suggests either the direct involvement of G_i or indirect involvement of G_o in the coupling of NPR-C to adenyly cyclase (32–35).

In this report we investigated whether the 37-amino acid cytoplasmic domain of NPR-C is involved in mediating pertussis toxin-sensitive signal transduction. Antibodies specific for the cytoplasmic domain inhibit signaling, and free cytoplasmic domain peptide inhibits adenyly cyclase in a manner consistent with G protein coupling. These data strongly implicate NPR-C in signal transduction.
NPR-C Cytoplasmic Domain Inhibits Adenylyl Cyclase

**EXPERIMENTAL PROCEDURES**

**Materials**—ATP, cAMP, and isoproterenol were purchased from Sigma. Creatine kinase (EC 2.7.3.2), myokinase (EC 2.7.4.3), and GTP·s were purchased from Boehringer Mannheim. [α-32P]ATP was from Amersham Corp. PT was from List Biochemicals (Campbell, CA). Rat ANP and the ring-deleted ANP analog cANP (4–23) were from Peninsula Laboratories (Belmont, CA). The purified protein derivative of tubulin was purchased from Statens Seruminstitut, Copenhagen, Denmark. Anti-rabbit horseradish peroxidase was from Sigma. Goat serum, biotinylated anti-rabbit antibody, the avidin/biotin blocking kit, and the ABC reagent were from Vector Laboratories (Burlingame, CA). Synthetic peptides R37A and TMC were synthesized and purified by standard solid phase techniques. K37E was custom synthesized by Peninsula Laboratories. The peptide was purified by reverse phase high performance liquid chromatography, analyzed by mass spectrometry, and its quantitation by amino acid analysis was performed to confirm the amino acid composition.

**Preparation of Heart Particulate Fractions**—The synthetic peptide termed R37A, corresponding to the 37-amino acid cytoplasmic domain of bovine NPR-C (see Table I) was conjugated to the purified protein derivative of tubulin with glutaraldehyde and used to immunize New Zealand White rabbits as described previously (20). The IgG fraction from goat serum was purified by protein A affinity chromatography (20).

**Western Blot Analysis**—Control samples were prepared from 293 cells expressing recombinant rat NPR-C using Triton X-100 non-ionic detergent as described previously (20). Rat heart membranes were prepared by homogenization in ice-cold 50 mM Hepes, 1 mM EDTA, 0.7 mg/ml pepstatin A, 0.5 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 10 mM sucrose, then centrifuged at 4°C for 10 min. The pellet was washed twice in 10 mM MgCl₂, 100 mM NaCl, 0.5 mM cAMP, 1 mM 3-isobutyl-1-methyloxanthine, 0.1 mM EDTA, 10 μM GTP·s, and an ATP-regenerating system consisting of 2 mM creatine phosphate, 0.1 mg of creatine kinase, and 0.1 mg of myokinase/ml in a final volume of 200 μl. Incubations were initiated with the addition of reaction mixture to the membranes (30–70 μl) which had been preincubated at 37°C for 10 min. The reactions, conducted in triplicate at 37°C for 10 min, were terminated by the addition of 0.6 μl of 120 mM zinc acetate, cAMP was purified by coprecipitation of other nucleotides with ZnCO₃ by the addition of 0.5 ml of 144 mM Na₂CO₃ and by subsequent chromatography, using the double column system (39). Under these assay conditions, adenylyl cyclase activity was linear with respect to protein concentration and time of incubation.

**Adenylyl Cyclase Activity Determination**—Adenylyl cyclase activity was determined by measuring [32P]cAMP formation from [α-32P]ATP as described previously (33, 37). The typical assay medium contained 50 mM glycylglycine, pH 7.5, 0.5 mM MgATP, [α-32P]ATP (1.5 × 10⁶ cpm), 5 mM MgCl₂, 100 mM NaCl, 0.5 mM cAMP, 1 mM 3-isobutyl-1-methyloxanthine, 0.1 mM EDTA, 10 μM GTP·s, and an ATP-regenerating system consisting of 2 mM creatine phosphate, 0.1 mg of creatine kinase, and 0.1 mg of myokinase/ml in a final volume of 200 μl. Incubations were initiated with the addition of reaction mixture to the membranes (30–70 μl) which had been preincubated at 37°C for 10 min. The reactions, conducted in triplicate at 37°C for 10 min, were terminated by the addition of 0.6 μl of 120 mM zinc acetate, cAMP was purified by coprecipitation of other nucleotides with ZnCO₃ by the addition of 0.5 ml of 144 mM Na₂CO₃ and by subsequent chromatography, using the double column system (39). Under these assay conditions, adenylyl cyclase activity was linear with respect to protein concentration and time of incubation.

**RESULTS**

**Antibodies Specific for the NPR-C Cytoplasmic Domain**—The binding of ANP to NPR-C leads to a PT-sensitive inhibition of adenylyl cyclase in several tissues (17, 18); therefore, the 37-amino acid cytoplasmic domain of NPR-C could mediate signal transduction. To address this, polyclonal rabbit antisera against the 37-amino acid sequence corresponding to the cytoplasmic domain of bovine NPR-C were raised (Table I). The purified protein derivative-coupled R37A peptide was very immunogenic, with serum titers of 1:500,000 when tested by Western blot to detect immunogenic, with serum titers of 1:500,000 when tested by Western blot. Recombinant rat NPR-C expressed in 293 cells was detected as a nonreduced homodimer of approximately 150 kDa and comigrated with NPR-C homodimer in a rat heart membrane fraction (Fig. 1A). Blocking of immunoreactivity with R37A peptide (Fig. 1B) demonstrates the specificity of anti-R37A and reveals a nearly identical pattern of nonspecific background in the heart sample on un-
blocked (Fig. 1A, lane 2) and blocked (Fig. 1B, lane 2) filters. The reactivity of anti-R37A, raised against the bovine NPR-C cytoplasmic domain sequence, with rat (Fig. 1) and human NPR-C is consistent with the high degree of conservation of this 37-amino acid cytoplasmic domain (Table I).

Effect of Anti-R37A on ANP-mediated Inhibition of Adenylyl Cyclase—To extend the pharmacology implicating NPR-C in the inhibition of adenylyl cyclase, the NPR-C-specific anti-R37A antibodies were tested for blocking this pathway. Using anti-R37A, and the human NPR-A-specific antibody A-4 as a control (20), heart particulate fraction was preincubated with antibodies at a 1:100,000 dilution (0.1 μg/ml) prior to assay of adenylyl cyclase activity (Fig. 2). In this ANP concentration-response assay for inhibition of adenylyl cyclase, there was 30–40% maximal inhibition of enzyme activity in the presence of control antibody A-4. The NPR-C-specific anti-R37A resulted in marked attenuation of the ANP effect, with at most 10% maximal inhibition of the enzyme at 10^{-6} M ANP. This experiment provides evidence for the involvement of the 37-amino acid cytoplasmic domain of NPR-C in mediating ANP-dependent inhibition of adenylyl cyclase. This result also suggests that NPR-C, and not another NPR with similar pharmacology, is mediating ANP signal transduction.

Effect of R37A and TMC on Adenylyl Cyclase Activity—The next series of experiments was designed to test the hypothesis that the cytoplasmic domain alone may mimic the ANP-dependent signaling of NPR-C. For this purpose, we used the R37A synthetic peptide and a 47-amino acid peptide, TMC (Table I), which consists of 10 additional residues from the transmembrane domain of NPR-C. The results shown in Fig. 3 demonstrate that R37A and TMC were equipotent and inhibited rat heart adenylyl cyclase in a concentration-dependent manner with an apparent K_i of about 1 nM. In comparison, ANP was more potent (K_i = 0.1 nM) and inhibited adenylyl cyclase activity by about 50–55% compared with 35–45% for R37A and TMC. Thus, the free cytoplasmic domain of NPR-C can inhibit adenylyl cyclase activity, whereas the NPR-C homodimer inhibits adenylyl cyclase in a ligand-dependent manner. The inhibitory effect of these peptides was not due to the presence of positive charges per se, since the R37A scrambled peptide K37E has the same amino acid composition and did not exert any inhibitory effect on adenylyl cyclase (Fig. 4).

Dependence of R37A- and TMC-mediated Inhibition of Adenylyl Cyclase on Guanine Nucleotides—The inhibitory effect of cANP (4–23) or ANP on adenylyl cyclase activity was determined as described under “Experimental Procedures” in the presence of 10 μM GTPγS. The basal adenylyl cyclase activity was (416 ± 10 pmol of cAMP/mg of protein/10 min. Values are the means ± S.E. of three separate experiments.

Fig. 1. Immunoblot of NPR-C. Membranes from 293 cells expressing recombinant rat NPR-C (lanes 1) or membranes from rat heart (lanes 2) were fractionated by nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed with anti-R37A antibody (panel A) or anti-R37A antibody in the presence of excess R37A peptide (panel B). Blots were developed with anti-rabbit peroxidase and chemiluminescence. The position of the immunoreactive NPR-C homodimer is indicated by an arrowhead on the left with molecular weight standards.

Fig. 2. Anti-R37A blocks ANP inhibition of adenylyl cyclase. Enzyme activity was determined in rat heart particulate fraction treated with NPR-C receptor antibody or control antibody specific for human NPR-A. Values are the means ± S.E. of three separate experiments. 10 μM GTPγS-stimulated adenylyl cyclase activity for control and anti-R37A antibody-treated samples was 664 ± 9 and 670 ± 9 pmol of cAMP/mg of protein/10 min, respectively.

Fig. 3. Inhibition of rat heart adenylyl cyclase activity. The effect of ANP and the two NPR-C cytoplasmic domain peptides R37A and TMC on adenylyl cyclase activity was determined as described under “Experimental Procedures” in the presence of 10 μM GTPγS. The basal adenylyl cyclase activity was 416 ± 10 pmol of cAMP/mg of protein/10 min. Values are the means ± S.E. of three separate experiments.
the intact receptor, the response should be sensitive to guanine nucleotides. Fig. 5 illustrates the effect of R37A on adenyl cyclase from heart particulate fractions in the absence and presence of various concentrations of GTPγS alone (basal) or in combination with 0.1 μM R37A.

Effect of PT on R37A- and TMC-mediated inhibition of adenylyl cyclase—Additional evidence for the role of a G protein-coupled pathway was obtained using PT. As shown in Fig. 6, R37A (panel A) and TMC (panel B) inhibited adenylyl cyclase activity in a concentration-dependent manner in heart particulate fractions, which was completely abolished by PT treatment. These results suggest that the inhibition of adenyl cyclase by R37A or TMC, like ANP or cANP(4–23), also is dependent on the presence of guanine nucleotides.

Effect of TMC on Adenylyl Cyclase Activity in Solubilized Membranes—To investigate if NPR-C or the cytoplasmic domain peptide TMC can inhibit adenyl cyclase in solubilized
membranes, heart particulate fraction was solubilized in nonionic detergent and used to assay for effects on adenylyl cyclase activity. 10 μM GTPγS stimulated enzyme activity in control and solubilized heart membranes by about 7- and 3-fold, respectively, whereas isoproterenol and c-ANP(4–23) were unable to elicit any inhibition of the enzyme activity. The simplest interpretation of the inhibition of adenylyl cyclase by TMC in the solubilized membrane fractions is that TMC may be (directly?) activating a G protein-coupled pathway.

Discussion

Previous observations, from our group and others, on ANP inhibition of adenylyl cyclase through a receptor with NPR-C-like pharmacology (17, 18), led us to design experiments to support further the role of this receptor. We raised polyclonal antibodies against the short 37-amino acid cytoplasmic domain of NPR-C (synthetic peptide R37A). The specificity of anti-R37A antiserum against NPR-C was established by Western blotting against recombinant NPR-C and rat heart particulate fraction. Blocking of ANP signaling by anti-R37A in a rat heart particulate fraction indicates that NPR-C is involved in signaling, and another unidentified receptor need not be invoked to account for this ANP signal transduction pathway.

Our studies exploring the effect of the two NPR-C cytoplasmic domain peptides R37A and TMC on signal transduction are consistent with previous reports on ANP signaling via a PT-sensitive G protein (17, 18). The inhibitory effect of these peptides on adenylyl cyclase was not due to the net positive charge present (i.e. amino acid composition) since the scrambled peptide K37E with the same composition as R37A but a different sequence did not inhibit adenylyl cyclase activity. These data also suggest that there is structural specificity of the ANP-C cytoplasmic domain peptides to exert the inhibitory effects on adenylyl cyclase. The inability of cANP(4–23) and isoproterenol to inhibit or stimulate adenylyl cyclase, respectively, in detergent-solubilized particulate fraction suggests that the NPR-C and β-adrenergic receptors are dissociated or uncoupled from adenylyl cyclase by detergent treatment. However, the inhibition of adenylyl cyclase by TMC in these detergent-solubilized membrane preparation lends itself to the interpretation that there may be a direct peptide/G protein interaction.

Receptor coupling to G proteins in hormonal inhibition of adenylyl cyclase is typically with seven transmembrane domain receptors such as the angiotensin II, α2-adrenergic, endothelin B, muscarinic 2 and 4 subtypes, or the dopamine D2 receptor, in which the third cytoplasmic loop plays a role in determining G protein coupling and specificity (41–43). However, not all pathways for G protein activation need necessarily involve a seven-transmembrane domain receptor. The neuronal protein GAP-43 stimulates GTP binding to Go, suggesting that an intracellular protein can activate a G protein pathway (44). In addition, mastoparan, a 14-amino acid wasp venom peptide, activates G proteins directly (45). The single transmembrane domain insulin and insulin-like growth factor II/mannose 6-phosphate receptors have also been reported to

**Table II**

| Additions          | CAMP/mg protein/10 min | % of control<sup>a</sup> | Solubilized membranes | % of control<sup>a</sup> |
|--------------------|------------------------|--------------------------|------------------------|--------------------------|
| None               | 59 ± 2                 | 100                      | 114 ± 6                | 100                      |
| GTPγS (10 μM)      | 440 ± 12               | 647 ± 20                 | 458 ± 13               | 100                      |
| GTPγS + isoproterenol (50 μM) | 401 ± 8               | 100                      | 449 ± 7                 | 98                       |
| GTPγS + acetate buffer<sup>c</sup> | 298 ± 6               | 74                       | 425 ± 28                | 100                      |
| GTPγS + cANP(4-23) (0.1 μM) | 279 ± 8               | 69                       | 268 ± 12               | 63                       |
| GTPγS + cANP(4-23) (0.1 μM) | 2,076 ± 101           | 3,519                    | 446 ± 8                | 105                      |

<sup>a</sup> Adenylyl cyclase activity was determined in control and solubilized heart membranes as described under "Experimental Procedures." Values are means ± S.E., of three experiments, each done in triplicate.

<sup>b</sup> Calculated as percent of adenylyl cyclase activity in the presence of 10 μM GTPγS or absence of GTPγS (none) for forskolin.

<sup>c</sup> Excipient control for TMC and cANP(4–23).
activate protein-coupled pathways (46–48). The relationship of these activation pathways to that reported here remains to be explored.

Regulation of cellular cAMP production is subject to an interplay of positive and negative signals of at least six isoforms of adenylyl cyclase (30, 31). Inhibition of cAMP production is sensitive to this pathway (49). Taken together, it may thus be suggested that NPR-C-mediated inhibition of rat heart adenylyl cyclase by cytoplasmic domain peptides may be through their interaction with Gα1 and Gα3 and not through Gβγ or Gβy.

In conclusion, our data provide evidence for the involvement of NPR-C in signal transduction and extend previous pharmacological studies on G protein-mediated inhibition of adenylyl cyclase with NPR-C-specific ANP variants. A short 37-amino acid cytoplasmic domain appears to be sufficient to activate this response. The role of this signal transduction pathway in the in vivo physiology of the natriuretic peptides ANP, BNP, or CNP remains to be explored fully. The use of guanylyl cyclase-specific natriuretic peptide variants (53) or the application of gene targeting techniques to eliminate receptor expression may help address this question.

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