Identification of the G protein-activating sequence of the single-transmembrane natriuretic peptide receptor C (NPR-C)

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Zhou, Huiping, and Karnam S. Murthy. Identification of the G protein-activating sequence of the single-transmembrane natriuretic peptide receptor C (NPR-C). Am J Physiol Cell Physiol 284: C1255–C1261, 2003; 10.1152/ajpcell.00520.2002.—Rat natriuretic peptide clearance receptor (NPR-C) contains four sequences capable of inhibiting adenylyl cyclase. We have undertaken mutational and deletion studies on the intracellular domain of rat NPR-C to determine which of these sequences is functionally relevant. Nine mutant receptors were constructed by deletion of 11 or 28 COOH-terminal residues or by site-directed mutagenesis of basic residues in a 17-amino acid sequence, R469RNHQESNIGKHREL485, corresponding to the main active peptide. Substitution of arginine residues (R469R470) flanking the NH2 terminus abolished G1 and G2 and PLC-β2 activities and inhibition of adenylyl cyclase. Substitution of one or two basic residues (H481 and/or R482 or R485) in the COOH-terminal motif (H481REL485) greatly decreased or abolished G protein and PLC-β activities and inhibition of adenylyl cyclase. This implies that sequences NH2-terminal to the motif or COOH-terminal to R485 could not sustain receptor activity in situ, although they exhibited activity when used as synthetic peptides. Deletion of the 11 COOH-terminal residues (E466 to A469) suggested an autoinhibitory function for this sequence. We conclude that the 17-amino acid sequence (R469 to R485) in the middle region of the intracellular domain of NPR-C is both necessary and sufficient for activation of G proteins and effector enzymes.

G PROTEIN-ACTIVATING SEQUENCES have been identified in the intracellular domains of various single-transmembrane receptors. Okamoto et al. (13) have shown that a 14-amino acid intracellular sequence of the human insulin-like (IGF) II/mannose 6-phosphate receptor activates pertussis toxin-sensitive G proteins (G12 > G11 > G13 > Gα). The sequence is characterized by the presence of two NH2-terminal basic residues and a COOH-terminal motif BBXXB, where B and X represent basic and nonbasic residues, respectively (13–16, 22). Synthetic short cationic peptides containing 10–26 amino acid residues with a pair of NH2-terminal basic residues and COOH-terminal BBXXB, BBXB, or BXB motifs activate G proteins, usually one or more isoforms of G1 (4, 9, 23). Similar active sequences have been identified in the cytoplasmic regions of seven-transmembrane muscarinic m1-m5 and adrenergic α2 and α1b receptors and in the COOH-terminal region of the 7- to 11-transmembrane polycystin-1 receptor (14, 19, 22, 26).

The single-transmembrane natriuretic peptide clearance receptor, NPR-C, has been shown to inhibit adenylyl cyclase in a G protein-dependent, pertussis toxin-sensitive fashion (1, 11, 18). A synthetic peptide corresponding to the entire 37-amino acid intracellular domain of NPR-C inhibited adenylyl cyclase in rat cardiac membranes, and a polyclonal antibody to this peptide blocked atrial natriuretic peptide (ANP)-induced inhibition of adenylyl cyclase (2). Our studies in smooth muscle cells have identified G11 and G12 as the G proteins selectively activated by NPR-C (9–11). NPR-C is widely expressed in vascular and visceral smooth muscle. In smooth muscle of the gut, NPR-C is coexpressed with NPR-B but not NPR-A (10, 11). In gastric and intestinal smooth muscle cells that also express nitric oxide synthase III (NOS-III), NPR-C activated NOS-III and inhibited adenylyl cyclase via the α-subunits of both G11 and G12 (10). In NOS-deficient cells, such as smooth muscle cells from guinea pig tenia coli, NPR-C inhibited adenylyl cyclase via the α-subunits of G11 and G12 and activated phospholipase C (PLC)-β3 via the βγ-subunits (11). The G protein-activating domain of NPR-C was determined by using receptor-derived synthetic peptides corresponding to NH2-terminal, COOH-terminal, and middle regions of the 37-amino acid intracellular domain of human NPR-C (9). A 17-amino acid sequence of the middle region (R469 to R485) that possesses two NH2-terminal arginine residues, R469R470, and the COOH-terminal motif, H481REL485 (BBXXB), selectively activated G11 and G12, inhibited adenylyl cyclase via the α-subunits, and activated PLC-β3 via the βγ-subunits in permeabilized tenia smooth muscle cells and in smooth muscle membranes in a similar fashion to the selective NPR-C ligand, eANP-4–23 (9). Cotransfection of NPR-C and NOS-III into COS-1 cells or transfection of NOS-III into cultured tenia coli muscle cells confirmed the ability of NPR-C to activate NOS-III (10).

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The intracellular domain of rat NPR-C is similar but not identical to that of human NPR-C and contains several sequences that could potentially activate G proteins. Several peptides corresponding to these sequences were shown to inhibit adenylyl cyclase in cardiac and vascular smooth muscle membranes, including a 17-amino acid sequence from the middle region of the intracellular domain of rat NPR-C that more than one region of the intracellular domain of human NPR-C (9, 17). The study raised the possibility that more than one region of the intracellular domain of rat NPR-C could participate in G protein activation. Although synthetic peptides are useful in locating G protein-activating sequences in the intracellular domain, they do not provide decisive evidence as to which sequence of the receptor in situ activates specific G proteins.

In the present study, we attempted to resolve this issue by deletion and site-directed mutagenesis of critical basic residues. Nine mutant NPR-C receptors were constructed in which single or dual basic amino residues in the NH2-terminal (BB) and COOH-terminal motif (BBXB) were mutated to Leu. The mutants were stably expressed in COS-1 cells, and the activities of G proteins were utilized.

**MATERIALS AND METHODS**

Construction of mutant receptor cDNAs. Mutant receptor cDNAs were constructed by the Megaprimer method (20), with three PCR primers used to perform two rounds of PCR. The product of the second PCR was used as one of the PCR primers for the second PCR. PCR was performed under standard conditions [10 mM KCl, 10 mM (NH4)2 SO4, 20 mM Tris•HCl, 2 mM MgSO4, pH 8.8, 200 μM dNTP, and 100 ng of each primer] in a final volume of 50 μl using 2.5 units of Taq DNA polymerase. The wild-type rat NPRC receptor cDNA was subcloned into pcDNA3 as was used in the template. COOH-terminal deletion mutant cDNAs were amplified by PCR using Taq DNA polymerase, and all the mutant cDNAs were subcloned into pcDNA3 expression vector. Mutants were sequenced to confirm that the mutagenesis or deletion was successful. The primer sequences are listed in Table 1.

The sequence of the G protein-activating peptide derived from the middle region of the intracellular domain was used as a guide in the construction of mutants. The sequences of the intracellular domain of rat wild-type and nine mutant NPR-C receptors are listed in Fig. 1. The mutations consisted of substitutions of leucine for single or adjacent basic amino acid residues at the NH2-terminal (R469 and/or R470) and COOH-terminal motif (H481 and/or R482 or R485).

Cell culture and stable expression of wild-type and mutant NPR-C. COS-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, penicillin (50 U/ml), streptomycin (50 μg/ml), and gentamicin (100 μg/ml) at 37°C in a humidified atmosphere of 95% air and 5% CO2. The pcDNA3 expression vector containing either wild-type or mutant cDNA NPR-C was transfected into COS-1 cells using Lipofectamine. Transfected cells were isolated in a medium containing 500 μg/ml geneticin (G418). G418-resistant cells were recultured, and confluent monolayers were screened for expression of wild-type and mutant NPR-C by RT-PCR and radioligand binding using [125I]ANP. Selection pressure for clonal cell lines was maintained by the addition of 100 μg/ml G418 to the culture medium. For all studies, transfected cells in monolayers at ~90% confluence were utilized.

**[125I]ANP binding assay.** Confluent cultures of COS-1 cells were detached with 0.25% trypsin and 1 mM EDTA. Cells were immediately centrifuged at 500 g for 5 min at 4°C. The cell pellet was washed with phosphate-buffered saline (PBS) and homogenized. The homogenate was centrifuged at 30,000 g for 30 min at 4°C, and the membrane pellet was suspended in 50 mM Tris•HCl containing 5 mM MgCl2, 0.5% BSA, 0.5% bacitracin, and 10 μg/ml aprotinin. Membranes (30 μg) were incubated with 50 pM [125I]labeled ANP at 25°C for 30 min in the presence or absence of various concentrations of unlabeled cANP4–23. The reaction was terminated with the addition of 5 ml of ice-cold PBS. The solution was filtered under vacuum through a Whatman GF/C glass filter, and the filter was then washed three times with 5 ml of PBS. The radioactivity retained on the filter was counted, and the specific binding was calculated as the difference between the counts in the presence and absence of the competitor.
total binding and nonspecific binding measured in the presence of 10 μM cANP4–23.

Assay of PLC-β activity. Inositol phosphates were measured as described previously using anion exchange chromatography (9–11, 25). COS-1 cells expressing wild-type or mutant rat NPR-C in six-well culture plates were labeled with myo-[2-3H]inositol in inositol-free DMEM (1 μCi/well) for 24 h. The cells were washed with PBS and treated with cANP4–23 for 60 s in 1 ml of 25 mM HEPES medium (pH 7.4) containing (in mM) of 115 NaCl, 5.8 KCl, 2.1 KH2PO4, 2 CaCl2, 0.6 MgCl2, and 14 glucose. The buffer was aspirated, and the reaction was terminated by the addition of 940 μl of chloroform–methanol–HCl (50:100:1). The samples were extracted with 340 μl of chloroform and 340 μl of H2O and centrifuged at 1,000 g for 15 min. The upper aqueous phase was applied to Dowex AG-1 column. The column was washed with 10 ml of H2O and 10 ml of 5 mM sodium phosphate. The [3H]inositol phosphates were eluted with 0.8 M ammonium formate–0.1 M tetraborate–60 mM ammonium formate. The [3H]inositol was obtained from NEN Life Science Products; polyclonal G protein antibodies were from Santa Cruz Biotechnology; and all other chemicals were from Sigma. DNA sequencing was done by MCV-VCU Nucleic Acids Core Facility.

Identification of receptor-activated G proteins by [35S]GTPγS binding assay. G protein activation by cANP4–23 was measured by an adaptation of the method of Okamoto et al. (12) as described previously (9–11). COS-1 cells were homogenized in 20 mM HEPES (pH 7.4) containing 2 mM MgCl2, 1 mM EDTA, and 2 mM 1,4-dithiothreitol (DTT). The homogenate was centrifuged at 30,000 g for 30 min at 4°C, and the membranes were solubilized at 4°C in 20 mM HEPES (pH 7.4) buffer containing 0.5% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS). The cell membranes were incubated with 100 nM [35S]GTPγS in a solution containing 10 mM HEPES (pH 7.4), 0.1 mM EDTA, and 10 mM MgCl2 for 20 min at 37°C in the presence or absence of agonist. The reaction was stopped with 10 volumes of 100 mM Tris-HCl (pH 8.0) containing 10 mM MgCl2, 100 mM NaCl, and 20 μM GTP. The membranes were incubated for 2 h on ice in wells precoated with specific antibodies to G12α and G13α. The wells were washed with phosphate buffer containing 0.05% Tween 20, and the radioactivity from each well was counted by liquid scintillation.

Construction of mutant receptors. The sequence of the synthetic G protein-activating peptide derived from the middle region (R469 to R485) of the intracellular

**RESULTS**

**Construction of mutant receptors.** The sequence of the synthetic G protein-activating peptide derived from the middle region (R469 to R485) of the intracellular
domain of rat NPR-C was used as a guide in the construction of the NPR-C mutants. The mutations consisted of substitutions of leucine for single or adjacent basic amino acid residues at the NH2-terminal and COOH-terminal motif of this sequence (Fig. 1). In seven mutants (mutant 3 to mutant 9), single or adjacent basic amino acid residues at the NH2-terminal (R469 and/or R470) and COOH-terminal motif (H481 and/or R482 or R485) were substituted with leucine. Mutants 1 and 2 consisted of deletions of 11 (E486 to A496, mutant 1) and 28 COOH-terminal amino acid residues (E486 to A496, mutant 9) to the active synthetic peptide completely inhibited Go11 and Go12 activities.

Stable expression and radioligand binding of wild-type and mutant NPR-C. Wild-type and all nine mutant receptors stably expressed in COS-1 cells bound [125I]ANP with similar affinities. The results are shown in Fig. 2Aa for leucine-substituted mutants 3 to 9 (see Fig. 1) and in Fig. 2Ab for COOH-terminal-deleted mutants 1 and 2. The IC50 values for inhibition of [125I]ANP binding by the selective NPR-C ligand, cANP4–23, in wild-type, and all nine mutant NPR-C were close to 1 nM (range: 0.8 ± 0.4 to 1.2 ± 0.3 nM) (Fig. 2, Aa and Ab). [125I]ANP binding in the absence of competitor was similar (2,195 ± 232 to 2,505 ± 302 cpm/mg protein) for wild-type and all nine mutant receptors, suggesting similar levels of expression.

G protein-activating potential of NPR-C mutants. In COS-1 cells expressing wild-type NPR-C, cANP4–23 (1 μM)-activated G11 and G12 (i.e., increased [35S]GTPγS binding to G11α and G12α by 401 ± 32% and 476 ± 28%, respectively) (Fig. 3). Deletion of 11 COOH-terminal amino acid residues (E486 to A496, mutant 1) did not affect Go11 activity but significantly increased Go12 activity (Fig. 3). However, deletion of the 17-amino acid sequence in the middle region (R469 to A496, mutant 2) corresponding to the active synthetic peptide completely inhibited Go11 and Go12 activities.

Substitution of single NH2-terminal arginine residues of this sequence (R469L or R470L, mutants 3 and 4) slightly reduced Go11 and Go12 activities (11 ± 3 to 22 ± 5%), whereas substitution of both residues (R469L and R470L, mutant 5) abolished Go11 and Go12 activities (Fig. 3). Substitution of H481 (H481L, mutant 6) or R482 (R482L, mutant 7) in the COOH-terminal motif decreased Go11 and Go12 activities by 35 ± 4 to 54 ± 6%, respectively, whereas substitution of both of these residues (mutant 8) abolished G protein activity (Fig. 3). Substitution of the ultimate arginine residue (R485L) in the COOH-terminal motif (mutant 9) also abolished G protein activity.

Activation of PLC-β by wild-type and mutant NPR-C. Activation of PLC-β by wild-type and mutant NPR-C closely paralleled activation of G11 and G12. cANP4–23 stimulated PLC-β activity in a concentration-dependent fashion (Fig. 4). PLC-β activity was previously shown to reflect activation of the PLC-β3 isofrom by the βγ-subunits of G11 and G12 (6–8, 11). Deletion of 11 COOH-terminal amino acid residues (E486 to A496, mutant 1) augmented PLC-β activity, shifting the concentration-response curve to the left (Fig. 4). However, deletion of 28 COOH-terminal amino acid residues,
and R470L, whereas substitution of both arginine residues (R469L and/or R482L, R485L). Values are means of 1 or 2 basic residues in the COOH-terminal motif (R469L and/or R470L). Induced by NPR-C with mutations of 1 or both arginine residues (H481L and/or R482L) had no effect on cANP4–23 (Fig. 4).

Substitution of single NH2-terminal arginine residues (R469L or R470L, mutants 3 and 4) had no effect on cANP4–23-stimulated PLC-β activity (Fig. 5), whereas substitution of both arginine residues (R469L and R470L, mutant 5) abolished PLC-β activity (Fig. 5). Substitution of H481L (H481L, mutant 6) in the COOH-terminal motif caused a slight decrease in PLC-β activity, whereas substitution of the adjacent arginine residue (R482L, mutant 7) abolished PLC-β activity at lower concentrations of cANP4–23 and inhibited activity at the highest concentration (1 μM) by 51 ± 3% (Fig. 5). Substitution of adjacent basic residues in the motif (H481L and R482L, mutant 8) abolished PLC-β activity (Fig. 5). Substitution of the ultimate arginine residue (R485L) in the motif (mutant 9) abolished PLC-β activity at all but the highest concentration of cANP4–23 (Fig. 5B).

Inhibition of adenylyl cyclase by wild-type and mutant NPR-C. As shown previously, inhibition of adenylyl cyclase activity by cANP4–23 was mediated by the α-subunits of Gi1 and Gi2 (2, 9, 10, 11, 13). Inhibition of forskolin-stimulated adenylyl cyclase activity via wild-type and mutant NPR-C was examined at the highest concentration of cANP4–23 (1 μM). At this concentration, cANP4–23 inhibited forskolin-stimulated adenylyl cyclase activity by 69 ± 2% in COS-1 cells expressing wild-type NPR-C (Fig. 6). Deletion of 11 COOH-terminal amino acid residues (E486 to A496, mutant 1) caused significantly greater inhibition of adenylyl cyclase activity (91 ± 3 vs. 69 ± 2% for wild-type NPR-C; P < 0.01) (Fig. 6). In contrast, deletion of 28 COOH-terminal amino acid residues, including the 17-amino acid sequence in the middle region (R469 to A496, mutant 2), abolished adenylyl cyclase by cANP4–23 (Fig. 6).

Substitution of single NH2-terminal arginine residues (R469L or R470L, mutants 3 and 4) significantly decreased the inhibition of adenylyl cyclase (39 ± 4 and 41 ± 4% vs. 69 ± 2% for wild-type NPR-C; P < 0.01) (Fig. 6), whereas substitution of both arginine residues (R469L and R470L, mutant 5) abolished inhibition of adenylyl cyclase (Fig. 6). Substitution of H481L (H481L, mutant 6) or R482L (R482L, mutant 7) in the COOH-terminal motif decreased the inhibition of adenylyl cyclase (35 ± 5 and 59 ± 4%, respectively, vs. 69 ± 2% for wild-type NPR-C), whereas substitution of both residues (H481L and R482L, mutant 8) abolished inhibition of adenylyl cyclase (Fig. 6). Substitution of the ultimate arginine residue (R485L) in the COOH-terminal motif (mutant 9) abolished inhibition of adenylyl cyclase (Fig. 6).
DISCUSSION

Four peptides derived from various regions of the intracellular domain of rat NPR-C were recently shown to inhibit adenylyl cyclase activity in rat heart and vascular smooth muscle membranes (17). All four peptides were characterized by a pair of NH2-terminal basic residues and variable COOH-terminal motifs—BBXXB, BBXB, BXB, and XB (R461-H472, R469-K480, H481-H492, and R469-R485) (Fig. 1). The last sequence (R469-R485), a 17-amino acid peptide derived from the middle region of the intracellular domain, was closely similar to the 17-amino acid peptide derived from the middle region of human NPR-C previously shown by us to activate selectively G1 and G2, stimulate PLC-β3 activity, and inhibit adenylyl cyclase activity (9). We have now shown by deletion and site-directed mutagenesis of rat NPR-C expressed in COS-1 cells that only this 17-amino acid sequence within the receptor in situ is capable of activating G proteins, specifically G1 and G2. Both the NH2-terminal arginine residues (R469R470) and the COOH-terminal motif (H481RELR485) were essential to enable the receptor in situ to activate G protein and effector enzymes. Furthermore, deletion studies indicated that the 11-amino acid COOH-terminal sequence of rat NPR-C possessed G protein inhibitory activity. A similar conclusion was reached in our earlier study from the use of the corresponding synthetic peptide (9). Deletion of the entire 28-amino acid COOH-terminal sequence, which included the active 17-amino acid sequence, abolished G protein or effector enzyme activities.

Substitution of either NH2-terminal arginine residue in the active 17-amino acid sequence had a minimal effect, whereas substitution of both arginine residues (R469R470) virtually abolished agonist-stimulated G1 and G2 and PLC-β activities or the inhibition of adenyl cyclase activity. This implied that no sequence COOH-terminal to these two arginine residues is capable by itself of sustaining receptor activity in situ, including sequence H481-H492, which was capable of inhibiting adenyl cyclase activity when used as a synthetic peptide (17).

Substitution of single amino acid residues in the COOH-terminal motif (H481RELH492 or BBXB) greatly decreased agonist-stimulated G1 and G2 and PLC-β activities or inhibition of adenyl cyclase activity. These single substitutions were more effective than the corresponding substitutions at the NH2-terminus of the active sequence. In effect, substitution of the ultimate arginine or adjacent basic residues of the COOH-terminal motif abolished G protein and PLC-β activities and inhibition of adenyl cyclase activity. This implied that no sequence NH2-terminal to this motif is capable of sustaining receptor activity in situ, including sequences K461-H472 and R469-K480, which were capable of inhibiting adenyl cyclase activity when used as synthetic peptides (17).

Deletion studies suggested that the 11 COOH-terminal amino acid sequence (E486–A496) adjacent to the BBXB motif harbored inhibitory activity, reflected in greater activation of G12 and PLC-β and greater inhibition of adenylyl cyclase upon deletion. Our previous studies showed that a synthetic peptide corresponding to this sequence decreased the ability of ANP4–23 or the active 17-amino acid peptide to activate G proteins (particularly G2) and PLC-β or inhibit adenylyl cyclase (9). It is possible that the BBXB motif (R469S471H492) within this sequence enables it to bind but not activate G proteins and thus function as a competitive inhibitor.

Although the nine NH2-terminal amino acid residues flanking the G protein-activating sequence were devoid of activity, they contain the only threonine residue in the intracellular domain. In preliminary studies, we have shown this residue to be critical to desensitization and internalization of NPR-C. Selective phosphorylation of this residue by cGMP-dependent protein kinase (PKG) induced translocation of NPR-C from the plasma membrane to the cytoplasm and inhibited ANP4–23-mediated stimulation of PLC-β activity (27). NPR-C is abundantly expressed in various cells and is often coexpressed with the receptor guanylyl cyclase, NPR-B and/or NPR-A, which exhibit relatively high affinity for ANP and can generate cGMP (3, 5, 21). Generation of cGMP would lead to PKG-dependent phosphorylation of NPR-C and internalization of both receptor and ligand, thereby reducing the availability of ANP. In gastrointestinal smooth muscle cells that express NOS-III, interaction of ANP with NPR-C induces G12-dependent activation of NOS and stimulation of cGMP that could lead to similar feedback phosphorylation and internalization of NPR-C (10, 24).

In summary, the truncated intracellular domain of NPR-C contains several short cationic peptide sequences flanked by basic residues or motifs. Synthetic peptides derived from these sequences are capable of activating G. Mutational and deletion studies of the intracellular domain of the receptor in situ, however, indicate that only one of these, the 17-amino acid sequence in the middle region of the intracellular domain consisting of two NH2-terminal arginine residues and the COOH-terminal motif, HRELH492, is capable of selectively activating G1 and G2. The study emphasizes that mutational and deletion studies on the receptor in situ are essential to define the functional significance of putative active sequences.

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REFERENCES


G PROTEIN-ACTIVATING SEQUENCE OF NPR-C