Identification of the G Protein-activating Domain of the Natriuretic Peptide Clearance Receptor (NPR-C)*

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We have shown recently that the 37-amino acid intracellular domain of the single-transmembrane, natriuretic peptide clearance receptor, NPR-C, which is devoid of kinase and guanylyl cyclase activities, activates selectively G_{i1} and G_{i2} in gastric and tenia coli smooth muscle. In this study, we have used synthetic peptide fragments of the N-terminal, C-terminal, and middle regions of the cytoplasmic domain of NPR-C to identify the G protein-activating sequence. A 17-amino acid peptide of the middle region (Arg^{469}-Arg^{485}), denoted Peptide 4, which possesses two N-terminal arginine residues and a C-terminal B-B-X-X-B motif (where B and X are basic and non-basic residues, respectively) bound selectively to G_{i1} and G_{i2}, activated phospholipase C-\beta3 via the \betay subunits, inhibited adenylyl cyclase, and induced smooth muscle contraction, in similar fashion to the selective NPR-C ligand, cANP4–23. A similar sequence (Peptide 3), but with a partial C-terminal motif, had minimal activity. Sequences which possessed either the N-terminal basic residues (Peptide 1) or the C-terminal B-B-X-X-B motif (Peptide 2) were inactive. Peptide 2, however, inhibited G protein activation and cellular responses mediated by the stimulatory Peptide 4 and by cANP4–23, suggesting that the B-B-X-X-B motif mediated binding but not activation of G protein, thus causing Peptide 2 to act as a competitive inhibitor of G protein activation.

The single-transmembrane natriuretic peptide clearance receptor, NPR-C,† possesses a 37-amino acid intracellular domain devoid of kinase and guanylyl cyclase activities (1, 2). Although truncated, the intracellular domain binds pertussis toxin-sensitive G proteins and activates various effector enzymes (3–6). Recent studies in visceral smooth muscle have identified the G proteins activated by NPR-C as G_{i1} and G_{i2} (7, 8). In tenia coli smooth muscle, NPR-C selectively bound G_{i1} and G_{i2} (G_{i2} > G_{i1}) and activated phospholipase C-\beta3 (PLC-\beta3) via the \betay subunits of both G proteins and inhibited adenylyl cyclase via the \alpha subunit (8). In gastric smooth muscle, which unlike tenia coli, expresses endothelial nitric-oxide synthase (eNOS) (9), NPR-C selectively bound G_{i1} and G_{i2} (G_{i2} > G_{i1}), activated eNOS, and inhibited adenylyl cyclase via the \alpha subunits of both G proteins and activated PLC-\beta presumably via the \betay subunits (7, 8). A synthetic peptide corresponding to the 37-amino acid intracellular domain of NPR-C inhibited adenylyl cyclase activity in cardiac membranes in a pertussis toxin-sensitive fashion implying that this domain was the locus of G protein binding and activation (10). The specific motifs within this domain responsible for G protein activation have not been identified.

Both single- and multitransmembrane receptors possess intracellular sequences capable of activating G proteins. Okamoto et al. (11, 12) have identified a 14-amino acid intracellular sequence (Arg^{2410}–Lys^{2425}) of the human insulin-like growth factor (IGF) II/mannose 6-phosphate receptor that activates G proteins with an order of potency of G_{i2} > G_{i1} > G_{s}. The sequence is characterized by the presence of two N-terminal basic residues and a C-terminal B-B-X-X-B motif, where B and X represent basic and non-basic residues, respectively. A 9-amino acid peptide sequence lacking the N-terminal basic residues inhibited activation of G proteins by both IGF-II and the 14-amino acid stimulatory peptide (13). Recently, a similar G_{i2}-activating sequence was identified in the C-terminal region of the 7- to 11-transmembrane polycystin-1 receptor (14). A consensus sequence (Arg^{2559}–Lys^{2733}) present in the terminal region of third cytoplasmic loop of the seven-transmembrane \beta2-adrenergic receptor couples preferentially to G_{s}; phosphorylation of Ser^{262} by cAMP-dependent protein kinase decreases coupling to G_{s} and enhances coupling to G_{i1} (15–17).

In the present study, we have used peptide fragments corresponding to the N-terminal, C-terminal, and middle regions of the cytoplasmic domain of NPR-C to determine the locus of G protein binding and activation. A 17-amino acid peptide of the middle region (Arg^{469}–Arg^{485}), which possesses the consensus sequence B-B-...-B-B-X-X-B, was shown to bind selectively to G_{i1} and G_{i2}, activate PLC-\beta3 via the \betay subunits, and inhibit adenylyl cyclase in similar fashion to the selective NPR-C ligand, cANP4–23. A C-terminal peptide (Gly^{479}–Ala^{496}), which included the B-B-X-X-B motif at its N-terminal, inhibited activation by the stimulatory peptide and cANP4–23.

EXPERIMENTAL PROCEDURES

Synthesis of Partial Sequences Corresponding to the Cytoplasmic Domain of NPR-C—Four peptide fragments corresponding to the N-terminal region (Peptide 1, Arg^{468}–Arg^{470}), C-terminal region (Peptide 2, Gly^{479}–Ala^{496}), and middle region (Peptide 3, Ile^{467}–Arg^{482}; and Peptide 4, Arg^{469}–Arg^{485}) of the 37-amino acid cytoplasmic domain of NPR-C were synthesized by the solid phase method and highly (95–99%) purified by high performance liquid chromatography (Chiron Technologies) (Fig. 1). The lyophilized synthetic peptides were dissolved in distilled water.

Preparation of Freshly Dispersed and Cultured Smooth Muscle Cells—Muscle cells were isolated from guinea pig tenia coli by sequential enzymatic digestion, filtration, and centrifugation as described previously (7, 9). After washing, the cells were allowed to disperse

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† The abbreviations used are: NPR-C, natriuretic peptide clearance receptor; PLC, phospholipase C; eNOS, endothelial nitric-oxide synthase; IGF, insulin-like growth factor; cANP4–23, [des-Gln^{18}, Ser^{20},Gln^{21},Leu^{22}]ANP4–23-NH_{2}; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; GTP, guanosine 5’-\beta,\gamma-imidodiphosphate.
spontaneously for 30 min and then harvested by filtration through 500-μm Nitex and centrifuged twice at 350 × g for 10 min. In some experiments, the cells were permeabilized by incubation for 5 min with saponin (35 μg/ml) in a low CaCl2 (100 mM) medium as described previously (7) and resuspended in saponin-free medium with 1.5 mM ATP and ATP-regenerating system (5 mM creatine phosphate and 10 units/ml creatine phosphokinase).

Dispersed muscle cells were cultured as described previously (7, 9) in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. The muscle cells in confluent primary cultures were trypsinized, re-plated at a concentration of 2.5 × 10^5 cells/ml, and cultured under the same conditions. All experiments were done on cells in first passage.

Identification of Receptor-activated G Proteins in Solubilized Membranes—G proteins selectively activated by the synthetic peptides were identified by an adaptation of the method of Okamoto et al. (18), as described previously (19, 20). Cultured muscle cells (2 × 10^6 cells/ml) were homogenized in 20 mM HEPES medium (pH 7.4). After centrifugation at 25,000 × g for 15 min, the membranes were solubilized at 4 °C in 20 mM HEPES medium (pH 7.4) and 1% CHAPS. The membranes were incubated with 60 ng of [35S]GTPγS in a medium containing 10 mM HEPES (pH 7.4), 100 μM EDTA, and 10 mM MgCl2 for 20 min at 37 °C in the presence or absence of peptides (100 μM). The reaction was stopped with 10 volumes of 100 mM Tris–HCl medium (pH 8.0) containing 10 mM MgCl2, 100 mM NaCl, and 20 μM GTP, and the solubilized membranes were incubated for 2 h on ice in wells precoated with specific antibodies to Gα10, Gα12, Gα13, Gαα, and Gα11α. The wells were washed three times with phosphate buffer containing 0.05% Tween 20, and the radioactivity from each well was counted.

Assay of PLC-β Activity in Muscle Membranes—PLC activity was determined as described previously (19) by a modification of the method Uhung et al. (21) in membranes from cultured tenia coli muscle cells prelabeled with myo[3H]inositol. The assay was initiated by addition of 0.4 mg of membrane protein to 25 mM Tris–HCl (pH 7.5), 0.5 mM MgCl2, 10 mM MgCl2, 300 mM free CaCl2, 100 μM GTP, 5 mM phosphocreatine, 50 units/ml creatine phosphokinase, in a total volume of 0.4 ml. After incubation at 31 °C for 60 s, the reaction was terminated with 0.6 ml 25% trichloroacetic acid. The supernatant was extracted four times with 2 ml of diethyl ether, and the amount of labeled inositol phosphates in the aqueous phase counted. The trichloroacetic acid-soluble radioactivity at time 0 (100–150 cpm) was subtracted from all values. PLC activity was expressed as counts/min/mg of protein.

Measurement of Contraction in Permeabilized Muscle Cells—Contraction was measured in permeabilized muscle cells by scanning micrometry, as described previously (7). A 0.25-ml aliquot of cells (10^6 cells/ml) was added to 0.1 ml of medium containing cAMP-23 (1 μM) or various concentrations of partial peptide sequences, and the reaction was terminated after 30 s with 1% acrolein. The effect of the partial peptide sequences on maximal contraction induced by cAMP-23 was also determined. Under each condition, the lengths of treated muscle cells were compared with the lengths of untreated control cells. Contraction was expressed in micrometers as the mean decrease in cell length from control.

Materials—[35S]GTPγS, and myo[3H]inositol were obtained from NEN Life Science Products; polyclonal antibodies to G proteins and PLC-β isoforms from Santa Cruz Biotechnology; and all other chemicals from Sigma.
had no significant effect (range of inhibition: 10 ± 12% to 14 ± 16%) (Fig. 5). As shown previously for smooth muscle receptors coupled to \( G_\text{i} \) or \( G_\text{o} \) (19, 20, 22–24), activation of PLC-\( \beta \) conforms to a pattern of preferential activation of this PLC-\( \beta \) isozyme by the \( \beta \gamma \) subunits of inhibitory G proteins.

**Effect of Peptides on Muscle Cell Contraction**—The ability of Peptides 1–4 to induce contraction paralleled their ability to stimulate phosphoinositide hydrolysis. Contraction was measured in saponin-permeabilized tenia coli smooth muscle cells by scanning micrometry and expressed as mean decrease in muscle cell length from control. Peptide 4 induced contraction in a concentration-dependent fashion with an \( \text{EC}_{50} \) of 0.4 ± 0.1 \( \mu \text{M} \) and a maximal contraction of 22.6 ± 0.4 \( \mu \text{M} \), whereas Peptide 3 was effective only at high concentrations (maximal contraction 7.2 ± 5.5 \( \mu \text{M} \)), whereas Peptide 2 inhibited PLC-\( \beta \) activity stimulated by cANP4–23.

FIG. 2. Concentration-dependent stimulation of PLC-\( \beta \) by Peptide 4. Smooth muscle membranes were incubated for 15 min with various concentrations of Peptides 1–4. PLC-\( \beta \) activity was expressed as \([\text{H}]\)inositol phosphate formation (counts/min/mg of protein). Peptide 3 showed minor activity at high concentrations; Peptides 1 and 2 were inactive. Values are means ± S.E. of four experiments. **, significant stimulation, \( p < 0.01 \); *, \( p < 0.05 \).

FIG. 3. Augmentatory effect of Peptide 4 and inhibitory effect of Peptide 2 on PLC-\( \beta \) activity stimulated by cANP4–23. Smooth muscle membranes were incubated for 15 min with Peptide 4 (1 \( \mu \text{M} \)) or Peptide 2 (10 \( \mu \text{M} \)) in the presence of various concentrations of cANP4–23. PLC-\( \beta \) activity was expressed as \([\text{H}]\)inositol phosphate formation (counts/min/mg of protein). Bar graphs represent effects of Peptide 4 (P4) and Peptide 2 (P2) alone. cANP4–23 stimulated PLC-\( \beta \) activity in a concentration-dependent fashion (\( \text{EC}_{50} \) and threshold concentrations 0.8 ± 0.2 \( \mu \text{M} \) and 1 \( \mu \text{M} \), respectively). Peptide 4 augmented PLC-\( \beta \) activity induced by cANP4–23, shifting the concentration-response curve to the left, whereas Peptide 2 inhibited PLC-\( \beta \) activity, shifting the concentration-response curve to the right (\( p < 0.01 \) at all concentrations of cANP4–23). Values are means ± S.E. of four experiments.

| Table I | Binding of [\( \text{[35S]}\)GTP\( \gamma \text{S} \) • Ga complexes to Ga antibodies (cpm/mg protein) |
|---------|-----------------------------------------------------------------------------------------------------------------|
| G\( \gamma \text{G} \) Ab | G\( \gamma \text{G} \) Ab | G\( \gamma \text{G} \) Ab | G\( \gamma \text{G} \) Ab | G\( \gamma \text{G} \) Ab |
| G\( \gamma \text{G} \) S alone | 2348 ± 303 | 2296 ± 180 | 1390 ± 370 | 1842 ± 263 | 1811 ± 431 |
| + Peptide 1 | 2288 ± 363 | 2586 ± 192 | 1645 ± 300 | 1850 ± 200 | 1925 ± 421 |
| + Peptide 2 | 2552 ± 223 | 2880 ± 386 | 1470 ± 350 | 1950 ± 228 | 1952 ± 450 |
| + Peptide 3 | 3692 ± 463* | 4450 ± 512b | 1545 ± 444 | 1886 ± 245 | 1906 ± 473 |
| + Peptide 4 | 7427 ± 524* | 10282 ± 1452* | 1967 ± 404 | 2571 ± 328 | 1943 ± 517 |

* \( p < 0.05 \).  
b \( p < 0.01 \) for difference from GTP\( \gamma \text{S} \) alone.
domain of the NPR-C accounts for the ability of this single-transmembrane receptor to activate pertussis toxin-sensitive G proteins in various tissues (2, 7, 10). The sequence possesses two N-terminal basic residues (Arg469, Arg470), and the C-terminal motif, B-B-X-X-B (where B = basic and X = nonbasic residue) (Fig. 1). A synthetic peptide with this sequence (denoted Peptide 4 in this study) activated selectively Gi1 and Gi2 in tenia coli smooth muscle, stimulated phosphoinositide hydrolysis by activating PLC-b3 via the βγ subunits of both G proteins, inhibited adenyl cyclase activity via the α subunits, and induced muscle contraction, mimicking in all instances the properties of the selective NPR-C ligand, cANP4–23. The peptide also enhanced the ability of cANP4–23 to activate Gi1 and Gi2, stimulate phosphoinositide hydrolysis, induce contraction, and inhibit forskolin-stimulated cAMP. The effects of Peptide 4 alone and in combination with cANP4–23 were concentration-dependent.

A C-terminal peptide (denoted Peptide 2), which included the B-B-X-X-B motif at its N-terminal (Fig. 1), had no effect by itself but it blocked activation of Gi1 and Gi2 and all cellular responses induced by Peptide 4 and by cANP4–23, suggesting that Peptide 2 bound to, but did not activate Gi1 and Gi2, thus acting as a competitive inhibitor of G protein activation. The ability of Peptide 2 to inhibit responses to Peptide 4 and cANP4–23 was concentration-dependent.

The muscle cells were highly sensitive to cANP4–23 (EC50 0.8 ± 0.2 and 0.6 ± 0.3 nM for activation of PLC-β and stimulation of muscle contraction, respectively). At an EC50 concentration, Peptide 4 augmented the PLC-β and contractile responses to all concentrations of cANP4–23 (Figs. 3 and 7). The augmentation was additive suggesting additional recruitment of Gi1 and Gi2 by Peptide 4. Peptide 2 inhibited PLC-β and contractile responses to all concentrations of cANP4–23 (Figs. 3 and 7).

The synthetic peptides were designed to include or exclude specific residues in the stimulatory consensus sequence. Thus, Peptide 1, which included at its C terminus the two arginine residues present in the N terminus of Peptide 4, had no effect. Peptide 3, which closely resembled Peptide 4 and included the two N-terminal arginine residues but only a part (i.e. B-B) of the C-terminal B-B-X-X-B motif, was only partially active at the highest concentrations (100 μM), emphasizing the requirement for a complete N-terminal motif. Peptide 2, which retained the complete B-B-X-X-B motif at its C terminus, maintained the ability to bind but not activate G proteins: the pattern emphasized the significance of the location of the B-B-X-X-B motif at the C terminus, as well as the requirement for N-terminal arginine residues.

As noted above (1, 2), NPR-C, unlike NPR-A or NPR-B, is devoid of an intracellular guanylyl cyclase domain. Its truncated intracellular sequence possesses a Gi1/Gi2 binding domain that induces activation or inhibition of other effector enzymes. Activation of NPR-C in tenia coli smooth muscle causes inhibition of adenyl cyclase and activation of PLC-β3, resulting in stimulation of inositol 1,4,5-trisphosphate-dependent Ca2+ release and muscle contraction (8). The activation of PLC-β3 is mediated by the βγ subunits of Gi1 and Gi2; this conforms to a pattern of preferential activation of this PLC-β isozyme by the βγ subunits of inhibitory G proteins, as shown for other smooth muscle receptors coupled to Gi1 (somatosta-

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**Fig. 4.** Concentration-dependent augmentation of PLC-β response to cANP4–23 by Peptide 4 and inhibition by Peptide 2. Smooth muscle membranes were incubated for 15 min with a maximal concentration of cANP4–23 (1 μM) in the presence of various concentrations of Peptides 1–4. PLC-β activity was expressed as percent change in PLC-β activity in response to cANP4–23 (4482 ± 293 counts/min/mg of protein). Values are means ± S.E. of four experiments. ***, significant stimulation or inhibition, p < 0.01; *, p < 0.05.

**Fig. 5.** Selective inhibition of Peptide 4-stimulated PLC-β activity by PLC-β3 and Gβ antibodies. PLC-β activity induced by Peptide 4 was measured in smooth muscle membranes, before and after treatment for 60 min with antibodies (10 μg/ml) to various G proteins and PLC-β isoforms. PLC-β activity was expressed as [3H]inositol phosphate formation (counts/min/mg of protein). Values are means ± S.E. of four experiments. **, significant inhibition, p < 0.01.
tin-3 receptors; Ref. 22), Gi2 (opioid receptors; Ref. 23), and Gi3 (adenosine A1 (24), muscarinic m2 (20), and purinergic P2Y2 receptors (19)). Activation or inhibition of other regulatory enzymes involved in cell signaling by the bg subunits of G proteins has been well documented (25–29).

Unlike tenia coli smooth muscle cells, gastric and intestinal smooth muscle cells express eNOS (9). Activation of NPR-C in these cells results in preferential activation of eNOS by Gi1/Gi2; the formation of nitric oxide causes sequential activation of soluble guanylyl cyclase and cGMP-dependent protein kinase and results in muscle relaxation (7). Thus, although NPR-C is devoid of a membrane-bound guanylyl cyclase domain, its activation by natriuretic peptides can result in relaxation of gastric and intestinal muscle that expresses a G protein-dependent, constitutive NOS (7, 9).

The potency of the G i2/Gi1-activating sequence of NPR-C (EC50; 1 μM for activation of PLC-β3 and 0.5 μM for stimulation of muscle contraction) was similar to that of the G i2-activating sequence located in the C-terminal region of the multitransmembrane polycystin-1 receptor (14). The potency of both sequences may be related to the presence of dual N-terminal arginine residues and arginine or lysine residues in the C-terminal motif. As noted by Okamoto et al. (11), substitution of one basic residue for another altered the potency of the Gi2-activating sequence of IGF II/mannose 6-phosphate receptor in the order of arginine > lysine > histidine. Preferential activation of Gi2/Gi1 appears to be a common feature of the intracellular consensus sequences not only of NPR-C, but also of the polycystin-1 and the IGF II receptors (11, 14).

FIG. 6. Concentration-dependent contraction of smooth muscle cells by Peptide 4. Freshly dispersed tenia coli muscle cells were permeabilized with saponin, and the effect of peptides on muscle cell length was determined by scanning micrometry. Peptide 4 caused concentration-dependent contraction of muscle cells (decrease in cell length from control; mean control length, 96 ± 2 μm), whereas Peptide 3 was effective only at high concentrations. Peptides 1 and 2 had no significant effect on muscle cell length. Values are means ± S.E. of four experiments. **, significant stimulation or inhibition, p < 0.01.

FIG. 7. Augmentatory effect of Peptide 4 and inhibitory effect of Peptide 2 on muscle contraction induced by cANP4–23. Freshly dispersed tenia coli muscle cells were permeabilized with saponin, and the effect of Peptide 4 (0.1 μM) or Peptide 2 (1 μM) on muscle cell length was determined by scanning micrometry in the presence of various concentrations of cANP4–23. Bar graphs represent the effects of Peptide 4 (P4) and Peptide 2 (P2) alone. cANP4–23 contracted muscle cells in a concentration-dependent fashion (EC50 and threshold concentrations 0.6 ± 0.3 nM and <10 μM, respectively). Peptide 4 augmented contraction induced by cANP4–23, shifting the concentration-response curve to the left, whereas Peptide 2 inhibited contraction induced by cANP4–23, shifting the concentration-response curve to the right (p < 0.01 at all concentrations of cANP4–23). Values are means ± S.E. of four experiments.

FIG. 8. Concentration-dependent augmentation of muscle contraction induced by cANP4–23 by Peptide 4 and inhibition by Peptide 2. Freshly dispersed tenia coli muscle cells were permeabilized with saponin and the effect of a maximal concentration of cANP4–23 (1 μM) on muscle cell length was determined by scanning micrometry in the presence of various concentrations of Peptide 4 and Peptide 2. Values are means ± S.E. of four experiments. **, significant stimulation or inhibition, p < 0.01; *, p < 0.05.
peptides to NPR-C, as a prelude to their recycling and degradation, activates G protein-dependent pathways linked to several effector enzymes. In smooth muscle cells (e.g. gastric and intestinal smooth muscle) that express eNOS, G protein activation leads to NO formation and muscle relaxation. In smooth muscle cells devoid of eNOS (e.g. tenia coli), G protein activation leads to phosphoinositide hydrolysis and muscle contraction.

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