C-Type Natriuretic Peptide Attenuates Evoked Dopamine Efflux by Influencing $G_o\alpha$

Satoshi Takida, Barbara J. Elmquist, George J. Trachte

Abstract—Natriuretic peptides suppress adrenergic neurotransmission by a mechanism sensitive to pertussis toxin, suggesting that GTP-binding proteins are involved in the response. The major GTP-binding proteins present in the pheochromocytoma (PC12) cells used in this report are $G_o\alpha$ and $G_v\alpha$. We tested the hypothesis that the more abundant GTP-binding protein, $G_o\alpha$, mediates natriuretic peptide effects in PC12 cells by selectively ablating $G_o\alpha$ from the cells with antisense oligodeoxynucleotides. The results indicate that a selective ablation of $G_o\alpha$ with this technique eliminated C-type natriuretic peptide (CNP) effects and suppressed dopamine efflux evoked by a depolarizing stimulus. However, the activation of guanylyl cyclase (GC) by CNP was sustained after the $G_o\alpha$ ablation. Further, $N^{\omega}$-nitro-l-arginine methyl ester suppressed evoked dopamine efflux equally in the presence and absence of $G_o\alpha$. These results suggest that CNP attenuates evoked catecholamine efflux from PC12 cells by a mechanism requiring $G_o\alpha$ but independent of GC activation. (Hypertension. 1999;33:124-129.)

Key Words: neurotransmission ■ GTP-binding proteins ■ oligodeoxynucleotides, antisense ■ pheochromocytoma cells ■ guanylyl cyclase

Atrial natriuretic peptide (ANP) was identified as a hypotensive and natriuretic entity secreted by atria. The active peptide was found to contain 28 amino acids, with a cyclic ring consisting of 17 amino acids. Subsequently, 2 other natriuretic peptides were discovered and were named brain natriuretic peptide and C-type natriuretic peptide (CNP). These natriuretic peptides were found to interact with 2 specific classes of membrane receptors, including membrane forms of guanylyl cyclase (GC) and a truncated receptor termed the natriuretic peptide C receptor (NPR-C). The GC natriuretic peptide receptors are designated GC-A and GC-B and contain an extracellular high-affinity binding site for ANP and CNP, respectively, a single transmembrane region, an intracellular protein kinase homology domain, and a cyclase catalytic domain. Ligand binding to these receptors results in the elevation of intracellular cGMP concentrations that are generally thought to mediate various direct hypotensive actions of the natriuretic peptides. The NPR-C is a lower molecular weight receptor. It binds all natriuretic peptides and contains a single transmembrane-spanning domain and a short cytoplasmic tail of 37 amino acids that is devoid of GC activity. The NPR-C has been purported to act solely as a hormonal buffer system mediating the metabolic clearance of natriuretic peptides from the circulation. Thus, of the 3 identified receptors for natriuretic peptide, 2 receptors mediate an increase in GC activity in response to natriuretic peptides whereas the third receptor is perceived to facilitate the entry of natriuretic peptides into the cytoplasm of cells but not to participate in mediating biological responses.

This simplified scenario attributing all biological effects to GC activation has been challenged by studies performed in the last decade. In vivo, no change in renal metabolic clearance rate occurs with administration of cANP, a specific ligand of the NPR-C, after a CNP infusion. These results imply that the NPR-C in canine kidney does not clear all natriuretic peptides. Further, overwhelming evidence in vitro suggests that the NPR-C functions as a signal-transducing entity. In particular, pretreatment of cells with pertussis toxin abolishes NPR-C–activated inhibition of both adenylyl cyclase and catecholamine efflux and stimulation of neutrophil migration, suggesting the involvement of a pertussis toxin–sensitive GTP-binding protein such as $G_o\alpha$ or $G_v\alpha$. The current study defines the GTP-binding protein involvement in the neuromodulatory effects of CNP with antisense oligodeoxynucleotides to ablate $G_o\alpha$ selectively. The oligodeoxynucleotide used to accomplish the $G_o\alpha$ ablation was selected to eliminate both forms of $G_o\alpha$ but to leave $G_v\alpha$ unaffected.

Antisense oligodeoxynucleotide approaches involve delivery of a short oligodeoxynucleotide with a sequence complementary to specific mRNA. It selectively binds to target sequences and interferes with the expression of a specific protein. The significant specificity derives from the
ability of the oligodeoxynucleotide to hybridize, by very specific Watson-Crick base pairing, to its corresponding mRNA codon. The properties of the targeted protein can be deduced from the resulting change of function in the cell. Thus, we examined natriuretic peptide effects on evoked dopamine efflux and GC activity to assess the relevance of Gα in these actions.

Methods

Cell Culture

Pheochromocytoma (PC12) cells were grown in DMEM supplemented with 10% FCS and 5% heat-inactivated horse serum. They were plated at a density of 1 million cells per 25-mL flask, coated with rat-tail collagen, and incubated at 37°C in a 95% air-5% CO2 humidified atmosphere. Differentiation of the cells was induced by addition of 1×10^{-5} g/L. 78 nerve growth factor, isobutylmethylxanthine, Pheochromocytoma (PC12) cells were grown in DMEM supplemented with 10 g/L (1%) FCS. The cells were used for experiments after 8 to 10 days of differentiation.

Materials

Human/rat CNP was purchased from Peninsula Laboratories. Nerve growth factor, isobutylmethylxanthine, Nα-nitro-l-arginine methyl ester (LNAME), dihydroxybenzylamine, and dopamine were purchased from Sigma Chem Co. Reduced serum media (Opti-MEM) were purchased from Life Technologies. Anti-Gα polyclonal antibody and antitubulin monoclonal antibody were generous gifts from Dr Timothy Walseth (University of Minnesota, Minneapolis, MN) and Dr Jon Holy (University of Minnesota-Duluth, Duluth, MN), respectively. The anti-Gα and anti-Gα were purchased from Neomarkers Inc.

Antisense Oligodeoxynucleotides

Antisense (CGCCTTGGCYYCCGTGAG) and missense (CTTCCTGGYACGGCCCGTAG) oligodeoxynucleotides were purchased from Oligos Etc Inc. The detailed properties of these oligodeoxynucleotides were not disclosed by the manufacturer because of patent pending. The antisense oligodeoxynucleotide sequence was shown previously to suppress Gα expression after microinjection into cells.

Oligodeoxynucleotides Transfection/Delivery

GS3815 (Glen Research) was prepared according to the manufacturer’s protocol. Oligodeoxynucleotides were dissolved in 1/20 vol of Opti-MEM, and GS cytofectin was mixed into another 1/20 vol of serum-free media. Both solutions were combined to form the oligocytofectin complex and incubated for 10 minutes. The remaining 9/10 vol of Opti-MEM containing 10 g/L (1%) FCS was added. The cells were exposed to the oligocytofectin complex for 12 hours. The final concentrations of oligodeoxynucleotide and GS cytofectin were 5×10^{-3} mol/L and 2.5×10^{-5} g/L, respectively. The cells were used in experiments 48 hours later.

SDS-PAGE/Western Blotting

The cells were digested with solubilizing buffer consisting of the following: 10 g/L (1%) Triton X-100; 5 g/L (0.5%) deoxycholic acid; 1 g/L (0.1%) sodium dodecyl sulfate; 1.5×10^{-5} mol/L sodium chloride; 5×10^{-2} mol/L Tris; and 1 mmol/L EDTA. Additionally, this buffer contained the following protease inhibitors: leupeptin, 2×10^{-3} g/L; antipain, 4×10^{-3} g/L; benzamidine, 2×10^{-5} g/L; and aprotinin, 18 trypsin inhibitory units per liter. The cell proteins resulting from this solubilization were separated from cellular debris by centrifugation (15 000g for 15 minutes at 4°C) and assayed by the Biorad DC protein assay. Aliquots were taken to equal protein concentrations of isobutylmethylxanthine. The cells were exposed to the antisense oligodeoxynucleotide (5×10^{-3} mol/L) in the depolarizing buffer for 5 minutes. At that point, the medium and cells were assayed for dopamine contents. Results are expressed as percent of control release.

Catecholamine Release and Measurement

Catecholamine efflux from cells was induced by depolarization with Krebs buffer as previously described. Cells were incubated with either CNP (10^{-11} g/L, 10^{-9}, or 10^{-7} mol/L) or L-NAME (2×10^{-8} or 2×10^{-7} mol/L) in the depolarizing buffer for 5 minutes. At that point, the medium and cells were assayed for dopamine contents. Results are expressed as percent of control release.

Statistics

The CNP or L-NAME effect on potassium chloride–induced catecholamine release and cGMP generation was assessed by 2-way ANOVA. Individual values were compared by Student’s t test with Dunnett’s correction for multiple comparisons.

Results

Figure 1 shows the effect of treatment with Gα antisense and missense oligodeoxynucleotides on cell contents of Gα and β-tubulin. Control cells also were included to assess nonspecific effects of oligodeoxynucleotide administration. The Gα migrated at an approximate molecular weight of 40 kDa, and the β-tubulin migration was consistent with a molecular weight of 56 kDa. The β-tubulin was probed to assess whether protein contents were equal in the various lanes. As seen in Figure 1, these levels were fairly consistent, with the control lane having somewhat more protein than the lanes loaded with proteins from cells treated with oligodeoxynucleotides. The cells exposed to the antisense oligodeoxynucleotide (5×10^{-5} mol/L) had no detectable Gα, whereas those exposed to the missense oligodeoxynucleotide exhibited Gα immunoreactivity. The control cells were not treated with oligodeoxynucleotides and they possessed both more protein, as indicated by β-tubulin immunoreactivity, and more Gα protein. These results are consistent with an
effective and specific ablation of the G_o,α with the antisense protocol used. The antisense oligodeoxynucleotide for G_o,α was specific and did not influence G_o,α_1 or G_o,α_2 (data not shown).

The antisense oligodeoxynucleotide treatment (5×10^{-8} mol/L) tended to reduce the efflux of dopamine from PC12 cells stimulated with 40 mmol/L potassium chloride. This effect was statistically significant when the data from all cells treated in this manner were pooled (N=10; P=0.03). Efflux of dopamine in response to a depolarizing stimulus averaged 22.6±3.4% in cells treated with the missense oligodeoxynucleotide and 17.6±2.6% in cells treated with the antisense oligodeoxynucleotide (data not shown). These results indicate a tendency for G_o,α ablation to reduce evoked release of catecholamines.

The inhibitory effect of CNP was intact in cells receiving the missense oligodeoxynucleotide, as seen in Figure 2. CNP maximally suppressed evoked dopamine efflux 49±12% at a concentration of 10^{-8} mol/L. The concentration producing a half-maximal effect averaged 1×10^{-11}±0.7×10^{-10} mol/L. In stark contrast, cells treated with the antisense oligodeoxynucleotide failed to respond to CNP with a reduction in evoked dopamine efflux. The response to CNP was converted to a potentiation of evoked dopamine efflux in the cells treated with the antisense oligodeoxynucleotide, although none of the potentiative responses differed significantly from the control release (P=0.24 at 10^{-9} and 10^{-8} mol/L). The curves were statistically different when compared by ANOVA (P=0.006), as were the slopes of the curves (P=0.03). These data indicate that G_o,α is essential for the inhibitory neuromodulatory response to CNP. The dopamine contents of the cells varied from 9 to 17 ng per culture, with no alteration in dopamine contents in cells exposed to the G_o,α antisense oligodeoxynucleotide relative to those exposed to the missense oligodeoxynucleotide (data not shown).

The GC activation occurring in response to natriuretic peptides is considered both to mediate biological responses to natriuretic peptides and to be independent of GTP-binding proteins. Therefore, we used this response to test for nonspecific effects of GTP-binding protein ablation. As seen in Figure 3, CNP stimulated cGMP accumulation in cells treated with either the missense or antisense oligodeoxynucleotide. Significant increases in cGMP accumulation were not observed at CNP concentrations <10^{-7} mol/L in either group. The 2 curves were not statistically different by ANOVA (P=0.19). A concentration-dependent effect of CNP was observed (P=0.0005), with the slopes of the 2 curves being similar (P=0.14). These results indicate that the GC response to CNP was intact after G_o,α ablation, as would be expected. Basal cGMP concentrations in the cultures were 7.7±1.7×10^{-12} moles per flask in cells treated with the missense oligodeoxynucleotide and 10.6±2.7×10^{-12} moles per flask in antisense-treated cells (data not shown). These values did not differ significantly.

The neuromodulatory effect of L-NAME, an agent acting independently of GTP-binding proteins, also was examined to ascertain nonspecific effects of the G_o,α knockdown. As seen in Figure 4, L-NAME was equally effective at reducing evoked dopamine efflux whether the cells had been treated with missense or antisense oligodeoxynucleotides. L-NAME suppressed evoked dopamine efflux to 80±10% and 81±8% of control in cells treated with the antisense or missense oligodeoxynucleotide, respectively. These responses represented concentration-dependent reductions in dopamine efflux in response to L-NAME (P=0.002), but the responses...
between groups did not significantly differ when compared by ANOVA (P = 0.71). Further, both lines had similar slopes (P = 0.84). Collectively, these results indicate that although the antisense treatment used suppresses neurotransmission, it does not prevent the activity of all neuromodulators.

Discussion

The present study for the first time determines that the $G_o\alpha$ subtype of GTP-binding protein mediates the signal-transduction pathway initiated by CNP to suppress neurotransmitter efflux. The neuromodulatory effect of CNP was ablated specifically by reducing the $G_o\alpha$ protein by use of the antisense technique (Figure 2). In contrast, the neuromodulatory effect of L-NAME was sustained in the absence of the $G_o\alpha$ protein (Figure 4), as was CNP activation of GC (Figure 3). These data suggest that natriuretic peptides act via a cGMP-independent mechanism to affect neurotransmission. This mechanism has been attributed to activation of the NPR-C with coupling to a pertussis toxin–sensitive GTP-binding protein. These novel data indicate that the GTP-binding protein appears to be $G_o\alpha$.

A number of studies have linked the NPR-C with a pertussis toxin–sensitive mechanism presumably involving inhibitory GTP-binding proteins. Specific ligands for the NPR-C suppress adenylyl cyclase activity and reduce adrenergic neurotransmitter release by pertussis toxin–sensitive mechanisms. More recently, another NPR-C selective natriuretic peptide analog was found to inhibit mitogen-activated protein kinase induced by endothelin-3, platelet-derived growth factor, or phorbol 12-myristate 13-acetate in cultured fetal rat astrocytes. Thus, there is support for a signal-transducing role of the NPR-C.

The PC12 cells contain mRNA for GC-A but not for GC-B. The PC12 cells also appear to possess the NPR-C, as indicated by radiolabeled ANP binding being displaced by cANP, a selective NPR-C binding agent. Collectively, these results indicate that PC12 cells express GC-A and NPR-C. We have taken advantage of this situation to differentiate neuromodulatory effects of CNP from GC stimulation. This is possible because of the dearth of GC-B in the PC12 cells. Indeed, CNP inhibited evoked dopamine efflux in a concentration range that failed to alter intracellular cGMP concentrations (Figures 2 and 3) in the present study, as well. The only identified natriuretic peptide receptor failing to alter GC activity is the NPR-C, suggesting that CNP inhibition of dopamine release is dependent on this receptor.

We examined L-NAME effects on the oligodeoxynucleotide-treated cells to assess the specificity of the antisense treatment. L-NAME, an NO synthetase inhibitor, is known to attenuate evoked catecholamine release in PC12 cells. In both antisense- and missense-treated cells, L-NAME suppressed dopamine efflux in the same manner (Figure 4), which indicates that the antisense oligodeoxynucleotide treatment does not affect this inhibitory pathway nonspecifically. This result, thus, ruled out the possibility of the antisense treatment nonspecifically impairing the neurosympathoinhibitory systems in the cells.

This report has implicated $G_o\alpha$ in the neuromodulatory signal-transduction pathway of CNP but has not addressed the effect of $G_o\alpha$ in promoting a reduction in evoked neurotransmitter efflux from the cells. This pathway has been addressed in previous work defining the effect as a reduction in the sensitivity of the exocytotic apparatus to calcium. Adrenergic tissue is thought to secrete neurotransmitter in response to an elevation in intracellular calcium concentra-
tion. Surprisingly, natriuretic peptides were observed to augment calcium entry into PC12 cells. These results are consistent with those obtained in adrenal glomerulosa tissue, in which natriuretic peptides suppress aldosterone secretion while augmenting calcium currents. This mechanism of action to suppress calcium sensitivity, rather than suppressing calcium currents, probably accounts for the inability of natriuretic peptides to eliminate evoked catecholamine efflux from the PC12 cells. Further, it could account for the potentiation of neurotransmitter efflux by CNP observed in antisense-treated cells (Figure 2).

The inhibitory effect of Goα ablation on evoked dopamine efflux is a novel observation. We have been unable to locate any other report of GTP-binding protein ablations with antisense techniques in neural tissue. Previous work with pertussis toxin revealed a tendency, but no significant effect, to reduce evoked dopamine efflux in these cells, although others have found pertussis toxin pretreatment to augment evoked catecholamine release. Intracellular injection of an antibody to Goα also enhanced evoked neurotransmitter efflux in the latter study. The Goα associates with exocytic granules and is anticipated to participate in the exocytotic process. The inhibitory effect of the Goα reduction observed in the present study has not been reported previously but would be consistent with a potential involvement of Goα in the exocytotic process.

The Goα ablation also tended to attenuate GC responses to CNP (Figure 3), although the reduction in activity was not statistically significant. Although GTP-binding proteins are not generally considered to interact with GC, Khurana and Pandey have shown that a GTP-binding protein activator, mastoparan, augments GC activity. Additionally, an antibody to Goα prevented the effect of the mastoparan, indicating that Goα is capable of augmenting GC activity. Our data are consistent with their report except that the results were not statistically significant in our study.

In conclusion, we demonstrate that the Goα subunit of heterotrimeric GTP-binding proteins is involved in the CNP signaling pathway to inhibit evoked catecholamine release from PC12 cells. This represents the first report identifying the GTP-binding protein involved in signal-transducing effects of natriuretic peptides. Further, this mediatory role of Goα appears to be involved with the NPR-C because the concentration range for neuromodulatory effects of CNP does not overlap with activation of GC. Thus, our results are consistent with the NPR-C mediation of the neuromodulatory action of CNP by an interaction with the Goα protein. Significantly, Goα ablation failed to alter either GC responses to CNP or neuromodulatory effects of L-NAME. These data indicate the utility of this antisense technique in providing a specific assessment of GTP-binding protein involvement in signal-transduction events.

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References


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