Nerve Growth Factor Decreases Soluble Guanylate Cyclase in Rat Pheochromocytoma PC12 Cells*

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Nitrergic nerve (NO) modulates neurotransmission in the central and peripheral nervous systems. NO acts, in part, by stimulating cGMP production by soluble guanylate cyclase (sGC), an obligate heterodimer composed of α and β subunits. To investigate mechanisms that regulate responsiveness to NO in the nervous system, sGC regulation was examined in a rat pheochromocytoma cell line (PC12) exposed to nerve growth factor (NGF). NGF decreased sGC α1 and β1 subunit mRNA and protein levels as well as NO-stimulated sGC enzyme activity. The NGF-mediated decrease in sGC subunit mRNA levels was blocked by 5′-deoxy-5′-methylthioadenosine (an inhibitor of NGF-induced tyrosine phosphorylation). NGF did not decrease sGC subunit mRNA levels in PC12 cells containing a mutant Ras protein that blocks Ras-dependent intracellular signaling. Incubation of PC12 cells with a transcription inhibitor (actinomycin D) or protein synthesis inhibitors (anisomycin or cycloheximide) attenuated the ability of NGF to decrease sGC subunit mRNA levels. Moreover, sGC subunit mRNA levels decreased more rapidly in NGF-treated cells than in actinomycin D-treated cells, suggesting that NGF decreases sGC subunit mRNA stability. Thus, NGF decreases sGC subunit mRNA levels via mechanisms that are dependent on protein tyrosine phosphorylation and Ras activation. The effect of NGF on sGC subunit mRNA stability appears to be transcription- and translation-dependent. Modulation of sGC subunit levels and enzyme activity in PC12 cells suggests that NO responsiveness may be regulated in the nervous system by NGF.

In central and peripheral nervous systems, nitric oxide (NO) has an important role as a physiologic messenger molecule (1, 2). The enzymes responsible for NO synthesis, NO synthases, are present in selected neuronal populations in the brain, retina, adrenal medulla, and intestine as well as nerve fibers in the posterior pituitary (3). Many of the effects of NO on neuronal functions are mediated by the intracellular second messenger, cGMP. cGMP regulates neurotransmitter release (4) and appears to have an important role in long term potentiation in hippocampal pyramidal neurons (5). In addition, cGMP has been reported to repress gonadotropin-releasing hormone gene expression in a hypothalamic cell line (6). Moreover, NO has been observed to increase viability of trophic factor-deprived PC12 cells and sympathetic neurons via a cGMP-dependent mechanism (7).

NO stimulates soluble guanylate cyclase (sGC) to synthesize cGMP. sGC is an obligate heterodimer composed of α and β subunits with two isoforms of each subunit identified in the rat genome: α1, α2, β1, and β2 (8). cGMP interacts with several intracellular targets including protein kinases, ion channels, and phosphodiesterases. cGMP is metabolized to relatively inactive GMP by phosphodiesterases.

Although the regulation of NO production in the nervous system has been extensively investigated, the mechanisms regulating responsiveness to NO are less completely understood. We (9) and others (10) observed that agents which increase intracellular cAMP decrease sGC subunit mRNA levels and decrease the ability of cells to synthesize cGMP in response to NO-donor compounds. Ujie et al. (11) reported that agents which increase intracellular cGMP concentrations also decrease sGC enzyme activity and subunit mRNA levels in rat medullary interstitial cells. Whether or not NO responsiveness is regulated in the nervous system has not been reported.

The rat pheochromocytoma cell line, PC12, is an extensively characterized model used for the study of cell differentiation and proliferation in response to receptor-mediated tyrosine kinase activation (12). Recently, Peunova and Enikolopov (13) reported that differentiation of PC12 cells in response to nerve growth factor (NGF) was associated with increased expression of NO synthases. In the present study, we investigated the effect of NGF on sGC function in PC12 cells. sGC subunit mRNA and protein levels as well as sGC enzyme activity decreased in PC12 cells exposed to NGF. Evidence is presented that NGF decreases sGC subunit mRNA levels via mechanisms that are tyrosine kinase- and Ras-dependent.

EXPERIMENTAL PROCEDURES

Materials—NGF (2.5 S, isolated from mouse submaxillary glands) was purchased from Collaborative Biomedical Products (Bedford, MA). Epidermal growth factor (EGF, isolated from mouse submaxillary glands), basic fibroblast growth factor (bFGF, isolated from bovine pituitary glands), sodium nitroprusside, actinomycin D, anisomycin, cycloheximide, 5′-deoxy-5′-methylthioadenosine (MeSAdo), Nω-nitro-L-arginine methyl ester (L-NAME), S-bromo-cGMP, dibutyryl cGMP, dibutyryl cAMP, forskolin, isobutylmethylxanthine (IBMX), phorbol 12-myristate 13-acetate (PMA), and A23187 were purchased from Sigma.

Cell Culture—PC12 rat pheochromocytoma cells were obtained from American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 culture medium supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, 112 units/ml penicillin, and 112

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units/ml streptomycin. Adherent cells were passaged every 3–4 days into 100-mm tissue culture dishes at a density of 1 x 10^6 cells/plate, and cells were used 2 days following passage.

M-M17-26, a PC12 cell line expressing a dominant inhibitory mutant Ras (a point mutation in codon 17 resulting in the substitution of serine by arginine) (14), was generously provided by Dr. G. M. Cooper (Dana Farber Cancer Institute, Boston, MA). M-M17-26 cells were maintained in the same medium as PC12 cells supplemented with genetin (G418, 0.4 mg/ml).

RNA Blot Hybridization—RNA was isolated from PC12 cells by the guanidine isothiocyanate-cesium chloride method (15). Fifteen μg of RNA were fractionated in 1.5% agarose-formaldehyde gels, transferred to MAGNA CHARGE membranes (Micron Separations, Westborough, MA), and cross-linked by exposure to UV light. Membranes were hybridized overnight at 42 °C with either a 32P-radiolabeled 0.9-kilobase EcoRI/SacI restriction fragment of the rat sGC a1 subunit cDNA or a 32P-radiolabeled 1.4-kilobase KpnI/BglII restriction fragment of the rat sGC β1 subunit (both cDNAs generously provided by Dr. M. Nakane, Abbott (16)). Membranes were washed at high stringency in a solution containing 3 mM sodium citrate, 30 mM sodium chloride, and 0.1% sodium dodecyl sulfate at 65 °C and were exposed to x-ray film. To quantitate the amount of RNA loaded on the agarose-formaldehyde gels, the membranes were subsequently hybridized with a 10-fold molar excess of a 32P-radiolabeled oligonucleotide complementary to rat 18 S ribosomal RNA (17). In some experiments, RNA blots were also hybridized with radiolabeled probes derived from cDNAs that encoded rat sGC a1 subunit (both cDNAs generously provided by Dr. M. Nakane, Abbott (16)), and cross-linked by exposure to UV light. Membranes were hybridized with radiolabeled probes derived from cDNAs that encoded rat c-jun or c-fos (both kindly provided by Dr. T. Curran, Roche Institute of Molecular Biology, Nutley, NJ (18)). Autoradiograms were scanned using a Color Image Scanner (Seiko Epson Corp., Japan). All RNA blots shown are representative of at least three similar experiments.

Measuring sGC Subunit Protein Levels—PC12 cells were washed twice with 10 ml of ice-cold phosphate-buffered saline and harvested by scraping with a rubber policeman into buffer that contained 50 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1 mM dithiothreitol, and 2 mM phenylmethylsulfonyl fluoride (TED buffer). Cell membranes were disrupted by passing through a 22-gauge needle 10 times. Cell extracts were centrifuged at 100,000 × g for 30 min at 4 °C. Cell supernatants containing 50 μg of protein were subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred electrophoretically to nitrocellulose filters (Micron Separations). Filters were blocked in phosphate-buffered saline containing 5% nonfat milk at room temperature for 1 h and then incubated with an antiserum directed against the rat a1 sGC subunit (provided by Dr. M. Nakane) or with an immunopurification-purified polyclonal antiserum directed against the sGC β1 subunit cDNA or a polyclonal antiserum directed against the sGC β1 subunit (both kindly provided by Dr. T. Curran, Roche Institute of Molecular Biology, Nutley, NJ (18)). Protein concentrations in cell extracts were measured using the Bio-Rad dye concentration reagent (Bio-Rad) and bovine serum albumin as a standard.

Soluble Guanylate Cyclase Enzyme Activity—sGC activity was measured as described previously (20). Briefly, PC12 cells were extracted in TED buffer, and cell supernatants were prepared as described above. Cell extracts (10 μg) were incubated in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 4 mM MgCl2, 0.5 mM IBMX, 7.5 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, and 1 mM GTP with or without 1 mM sodium nitroprusside for 10 min at 37 °C. The reaction was terminated by addition of 0.9 ml of ice-cold 0.05 M HCl and boiling for 3 min. The concentration of cGMP in the reaction mixture was measured using a commercial radioimmunoassay kit (Biomedical Technologies Inc., Stoughton, MA). sGC enzyme activity is expressed as pmol of cGMP produced/min/mg of protein in the cell extract supernatant.

RESULTS

Characterization of Soluble Guanylate Cyclase Regulation by Nerve Growth Factor in a Rat Phaeochromocytoma Cell Line

NGF Decreases sGC Subunit mRNA Levels in PC12 Cells—To investigate the effect of NGF on sGC subunit gene expression,
sGC subunit protein levels were measured in PC12 cells incubated in the presence and absence of NGF. Consistent with the observation that prolonged exposure to NGF was necessary to decrease NO-stimulated sGC enzyme activity, decreased sGC subunit protein levels were evident in PC12 cells incubated with NGF for 24 h but not in cells exposed for 2, 4, and 8 h (data not shown). The effect of NGF on levels of both sGC subunits was dose-dependent; decreased subunit protein levels were detected in cells exposed to NGF in concentrations of 10 ng/ml or greater (Fig. 3).

**Signal Transduction Pathways Regulating sGC Subunit Gene Expression in PC12 Cells Exposed to NGF**

**Modulation of sGC Subunit Gene Expression Is Tyrosine Phosphorylation-dependent**—The biological response to NGF is initially by autophosphorylation of its high affinity receptor (p140) on tyrosine residues (21). To determine whether the mechanisms involved in the regulation of sGC subunit gene expression are tyrosine phosphorylation-dependent, sGC subunit mRNA levels were measured in PC12 cells pretreated with 3 mM MeSAdo (a methyltransferase inhibitor that inhibits tyrosine phosphorylation of the NGF receptor as well as other proteins in PC12 cells exposed to NGF) (21). At this concentration, MeSAdo effectively inhibited NGF-induced tyrosine phosphorylation in PC12 cells (data not shown). MeSAdo also blocked the NGF-induced decrease in sGC subunit mRNA levels (Fig. 4). Incubation of PC12 cells with two other growth factors that signal through receptor tyrosine phosphorylation, EGF and bFGF, in concentrations sufficient to induce protein tyrosine phosphorylation (22) and c-fos gene expression in PC12 cells (14), failed to alter sGC subunit gene expression (Fig. 5).

**Regulation of sGC Subunit Gene Expression by NGF Is cGMP-, cAMP-, Calcium-, NO-, and Protein Kinase C-independent**—To further characterize the intracellular signaling mechanisms participating in the regulation of sGC subunit gene expression by NGF, sGC subunit mRNA levels were measured in PC12 cells exposed to agents that modulate several regulatory pathways. Incubation of PC12 cells with membrane-permeable cGMP analogues, dibutyryl cGMP and 8-bromo-cGMP, did not decrease sGC subunit mRNA levels (Fig. 5). Moreover, pretreatment of PC12 cells with 1 mM l-NAME, a NO synthase inhibitor did not attenuate the ability of NGF to decrease sGC subunit mRNA levels, suggesting that NO synthase activity did not account for the effect of NGF (Fig. 6).
PC12 cells were also exposed to PMA (100 nM), an activator of protein kinase C, and A23187 (5 μM), a calcium ionophore. sGC subunit mRNA levels were not altered in PC12 cells exposed to either PMA or A23187 (Figs. 5 and 6). In addition, pretreatment of PC12 cells with 10 μM bisindolylmaleimide I, a protein kinase C inhibitor, did not block the effect of NGF on sGC subunit gene expression (data not shown).

To determine the effect of cAMP on sGC subunit mRNA levels in PC12 cells, cells were incubated for 4 h with 1 μM dibutyryl cAMP (a membrane-permeable cAMP analogue), 10 μM forskolin (an adenylate cyclase agonist), or 1 mM IBMX (a phosphodiesterase inhibitor). All three agents decreased sGC subunit mRNA levels (Fig. 5). However, incubation of PC12 cells with 100 ng/ml NGF for 1–30 min did not increase intracellular cAMP levels, whereas 10 μM forskolin increased intracellular cAMP levels 15-fold after 30 min (data not shown). These results suggest that although increased intracellular cAMP concentrations can decrease sGC subunit mRNA levels, they do not account for the effect of NGF on sGC subunit gene expression.

NGF Destabilizes Soluble Guanylate Cyclase Subunit mRNAs—NGF binds to its receptor on the cell surface, activating a signaling cascade that leads to the regulation of target gene expression. In the context of this study, NGF was found to decrease sGC subunit mRNA levels in PC12 cells, which is consistent with previous observations (14). The mechanism by which NGF decreases mRNA stability has been the subject of investigation in this study.

Molecular Mechanisms Involved in the Regulation of sGC Subunit Gene Expression in NGF-treated PC12 Cells

Destabilization of sGC Subunit mRNAs by NGF Is Dependent on Gene Transcription—To investigate the role of mRNA stability on the effect of NGF on sGC subunit mRNA levels, we examined the effects of actinomycin D, an RNA polymerase inhibitor, on sGC subunit gene expression in PC12 cells. The levels of sGC α1 and β1 subunit mRNAs did not change in PC12 cells exposed to 10 μM actinomycin D for up to 6 h. In contrast, c-jun mRNA levels decreased more than 50% within 1 h (Fig. 8, left panel). sGC subunit levels decreased more rapidly in PC12 cells exposed to NGF than in cells exposed to actinomycin D (see Fig. 1, Panel A), suggesting that NGF decreases sGC subunit mRNA stability. Moreover, incubation underlie the presence or absence of 100 ng/ml NGF for 4 h. RNA was extracted and hybridized with sGC α1 and β1 subunit cDNA probes and with an oligonucleotide complementary to 18 S ribosomal RNA.

FIG. 6. Regulation of sGC subunit gene expression by NGF is NO- and calcium-independent. PC-12 cells pretreated with l-NAME (1 mM) or A23187 (5 μM) for 15 min were incubated with or without 100 ng/ml NGF for 4 h. RNA was extracted and hybridized with sGC α1 and β1 subunit cDNA probes and with an oligonucleotide complementary to 18 S ribosomal RNA.

FIG. 7. NGF decreases sGC subunit mRNA levels via mechanisms dependent on Ras activation. Panel A, RNA was extracted from wild-type PC12 cells (PC12) and mutant PC12 cells (M-M17-26) expressing a dominant inhibitory Ras that were incubated in the presence or absence of 100 ng/ml NGF for 4 h. RNA was hybridized with sGC α1 and β1 subunit cDNA probes and with an oligonucleotide complementary to 18 S ribosomal RNA. Panel B, M-M17-26 cells were incubated with NGF (100 ng/ml), dibutyryl cAMP (1 mM), forskolin (10 μM), or IBMX (1 mM) for 4 h. RNA was extracted and hybridized with sGC α1 and β1 subunit cDNA probes and with an oligonucleotide complementary to 18 S ribosomal RNA. Panel C, RNA extracted from M-M17-26 cells incubated with 100 ng/ml NGF for 15, 30, 60, and 120 min was hybridized with a c-fos cDNA probe and with an oligonucleotide complementary to 18 S ribosomal RNA.
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The effect of NGF on sGC subunit mRNA levels is dependent on protein synthesis. PC12 cells were incubated with cycloheximide or anisomycin in the presence (+) or absence (−) of 100 ng/ml NGF for 4 h. RNA was extracted and hybridized with sGC α1 and β1 subunit cDNA probes and with an oligonucleotide complementary to 18 S ribosomal RNA.

FIG. 8. Actinomycin D blocks the effect of NGF on sGC subunit gene expression. PC12 cells were incubated with 10 μM actinomycin D in the absence (left panel) or presence of 100 ng/ml NGF (right panel) for 0, 1, 2, 3, 4, and 6 h. RNA was extracted and hybridized with sGC α1 and β1 subunit and c-jun cDNA probes and with an oligonucleotide complementary to 18 S ribosomal RNA.

Of PC12 cells with actinomycin D blocked the ability of NGF to decrease sGC subunit mRNA levels (Fig. 8, right panel). These results suggest that NGF decreases the stability of sGC subunit mRNAs via a transcription-dependent mechanism.

Destabilization of sGC Subunit mRNAs by NGF Is Dependent on Protein Synthesis—To further examine the mechanisms by which NGF decreases sGC subunit mRNA stability, PC12 cells were pretreated with cycloheximide or anisomycin, protein synthesis inhibitors, before exposure to NGF. Inhibition of protein synthesis completely blocked the ability of NGF to decrease sGC β1 subunit mRNA levels and partially blocked the decrease in α1 subunit mRNA levels (Fig. 9). These data suggest that NGF decreases sGC subunit mRNA levels through mechanisms that involve both RNA transcription and de novo protein synthesis.

DISCUSSION

Soluble guanylate cyclase is a critical component in NO-mediated signal transduction. In this study, regulation of sGC subunit gene expression was investigated in a rat pheochromocytoma cell line, PC12 cells, exposed to the neurotrophic factor NGF. NGF decreased levels of both sGC α1 and β1 subunit mRNAs in a dose- and time-dependent manner. The half-maximal effect of NGF on sGC subunit gene expression was observed between 1 and 10 ng/ml, which is consistent with the reported dissociation constants ($K_d$) of the two classes of NGF receptors (approximately $10^{-9}$ M, 25 ng/ml) (23, 24) and the EC$_{50}$ for the activation of mitogen-activated protein kinase by NGF (3 × 10^{-10} M, 10 ng/ml) (25) in PC12 cells. Decreased sGC subunit mRNA levels were observed within 2 h after addition of NGF and reached lowest levels within 4 h. Decreased sGC subunit mRNA levels were associated with decreased sGC subunit protein levels and NO-activated enzyme activity. However, the decrease in subunit protein levels and enzyme activity was detectable only after 24 h of continuous exposure to NGF. These results suggest that both sGC subunits are relatively stable cellular proteins in PC12 cells.

Although two forms of NGF receptor p75 and p140, are expressed in PC12 cells, one form, p140, appears to be required for NGF-induced receptor tyrosine kinase activity, Ras activation, and immediate early gene expression (including c-fos) (26). MeSAdo, a methyltransferase inhibitor, inhibits NGF-induced tyrosine kinase activation of the NGF receptor as well as other cellular proteins (21). MeSAdo blocked the ability of NGF to decrease sGC subunit mRNA levels. These observations suggest that receptor tyrosine kinase activation, the initiating event in NGF signal transduction, is involved in the NGF regulation of sGC subunit gene expression in PC12 cells. Moreover, the tyrosine kinase-mediated regulation of sGC subunit gene expression appeared to be NGF-selective, because EGF and bFGF, agonists for two other receptor tyrosine kinases in PC12 cells, failed to modulate sGC subunit mRNA levels.

NGF is known to generate cellular responses via multiple signal transduction pathways. It has been reported that NGF increases the half-life of GAP43 mRNA in PC12 cells through a protein kinase C-dependent mechanism (27). Protein kinase C activation alone was insufficient to account for the NGF-induced decrease in sGC subunit mRNA levels because incubation of PC12 cells with PMA, a protein kinase C agonist, did not alter sGC subunit gene expression. Moreover, the inability of bisindolylmaleimide I, a protein kinase C inhibitor, to block the effect of NGF on sGC subunit mRNA levels suggested that protein kinase C activation was not required for the regulation of sGC subunit gene expression by NGF.

Similar to observations in rat fetal lung fibroblasts (9) and rat aortic smooth muscle cells (10), agents that increase intracellular cAMP were found to decrease sGC subunit gene expression in PC12 cells. However, consistent with the observations of Hatanaka et al. (28) and Buskirk et al. (29), cAMP levels were not increased in PC12 cells exposed to NGF. These results suggested that regulation of sGC gene expression by NGF is not dependent on cAMP.

Several observations led us to consider the possibility that NO and cGMP may mediate the effect of NGF on sGC subunit gene expression. First, Peunova and Enikolopov (13) observed that NGF stimulated NO synthase expression in PC12 cells. Second, Ujije et al. (11) reported that NO donor compounds and agents that increase intracellular cGMP levels decreased sGC subunit mRNA and enzyme levels in rat medullary interstitial cells. Finally, we recently observed that sGC subunit mRNA and protein levels and sGC enzyme activity were decreased in rat pulmonary artery smooth muscle cells exposed to NO-donor compounds. In the present study, incubation of PC12 cells with membrane-permeable cGMP analogues did not decrease sGC subunit mRNA levels. Moreover, pretreatment of
PC12 cells with l-NAME did not block the effect of NGF on sGC subunit mRNA levels. These results suggested that the effect of NGF on sGC subunit gene expression was not mediated by NO or cGMP.

Ras appears to have an important role in the regulation of sGC subunit mRNA levels by NGF. Ras is located at the inner surface of the plasma membrane and transduces signals from tyrosine kinase receptors to intracellular target molecules (30). Intrinsic GTPase activity regulates the levels of active (GTP-bound) and inactive (GDP-bound) Ras. To investigate the role of Ras in the regulation of sGC subunit gene expression, the M-M17-26 cell line (14), a PC12 cell line stably transfected with the mutant p21(Asn-17)Hras gene, was used. The encoded mutant Ras is inactive due to a high affinity for GDP and likely competes with normal Ras for guanine nucleotide exchange factors, sequestering them into nonfunctional complexes (19). Incubation of M-M17-26 cells with NGF did not decrease sGC subunit mRNA levels, suggesting that the NGF effect was Ras-dependent. The presence of a functional NGF receptor in M-M17-26 cells was confirmed by the observations that NGF receptor-mediated regulation of sGC subunit gene expression was Ras-dependent. The presence of a functional NGF receptor in M-M17-26 cells was confirmed by the observations that NGF receptor-stimulated tyrosine kinase activity and Ras activation. The effect of NGF on sGC subunit mRNA levels did not depend on NO, cGMP, or protein kinase C. Although agents that increase cAMP levels decreased sGC subunit mRNA levels, NGF did not decrease cAMP levels, suggesting that NGF-mediated regulation of sGC subunit gene expression is cAMP-independent.

NGF decreased sGC α1 and β1 subunit mRNA levels coordinately, at least in part by decreasing mRNA stability. Inhibitors of RNA transcription and protein synthesis attenuated the ability of NGF to decrease sGC subunit levels, suggesting that NGF induces synthesis of a factor that selectively decreases sGC subunit mRNA stability. The decrease in sGC function in PC12 cells exposed to NGF appeared to precede the induction of NO synthases. These results suggest that NGF-induced changes in NO responsiveness as well as NO synthesis may contribute to the neuronal differentiation of PC12 pheochromocytoma cells.

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