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NGF REGULATES ADRENERGIC EXPRESSION

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ABSTRACT

The mechanism by which nerve growth factor (NGF) regulates adrenergic expression was examined in PC12 cells transfected with a rat phenylethanolamine N-methyltransferase (PNMT) promoter-luciferase reporter gene construct pGL3RP893. NGF treatment increased PNMT promoter-driven luciferase activity in a dose- and time-dependent fashion. Induction was attenuated by inhibition of the extracellular-signal-regulated-kinase (ERK) mitogen-activated protein kinase (MAPK) pathway (~60%) but not by inhibition of the protein kinase A (PKA), protein kinase C (PKC), phosphoinositol kinase (PI₃K) or p38 MAPK pathways. Deletion PNMT promoter-luciferase reporter gene constructs showed that the NGF responsive sequences lay within the proximal -392 bp of PNMT promoter, wherein binding elements for Egr-1 (-165 bp) and Sp1 (-48 bp) reside. Western analysis further showed that NGF increased nuclear levels of Egr-1, but not Sp1 or PKA-C, the catalytic subunit of PKA. Gel mobility shift assays showed increased potential for Egr-1, but not Sp1, protein-DNA binding complex formation. Mutation of either the Egr-1 or Sp1 binding sites in the PNMT promoter attenuated NGF activation. NGF, combined with PACAP, another PNMT transcriptional activator, cooperatively stimulated PNMT promoter driven-luciferase activity beyond levels observed with either neurotrophin alone. Finally, post-transcriptional control appears another important mechanism by which neurotrophins regulate the adrenergic phenotype. Independently NGF and PACAP and the combination stimulated both intron-retaining and intronless PNMT mRNA and PNMT protein but to a different extents.

Phenylethanolamine N-methyltransferase (PNMT, EC 2.1.1.28), the final enzyme in catecholamine biosynthesis, converts norepinephrine to epinephrine, thereby serving as a marker of the adrenergic phenotype (Wong, 2003). PNMT is expressed predominantly in adrenal medullary chromaffin cells (Anderson, 1993), with limited expression in specific brainstem neurons (Foster et al., 1985) and cardiac myocytes (Ebert et al., 1996).

Adrenal chromaffin cells arise from sympatho-adrenal (SA) progenitors of neural crest derivation. Neurotrophic factors, such as nerve growth factor (NGF), promote SA progenitor cell differentiation to sympathetic neurons and ensure survival of the latter (Anderson, 1993). In general, pre-ganglionic sympathetic neurons innervate post-ganglionic noradrenergic neurons and noradrenergic and adrenergic chromaffin cells in the adrenal medulla (Anderson, 1993; Muller and Unsicker, 1986). While NGF and other neurotrophins are present in developing and mature adrenal chromaffin cells (Suter-Crazzolara et al., 1996), their ability to regulate adrenergic expression in these cells appears controversial. NGF has been shown to increase PNMT activity in bovine chromaffin cells (Acheson et al., 1984) but not rat chromaffin cells (Muller and Unsicker, 1986). It does not appear to induce PNMT expression in PC12 cells derived from rat adrenal medullary pheochromocytomas (Unsworth et al., 1999).

The neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) is another potent neurotrophin involved in the differentiation of sympathoadrenal cell lineage (Grumolato et al., 2003), and it functions as a neurotransmitter as well when released from the splanchnic nerve innervating the adrenal medulla (Guo and Wakade, 1994). In cultured adrenal bovine chromaffin cells, PACAP increases the expression of

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all the catecholamine biosynthetic enzyme genes, including that of PNMT (Choi et al., 1999), and has been shown to stimulate PNMT promoter-driven luciferase activity in PC12 cells transfected with rat PNMT-promoter luciferase reporter gene constructs (Wong et al., 2002). Recent findings suggest that in their developmental role as neurotrophins, NGF and PACAP may act in a complementary fashion during the differentiation of sympathoadrenal cells based on common and separate effects on neuroendocrine and neural marker genes and associated transcription factors (Grumolato et al., 2003). However, whether they function together to regulate adrenergic expression is unknown.

The current study was undertaken to delineate the effects of NGF on adrenergic expression, investigate the underlying mechanism for NGF-induced changes and determine whether NGF and PACAP act cooperatively to control adrenergic function. Results show that NGF increases rat PNMT promoter-driven gene transcription in PC12 cells. NGF induction of PNMT promoter activity is mediated via extracellular-signal-regulated-kinase (ERK) mitogen-activated protein kinase (MAPK) via downstream effects on the PNMT gene transcriptional activators Egr-1 and Sp1. In addition, NGF can interact with the polypeptide PACAP to synergistically activate PNMT promoter-driven gene expression beyond activation that occurs with either neurotrophin alone. Finally, both NGF and PACAP induce two forms of PNMT mRNA in PC12 cells, intron-retaining and intronless (Unsworth et al., 1999), but together ensure PNMT primary transcript splicing predominantly to fully processed message.

MATERIALS AND METHODS

Cell Culture

Rat pheochromocytoma-derived PC12 cells (Dr. Daniel O'Connor, Department of Medicine, University of California, San Diego, CA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corp., Carlsbad, CA) supplemented with 5% equine serum (Hyclone Inc., Logan, UT), 5% bovine calf serum (Hyclone Inc., Logan, UT) and gentamycin sulfate (50 µg/ml; Sigma-Aldrich Corp., St. Louis, MO) and maintained in a humidified incubator at 37°C under an atmosphere of 5% CO₂-95% air (Tai et al., 2001). Prior to transfection, medium was exchanged to DMEM containing charcoal-treated sera. For the transfection studies, cells were seeded into 24-well tissue culture plates at a density of 1x10⁵ cells/well. To isolate nuclear protein and total RNA, cells were grown in 100 mm culture dishes to a density of 5x10⁵ - 1x10⁷ cells/dish before drug treatment. NGF (0-100 ng/ml), PACAP (10 nM) and the signaling pathway drugs, H-89 (30 µM), a selective protein kinase A (PKA) inhibitor (Chijiwa et al., 1990; Tai and Wong, 2002); forskolin (10 µM), an adenylate cyclase activator; GF109203X (100 nM), a protein kinase C (PKC) inhibitor (Tai and Wong, 2002; Tsuji et al., 2001); phorbol-12-myristate-13-acetate (PMA, 80 nM), a PKC activator at low concentrations (Morita et al., 1995); UO126 (10 µM), an ERK1/2 MEK inhibitor (Hamelink et al., 2002; Harada et al., 2001; Hou et al., 2003); SB203580 (50 µM), a p38 MEK inhibitor (Cheng et al., 2000); Wortmanin and LY294002 (10-50 µM), phosphatidylinositol 3-kinase inhibitors (Chang et al., 2003; Ha et al., 2003; Tsuji et al., 2001) and U73122 (50 µM), a phospholipase C inhibitor (Hamelink et al., 2002), were obtained from Sigma-Aldrich Corp. (St. Louis, MO). Initial treatment concentrations were based on literature values, and dose

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response curves and time courses executed to optimize treatment conditions (data not shown).

Plasmids

Wild-type (pGLRP893) and nested deletion PNMT promoter-luciferase reporter gene constructs (pGL3RP442, pGL3RP392 and pGL3RP60) were generated as described previously (Ebert et al., 1994; Tai et al., 2001). PNMT promoter-luciferase reporter gene constructs containing mutations of the -165 bp Egr-1 (pGL3RP893mutEgr-1) and the -168 and/or -48 bp Sp1 (pGL3RP893mutSp1A, pGL3RP893mutSp1B and pGL3RP893mutSp1A/B) binding sites were generated by site-directed mutagenesis (Ebert and Wong, 1995; Her et al., 2003; Tai and Wong, 2003). The pRSV-LacZ plasmid containing the β -galactosidase gene was used as a normalization control to correct for variable transfection efficiency.

Transient Transfection Assays

Transient transfections were performed as previously with minor modifications (Tai et al., 2001). Briefly, PC12 cells grown in 24-well tissue culture plates were transfected for 3 h with 1.0 μ g of wild-type or mutant PNMT promoter-luciferase reporter gene construct and 0.3 μ g of pRSV-LacZ, using the polyethylenimine method (Boussif et al., 1995). Following transfection, cells were washed with phosphate-buffered saline (PBS, pH 7.4), culture medium replaced with fresh DMEM (containing charcoal-treated sera) and cells maintained for 24 h, followed by drug treatment for 24 h unless otherwise specified.

Luciferase and β -Galactosidase Assays

Culture medium was removed, cells rinsed twice with PBS, and then lysed with 100 μ l of cell lysis buffer by subjecting to a freeze-thaw cycle. Lysates were centrifuged (1,000 g for 10 min) and 20 μ l of supernatant assayed for luciferase activity as previously described (Ebert et al., 1994) using a microplate luminometer (Dynex Technologies, Chantilly, VA). Protein in the cell lysates was quantified by the method of Bradford (Bradford, 1976) and luciferase activity expressed per μ g protein. β -galactosidase activity was determined to correct for variation in transfection efficiency (Ebert et al., 1994) and luciferase activity expressed relative to β -galactosidase. The ratio of luciferase to β -galactosidase activity for the wild-type or the mutant deletion PNMT promoter-luciferase reporter gene construct, as appropriate, was set to unity, and all values from treatment samples expressed relative to it.

Cytosolic and Nuclear Protein Extraction

Protein extracts from PC12 cells were prepared as previously described (Tai et al., 2001). Briefly, following treatment with 50 ng/ml NGF, 10 nM PACAP or the combination, cells were washed with ice-cold PBS, pelleted in microfuge tubes by centrifugation (1,000 g for 5 min) and lysed by resuspension in 400 μ l of 10 mM HEPES-KOH (pH 7.9) containing 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors (Complete, Mini-EDTA free protease inhibitor cocktail tablets, Boehringer Ingelheim, Ridgefield, CT). After incubation on ice for 10 min, nuclei were collected by centrifugation (1,000 g for 5 min) and the supernatant retained as cytosolic protein extract. Pelleted nuclei were then lysed by resuspension in 100 μ l of 20 mM HEPES-KOH (pH 7.9) containing 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM

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PMSF and protease inhibitors. Nuclear protein extract was isolated as the supernatant recovered after centrifugation (17,000 g for 2 min) at 4°C. Protein concentrations for cytosolic and nuclear extracts were determined as above and stored at -70°C until use.

Western Blot Analysis

Cytosolic or nuclear proteins (10-20 µg) were resolved on 10-12% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and then transferred to nitrocellulose membranes (Tai et al., 2001). Membranes were blocked overnight at 4°C with 10% skim milk in 20 mM Tris-HCl, 137 mM NaCl, 0.05% Tween-20, pH 7.2 (TBS-T). After washing with TBS-T (1 x 15 min, 2 x 10 min at room temperature), membranes derived from cytosolic extracts were incubated with anti-bovine PNMT antibody (1:5000, (Wong et al., 1987) while membranes derived from nuclear extracts were incubated with rat polyclonal Egr-1 (1:1000, C-19, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Sp1 (1:5000, PEP2, Santa Cruz Biotechnology, Inc.) or mouse monoclonal PKA-C (1:500, Transduction Laboratories, Lexington, KY) antibodies for 1 h at room temperature. Membranes were again washed with TBS-T (1 x 15 min, 2 x 10 min) and then incubated with horseradish peroxidase conjugated anti-rabbit or anti-mouse IgG (1:5000, Santa Cruz Biotechnology, Inc.) as appropriate for 1 h at room temperature. After a final wash with TBS-T (1 x 15 min, 2 x 10 min), proteins were detected by enhanced chemiluminescence (Santa Cruz Biotechnology, Inc.) using Hyperfilm (Amersham Biosciences Corp., Piscataway, NJ).

Gel Mobility Shift Assay. Gel mobility shift assays (GMSAs) were performed using the nuclear protein extracts described above and double-stranded oligonucleotides encoding the -165 bp Egr-1 binding element in the rat PNMT promoter

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(5'CCTCCCCGCCCCCGCGCGTCC3', -160 to -180 bp) (Ebert et al., 1994) or a consensus Sp1 binding element (5'TAGAGGGGCGGGGCTCTAGAC3', (Christy and Nathans, 1989) 5' end-labeled with [γ -³²P]dATP using T4 polynucleotide kinase as previously described (Ebert et al., 1994; Tai et al., 2001). Protein-DNA complexes were separated on 5% polyacrylamide gels and visualized by autoradiography using Kodak X-Omat LS film (Fisher Scientific, Springfield, NJ).

RT-PCR

Radioactive reverse transcription-polymerase chain reaction (RT-PCR) was performed as previously (Her et al., 2003). Briefly, total RNA was extracted from control and drug-treated PC12 cells using Tri-ReagentTM (Sigma-Aldrich Corp., St. Louis, MO) as per manufacturer. All samples were treated with DNaseI (1 unit/2 μ g of total RNA; Ambion, Inc., Austin, TX) for 30 min at 37°C before use. One μ g of total RNA was reverse transcribed with StrataScriptTM (Stratagene, La Jolla, CA) according to vendor. PCR was performed in a total volume of 25 μ l containing 100 ng of RT product, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 200 nM dNTPs and 0.2 μ M sense and anti-sense primers, 0.1 μ Ci [α -³²P]dATP and 2 units of Taq DNA polymerase (Promega Corp., Madison, WI). The following primer sets were used for PNMT and glyceraldehyde phosphate dehydrogenase (GAPD):

PNMT

sense, 5'-CAGACTTCTTGGAGGTCAACCTG-3'

anti-sense, 5'-TTATTAGGTGCCACTTCGGGTG-3')

GAPDH

sense, 5'-ATGCTGGTGCTGAGTATGTGCG-3'

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anti-sense, 5'-CATGTCAGATCCACAACGGATAC-3')

Reactions were incubated for 1 min at 94°C, 1 min at 61°C and 1 min at 72°C in a PTC-200 DNA Engine Thermocycler (MJ Research, Inc., Waltham, MA), repeating these cycles 35 times for PNMT and 18 times for GAPDH. PNMT and GAPDH amplicons were combined, resolved on 5% polyacrylamide gels and visualized by autoradiography.

Data Analysis

All data are presented as the mean \pm SEM. Experiments were repeated at least three times, with 6 replicates per group. Statistical significance between experimental and control groups was determined by one-way ANOVA followed by post-hoc comparisons using Student-Newman-Keuls multiple comparisons test to compare values against each other or Dunnett's comparison test to compare treatment groups against controls. Values of $p \leq 0.05$ were considered statistically significant.

RESULTS

NGF Activation of the PNMT Promoter

To determine the effects of NGF on adrenergic expression, PC12 cells transfected with a construct containing the proximal 893 bp of the rat PNMT promoter (Fig. 1A) upstream of the firefly luciferase reporter gene (pGL3RP893) were treated with varying concentrations of NGF up to 100 ng/ml and PNMT promoter-driven luciferase reporter gene expression determined after 24 h (Fig. 1B). NGF activated the PNMT promoter in a dose-dependent fashion with induction reaching maximum values between 5-100 ng/ml. As the 50 ng/ml concentration is consistent with previously reported investigations, it was used for all subsequent studies (Unsworth et al., 1999).

A time course for NGF induction was then performed. As shown in Fig. 1C, NGF increased luciferase activity ~3.0-fold at 6 h. Luciferase expression was even higher at 24 hr, with an ~6.0-fold elevation above basal levels and a 2.0-fold higher increment than that observed at 6 h. Thus, NGF stimulates PNMT promoter-driven gene transcription in both a dose- and time-dependent fashion.

Signaling Pathways Associated with NGF Activation of the PNMT Promoter

NGF activates several intracellular signaling pathways in PC12 cells, including those of PKA, PKC, p38 and ERK1/2 MAPK (Hama et al., 1986; Schubert et al., 1977; Xing et al., 1996). To determine whether any of these might be involved in NGF activation of the PNMT gene promoter, the effects of specific inhibitors and activators of these pathways were examined in PC12 cells transfected with the pGL3RP893 construct. Pre-treatment of cells with the PKA inhibitor H-89 (30 μ M) for 1 h did not alter NGF activation of the PNMT promoter (Fig. 2A) but treatment of the transfected cells

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with NGF in combination with the adenylate cyclase activator, forskolin (10 μ M), increased luciferase activity beyond the additive effects of either drug alone (~12.0-fold versus 5.0- and ~2.6-fold respectively, Fig. 2B). Similarly, the PKC inhibitor (GF109203X, 100 nM) had no significant effect on NGF activation of the PNMT promoter (Fig. 3A) while the PKC activator PMA potentiated NGF-induced PNMT promoter-driven gene expression, increasing luciferase ~2.5-fold beyond levels observed with NGF alone (Fig. 3B). However, in contrast to forskolin, PMA did not independently activate the PNMT promoter. Pre-treatment of the transfected cells with the p38 MAPK inhibitor SB203580 (10 μ M), two IP₃K inhibitors, Wortmanin (0.1-1.0 μ M) and LY294002 (10-50 μ M) or the phospholipase C inhibitor U73122 (5 μ M), did not attenuate NGF induction of the PNMT promoter as well (data not shown), but the MEK inhibitor UO126 (10 μ M), which inhibits the ERK1/2 pathway, reduced the NGF-mediated increase in luciferase activity by ~60% (Fig. 4). Thus, PNMT promoter activation by NGF appears orchestrated in part through activation of the ERK1/2 MAPK pathway but does not directly involve the p38, PKA, PKC, PI₃K or PLC pathways. In addition, PKA and PKC pathway activation apparently can act cooperatively to potentiate NGF stimulation of PNMT promoter-driven transcription.

NGF Responsive Regions in the PNMT Promoter

To identify DNA sequences within the proximal -893 bp of the PNMT promoter sensitive to NGF stimulation, nested deletion mutant PNMT promoter-luciferase reporter gene constructs generated by 5' exonuclease digestion of the PNMT promoter were transfected into PC12 cells and the effects of NGF on promoter activity examined (Fig. 5). NGF induction of PNMT promoter-driven luciferase expression for each of the

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deletion constructs was expressed relative to respective control as we have previously shown that basal luciferase activity for each construct varies depending on the complement and amount of transcription factors present in the cells relative to the corresponding binding elements within the promoter sequences of interest (Her et al., 1999). No significant differences were observed in NGF activation of luciferase in cells transfected with the wild-type pGL3RP893 construct or two of the truncated constructs, pGL3RP442 and pGL3RP392. However, a marked attenuation in luciferase induction (~63%) by NGF was observed with the shortest construct, pGL3RP60. Thus, the DNA sequences proximal to -392 bp are likely important for the effects of NGF on PNMT promoter-driven gene expression.

Role of Egr-1 and Sp1 in NGF Activation of the PNMT Promoter

The proximal -392 bp of the rat PNMT promoter contains functional consensus binding elements for the immediate early gene transcription factor, Egr-1 (-165 bp), and the ubiquitous transcription factor, Sp1 (-148 bp and -48 bp), with the -48 bp Sp1 site being the more functionally significant (Ebert et al., 1994). The effects of NGF on nuclear Egr-1 and Sp1 expression were therefore examined by analyzing nuclear protein extracts from control or NGF-treated PC12 cells by western blot analysis using primary antibodies specific for each transcription factor. Changes in nuclear levels of the catalytic subunit of PKA (PKA-C) were concurrently examined to confirm that PKA signaling does not contribute to NGF-induced PNMT promoter activity. As shown in Fig. 6A, basal levels of Egr-1 protein are very low in PC12 cell nuclei but NGF treatment of the cells markedly and rapidly induces nuclear expression of this transcription factor. Changes in Egr-1 are time-dependent and transient, with maximum stimulation

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occurring at 60 min, followed by restoration to basal values by 240 min after initial drug exposure. In contrast, nuclear levels of Sp1 in the PC12 cells are very high but expression of this transcription factor is not altered by exposure of the cells to NGF. In addition, NGF had no effect on nuclear expression of PKA-C, consistent with the absence of PKA participation in NGF activation of the PNMT promoter.

Since both Egr-1 and Sp1 must be phosphorylated to bind to their consensus elements and activate gene expression and antibodies specific for the phosphorylated transcription factors were unavailable, nuclear extracts isolated from NGF-treated PC12 cells were also assessed for Egr-1 and Sp1 protein-DNA complex formation using GMSAs (Fig. 6B). While protein-DNA complex formation for control samples using the double-stranded oligonucleotide containing the -165 bp Egr-1 binding element from the rat PNMT promoter was below the detection sensitivity of the GMSA assay, a very strong protein-DNA complex band was observed using nuclear extracts from NGF-treated PC12 cells. Previous GMSA analysis employing this oligonucleotide probe and Egr-1 antibodies demonstrated that for PC12 cells, the protein bound is Egr-1 (Tai et al., 2001). In contrast, no change in protein-DNA complex formation was apparent in GMSAs performed with the Sp1 double-stranded oligonucleotide, a probe shown to be specific for Sp1 protein binding with PC12 cells (Her et al., 1999).

To further investigate the role of Egr-1 and Sp1 in NGF-mediated PNMT promoter-driven gene expression, the effects of mutation of the -165 bp Egr-1 (pGL3RP893mutEgr-1), the -168 bp Sp1 (pGL3RP893mutSp1A), the -48 bp Sp1 (pGL3RP893mutSp1B) or both Sp1 sites (pGL3RP893mutSp1A/B) on NGF-elicited responses were assessed in transient transfection assays. As with the deletion

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mutants, luciferase activity was expressed relative to respective unstimulated controls to ensure correction for any differences in basal luciferase activity due to the mutations. Mutation of the -165 bp Egr-1 site attenuated NGF-stimulation of the PNMT promoter ~38% (Fig. 7A) while mutation of the upstream -168 bp Sp1 site decreased PNMT promoter activation 24%. However, when the proximal -48 bp Sp1 site or both Sp1 sites were mutated, NGF induction of the PNMT promoter was completely eliminated (Fig. 7B).

Cooperative Induction of PNMT Promoter-driven Gene Expression by NGF and PACAP

Recent findings suggest that NGF and PACAP can act additively or cooperatively to regulate cellular processes, including gene expression (Grumolato et al., 2003; Hashimoto et al., 2000; Sakai et al., 2001; Yuhara et al., 2003). Since PACAP, like NGF, also activates PNMT promoter-driven gene transcription (Wong et al., 2002), the combined effect of NGF and PACAP on PNMT promoter activity was examined in PC12 cells transfected with the pGL3RP893 construct (Fig. 8A). While NGF (50 ng/ml) and PACAP (10 nM) independently stimulated luciferase activity 6.5 and ~5.0 respectively in the transfected PC12 cells after 24 h of drug exposure, simultaneous treatment with these neurotrophins increased luciferase activity ~16.0-fold. Thus, NGF and PACAP apparently interact synergistically to stimulate PNMT promoter-driven gene expression beyond the independent activation by either alone.

As shown in Fig. 2, the adenylate cyclase activator forskolin incrementally elevated PNMT promoter-driven luciferase reporter gene expression in PC12 cells above the rise induced by NGF alone. One of the major signal transducers associated with PACAP-mediated gene activation is cAMP via both PKA-dependent and

-independent pathways (Vaudry et al., 2002). To further examine the role of adenylate cyclase and cAMP in the synergistic activation of PNMT promoter-driven gene expression, pGL3RP893-transfected PC12 cells were pre-treated with 30 μ M H89 for 1 hr, followed by treatment with NGF (50 ng/ml) and PACAP (10 nM) or the combination for 24 hr (Fig. 8B). H89 did not inhibit NGF-mediated PNMT promoter stimulation as shown earlier and only partially attenuated PNMT promoter induction by PACAP ($p \leq 0.01$) or the combination of NGF and PACAP ($p \leq 0.01$). These results suggest that synergistic effects of NGF and PACAP on the PNMT promoter are evoked through cAMP/PKA-dependent as well as cAMP/PKA-independent signaling mechanisms.

Effect of NGF and PACAP on Endogenous PNMT mRNA and Protein Expression

To demonstrate that NGF and PACAP are important and effective regulators of the endogenous PNMT gene as well, PNMT mRNA was quantified in total RNA isolated from PC12 cells treated with NGF, PACAP or the combination of NGF and PACAP for 24 h using radioactive RT-PCR (Fig. 9). As previously reported (Unsworth et al., 1999), PC12 cells express very low levels of the two forms of PNMT mRNA, intron-retaining and intronless (fully processed message). NGF treatment increased the expression of both forms, with fully processed PNMT mRNA being the predominant species. PACAP treatment increased both forms of PNMT mRNA as well but to an equivalent extent. When cells were treated with NGF and PACAP together, two forms of PNMT mRNA were again detectable but PACAP appeared to limit PNMT mRNA induction. Intronless message levels were equal to those observed with PACAP alone while intron-retaining PNMT mRNA was lower.

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PNMT protein was also examined in cytosolic protein extracts isolated from identically treated cells to determine if mRNA and protein changes corresponded. As shown in Fig. 9, PNMT protein, similar to PNMT mRNA, was barely detectable in PC12 cells. Treatment with NGF, PACAP or the combination increased PNMT expression markedly. Together, these findings suggest disparity between transcriptional and translational changes, indicating that post-transcriptional events impose additional constraints on PNMT expression.

DISCUSSION

Neurotrophic factors such as NGF promote the differentiation of sympatho-adrenal progenitor cells into sympathetic neurons and are also capable of dedifferentiating chromaffin cells toward a sympathetic neuronal phenotype, whereby they lose their competence to express PNMT (Anderson, 1993). Yet PNMT activity can be detected in the sympathetic ganglia of neonatal and adult rats and increases in response to NGF treatment (Liuzzi et al., 1977a; Liuzzi et al., 1977b). Similarly, NGF can induce PNMT activity in the rat adrenal medulla (Angeletti et al., 1972) and in cultured bovine adrenal chromaffin cells (Acheson et al., 1984), and it also increases steady-state levels of PNMT mRNA expression in PC12 cells (Unsworth et al., 1999). The present study now provides evidence that these changes in NGF regulated adrenergic expression in PC12 cells are at least in part mediated through an increase in PNMT promoter-driven gene transcription. Furthermore, while NGF activates a number of intracellular signaling pathways, including those of PKA (Gur et al., 2002; Schubert et al., 1977; Vaudry et al., 2002), PKC (Gur et al., 2002; Hama et al., 1986; Sakai et al., 2004), p38 MAPK (Dohi et al., 2002; Gur et al., 2002; Sakai et al., 2004; Vaudry et al., 2002; Xing et al., 1996) and ERK1/2 MAPK (Dohi et al., 2002; Gur et al., 2002; Sakai et al., 2004; Vaudry et al., 2002; Xing et al., 1996), only the ERK1/2 MAPK pathway appears involved in PNMT promoter activation by NGF. In the case of PKA and PKC signaling, we provide direct evidence that activation of these pathways produces an incremental rise in NGF-mediated PNMT promoter driven luciferase expression (Fig. 2 and 3).

Deletion mutation analysis of the rat PNMT promoter further revealed that the DNA sequences conferring NGF sensitivity reside within the proximal -392 bp of sequence upstream of the transcription initiation start site. This same region has previously been implicated in the cAMP sensitivity of the PNMT gene (Tai et al., 2001). It contains a consensus binding site for the immediate early gene transcription factor Egr-1 at -165 bp (Ebert et al., 1994), which if mutated, markedly attenuates the cAMP-responsiveness of the PNMT promoter (Tai et al., 2001; Wong and Tai, 2002). While the present study also implicates Egr-1 in NGF-mediated activation of the PNMT promoter, ERK1/2 MAPK pathway signaling appears to underlie Egr-1 induction of these changes. The latter agrees with earlier reports demonstrating that NGF stimulation of Egr-1 expression in PC12 cells (Sukhatme et al., 1988) can occur via ERK1/2 MAPK (Harada et al., 2001), independent of PKA (Ginty et al., 1991), and is a key step in NGF-mediated neuritogenesis of PC12 cells to sympathetic-like neurons. However, these results do not preclude the possibility that sequences upstream of the Egr-1 site may also be required for NGF activation, one candidate being the glucocorticoid receptor (GR) (Tai et al., 2002). We have previously shown that Egr-1 bound to the -165 bp consensus element can interact cooperatively with GRs bound to overlapping glucocorticoid response elements at -773 and -759 bp to markedly stimulate PNMT promoter-driven luciferase expression beyond levels observed with either transcription factor alone.

In the presence of a mutated -165 bp Egr-1 site, residual NGF activation of PNMT promoter-driven luciferase expression was still apparent, suggesting that other transcription factor(s) may bind to DNA elements within the proximal -392 bp of

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promoter sequences to contribute to NGF induction of the promoter. The zinc finger protein Sp1 participates in the transcriptional regulation of several other NGF-responsive genes (Liu et al., 2001; Melnikova and Gardner, 2001). Functional Sp1 binding elements are located at -48 bp and -168 bp in the rat PNMT promoter (Ebert and Wong, 1995), and we have shown that the -48 bp Sp1 site contributes to the cooperative activation of the PNMT promoter by PKA and PKC (Tai and Wong, 2003). Although NGF did not alter Sp1 protein levels in PC12 cells, Sp1 must be phosphorylated to bind to its consensus element and activate transcription (Her et al., 2003; Kadonaga et al., 1987; Tai and Wong, 2003), and recent findings indicate that NGF does increase Sp1-DNA binding (Liu et al., 2001; Melnikova and Gardner, 2001). The present study showed that site-directed mutation of the -168 bp Sp1 site attenuated NGF activation of the PNMT promoter while mutation of the -48 bp Sp1 site or both Sp1 sites completely eliminated NGF activation. Thus, the Sp1 binding sites are apparently necessary for maximum PNMT promoter induction. However, no changes in Sp1-DNA complex formation were observed following NGF treatment. Together these results suggest several possibilities. First, Sp1 may subserve a permissive role in stimulus-induced PNMT promoter activation. Consistent with the latter, we have previously shown that the shortest PNMT promoter sequence permitting reporter gene expression is the 60 bp of sequence proximal to the site of transcription initiation (+1) (Ebert et al., 1994) containing the -48 bp Sp1 consensus element (Her et al., 2003). Second, the upstream -168 bp Sp1 and -165 bp Egr-1 sites overlap so that Sp1 and Egr-1 can compete for binding. However, this distal Sp1/Egr-1 site predominantly functions as an Egr-1 element (Ebert et al., 1994; Ebert and Wong, 1995) in agreement with the findings

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reported here, albeit elimination of both Sp1 sites does prevent NGF activation. Overlapping Sp1/Egr-1 binding elements are a common motif, and Sp1 and Egr-1 competition for binding is thought to be an important gene regulatory mechanism (Her et al., 2003). This motif occurs in the PNMT promoter of other species as well (mouse, -45/-43 and -165/-162; human, -93/-92 and bovine, -90/-89) as identified through Transfac database (version 6.0) analysis. In these species, the PNMT gene shares 58, 41 and 51% identity respectively with the rat gene in the 5' and 3' non-coding and intronic sequences and 90, 76 and 83% identity respectively in the coding region (Suh et al., 1994). Thus, activation of the PNMT promoter by NGF appears an important biological mechanism for regulating adrenergic responses. Third, we have previously demonstrated that several of the PNMT transcriptional activators, depending on the particular stimulus, seem to interact cooperatively to activate the PNMT promoter. Sp1, Egr-1, the GR and AP2 bind as dimers to consensus sites in the promoter, and thereby may impose alterations in promoter structure that facilitate transcription factor interaction. Finally, these possibilities are not mutually exclusive (Ebert et al., 1998; Wong et al., 1998).

Trophic factors, like NGF, can also work cooperatively to regulate cell differentiation and function. Recent studies have shown that PACAP complements the actions of NGF in chromaffin cell development (Grumolato et al., 2003), forebrain cholinergic neuronal survival (Yuhara et al., 2003), PC12 and hippocampal neuronal survival (Lee et al., 2002) and neuritogenesis (DiCicco-Bloom et al., 2000; Sakai et al., 2004), effects likely orchestrated through gene expression. NGF and PACAP have also been shown to cooperatively activate several genes, including the choline

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acetyltransferase gene (Yuhara et al., 2003) and the PACAP gene itself (Hashimoto et al., 2000; Sakai et al., 2001). Findings described in this report now demonstrate that NGF and PACAP synergistically activate yet another gene, the PNMT gene. PACAP activation of the PNMT promoter is controlled in part through the cAMP-PKA and ERK1/2 MAPK pathways (Wong et al., 2002). Extending those findings, we demonstrate here that PKA signaling can incrementally increase PNMT promoter stimulation above levels induced by NGF via the ERK1/2 MAPK pathway suggesting that synergistic activation of the PNMT promoter by NGF and PACAP may occur through interaction of PKA and ERK1/2 signaling. Both signal transduction pathways have previously been shown to participate in the synergistic induction of the PACAP gene (Hashimoto et al., 2000). ERK1/2 has also been shown to contribute to the synergistic effects of NGF and PACAP in cell differentiation and neurite outgrowth (Sakai et al., 2004). Studies describing cooperative neurogenesis and neuroprotective effects of NGF and PACAP suggest that the TrkA receptor may also provide crosstalk between these two neurotrophins (DiCicco-Bloom et al., 2000; Lee et al., 2002). NGF binding to TrkA rapidly activates tyrosine kinase signaling downstream. PACAP also activates tyrosine kinase activity through TrkA receptor activation but more slowly and indirectly via PACAP type 1 receptors. Taken together, these findings suggest that activation of both convergent and divergent signaling mechanisms may account for the cooperative effects of NGF and PACAP on gene regulation and a variety of biological processes.

Finally, NGF has previously been reported to regulate PNMT gene expression via differential RNA processing in rat brainstem and adrenal medulla-derived PC12 cells

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(Unsworth et al., 1999). With respect to NGF, 500 and 600 bp mRNAs are produced, the latter arising due to retention of intron 2. However, there is no accompanying increase in PNMT enzymatic activity in the PC12 cells. Consistent with those findings, we show that NGF treatment of PC12 cells stimulates the expression of both intronless and intron-retaining forms of PNMT message, with the former predominating. We extend those findings by demonstrating that another neurotrophic factor, PACAP, alone or in combination with NGF induces both forms of mRNA as well. The relative proportion of short and long forms depends on the neurotrophin and duration of exposure (data not shown). Previously, we reported that the PNMT transcriptional activator Sp1 selectively stimulates the production of intronless PNMT mRNA (Her et al., 2003) and are now examining its potential role in the differential processing of PNMT mRNA by NGF and PACAP. Analysis of NGF, PACAP and NGF/PACAP-induced changes in PNMT protein further showed that these neurotrophins appear to regulate PNMT protein synthesis as well.

In summary, the neurotrophin NGF, independently and cooperatively with PACAP, regulates adrenergic expression via transcriptional and post-transcriptional control of the PNMT gene. Synergistic induction of the PNMT promoter by NGF and PACAP is mediated via the activation of the ERK1/2 MAPK and PKA pathways, with downstream participation of Egr-1 and Sp1 through their interaction with consensus binding sites at -165 bp and -168 and -48 bp respectively. Finally, NGF and PACAP appear to further influence adrenergic expression post-transcriptionally by regulating processing of the PNMT primary transcript to intronless and intron-retaining forms of PNMT mRNA and synthesis of PNMT protein.

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FOOTNOTES

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LEGENDS FOR FIGURES

FIG. 1. DOSE AND TIME DEPENDENCY OF NGF INDUCTION OF THE PNMT PROMOTER. PC12 cells were transfected with the wild-type pGL3RP893 PNMT promoter-luciferase reporter gene construct. A. Schematic of proximal -893 bp of rat PNMT promoter subcloned into the pGL3 plasmid reporter vector (Promega Biosciences Inc., Madison, WI) showing identified regulatory response elements (Ebert et al., 1994; Ebert et al., 1998; Ebert and Wong, 1995; Her et al., 1999; Ross et al., 1990; Wong et al., 1998). B. Transfected cells were treated with varying doses of NGF from 0 to 100 ng/ml and luciferase activity determined after 24 h. C. Transfected cells were treated with 50 ng/ml NGF for times up to 24 h and luciferase activity determined. Luciferase expression at 6 or 24 h are depicted. Luciferase activity was expressed relative to untreated control values set to unity. Data are presented as the mean \pm SEM (n=6; significantly different from respective control: ***, $p \leq 0.001$; significantly different from 6 hr treatment: +++, $p \leq 0.001$). Experiments were replicated at least 3 times.

FIG. 2. ROLE OF cAMP-PKA PATHWAY IN NGF ACTIVATION OF THE PNMT PROMOTER. (A) PC12 cells transfected with pGL3RP893 PNMT were pre-treated with the PKA inhibitor, H-89 (30 μ M), for 1 h, followed by 50 ng/ml NGF for 24 h. (B) PC12 cells transfected with pGL3RP893 were treated with 50 ng/ml NGF and/or the adenylate cyclase activator, forskolin (10 μ M) for 24 h. Luciferase activity was determined and expressed relative to untreated control values set to unity. Data are expressed as the mean \pm SEM (n=6; significantly different from respective control: ***, $p \leq 0.001$;

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significantly different within a treatment group: +++, $p \leq 0.001$). Experiments were replicated at least 3 times.

FIG. 3. ROLE OF PKC PATHWAY IN NGF ACTIVATION OF THE PNMT PROMOTER.

A. PC12 cells transfected with pGL3RP893 were pre-treated with the PKC inhibitor, GF109203X (100 nM), for 1 h, followed by 50 ng/ml NGF for 24 h. B. PC12 cells transfected with pGL3RP893 were treated with 50 ng/ml NGF and/or the PKC activator, PMA (80 nM), for 24 h. Luciferase activity was determined and expressed relative to untreated control values set to unity. Data are expressed as the mean \pm SEM (n=6; significantly different from respective control: ***, $p \leq 0.001$; significantly different within a treatment group: +++, $p \leq 0.001$). Experiments were replicated at least 3 times.

FIG. 4. ROLE OF ERK1/ERK2 MAPK IN NGF ACTIVATION OF THE PNMT

PROMOTER. PC12 cells transfected with pGL3RP893 were pre-treated with the MEK inhibitor, UO126 (10 μ M), for 1 h, followed 50 ng/ml NGF for 24 h. Luciferase activity was determined and expressed relative to untreated control values set to unity. Data are expressed as the mean \pm SEM (n=6; significantly different from respective control: ***, $p \leq 0.001$; significantly different within a treatment group: +++, $p \leq 0.001$).

Experiments were replicated at least 3 times.

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FIG. 5. NGF RESPONSIVE REGIONS OF THE PNMT PROMOTER. PC12 cells were transfected with the wild-type construct pGL3RP893 or the nested deletion PNMT promoter-luciferase reporter gene constructs pGL3RP442, pGL3RP392 or pGL3RP60 and treated with 50 ng/ml for 24 h. Luciferase activity was determined and expressed relative to untreated control values set to unity. Data are expressed as the mean \pm SEM (n=6; significantly different from respective control: **, $p \leq 0.01$; ***, $p \leq 0.001$; significantly different from other constructs within a treatment group: ###, $p \leq 0.001$). Experiments were replicated at least 3 times.

FIG. 6. EFFECT OF NGF ON EGR-1 AND SP1 PROTEIN AND PROTEIN-DNA COMPLEX FORMATION. A. PC12 cells were treated with 50 ng/ml NGF for 0-360 min, nuclear protein extracts prepared and separated on 10% SDS-polyacrylamide gels, followed by ECL western analysis with PKA-C (1:500, Transduction Laboratories), Egr-1 (1:1000, Santa Cruz Biotechnology, Inc.) and Sp1 (1:5000, Santa Cruz Biotechnology, Inc.) antibodies. Representative fluorogram from 3 replicates. B. Nuclear extracts isolated from PC12 cells exposed to NGF for 60 min were subjected to GMSA analysis using ^{32}P -labeled double-stranded oligonucleotides encoding the -165 bp Egr-1 binding element in the PNMT promoter (5'CCTCCCGCCCCGCGGTCC3') and consensus Sp1 binding element (5'-TAGAGGGGCGGGGCTCTAGAC3') as described in "Materials and Methods". Protein-DNA complexes, following separation on 5% polyacrylamide gels, were visualized by autoradiography.

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FIG. 7. ROLE OF EGR-1 and SP1 IN NGF ACTIVATION OF THE PNMT PROMOTER.

A. PC12 cells were transfected with the wild-type construct pGL3RP893 or a construct containing a mutated -165 bp Egr-1 binding element, pGL3RP893mutEgr-1. Following transfection, cells were treated with 50 ng/ml NGF for 24 h. Luciferase activity was determined and expressed relative to untreated control values set to unity. Data are presented as the mean \pm SEM (n=6; significantly different from respective control: ***, $p \leq 0.001$; significantly different within NGF treatment group: ++, $p \leq 0.01$). Experiments were replicated at least 3 times. B. PC12 cells were transfected with the wild-type construct pGL3RP893 or constructs containing mutations in the -168 bp (pGL3RP893mutSp1A), -48 bp (pGL3RP893mutSp1B) or both (pGL3RP893mutSp1A/B) Sp1 binding sites and treated with 50 ng/ml NGF for 24 h. Luciferase activity was determined and expressed relative to untreated control values set to unity. Data are expressed as the mean \pm SEM (n=6; significantly different from respective control: *, $p \leq 0.05$; ***, $p \leq 0.001$; significantly different from pGL3RP893mutSp1A: a, $p \leq 0.001$; significantly different from pGL3RP893mutSp1B: b, $p \leq 0.001$).

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FIG. 8. SYNERGISTIC ACTIVATION OF THE PNMT PROMOTER BY NGF AND PACAP. A. PC12 cells transfected with pGL3RP893 were treated with 50 ng/ml NGF and/or 10 nM PACAP for 24 h and luciferase activity determined and expressed relative to untreated control values set to unity. Data are presented as the mean \pm SEM (n=6; significantly different from control: ***, $p \leq 0.001$; significantly different from NGF treatment group: a, $p \leq 0.001$; significantly different from PACAP treatment group: b, $p \leq 0.001$). B. PC12 cell transfected with pGL3RP893 were pre-treated with 30 μ M H-89, followed by treatment with NGF and/or PACAP as above and luciferase activity determined and expressed relative to untreated control values set to unity. Data are presented as the mean \pm SEM (n=6; significantly different from control: ***, $p \leq 0.001$; significantly different from PACAP or NGF/PACAP treatment group without H-89 pre-treatment: **, $p \leq 0.001$).

FIG. 9. EFFECT OF NGF AND/OR PACAP ON ENDOGENOUS PNMT mRNA and PROTEIN EXPRESSION. PC12 cells were treated with 50 ng/ml NGF, 10 nM PACAP or the combination of NGF and PACAP for 24 h. Total RNA was extracted as described in "Materials and Methods" and PNMT and GAPDH mRNAs amplified by radioactive RT-PCR. Amplicons were resolved on 5% polyacrylamide gels, followed by autoradiography. Cytosolic cell extracts were prepared as described in "Materials and Methods" and western analysis performed to analyze PNMT protein using rabbit anti-bovine PNMT antibody (Wong et al., 1987). Representative autoradiogram or fluorogram from 9 replicates.

Fig. 1

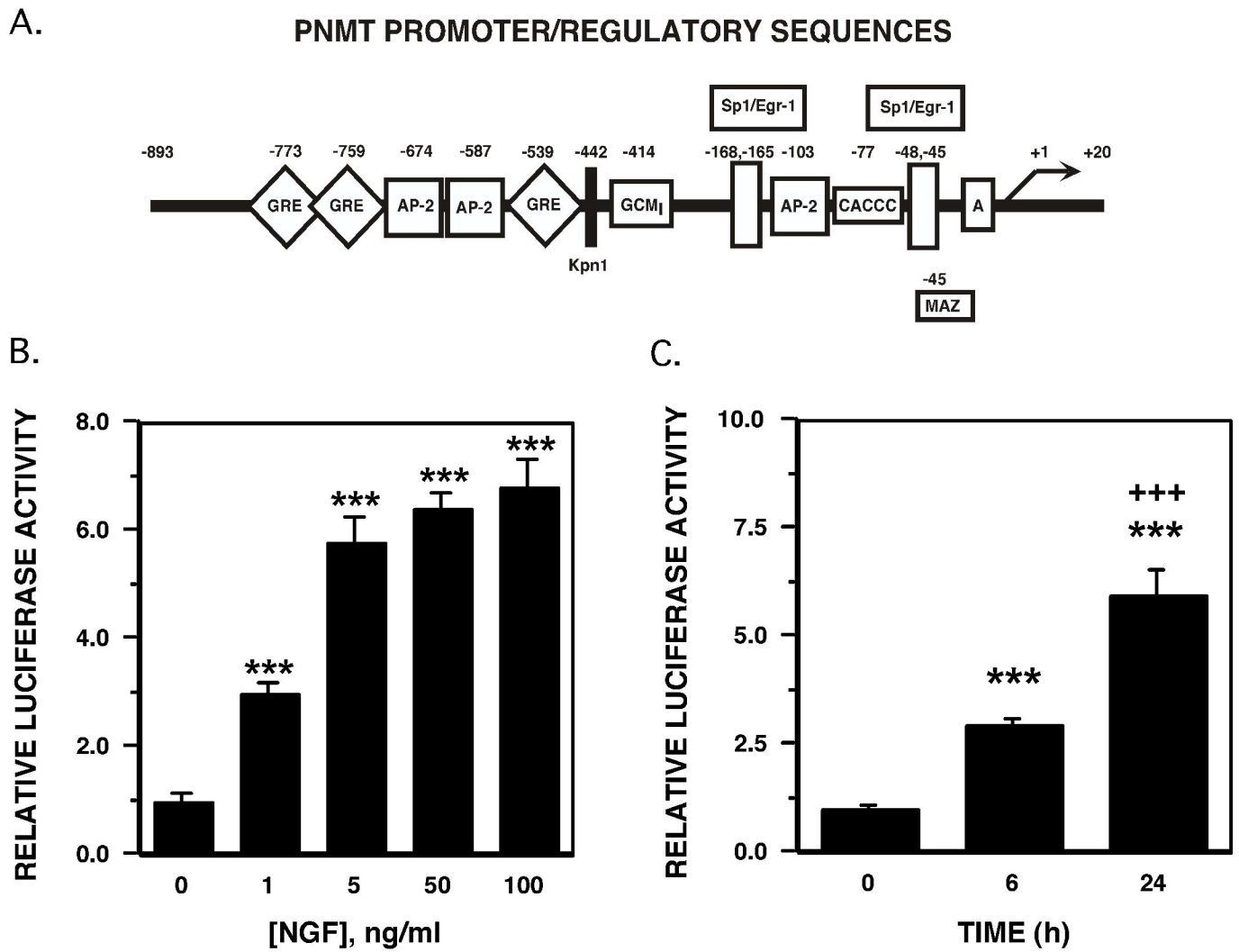


Fig. 2

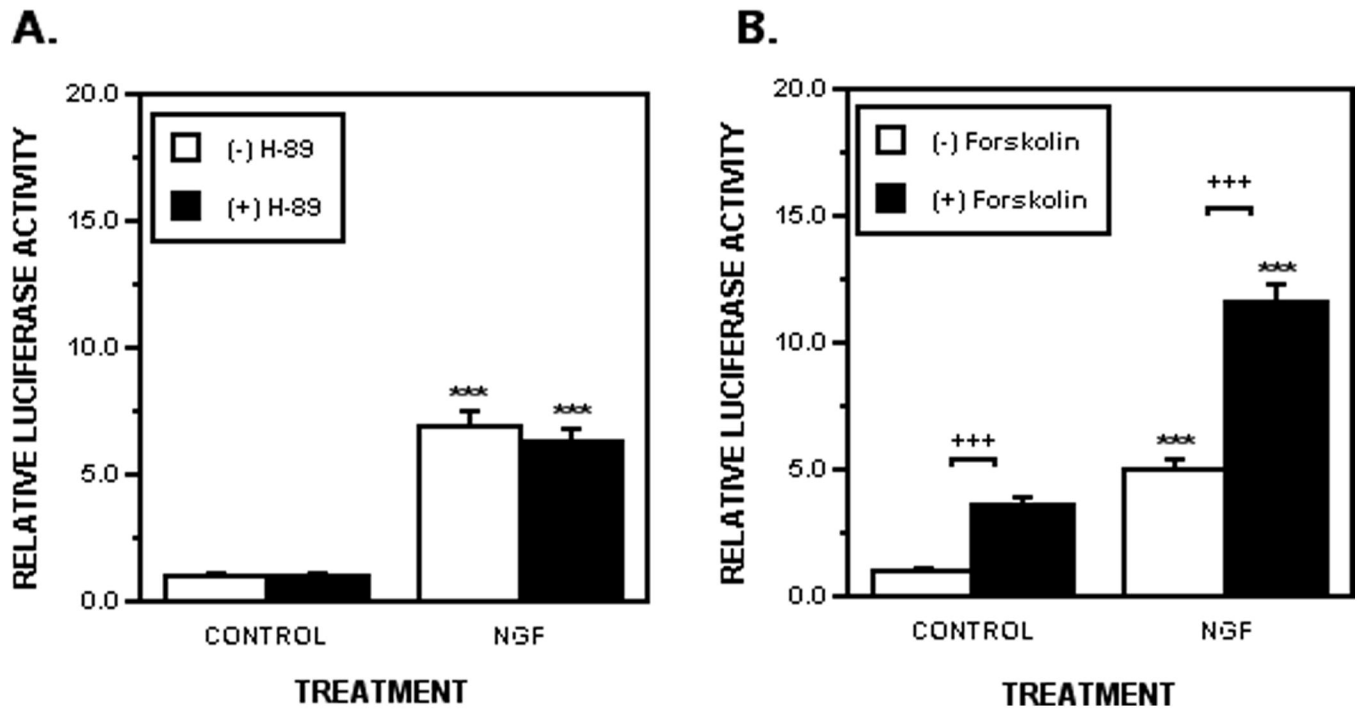


Fig. 3

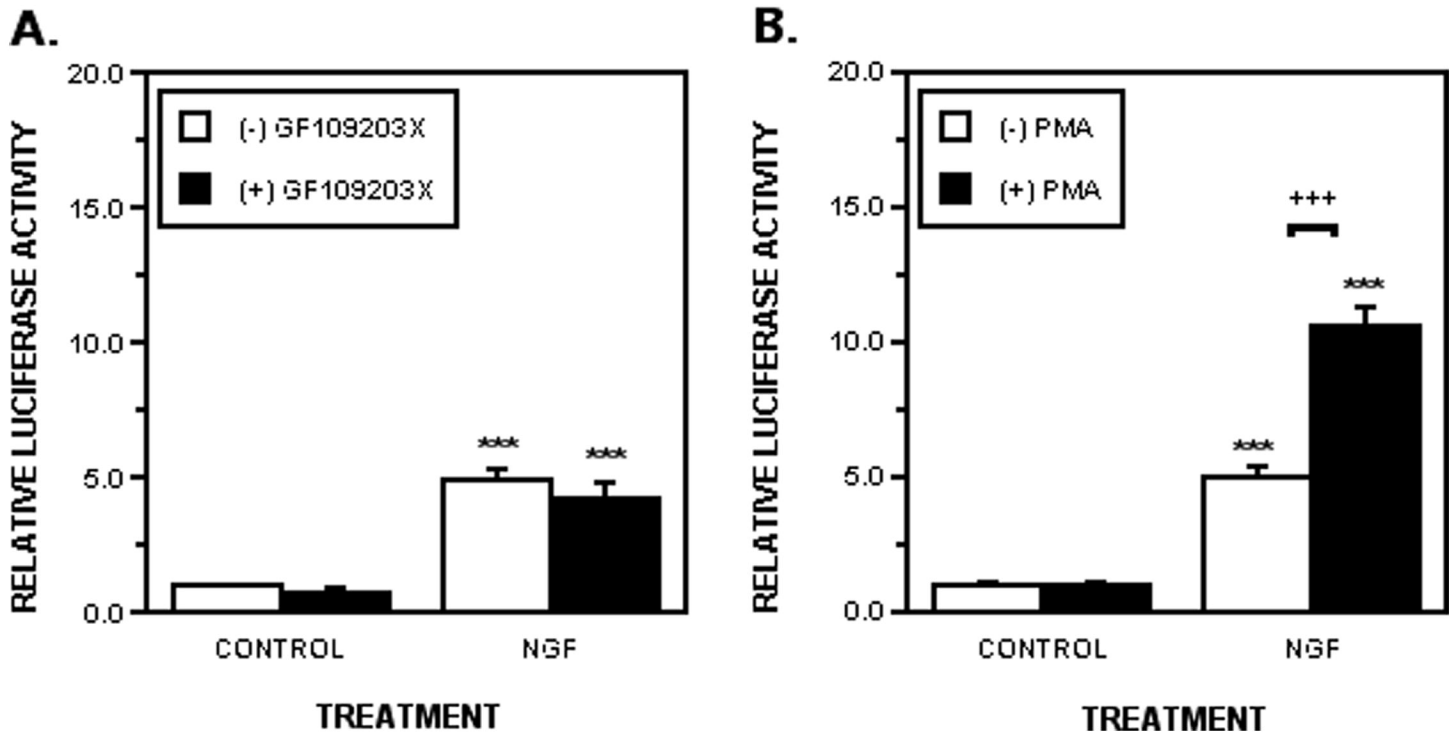


Fig. 4

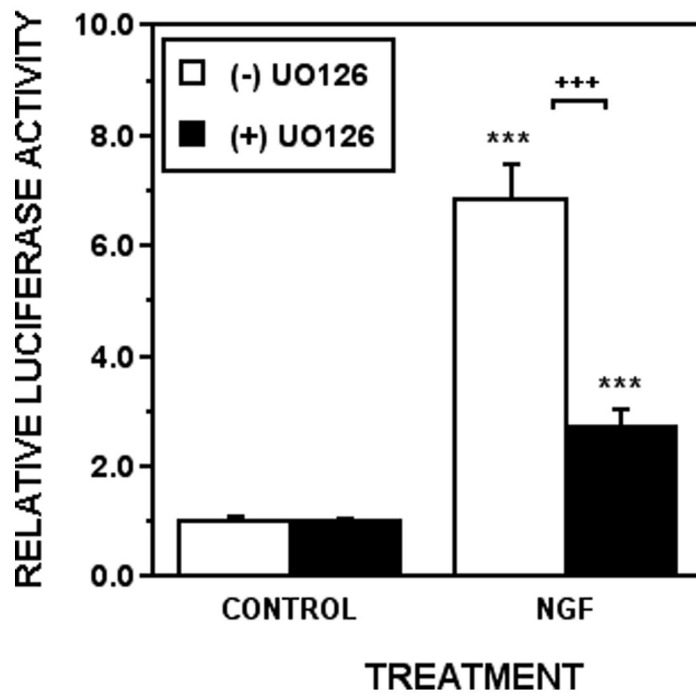


Fig. 5

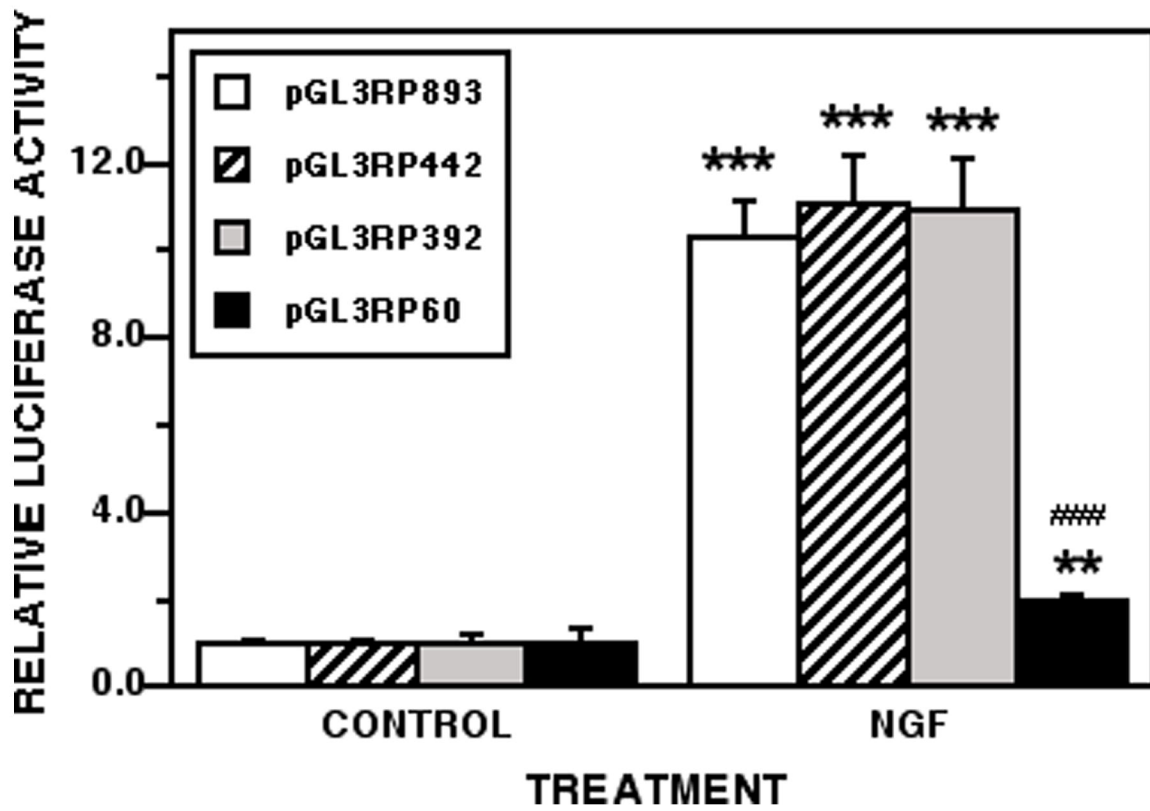


Fig. 6

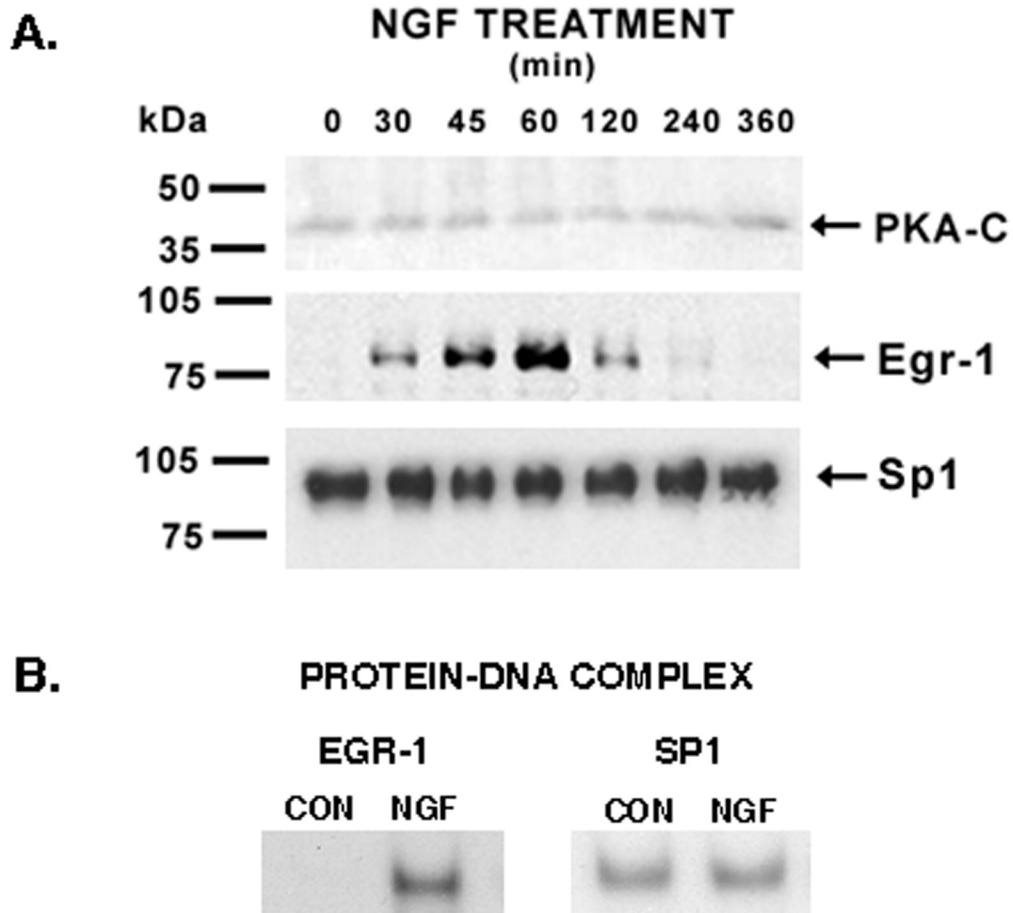


Fig. 7

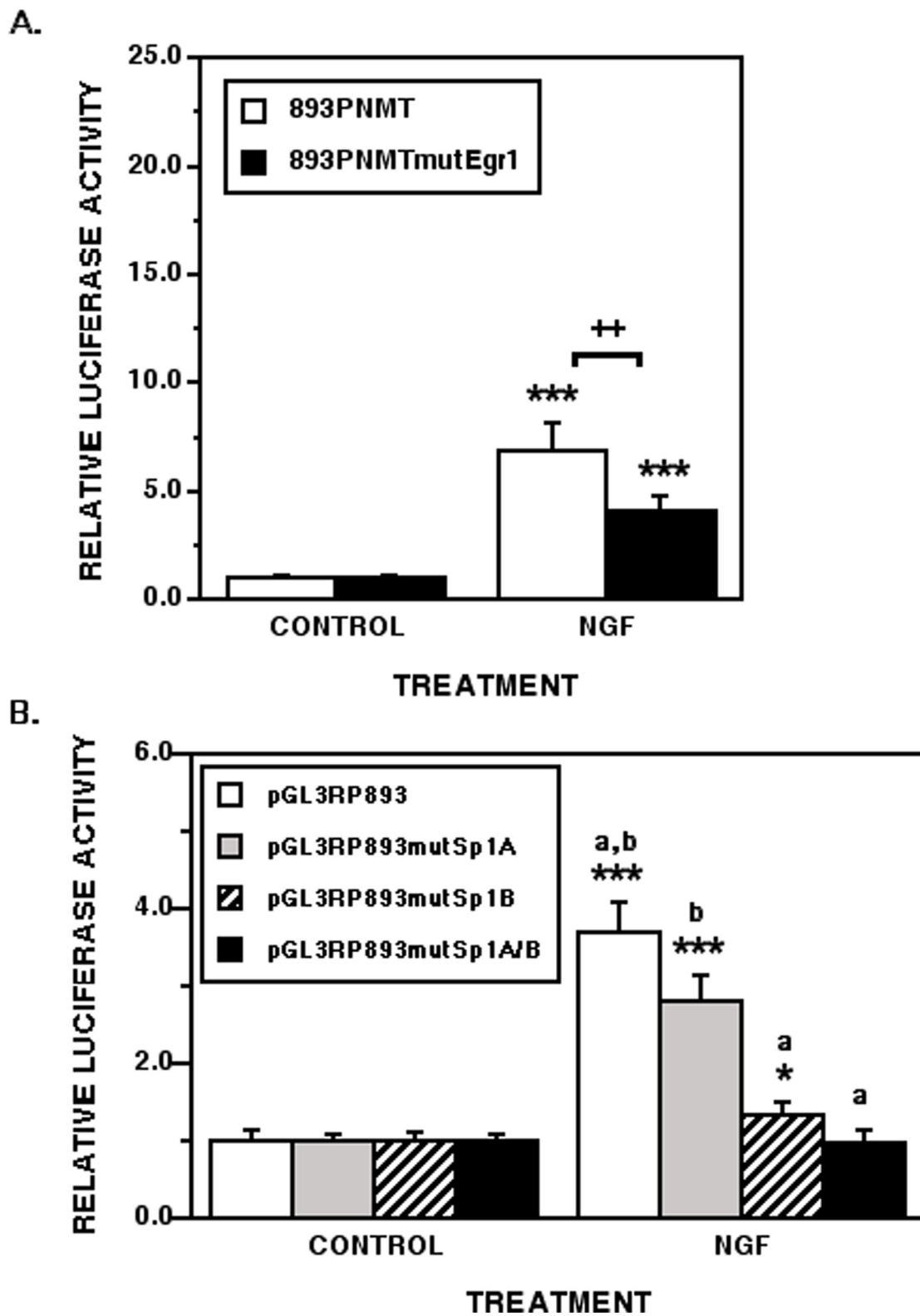


Fig. 8

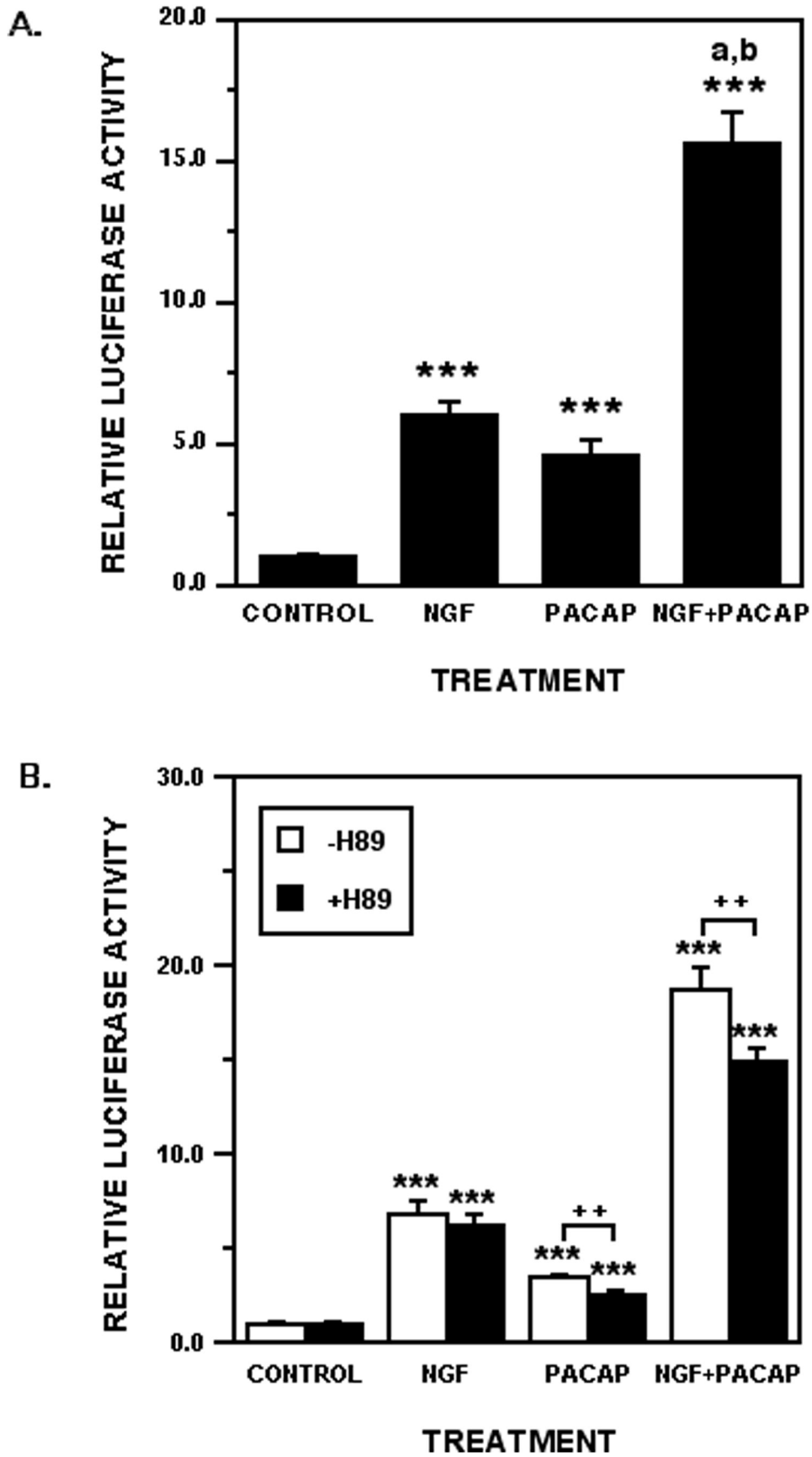


Fig. 9

