The Journal of Neuroscience, June 1995, 15(6): 4580–4591

Differential Regulation of Sympathetic Neuron Neuropeptide Y and Catecholamine Content and Secretion

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Cultured principal neurons of the superior cervical ganglion (SCG), which coexpress high levels of catecholamines and neuropeptide Y (NPY), were used as a model to simultaneously examine whether sympathetic neuronal peptide and transmitter content or secretion are differentially regulated. Accumulation of NPY immunoreactivity and the dopamine metabolites DOPAC and HVA in SCG neuronal conditioned culture medium was used as an index of NPY and catecholamine secretion, respectively. Release of NPY and catecholamines was linear with time; SCG neurons exhibited a basal NPY secretory rate of approximately 0.9–3 fmol NPY immunoreactivity/10^4 cells/hr, and basal DOPAC plus HVA accumulation was about 10–20 pmol total metabolites/10^4 cells/hr. While sympathetic neuronal NPY and total catecholamine cell content increased more than 6–10-fold by 14 d of culture, secretion remained constant. Depolarization stimulated the rate of NPY secretion 18-fold, whereas medium catecholamine metabolite levels increased 3-fold. Activation of intracellular signaling pathways was shown to be an important point of regulation of sympathetic neuron peptide and transmitter content and secretion. Differential regulation of SCG neuron NPY and catecholamine expression was second messenger system specific. Activation of the protein kinase A pathway with the cAMP analog dibutyryl cAMP, or the adenylyl cyclase activator forskolin, produced a concentration-dependent, sustained stimulation of NPY secretion; maximal stimulation resulted in decreased cellular NPY content. Parallel stimulated neuronal catecholamine release was observed, but in contrast to NPY, total cellular catecholamine content was also increased. Regulation of the protein kinase C pathway with phorbol myristate acetate (PMA) stimulated SCG neuronal NPY secretion to a lesser degree than activation of protein kinase A, but did not alter cellular NPY levels. PMA minimally stimulated catecholamine release and content. NPY secretion induced by the calcium ionophore A23187 was paralleled by a concomitant decrease in cellular NPY. A23187 decreased catecholamine release, but did not change cellular total catecholamine levels. The magnitude of the secretory responses of sympathetic neurons to these regulators was far greater than changes in NPY or catecholamine content, biosynthesis or mRNA levels, suggesting that release is a primary site of regulation. The independent regulation of sympathetic neuronal NPY and catecholamine content and release is consistent with the fundamental differences in the biosynthetic pathways, vesicular compartmentalization, uptake and metabolism of neuropeptides and neurotransmitters.

[Key words: neuropeptide Y, catecholamine, sympathetic neuron, superior cervical ganglion, secretion, regulation]

The coexpression of neuropeptides and neurotransmitters in individual neurons encodes a vast set of distinct signals to target cells and ultimately contributes to the functional diversity of the nervous system. The neurobiology of peptidergic systems, however, differs fundamentally from classical transmitter systems. Neuronal bioactive peptide levels are frequently more than two orders of magnitude lower than levels of small molecule neurotransmitters. Moreover, neuropeptides are synthesized in the neuronal soma and transported to the axon nerve terminals, whereas neurotransmitters are synthesized in the terminals proper (Sossin et al., 1989; Kelly, 1991, 1993; Sudhof and Jahn 1991). Although these processes are understood, few neuronal systems are directly amenable to experimental studies of the coordinate or differential regulation of neuropeptide and neurotransmitter expression.

Neuropeptide Y (NPY), a highly expressed 36 amino acid α-amidated peptide (Tatemoto et al., 1982; Allen et al., 1983; O'Donohue et al., 1985; DeQuidt and Emson, 1986), and catecholamines are frequently colocalized in cells of sympathoadrenal lineage (O'Donohue et al., 1985; Landis, 1988). *In vivo*, NPY and catecholamines are co-released from sympathetic neurons and adrenal chromaffin cells (Winkler and Carmichael, 1982; Allen et al., 1984). Increased neuronal activity elevates plasma levels of sympathetic neuronal NPY and norepinephrine, and activation of the adrenal medulla stimulates both NPY and epinephrine secretion into the circulation (O'Donohue et al., 1985; Lundberg et al., 1986, 1989). NPY elicits potent dose-dependent vasoconstriction and modulates the vasoconstricting actions of norepinephrine (Lundberg et al., 1982; Franco-Cereceda and Lundberg, 1987). The relative abundance of catecholamines and NPY in sympathoadrenal cells has proven useful for studying how anterograde and retrograde signals regulate neurotransmitter or neuropeptide expression.

Received July 21, 1994; revised Jan. 11, 1995; accepted Jan. 19, 1995.

This work was supported by National Institutes of Health Grants HD-27468 and NS-01636 (to V.M.) and National Science Foundation Grant DIR-9116229 (to V.M. and K.M.B.). We thank Drs. Carson J. Cornbrooks, Ellen B. Cornbrooks, and Richard E. Mains for initial help in the development of the cell culture system. Dr. Richard E. Mains is gratefully acknowledged for the generous gift of NPY antiserum JH3. The encouragement of Drs. Betty A. Eipper and Richard E. Mains throughout the development of this project is greatly appreciated.

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Rat PC12 pheochromocytoma and bovine adrenal medullary chromaffin cells, for example, have been widely used to examine the regulation of tyrosine hydroxylase expression, the rate-limiting catecholamine biosynthetic enzyme. In these cell types, cAMP analogs and activators of the cAMP–protein kinase A second messenger pathway increase tyrosine hydroxylase activity, biosynthesis, gene transcription, and mRNA levels (Joh et al., 1978; Lewis et al., 1987, Zigmond et al., 1989; Kim et al., 1993). Stimulation of sympathoadrenal cell protein kinase C with phorbol esters increases synthesis of dihydroxyphenylalanine (DOPA), the catecholamine biosynthetic intermediate, and transcription of the tyrosine hydroxylase gene (Zigmond et al., 1989; Vyas et al., 1990; Icard-Liepkalns et al., 1992). Other regulators, including calcium and calcium ionophores, have been suggested to stimulate the rate of tyrosine hydroxylation and activate tyrosine hydroxylase (Tachikawa et al., 1986; Zigmond et al., 1989).

Regulation of NPY expression has not been as well examined. Pro-NPY mRNA levels are induced in PC12 cells and N18TG-2 neuroblastoma cells by activation of either the protein kinase A or protein kinase C pathways, or by stimulation of the glucocorticoid receptor system (Higuchi et al., 1988; Sabol and Higuchi, 1990). In contrast to the PC12 and N18TG-2 cells, pro-NPY mRNA expression in NG108-15 neuroblastoma × glioma hybrid cells is inhibited following protein kinase A activation (Higuchi et al., 1988).

The results of the few regulatory studies of NPY and catecholamines in primary cultured neuronal cells have often differed from results using tumor cell lines, and may reflect the different cell types or measures examined. Among primary cultured neuron systems, dissociated neurons from the superior cervical ganglion (SCG) have been widely examined (Mains and Patterson, 1973a–c; Kessler, 1985; Landis, 1988; Marek and Mains, 1989, 1990). Most principal neurons of the SCG are catecholaminergic and almost two-thirds of the neurons coexpress norepinephrine and NPY (Jarvi et al., 1986; Hokfelt et al., 1987; Landis, 1988; Freidin et al., 1993). The high levels of NPY and catecholamine expression in cultured SCG neurons permit regulatory and biosynthetic studies, and cultured isolated SCG neurons have served as a unique model system to investigate the regulation of both neuropeptide and classical transmitter expression in the absence of preganglionic inputs or target cell influences. Unlike the pheochromocytoma and neuroblastoma cell lines, chronic activation of SCG neuron protein kinase A elevates both pro-NPY and tyrosine hydroxylase mRNA levels without affecting neuronal neuropeptide or catecholamine content or biosynthesis (Marek and Mains, 1990; Freidin et al., 1993). On the other hand, stimulation of protein kinase C decreases SCG neuronal norepinephrine and NPY biosynthesis without altering either pro-NPY or tyrosine hydroxylase mRNA (Marek and Mains, 1990). These results suggest that regulation of NPY and catecholamines is tissue specific, and implicate post-transcriptional mechanisms as important determinants of neuropeptide and neurotransmitter expression.

Although these results appear to suggest that the various parameters of NPY and catecholamine expression are differentially regulated, none of the previous studies have considered the importance of neuronal NPY or catecholamine secretion. In the current studies, we have identified secretion as a critical parameter in the regulation of SCG neuropeptide and neurotransmitter expression. We have established enriched primary SCG neuronal cultures and characterized both basal and stimulated NPY and catecholamine secretion. Furthermore, we have demonstrated that activation of specific second messenger systems differentially regulates SCG cellular neuropeptide and neurotransmitter levels and secretion.

Materials and Methods

Cell culture. Primary superior cervical ganglion neuron cultures were prepared by a modification of previously described methods (Marek and Mains, 1989; Higgins et al., 1991). Untimed pregnant Sprague–Dawley rats (Charles River, Quebec, Canada) given ad libitum access to commercial rat chow and tap water were maintained on a 12 hr light/12 hr dark cycle. SCG from neonatal rats from three to four litters, typically 35–50 animals (70–100 ganglia), were dissociated together, yielding a pooled population of cells. The ganglia were dissected free of surrounding connective tissue, coarsely blocked and dissociated in Dulbecco's modified Eagle medium/air (DMEM; May and Eipper, 1986) containing 4 mg/ml collagenase (CLS 2, 171 U/mg; Worthington Biochemical Corp., Freehold, NJ), 1 mg/ml hyaluronidase (1897 U/mg; Worthington Biochemical Corp., Freehold, NJ), 10 μg/ml DNase (type I, 2000 Kunitz/mg; Sigma Chemical Co., St. Louis, MO), and 10 mg/ml bovine serum albumin (BSA) by gentle agitation on a variable rate stirring plate at 300 rpm for 20 min at 37°C. The tissue fragments were collected by centrifugation, further dissociated with 3 mg/ml trypsin (225 U/mg; Worthington Biochemical Corp., Freehold, NJ) for 3 min, and dispersed into isolated cells by trituration. The resulting cell suspension was filtered through a 100 μm nylon mesh, and the cells plated onto double-type I collagen (Collaborative Research, Bedford, MA) coated multiwell culture plates (Higgins et al., 1991); the cell yield was typically 65–70%. Neuronal NPY content and secretion were identical in cells cultured on different substrata; however, collagen best facilitated plating efficiency for long term culture. The cells were cultured in DMEM-Ham's F-12 medium (1:1) containing 5% rat serum (GIBCO-BRL Life Technologies, Inc., Grand Island, NY), 10% Nu-Serum (Collaborative Research, Bedford, MA), 32 ng/ml 2.5S nerve growth factor (NGF; Collaborative Research, Bedford, MA), 15 mM HEPES, 1.2 gm/liter sodium bicarbonate, 4.5 gm/liter glucose, and 50 μg/ml gentamicin. SCG cells plated at initial densities of 1.9×10^3 to 1.5×10^4 cells/cm^2 exhibited similar NPY and catecholamine per neuron; the secretory rate as a percent of the cell content also remained unchanged. For all subsequent experiments, the cells were plated at an initial density of 1.5×10^4 neurons/cm^2 and incubated at 37°C in a humidified atmosphere of 95% air, 5% CO_2. Thirty-six hours following plating, the cultures were treated with 10 μM cytosine β-D-arabinofuranoside (ARA-C; Sigma Chemical Co., St. Louis, MO) for 24 hr to minimize non-neuronal cell proliferation. The SCG cells were then maintained in a modified complete serum-free medium (CSFM; 250 μl CSFM/1.5×10^4 cells) consisting of DMEM-Ham's F-12 medium (1:1) containing 10 μg/ml insulin, 20 μg/ml transferrin, 1 mg/ml BSA, 10 nM T_3, 32 ng/ml 2.5S NGF, trace elements (0.5 nM $MnCl_2 \cdot 4H_2O$, 0.5 nM $(NH_4)Mo_4O_{24} \cdot 4H_2O$, 0.25 nM $SnCl_2$, 25 nM $NaVO_4 \cdot 4H_2O$, 0.25 nM $NiSO_4 \cdot H_2O$, 15 nM H_2SeO_3, 25 nM $Na_2SiO_3 \cdot H_2O$, and 5 nM $CdSO_4$), 15 mM HEPES, 1.2 gm/liter sodium bicarbonate, 4.5 gm/liter glucose, and 50 μg/ml gentamicin (May and Eipper, 1986; Higgins et al., 1991); the culture medium was replaced completely every 48 hr.

For each treatment paradigm, replicate cultures from a single dissociation were used for control and treated neurons. Unless indicated otherwise, cells were incubated in 250 μl CSFM/1.5×10^4 cells containing vehicle or drug beginning on day 9 of culture. Each treatment paradigm was repeated two to five times. Each trial used 3–10 individual replicate cultures per data point. Dibutyryl cAMP (dBcAMP; Boehringer Mannheim Corp., Indianapolis, IN) was prepared in culture medium and filtered through a 0.22 μm filter before use. Phorbol myristate acetate (PMA; Calbiochem Corp., San Diego, CA) was prepared in dimethyl sulfoxide (DMSO) as a 1 mM stock; A23187 (Calbiochem Corp., San Diego, CA) was prepared as a 10 mM stock solution in DMSO. Forskolin (Calbiochem Corp., San Diego, CA) was prepared in DMSO as a 200 mM stock. Cholera toxin and pertussis toxin were obtained from List Biological Laboratories, Inc. (Campbell, CA) and dissolved in sterile water as 1 mg/ml and 100 μg/ml stock solutions, respectively.

Peptide level analysis. Following specific treatment paradigms, the conditioned culture medium was removed, centrifuged to remove debris and immediately stored at −85°C until time of assay. The SCG cells were extracted in 5 *N* acetic acid containing 2 mg/ml BSA and 30 μg/ml phenylmethylsulfonyl fluoride (PMSF). The cell extracts were frozen

and thawed three times, lyophilized and resuspended in 100 mM sodium phosphate buffer, pH 7.6, containing 1% Triton X-100 and 30 μg/ml PMSF (May and Eipper, 1986).

To determine cellular and secreted peptide levels, double antibody radioimmunoassays were performed in 50 mM sodium phosphate, pH 7.6, containing 2.5 mg/ml BSA in a final assay volume of 200 μl with 1:100,000 dilution of rabbit antiserum JH3 directed against synthetic NPY (obtained from Dr. R. E. Mains, The Johns Hopkins University, Baltimore, MD; Braas et al., 1994a). Samples were incubated with antiserum for 24 hr at 4°C prior to the addition of ^{125}I-NPY (Amersham Corp., Arlington Heights, IL). Competitive binding was allowed to proceed for an additional 24 hr at 4°C, and bound ligand was separated from free by double-antibody immunoprecipitation (May and Eipper, 1986; Braas et al., 1994a; 1994b). The assay exhibited a midpoint of 10–15 fmol. Each sample was assayed in triplicate to quintuplicate. In previous peptide stability tests, more than 85% of radiolabeled pro-NPY-related peptides was quantitatively recovered 48 hr after addition to the SCG cultures (Marek and Mains, 1989). The cumulative secreted peptide levels were calculated as the sum of the NPY immunoreactive material secreted per well for each 24 or 48 hr period within the specific treatment period.

Catecholamine level analysis. Cell culture medium was removed, centrifuged to remove debris, diluted with an equal volume of 100 mM perchloric acid containing 0.15 mM EDTA and frozen at −85°C until time of assay. For most studies of SCG catecholamine expression, samples of the culture media from individual wells were processed for both NPY and catecholamine levels. The cells were extracted in 100 mM perchloric acid containing 0.15 mM EDTA, frozen and thawed three times, sonicated and centrifuged to remove particulate matter. All medium and cell samples were centrifugally ultrafiltered using Ultrafree-MC Durapure filter units (Millipore Corp., Bedford, MA) prior to analysis.

Electrochemical catecholamine assays using reversed phase high performance liquid chromatography were used to measure cellular and secreted biogenic amines (Marek and Mains, 1989, 1990). Samples were fractionated using a Sensitive Manipulation and Recovery Technology (SMART) microanalytical system (Pharmacia LKB Biotechnology, Piscataway, NJ) with a Pharmacia 5 μm reversed phase Sephasil C_{18} SC 2.1/10 column at a solvent flow rate of 150 μl/min. Catecholamine and metabolite levels were quantitated following electrochemical detection with a Hewlett Packard 1049A electrochemical detector with a glassy carbon electrode. The mobile phase was composed of 75 mM monochloroacetic acid, pH 3.1, containing 0.67 mM Na_2EDTA, 9.26 mM octane sulfonic acid, and 0.5% tetrahydrofuran. The elution positions of known standards were confirmed using a second solvent system. Catecholamine levels were quantitated by peak area using the peak integration data evaluation program of SMART MANAGER operating software (version 1.41). At a flow rate of 150 μl/min and an oxidation potential of +0.70 V (50 nA full scale), the catecholamine detection limit (signal two times background) was less than 250 fmol. Standard curves were constructed from the evaluation of five to eight known concentrations of each standard compound, and were linear from less than 250 fmol to over 50 pmol of catecholamine or metabolites; sample amine levels were determined by comparison to standard curves. Treatment of the cultures with monoamine oxidase inhibitors or catecholamine uptake inhibitors was detrimental to the cells under long term conditions and was not used in subsequent studies (Sved, 1990). The cumulative medium catecholamine metabolite levels were calculated as the sum of the material accumulated per well for each 24 or 48 hr period within the specific treatment period.

Immunocytochemistry. SCG cells were fixed in 4% paraformaldehyde and immunocytochemically stained using a modification of the avidin–biotin–peroxidase complex technique (May et al., 1990; Braas et al., 1992; 1994a). Nonspecific binding was blocked with 1:200 normal goat serum (NGS) in 0.1 M sodium phosphate buffer, pH 7.5, and the cells were incubated in 1:50,000 anti-NPY (JH3) or 1:10,000 anti-tyrosine hydroxylase (Eugene Tech International, Inc., Allendale, NJ) in 1:200 NGS for 48 hr at 4°C. Following washing and blocking, the neurons were incubated with 1:400 biotinylated goat anti-rabbit IgG in 1:200 NGS for 60 min. The cultures were subsequently washed, blocked and incubated with 1:200 avidin–biotin–peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA) in 0.1 M sodium phosphate buffer for 60 min. Rinsed cells were reacted with diaminobenzidine and hydrogen peroxide as peroxidase reaction substrates. Morphometric analyses were performed as previously described (May and Eipper, 1986; Braas et al., 1994a). Immunocytochemically stained SCG cultures were viewed under a 10× objective and a 10× ocular lens with a calibrated grid reticule positioned in one eyepiece (Braas et al., 1994a). Stained cells within random grid areas were enumerated.

Data analysis. Analysis of variance (ANOVA) was used to determine differences among treatments. Newman-Keuls test was used in post hoc analysis to identify which treatments differed from the others. Significance of changes in secretion and cell content were evaluated; a P value of less than 0.05 was considered significant. All values are expressed as the mean ± standard error of the mean (SEM). For data points without apparent error bars, the error bars are within the symbols. Secretory rates were calculated using the SIGMAPLOT FOR WINDOWS version 1.01 software (Jandel Scientific, San Rafael, CA).

Results

Superior cervical ganglion neuronal NPY and catecholamine secretion remains constant, whereas content increases with time in culture

Initial studies were conducted to establish the basal NPY secretory rate in primary cultured SCG neurons. Examination of the cumulative NPY secreted by the sympathetic neurons revealed that the peptide secretory rate remained constant over the entire culture period, with the neurons exhibiting a secretory rate of 0.89 fmol/10^4 cells/hr (5.3 fmol/ml medium/hr; Fig. 1*A*). Basal secretory rates among cultures from different dissociations generally ranged from approximately 0.9–3 fmol/10^4 cells/hr. In contrast to the stable basal NPY secretion, cellular NPY content increased more than 10-fold with time in culture (Fig. 1*B*). The increased neuronal cell NPY content closely paralleled the increased biosynthesis of NPY and catecholamines reported in previous studies (Mains and Patterson, 1973a–c; Marek and Mains, 1989). At early culture periods, the basal release of NPY material into the culture medium represented a secretory rate of 0.3% of the cellular content/hr, which was comparable to hormonal secretory rates of cultured endocrine cells (May and Eipper, 1986). As the cellular NPY content increased with time in culture, but secretion remained constant, the secretory rate of NPY expressed as a function of culture NPY content declined to less than 0.05% of cell content/hr.

Matched cultured SCG cells were immunocytochemically stained for NPY to identify whether the population of SCG neurons expressing NPY immunoreactivity increased throughout the 28 d of culture. NPY immunoreactivity was observed in neuronal processes, fiber varicosities and soma of a subpopulation of neurons (Fig. 2*A*,*B*). Fifty-six percent of the SCG neurons exhibited NPY immunoreactivity throughout the entire culture period, which was similar to values reported for intact adult SCG and cultured neurons (Jarvi et al., 1986; Hokfelt et al., 1987; Marek and Mains, 1989; Freidin et al., 1993). Thus, when the amount of NPY per cell was calculated based on 56% of the population, each neuron contained approximately 0.5 fmol of NPY immunoreactive material after 14 d of culture.

The coexpression of NPY and catecholamines in SCG neurons has permitted us to simultaneously examine regulation of both neuropeptide and neurotransmitter content and secretion in individual cell cultures. Primary cultured SCG neuron total catecholamine/metabolite content increased five- to sixfold by 3 weeks of culture to 17.4 fmol of total catecholamine/metabolite per neuron (data not shown). These levels represented 70% of adult SCG neuronal catecholamine levels and were in close agreement with previous estimates of maximal SCG catecholamine content in culture (Iacovitti et al., 1982; Kessler, 1985). The developmental profile of catecholamine expression was similar to that for norepinephrine biosynthesis (Mains and Patterson,

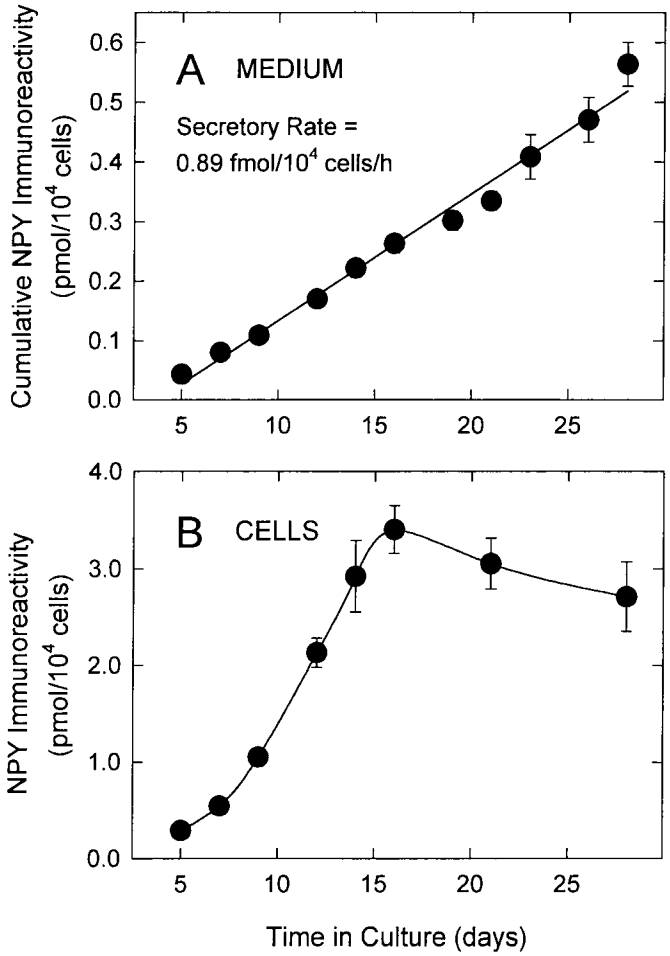

Figure 1. Developmental expression of cultured SCG neuronal NPY content and secretion. SCG neurons from neonatal rats were plated at a density of 1.5 × 10^4 cells/cm^2, and were cultured in 250 μl CSFM/1.5 × 10^4 cells. On the indicated days after plating, the cells were extracted and the conditioned culture medium collected for NPY radioimmunoassay. Data for neuronal cumulative NPY secreted (*A*) and NPY content (*B*) represent the mean NPY immunoreactivity of five cultures assayed in triplicate ± SEM.

1973a; 1973b; Marek and Mains, 1989). While NPY was localized to a subpopulation of sympathetic neurons, the catecholamine biosynthetic enzyme tyrosine hydroxylase was localized in nearly every neuron at all culture times (Fig. 2*C*). When SCG neurons were cultured in CSFM, which does not contain the dopamine β-hydroxylase cofactor ascorbate, the ratio of norepinephrine to dopamine in the neurons declined rapidly within 48 hr, reflecting diminished norepinephrine biosynthetic capability and consequential cellular accumulation of dopamine. By day 13 of culture, the cellular content of catecholamines and metabolites (norepinephrine:dopamine:epinephrine:3,4-dihydroxyphenylacetic acid:homovanillic acid or NE:DA:E:DOPAC:HVA) was 3.9:1.0:0.04:0.7:0.8. Since the ratios of cellular catecholamines and metabolites were stable, even in long term cultures in the absence of added ascorbate, compounds other than ascorbate may also serve as cofactors for dopamine-β-hydroxylase in norepinephrine production.

Although norepinephrine was stable in CSFM in the absence of cells, it was rapidly metabolized in the presence of SCG cells; neither norepinephrine nor its metabolites could be measured in conditioned culture medium. While dopamine was rapidly metabolized to DOPAC and HVA in the presence of SCG cells, the metabolites were stable in the culture medium (Fig. 3*A,B*). Greater than 65% of exogenously added dopamine to the SCG cultures was quantitatively recovered as DOPAC and HVA. The accumulation of both metabolites in medium from SCG neuronal cultures was stable throughout the 24–48 hr time periods examined for NPY secretion (Fig. 3*B*). DOPAC and HVA accumulated in the medium linearly with time at a rate of approximately 11–18 pmol/10^4 cells/hr and could be used as an index of SCG catecholamine release into the medium (Fig. 3*B,C*).

Depolarization differentially increases sympathetic neuron NPY secretion and medium DOPAC and HVA levels

To determine whether SCG neuronal NPY and catecholamine secretion were differentially stimulated by depolarization, cells were cultured in the presence of 40 mM potassium. Depolarization elicited a sustained 18-fold increase in the NPY secretory rate (Fig. 4*A*). The NPY secretory rate increased from 3.0 fmol/10^4 cells/hr under basal conditions to 54 fmol/10^4 cells/hr in the presence of high potassium concentrations. There were no apparent changes in neuronal morphology during the treatment period, although treatment periods greater than 96 hr diminished the secretory response.

While depolarization increased the NPY secretory rate 18-fold, incubation of SCG cultures with 40 mM potassium elicited only a threefold increase in medium dopamine metabolite levels, which most likely reflected an augmentation in neuronal catecholamine release (Fig. 4*B*). The rate of DOPAC and HVA accumulation increased from a basal 14 pmol/10^4 cells/hr to 41 pmol/10^4 cells/hr under depolarizing conditions. The elevated DOPAC and HVA levels were sustained for 96 hr, but like NPY, the levels of stimulation were significantly decreased at time points greater than 96 hr. Although the depolarization-induced increase in medium DOPAC and HVA levels was considerably less than the induced increase in NPY release, this may have resulted from the inherent differences between neuropeptide and catecholamine biosynthesis, uptake, or metabolism.

Regulation of sympathetic neuron NPY content and secretion is second messenger system specific

Since activation of different second messenger systems elicits tissue-specific regulation of NPY biosynthesis and mRNA expression, we compared the effects of protein kinase A or protein kinase C activation and calcium ionophores on SCG neuronal NPY content and secretion. Previous studies demonstrated that stimulation of intracellular signaling pathways elicits tissue-specific regulation of neuroendocrine NPY expression. In addition, the reported effects were dependent upon the parameter of NPY expression measured. While treatment of primary SCG cultures with cAMP analogs augmented NPY mRNA levels, neither NPY content nor biosynthesis appeared altered (Marek and Mains, 1990; Freidin et al., 1993). In contrast, treatment with phorbol esters did not alter NPY mRNA levels, but modestly decreased NPY biosynthesis (Marek and Mains, 1990). Therefore, the function of protein kinase A or C pathway activation in the regulation of SCG NPY has been uncertain. None of the studies to date, however, examined NPY secretion, which may have contributed to these apparent discrepancies.

Dibutyryl cAMP activation of the protein kinase A second messenger pathway elicited differential effects on SCG culture NPY content and release; NPY secretion was elevated, whereas cellular NPY content was decreased (Fig. 5*A,B*). NPY secretion

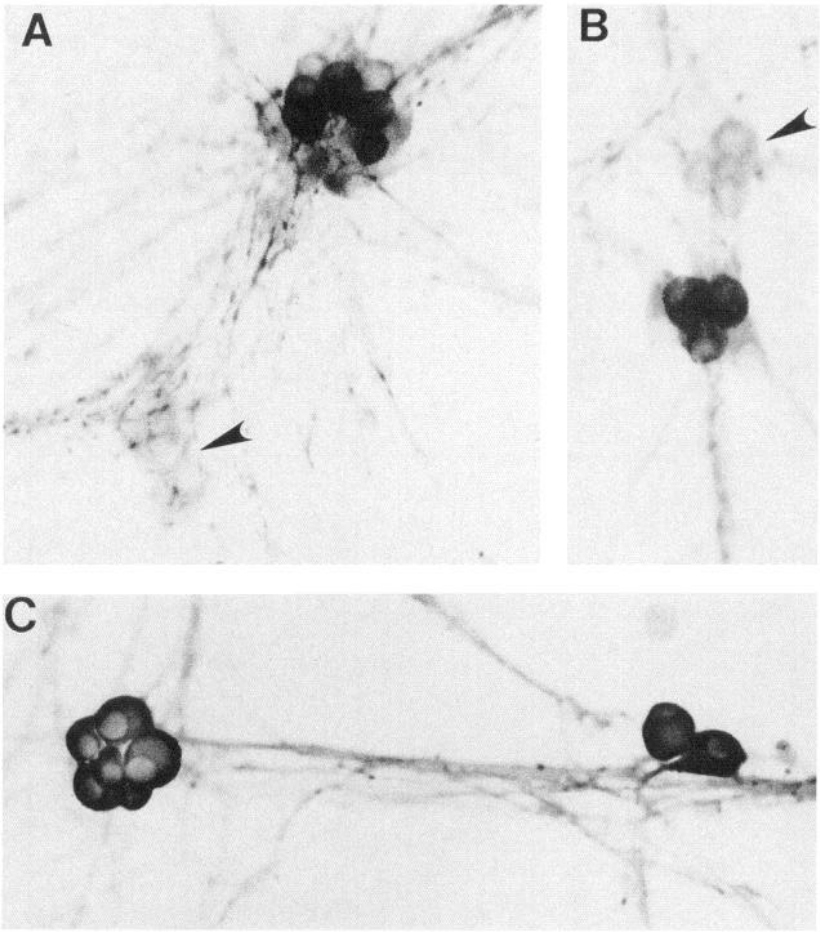

Figure 2. NPY is localized to a stable subpopulation of sympathetic neurons. Cultured SCG neurons were fixed with paraformaldehyde and immunocytochemically stained with 1:50,000 anti-NPY (*A*, *B*) or 1:10,000 anti-tyrosine hydroxylase (*C*) using the avidin–biotin–peroxidase complex technique. The number of neurons expressing NPY or enzyme immunoreactivity was determined as described in Materials and Methods. NPY immunoreactivity was localized to the processes, varicosities and soma of 56% of the cultured neurons, whereas tyrosine hydroxylase immunoreactivity was observed in nearly all of the cells. *Arrowheads*, Unstained neurons.

was increased maximally over 10-fold from 2.2 fmol/10^4 cells/hr to 35 fmol/10^4 cells/hr with 3.2 mM dBcAMP (Fig. 5*A*). At dBcAMP concentrations that maximally stimulated secretion, cellular NPY content was diminished from 7.7 pmol/10^4 cells to 4.9 pmol/10^4 cells (Fig. 5*B*). Low concentrations of dBcAMP (100 μM) increased sympathetic neuronal NPY content approximately 20% compared to control cells, suggesting that low but sustained stimulation may increase NPY synthesis and storage. Total SCG NPY production in the dBcAMP-treated cultures (cumulative secreted NPY plus cellular NPY content) was greater than control cultures, and suggested increased NPY biosynthesis following protein kinase A stimulation. Sustained stimulated secretion was observed in cultures treated with dBcAMP for 21 d (data not shown).

Similar sustained stimulatory responses were elicited following activation of the cAMP-protein kinase A pathway with forskolin. The diterpene compound forskolin, which activates the catalytic subunit of adenylyl cyclase, maximally increased SCG neuronal NPY secretion approximately fivefold (Fig. 5*C*); secretion increased from 6.6 fmol NPY/10^4 cells/hr to 34 fmol NPY/10^4 cells/hr with 10 μM forskolin. Consistent with dBcAMP-elicited decreased cellular NPY, forskolin diminished sympathetic neuronal NPY content 45% at concentrations that maximally stimulated secretion, suggesting that maximal stimulatory secretory rates exceeded the biosynthetic rates leading to depletion of intracellular neuropeptide stores (Fig. 5*D*). Increasing cellular cAMP levels following ADP-ribosylation of the guanine nucleotide binding protein G_s with cholera toxin increased NPY release approximately fourfold with all the concentrations tested (0.1 ng/ml to 10 μg/ml); the effects of pertussis toxin were much lower, eliciting less than a twofold increase in NPY secretion (data not shown).

Activation of the diacylglycerol-protein kinase C pathway with the phorbol ester PMA stimulated sustained SCG neuronal NPY secretion, but to a lesser degree than activation of protein kinase A (Fig. 6*A*). Maximal stimulated release was attained with 32 nM PMA. Concentrations of PMA higher than 32 nM diminished the levels of stimulated secretion. Unlike protein kinase A stimulation, activation of the protein kinase C pathway did not change SCG cellular NPY levels at any concentration of PMA (Fig. 6*B*); consequently, total NPY production (cumulative secreted NPY plus cellular NPY content) was augmented by PMA. The differences in cellular NPY levels between d BcAMP and PMA treated cultures may be a consequence of the differential regulation of neuropeptide production/secretion coupling between the two intracellular second messenger pathways.

NPY secretion induced by the calcium ionophore A23187 was paralleled by a concomitant decrease in cellular NPY (Fig. 6*C,D*). A23187 maximally stimulated NPY release less than threefold; secretion increased from 5.5 fmol NPY/10^4 cells/hr in control neurons to 15 fmol NPY/10^4 cells/hr with 100 nM A23187 (Fig. 6*C*). The calcium ionophore decreased the cellular NPY content (Fig. 6*D*) equivalent to the amount of NPY secreted. Neuronal NPY levels were decreased more than 50% with 32 nM A23187 compared to control cells; 100 nM A23187 decreased cellular NPY content to 20% of control. A23187 did

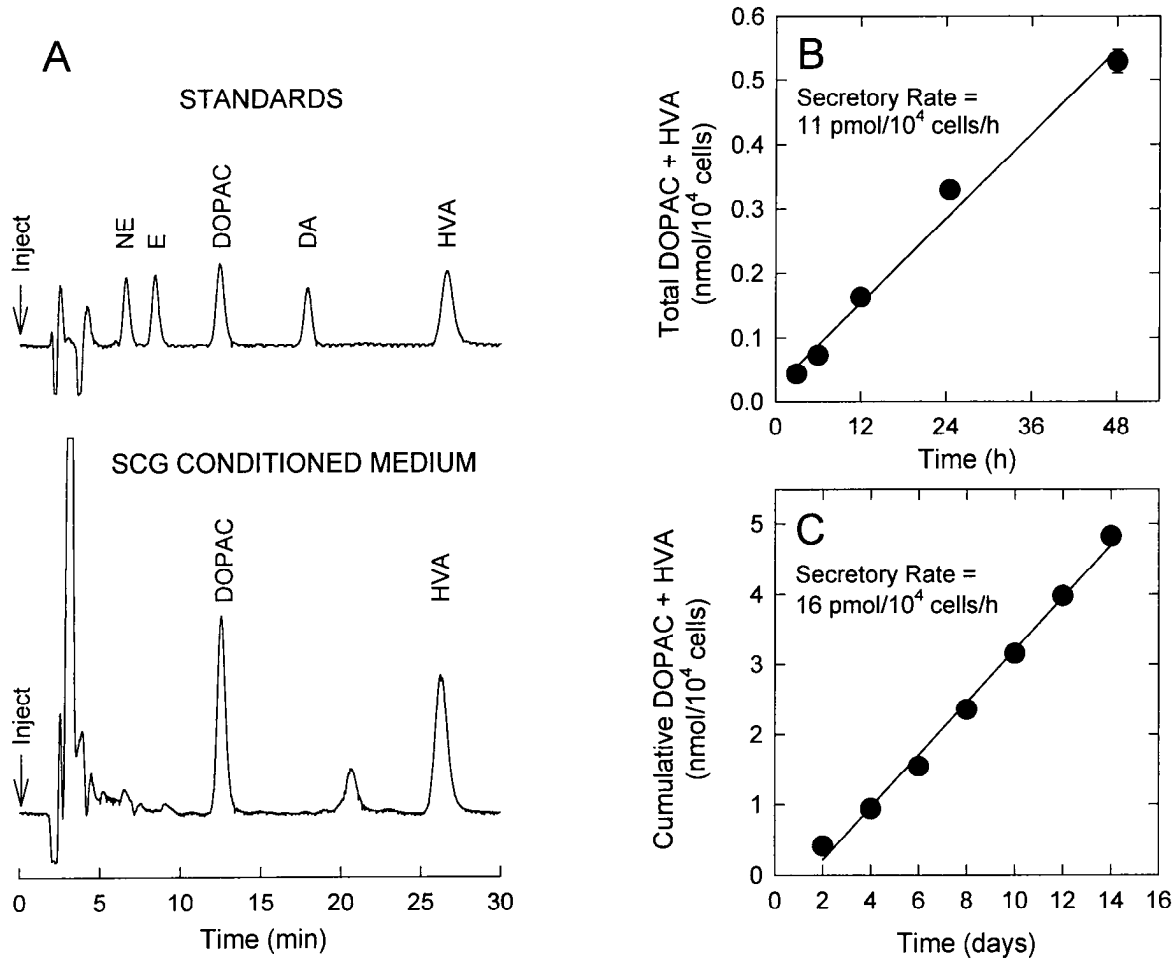

Figure 3. Accumulation of the catecholamine metabolites DOPAC and HVA in SCG culture medium is stable and linear with time. Sympathetic neurons plated at an initial density of 1.5×10^4 cells/cm^2 were cultured in 250 μl medium/1.5×10^4 cells beginning on day 9 following plating. The conditioned medium was analyzed for monoamine and metabolite levels using electrochemical detection coupled to reversed phase HPLC with a monochloroacetic acid/octane sulfonic acid/tetrahydrofuran solvent system. The HPLC profile showed that the primary catecholamine metabolites in the conditioned medium were DOPAC and HVA (*A*). At the indicated times, the conditioned medium was removed for metabolite assay (*B*). Accumulation of both metabolites was stable across the 48 hr times used for NPY analysis. Separate cultures were incubated in CSFM for 14 d beginning on day 9 of culture (*C*); the conditioned medium was collected for metabolite analysis and replaced with fresh CSFM each 48 hr. Data represent the mean total (*B*) or cumulative (*C*) nanomoles of DOPAC plus HVA per 10^4 cells from four to five cultures ± SEM. *NE*, Norepinephrine; *E*, epinephrine; *DOPAC*, 3,3-dihydroxyphenylacetic acid; *DA*, dopamine; *HVA*, homovanillic acid.

not change total NPY production (cumulative NPY secreted plus culture NPY content) from control cultures, whereas activation of the protein kinase A or protein kinase C pathways increased total NPY. These results suggested that under our culture conditions, the calcium ionophore had no apparent effects on NPY synthesis. Cultured neurons are acutely sensitive to cellular calcium levels (Johnson et al., 1992), and under current culture conditions, continuous treatment of the cultures for more than 4 d with concentrations of A23187 of 1 μM or greater were cytotoxic.

Sympathetic neuron NPY and catecholamine content and secretion are differentially regulated

To directly compare the regulation of NPY and catecholamine release from SCG neurons, cultures were treated with a maximal effective dose of each second messenger system activator. The conditioned medium from individual cultures was assayed for both NPY and catecholamines. Activation of the protein kinase A pathway with either dBcAMP or forskolin elicited parallel neuronal NPY and catecholamine release (Fig. 7*A*,*C*). Similar increases in NPY immunoreactivity and dopamine metabolite levels in SCG neuron conditioned medium suggested similar regulation of both neurotransmitter and neuropeptide secretion with protein kinase A activation.

Unlike protein kinase A stimulation, medium DOPAC and HVA levels were unchanged at 48 hr (data not shown) and increased to 160% of control following 96 hr of treatment with PMA (Fig. 7*C*). In contrast, PMA increased NPY secretion to over 300% of control at both times (Fig. 7*A*). Medium DOPAC and HVA levels from cultures treated with the calcium ionophore A23187 were reduced to approximately 65–80% of control levels, whereas NPY release was increased to approximately 200–300% of control.

The differential regulation of neuropeptide and neurotransmitter was more dramatic in the changes elicited in the intracellular levels. Maximal activation of the protein kinase A pathway decreased NPY content to approximately 40–45% of control, but increased total catecholamine content to 175–300% of

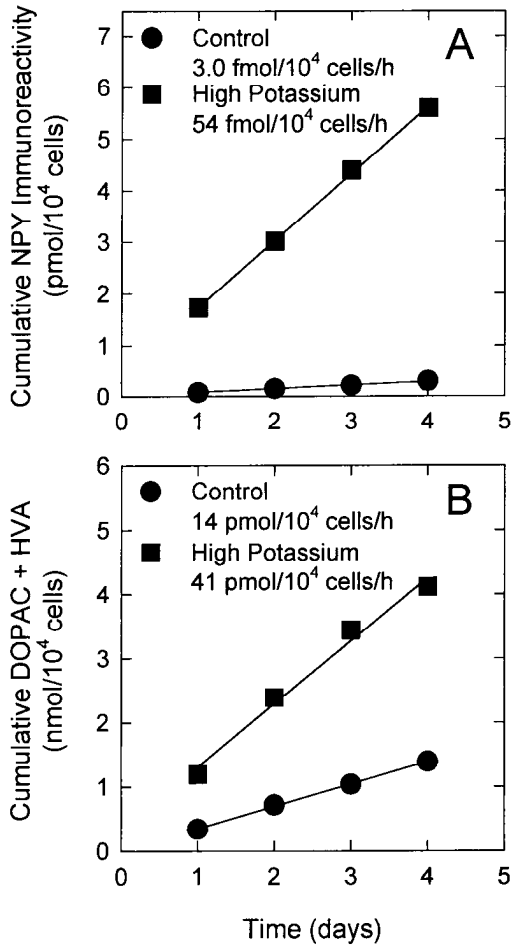

Figure 4. Depolarization increases sympathetic neuron NPY secretion and medium catecholamine metabolite levels. Dissociated SCG cells were plated at an initial density of 1.5×10^4 cells/cm^2. Nine days after plating, SCG neurons were incubated in 250 μl CSFM/1.5×10^4 cells containing 40 mM NaCl (control, ●) or 40 mM KCl (high potassium, ■) for 96 hr. Each 24 hr, conditioned medium was collected for NPY radioimmunoassay and catecholamine metabolite analysis, and replaced with fresh medium containing NaCl or KCl. Data represent the mean cumulative pmol of NPY immunoreactivity secreted per 10^4 cells or nmol total catecholamine metabolite per 10^4 cells from five cultures ± SEM.

control (Fig. 7*B,D*). Activation of the protein kinase C pathway resulted in no change in NPY levels, but increased total catecholamines by 20%. Calcium mobilization decreased NPY content to 50% of control, whereas total catecholamine was unaltered. Furthermore, activation of each of these second messenger systems differentially regulated each of the catecholamines and metabolites (Fig. 8). For example, stimulation of protein kinase A decreased cellular norepinephrine, but increased neuronal dopamine, epinephrine, DOPAC, and HVA; A23187 also decreased norepinephrine, but increased dopamine, epinephrine, DOPAC, and HVA leading to a net unaltered total cellular catecholamine level. The increase in dopamine content most likely reflected increased catecholamine biosynthesis in the absence of the dopamine-β-hydroxylase cofactor, ascorbic acid, and the increase in DOPAC may be indicative of elevated dopamine turnover. Many studies, however, have also demonstrated augmented neurotransmitter uptake by transporters following increased synaptic activity and activation of specific intracellular signaling mechanisms including cAMP-protein kinase A (Kadowaki et al., 1990; Desnos et al., 1992; Amara and Kuhar, 1993). The increases in cellular DOPAC and HVA may have reflected increased neuronal catecholamine reuptake and metabolism, although the mechanisms underlying the changes in cellular dopamine metabolite levels remain to be investigated.

Discussion

Cultured superior cervical ganglion neurons have been a well studied *in vitro* neuronal system with respect to neuronal survival, development, degeneration, neurotransmitter production, neuropeptidergic expression, neurochemical phenotypic plasticity, and receptor-mediated functions. The high levels of catecholamine and NPY expression in primary SCG cultures permit a direct means of examining how environmental conditions and regulatory factors coordinately or independently regulate transmitter and bioactive peptide production in neuronal cells. Although neuronal secretion is one of the defining parameters of neuronal function, catecholamine and NPY release in SCG cultures has not been examined previously. In our current studies we characterized NPY and catecholamine secretion from cultured SCG neurons and identified secretion as a central point of regulation of peptide and transmitter expression. Furthermore, we demonstrated second messenger system specific differential regulation of neuronal NPY and catecholamine content and release.

We have analyzed NPY and catecholamine levels in microcultures and conditioned medium, and established for the first time SCG neuron basal and stimulated peptide and transmitter secretory rates. When primary SCG neurons were cultured in defined medium (1.5×10^4 cells/cm^2; 250 μl of defined medium/1.5×10^4 cells), basal NPY secretion was approximately 0.9–3 fmol/10^4 cells/hr, whereas catecholamine release was about 10–20 pmol/10^4 cells/hr. Secretion of both peptide and transmitter was constant over time in culture.

The basal NPY secretory rate, expressed as a function of neuronal NPY content, diminished as cellular neuropeptide levels increased with time in culture. Basal NPY secretion was less than 0.05% of cell content/hr in mature cultures, which is relatively low compared to neuronal tumor cell lines and endocrine cells. However, this is similar to the results of Marek and Mains (1989, 1990), who reported that less than 0.05% of biosynthetically labeled NPY material/hr accumulated in conditioned medium from cultured SCG neurons. These low basal secretory rates are not due to peptide metabolism, since NPY is stable in conditioned medium (Marek and Mains, 1989), but instead are indicative of low basal secretion in unstimulated cultures. The low basal rates suggest that the primary determinants of NPY secretion are dependent essentially on the interactions of exogenous neuroregulatory signals.

By birth, nearly all of the principal neurons of the SCG express catecholaminergic markers and synthesize predominantly norepinephrine (Hendry, 1977; Landis, 1988). During early development, many SCG neurons exhibit concomitant expression of NPY, and SCG levels of catecholamine and NPY increase in parallel more than 10-fold during postnatal development. Our primary cultured SCG neurons exhibited similar parallel increased cellular NPY and catecholamine content. NPY and norepinephrine biosynthesis display comparable developmental profiles (Mains and Patterson, 1973b; Marek and Mains, 1989). In the adult rat, NPY is synthesized and stored in approximately 60–70% of the principal SCG neurons (O'Donohue et al., 1985;

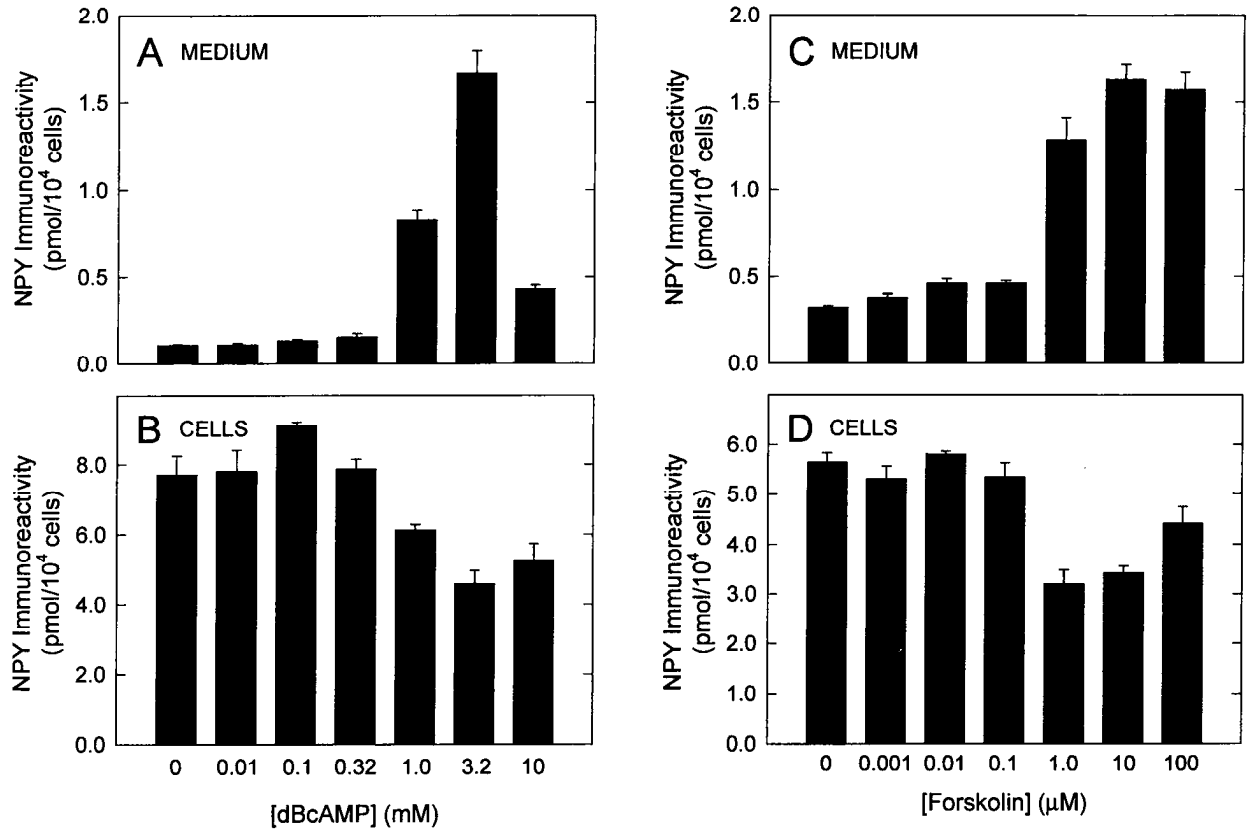

Figure 5. cAMP analogs and stimulation of adenylyl cyclase elicit concentration-dependent increased NPY secretion and decreased neuronal NPY content. Primary SCG neurons plated at 1.5×10^4 cells/cm^2 were cultured in 250 μl medium/1.5×10^4 cells containing vehicle (0) or the indicated concentrations of dBcAMP (*A, B*) or forskolin (*C, D*) for 96 hr beginning on day 9 of culture. The conditioned medium (*A, C*) from the final 48 hr, and cell extracts (*B, D*) were assayed for NPY immunoreactivity. Data represent the mean picomoles of NPY immunoreactivity per 10^4 cells ± SEM; n = 5–7 cultures for each concentration.

Jarvi et al., 1986; Landis, 1988). Approximately 50% of the neuronal population of mature SCG cultures also contain NPY (Marek and Mains, 1989). We observed a stable population (56%) of NPY immunoreactive neurons throughout the developmental time course of the cultures and therefore, the elevated NPY content in mature cultures was due to an increase in NPY per neuron, not an increase in the number of neurons expressing NPY.

Substances that regulate neuronal transmitter and peptide expression arise from a variety of sources including presynaptic signals, hormones, glial and supporting cell factors, hematopoietic factors and target tissue factors (Landis, 1988; Patterson and Nawa, 1993). The effects of presynaptic inputs to the SCG, that is depolarization, elicited sustained stimulated NPY and catecholamine secretion from cultured neurons; NPY release was stimulated greater than catecholamine secretion. Depolarization has also been shown to modulate SCG neuronal NPY and tyrosine hydroxylase mRNA and biosynthesis, as well as neurochemical phenotypic expression (Zigmond et al., 1989; Sun et al., 1992).

Stimulated secretory rates appear to be important components of second messenger system regulation of SCG neuropeptide and neurotransmitter expression. Activation of intracellular signaling pathways had specific and differential effects on NPY and catecholamine content and release. In previous studies, protein kinase A pathway activation increased N18TG-2 neuroblastoma and PC12 pheochromocytoma cell pro-NPY mRNA levels (Higuchi et al., 1988); SCG neuronal pro-NPY and tyrosine hydroxylase mRNA were increased approximately 50% by dBcAMP, whereas NPY and norepinephrine biosynthesis was unchanged (Marek and Mains, 1990). Since SCG culture NPY content was also unaltered by cAMP analogs (Freidin et al., 1993), the role of the cAMP–protein kinase A pathway in SCG NPY expression has remained unclear. We have shown that a primary neuronal response to cAMP–protein kinase A activation was stimulated secretion, which may account for the previous apparent discrepancies. NPY and catecholamine secretion was concomitantly increased over 500% by protein kinase A activation. Cellular NPY content remained relatively unchanged while NPY secretion was elevated under moderate stimulatory conditions, suggesting that NPY biosynthesis and secretion may be closely coupled to maintain constant cellular neuropeptide levels. Under maximally stimulated secretory conditions, SCG cellular NPY content was diminished approximately 40–45%, indicating that neuronal secretion exceeded cellular neuropeptide production levels. In contrast, SCG culture total catecholamine content (NE + DA + E + DOPAC + HVA) increased in concert with catecholamine release in response to cAMP–protein kinase A activation. SCG neuronal dopamine levels were elevated, and most likely reflected stimulated dopamine biosynthesis in cells cultured in the absence of ascorbate, and the elevated DOPAC and HVA levels may have reflected increased reuptake and/or metabolism. Under moderately stimulatory concentrations of dBcAMP or forskolin, cellular norepinephrine was unaltered, whereas at maximally

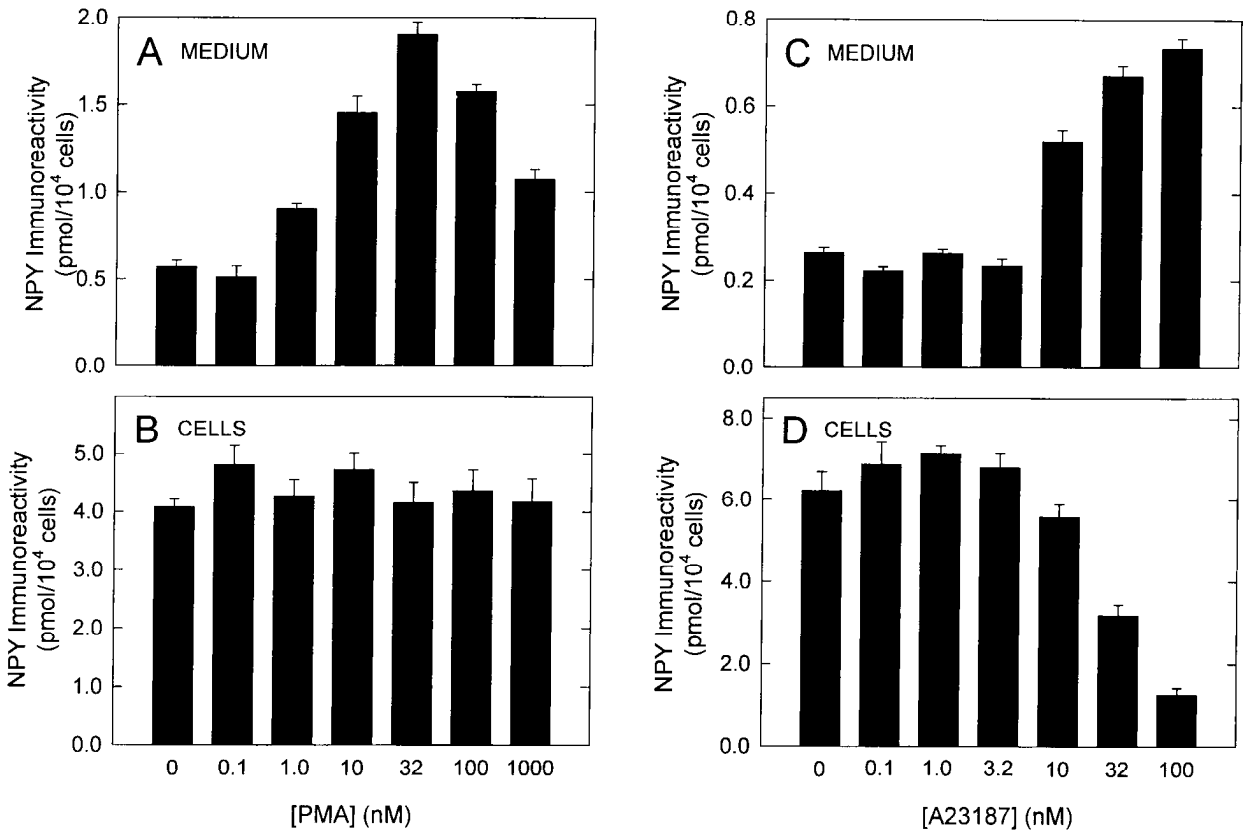

Figure 6. Regulation of SCG NPY content and secretion is intracellular signaling pathway specific. Sympathetic neurons were incubated in 250 μl medium/1.5 × 10^4 cells containing vehicle (0) or the indicated concentrations of PMA (*A, B*) or A23187 (*C, D*) for 96 hr beginning on day 9 of culture. The conditioned medium (*A, C*) from the final 48 hr, and cell extracts (*B, D*) were assayed for NPY immunoreactivity. Data represent the mean pmol of NPY immunoreactivity per 10^4 cells ± SEM; n = 5–7 for each concentration.

stimulatory concentrations, norepinephrine levels were decreased. In other studies, norepinephrine biosynthesis in SCG neurons cultured in the presence of ascorbate was unchanged following dBcAMP treatment (Marek and Mains, 1990). The differences in SCG NPY and catecholamine content following stimulation of the cAMP–protein kinase A pathway may reflect differential cellular responses to a common regulator.

The regulation of sympathetic neuron NPY expression was also second messenger system specific. Activation of the protein kinase C pathway with PMA stimulated SCG neuronal NPY secretion, but to a lesser degree than activation of protein kinase A. Unlike protein kinase A stimulation, activation of protein kinase C did not change cellular NPY levels at any PMA concentration. Furthermore, stimulation of the two intracellular signaling pathways produced different patterns of sympathetic neuronal catecholamine levels and secretion. Catecholamine and metabolite levels in neurons and conditioned medium were minimally increased or unchanged by protein kinase C activation. In contrast, stimulation of the protein kinase A pathway increased both total catecholamine/metabolite content and release; neuronal NPY levels were decreased under identical conditions.

Chronic treatment with PMA has been shown to increase pro-NPY mRNA levels and DOPA synthesis in PC12 pheochromocytoma cells (Higuchi et al., 1988; Zigmond et al., 1989; Sabol and Higuchi, 1990). On the other hand, primary cultured SCG neurons under comparable treatment paradigms displayed no changes in either pro-NPY or tyrosine hydroxylase mRNA, but exhibited diminished NPY and norepinephrine biosynthesis (Marek and Mains, 1989). In our studies, PMA stimulated NPY secretion, but did not alter neuronal NPY content, whereas cellular and released catecholamine and metabolite levels were unchanged or minimally increased. Moreover, the effects of long term PMA treatment on the downregulation of SCG neuronal protein kinase C and the consequences of these changes for NPY or catecholamine production has not been investigated.

Modulation of intracellular calcium levels has been shown to regulate a variety of cellular functions in different neuroendocrine cell types. Long term treatment of PC12 and neuroblastoma cell lines with the calcium ionophore A23187 modestly increased pro-NPY mRNA expression, but augmented neuropeptide mRNA levels dramatically upon concerted treatment with other secretagogues (Higuchi and Sabol, 1988). In our studies, NPY secretion induced by A23187 was paralleled by a concomitant decrease in cellular NPY. Activation of the protein kinase A and C pathways increased total NPY production (cumulative NPY secreted plus cellular NPY content), whereas A23187 did not change total NPY. The effects of A23187 on NPY expression therefore appear to be primarily secretory. In contrast to its effects on NPY expression, A23187 reduced catecholamine secretion without changing cellular content, and may reflect changes in SCG neurotransmitter biosynthesis, uptake, and metabolism. Excessive calcium influx has been suggested to mediate excitotoxic death of neurons (Choi, 1988; Johnson, 1992). Under our treatment conditions, SCG neurons incubated with A23187 were morphologically unaltered, appeared normal in trypan blue viability tests and demonstrated stimulated NPY

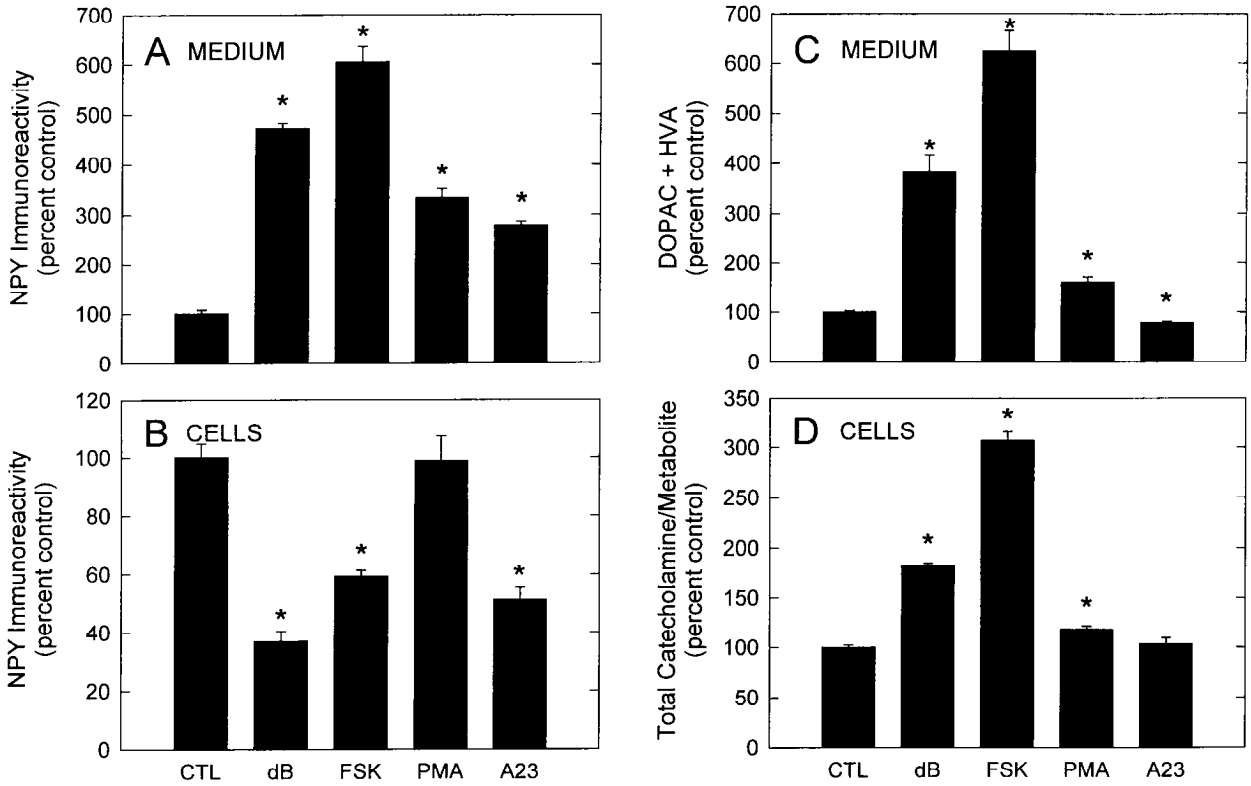

Figure 7. Stimulation of intracellular signaling pathways differentially regulates neuronal NPY and catecholamine content and secretion. SCG cultures were incubated in 250 μl CSFM 1.5 × 10^4 cells containing vehicle (control, *CTL*), 1.8 mM dBcAMP (*dB*), 10 μM forskolin (*FSK*), 32 nM PMA or 32 nM A23187 (*A23*) for two consecutive 48 hr treatment periods. The conditioned medium from the final 48 hr from individual cultures was assayed for both NPY immunoreactivity (*A*) and DOPAC and HVA levels (*C*); cell extracts from half of the cultures were also analyzed for NPY content (*B*), and half were analyzed for total catecholamine/metabolite levels (*D*). Data represent the mean percentage of the control NPY immunoreactivity (*A, B*) or total catecholamine/metabolite levels (*C, D*) ± SEM; for each treatment $n = 5$ for NPY and $n = 10$ for DOPAC/HVA. *, Significantly different from control by analysis of variance and Newman-Keuls test ($P < 0.01$).

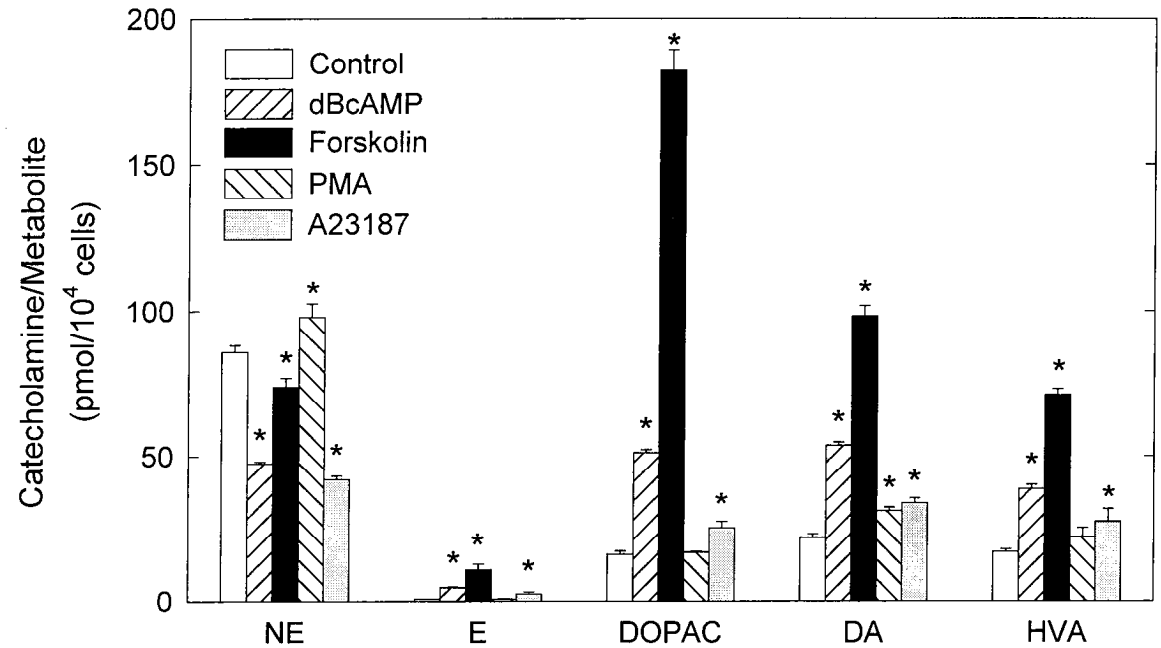

Figure 8. Stimulation of intracellular signaling pathways differentially regulates neuronal catecholamine and metabolite levels. The cell extracts of the sympathetic neuronal cultures treated with vehicle (control, *CTL*), 1.8 mM dBcAMP (*dB*), 10 μM forskolin (*FSK*), 32 nM PMA or 32 nM A23187 (*A23*), as described in Figure 7, were assayed for catecholamine/metabolite levels. Data represent the mean picomoles of catecholamine or metabolite per 10^4 cells ± SEM; for each treatment $n = 5$. *, Significantly different from control by ANOVA and Newman-Keuls test ($P < 0.01$). *NE*, Norepinephrine; *E*, epinephrine; *DOPAC*, 3,3-dihydroxyphenylacetic acid; *DA*, dopamine; *HVA*, homovanillic acid.

secretory responses; however, it is unknown whether other specific cell functional deficits were present that influenced peptide or transmitter expression.

The differential regulation of NPY and catecholamine content and release is most likely a reflection of the dichotomy between neuropeptide and neurotransmitter biosynthesis and storage. Electron microscopic and biochemical studies have established the compartmentalization of coexisting neurotransmitters and neuropeptides into two vesicle populations (Thureson-Klein, 1983; Fried et al., 1985; Kelly, 1993); synaptic vesicles contain classical neurotransmitters, whereas secretory granules contain neuropeptides. In sympathetic neurons, synaptic vesicles comprise approximately 95% of the vesicle population, while peptide-containing secretory granules represent only 5% of the population (Thureson-Klein, 1983; Fried et al., 1985). Catecholamines are synthesized at the axon terminal, where synaptic vesicles are refilled by an uptake mechanism. These vesicles then either dock along the plasma membrane in preparation for release, or are translocated to the reserve pool (Sossin et al., 1989; Sudhof and Jahn, 1991; Kelly, 1991, 1993). In contrast, neuropeptides are synthesized at sites distant from the terminal and following release, secretory granule membranes are recycled to the Golgi complex. The existence of a readily releasable or reserve pool of secretory granules and the details of granule mobilization remain uncertain. Differences in the biosynthetic pathways, vesicular compartmentalization, storage, uptake, and metabolism of neuropeptides and neurotransmitters support independent mechanisms of NPY and catecholamine regulation. Low frequency stimulation of the splenic nerve, for example, preferentially releases catecholamine from synaptic vesicles, while NPY secretion from secretory granules is dependent on higher stimulatory frequencies and patterns (Lundberg et al., 1986). In our studies, the SCG neuronal basal catecholamine secretory rate was over 5000-fold higher than that for NPY; the secretory rates of NPY and catecholamines expressed as a percentage of the cell content were approximately 0.05% and 10%, respectively. The differences in these basal secretory rates may be related to the functional compartmentalization, relative vesicle population sizes and independent biosynthetic pathways of neuropeptides and neurotransmitters. Moreover, the differences in depolarization-induced release of NPY and catecholamine (18-fold vs 3-fold, respectively) may be related to higher basal catecholamine secretion and slower NPY turnover. The two neurochemicals demonstrated important differences not only in the regulation of secretory responses, but also in the regulation of intracellular peptide and transmitter profiles. Again, the differential regulation of neuronal NPY and catecholamine content by specific intracellular signaling pathways may represent fundamental differences in sites of biosynthesis, biosynthetic rates, vesicle populations, storage, uptake, metabolism, and release mechanisms.

In summary, the current studies have demonstrated independent regulation of sympathetic neuronal NPY and catecholamine content and release. We have established the basal secretory rates for the primary transmitter and peptide in principal neurons of the SCG, and demonstrated that secretion of both NPY and catecholamines was differentially stimulated by depolarization. Activation of intracellular signaling pathways was shown to be an important point of regulation of sympathetic neuropeptide and neurotransmitter content and release. Regulation was second messenger system specific; stimulation of protein kinase A, protein kinase C, or intracellular calcium differentially affected SCG neuron NPY and catecholamine content and secretion. Th magnitude of the secretory responses of SCG neurons to thes regulators was far greater than other components of NPY c catecholamine expression, including content, biosynthesis an mRNA levels, suggesting that release is a primary site of mod ulation. Consequently, sympathetic neuron secretion may be sensitive indicator of peptide or transmitter expression, and a important measure to consider in understanding the mechanism of regulation. The independent control of sympathetic neuron NPY and catecholamine content and release is consistent wit the differential biosynthetic pathways, vesicular compartmental ization, storage, uptake, and metabolism of neuropeptides versu neurotransmitters.

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