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Differentiation Induces Pituitary Adenylate Cyclase-Activating Polypeptide Receptor Expression in PC-12 Cells

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SUMMARY

The two forms of pituitary adenylate cyclase-activating polypeptide (PACAP), PACAP27 and PACAP38, are neuropeptide hormones related to the vasoactive intestinal peptide/secretin/glucagon family of peptides. PACAP receptors that are positively coupled to adenylyl cyclase and phospholipase C have been recently identified. We have investigated the expression of PACAP-Rs in undifferentiated and differentiated PC-12 cells. PACAP27 and PACAP38 failed to significantly increase cAMP or [^{3}H]inositol monophosphate levels in undifferentiated PC-12 cells treated with vehicle, insulin-like growth factor I, or epidermal growth factor but greatly elevated levels after differentiation with nerve growth factor (NGF) or basic fibroblast growth factor. PACAP responsiveness increased significantly after 24 hr of NGF treatment, reaching a maximum within 4 days. At this time of differentiation, the effect of PACAP was dose dependent between 1 nM and 0.1 μM, whereas vasoactive intestinal peptide, at the maximal dose of 10 μM, slightly increased cAMP formation and failed to affect [^{3}H]inositol monophosphate content. Radioreceptor assays, performed with ^{125}I-PACAP27, revealed the induction of high affinity type I PACAP receptors in differentiated PC-12 cells. Using reverse transcription-polymerase chain reaction methodology, we showed the absence of type I PACAP receptor mRNAs in undifferentiated PC-12 cells and the expression of PACAP-R-hop mRNA after NGF or basic fibroblast growth factor treatment. The increased PACAP responsiveness induced by these growth factors in PC-12 cells may therefore result from the expression of the PACAP-R-hop isoform, positively coupled to both adenylyl cyclase and phospholipase C.

PACAP is a member of the secretin/glucagon/VIP family of peptides and exists in two α-amidated forms, PACAP38 and PACAP27, which share the same 27 amino-terminal amino acids and arise from a precursor peptide by post-translational processing (1–4). PACAP is present not only in distinct areas of the central nervous system but also in peripheral tissues, such as lung, testis, pancreas, gut, and adrenal gland (5–7). In these tissues, PACAP binding sites can be divided into at least two types, based on their relative affinities for PACAP and VIP (8–11). Type I PACAP receptors specifically bind PACAP, whereas type II PACAP receptors bind both PACAP and VIP, with similar affinities.

The cDNAs of five type I PACAP receptors have been recently isolated and shown to originate from a common gene by an alternative splicing mechanism (12–17). The shortest cDNA form encodes a 467-amino acid protein with the seven transmembrane domains characteristic of G protein-coupled receptors. The other cDNAs diverge from each other in the presence of either one or two cassettes, designated "hip" and "hop" (16). The insertion of these cassettes occurs in the carboxyl-terminal end of the third intracellular loop, a domain considered crucial for interaction with G proteins (18). Indeed, when transiently expressed in porcine renal epithelial LLC-PK$_1$ cells or in *Xenopus* oocytes, the PACAP receptor splice variants differentially regulate cAMP and inositol phosphate levels upon PACAP stimulation, thus suggesting coupling to different G proteins (16). This possibility is consistent with the pharmacology of PACAP receptors in undifferentiated rat pheochromocytoma PC-12 cells, where PACAP27 and PACAP38 were previously shown to elicit cAMP formation and inositol phospholipid hydrolysis (11, 19).

The PC-12 cell line has been extensively used as a model system for peripheral neuronal differentiation (20, 21). In serum-containing medium, PC-12 cells undergo mitosis and display properties of adrenal chromaffin cells. Addition of

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ABBREVIATIONS: PACAP, pituitary adenylate cyclase-activating polypeptide; VIP, vasoactive intestinal peptide; InsP, inositol monophosphate; IGF-I, insulin-like growth factor I; EGF, epidermal growth factor; NGF, nerve growth factor; FGF, fibroblast growth factor; RT, reverse transcription; PCR, polymerase chain reaction; PGK, phosphoglycerate kinase 1; bp, base pair(s); FBS, fetal bovine serum; PACAP receptor, pituitary adenylate cyclase-activating polypeptide receptor.

NGF or FGF induces transformation of PC-12 cells to nondividing sympathetic neuron-like cells (22, 23). Treatment with IGF-I or EGF instead stimulates proliferation of PC-12 cells but not differentiation (24, 25).

In this study we have investigated how phenotypic alteration of PC-12 cells affects PACAP receptor expression. We report that NGF and FGF, but not IGF-I or EGF, induce the functional and molecular expression of the PACAP-R-hop variant in PC-12 cells.

Experimental Procedures

Materials. Synthetic PACAP27 and PACAP38 were purchased from Calbiochem (Milano, Italy). VIP, EGF_{20-31}, carbamylcholine, bacitracin, aprotinin, and bovine serum albumin were obtained from Sigma (Milano, Italy). Mouse β-NGF was a gift from Prof. Angelucci (University of Rome, Italy). Human recombinant basic FGF and IGF-I were generously provided by Pharmacia Farmitalia Carlo Erba (Milano, Italy). Dulbecco's modified Eagle's medium, horse serum, and FBS were from Gibco-BRL (Milano, Italy). Tissue culture dishes were from Nunc (Kamstrup, Denmark). *myo*-[2-^{3}H]Inositol and ^{125}I-PACAP27 were from New England Nuclear-DuPont (Milano, Italy). Oligonucleotides used for RT-PCR were synthesized by Pharmacia Biotech (Milano, Italy). All of the following materials were purchased from Boehringer Mannheim (Milano, Italy): RNase-free DNase, Moloney murine leukemia virus reverse transcriptase and its buffer, a deoxynucleoside triphosphate mixture, *Thermus aquaticus* polymerase and its buffer, and DNA markers VI.

Cell cultures. PC-12 cells were purchased from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% horse serum, 5% FBS, 100 μg/ml streptomycin, and 100 units/ml penicillin. Cells were passaged at 1-week intervals by dissociation with trypsin-EDTA, with a midweek change of medium. Starting at day 1, medium was aspirated and replenished every other day. NGF (40 ng/ml), FGF (40 ng/ml), IGF-I (25 ng/ml), EGF_{20-31} (100 μM), or vehicle was added after each change of medium.

Measurement of inositol phospholipid hydrolysis. The rate of inositol phospholipid hydrolysis was estimated by measuring the accumulation of [^{3}H]InsP in the presence of Li^+, which blocks the conversion of InsP to free inositol (26, 27). Cultured PC-12 cells were seeded in 35- × 10-mm dishes at 500,000/dish, in complete medium supplemented with 20 nM *myo*-[2-^{3}H]inositol (specific activity, 23.45 Ci/mmol) to label membrane inositol phospholipids. Forty-eight hours later, dishes were washed twice with 1 ml of Krebs/Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11.7 mM glucose) containing 10 mM LiCl (equilibrated with 95% O_2/5% CO_2 to a final pH of 7.4 and prewarmed to 37°). The cells were then incubated for 20 min at 37° with 900 μl of buffer and for an additional 60 min in the presence of the drug. The buffer was removed by aspiration and the reaction (release of [^{3}H]InsP from membrane phospholipids) was stopped with 1.5 ml of ice-cold methanol/H_2O_2 (1:1, v/v). After the cells were harvested, the suspension was added to 0.75 ml of chloroform and centrifuged at 500 × *g* for 2 min, to facilitate phase separation. The amount of [^{3}H]InsP present in the aqueous phase was estimated as described previously (27). Eight hundred microliters of the aqueous phase were added to columns containing 1 ml of Dowex 1X8 resin (100–200 mesh, formate form; Bio-Rad), and the phosphate esters were eluted by stepwise addition of formate solutions of increasing strength. InsP was eluted with 0.2 M ammonium formate plus 0.1 M formic acid. The fractions were collected and the radioactivity was measured by liquid scintillation counting. Proteins were measured according to the method of Lowry *et al.* (28).

Intracellular cAMP determination. To measure intracellular cAMP content, PC-12 cells were preincubated for 20 min in Krebs/Henseleit buffer (equilibrated with 95% O_2/5% CO_2 to a final pH of 7.4 and prewarmed to 37°). After incubation of the cells with the drug for an additional 20 min, the medium was aspirated and the intracellular cAMP was extracted with 1 ml of ice-cold 0.4 N $HClO_4$. After the cells were harvested, the suspension was centrifuged at 500 × *g* for 2 min. Aliquots of the supernatant were neutralized with 0.5 N NaOH and used for cAMP determination with a ^{125}I-cAMP radioimmunoassay kit from New England Nuclear-DuPont.

Binding studies. PC-12 cells were washed with Tris buffer, pH 7.4, containing 0.1% bovine serum albumin, 5 mM $MgCl_2$, 0.5 μg/ml bacitracin, and 100 kallikrein-inactivating units/ml aprotinin (binding buffer), for 10 min. For competition studies, cells were incubated with 200 pM ^{125}I-PACAP27 (specific activity, 2200 Ci/mmol) in the presence of various concentrations of unlabeled PACAP27, PACAP38, or VIP. At the end of the incubation, PC-12 cells were washed with cold binding buffer and solubilized with 0.5 N NaOH, and the radioactivity was counted. Binding data were analyzed using the computer program LIGAND, provided by Dr. Peter J. Munson (National Institutes of Health, Bethesda, MD).

Analysis of PACAP receptor mRNAs by RT-PCR. Primers used to identify type I PACAP receptor mRNAs by RT-PCR were based on the reported sequence for the rat PACAP-R-hip-hop1 isoform (16) and are represented in Fig. 1. Oligonucleotide sequences

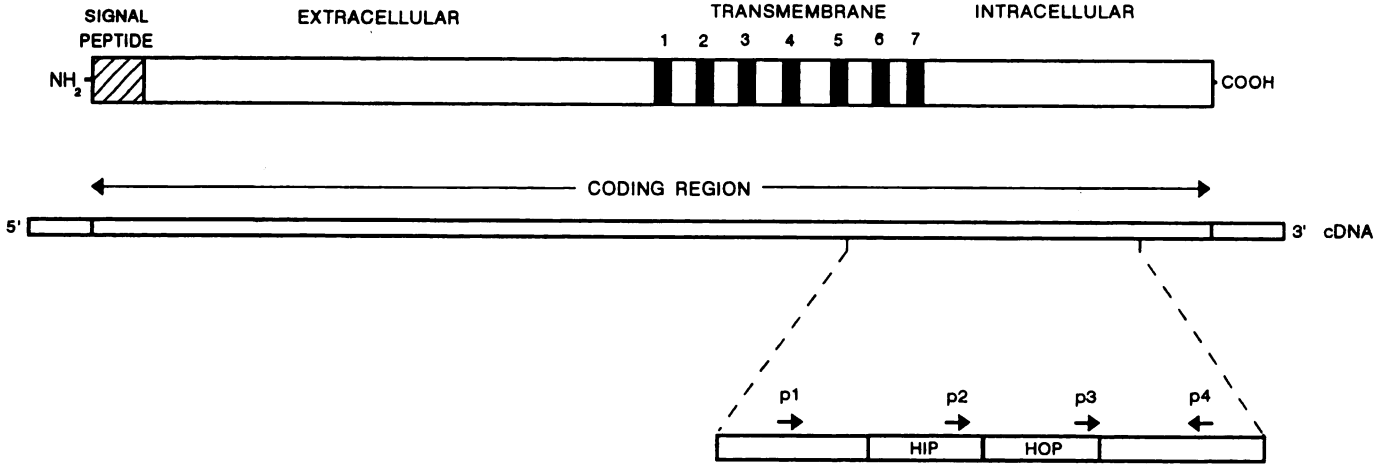

Fig. 1. Schematic representation of the PACAP-R-hip-hop1 protein, the corresponding cDNA, and the region selected for PCR amplification. Four primers (three forward, p1–p3, and one reverse, p4) were designed on the basis of the rat PACAP-R-hip-hop1 cDNA sequence (16). The expected PCR products using primer pairs p1/p4, p2/p4, and p3/p4 are described in Experimental Procedures.

and locations of the primers with respect to the translation initiation site were as follows: p1, 5′-CATCCTTGTACAGAAGCTGC-3′ (forward primer, matching the beginning of the third intracellular loop and corresponding to bases 984-1003); p2, 5′-CCTCAGACCAGCATTCACC-3′ (forward primer, matching the end of the hip cassette and corresponding to bases 1100–1118); p3, 5′-TCCACCATTACTCTACGGCT-3′ (forward primer, matching the end of the hop cassette and corresponding to bases 1198–1217); and p4, 5′-GGTGCTTGAAGTCCATAGTG-3′ (reverse primer, matching a region of the cytoplasmic tail and corresponding to bases 1437–1456). The following are the sizes of the expected PCR products using primer pair p1/p4: 305 bp for the PACAP-R, 386 bp for PACAP-R-hop2, 389 bp for PACAP-R-hip and PACAP-R-hop1, and 473 bp for PACAP-R-hip-hop1. The predicted PCR products using primer pair p2/p4 are 273 bp for PACAP-R-hip and 357 bp for PACAP-R-hip-hop1, whereas those obtained with primer pair p3/p4 are 256 bp for PACAP-R-hop2 and 259 bp for either PACAP-R-hop1 or PACAP-R-hip-hop1.

Primers for rat PGK (29) were used as an internal control (for the integrity of RNA) in each PCR. The primers used to detect rat PGK were 5′-AGGTGCTCAACAACATGGAG-3′ (forward primer, corresponding to bases 737–756) and 5′-TACCAGAGGCCACAGTAGCT-3′ (reverse primer, corresponding to bases 919–938), which generated a 182-bp cDNA fragment. Total RNA from PC-12 cells was isolated by the acid-guanidinium isothiocyanate method (30) and treated with RNase-free DNase to remove any residual genomic DNA. Single-stranded cDNAs were synthesized using total RNA (1 μg), Moloney murine leukemia virus reverse transcriptase, and the reverse primers for PACAP receptors and PGK. To the cDNA reaction was added a PCR master mixture, to yield the following final concentrations: 1 μM specific primers, 200 μM deoxynucleoside triphosphates, 2.5 units of *T. aquaticus* DNA polymerase, and *T. aquaticus* buffer containing 1.5 mM $MgCl_2$. PCR was performed with a Perkin-Elmer/Cetus thermal cycler (45 cycles of 95° for 30 sec, 61° for 30 sec, and 72° for 45 sec). At the end of the PCR, samples were kept at 72° for 10 min for final extension and were stored at 4°. Amplification products arising from RT-PCR were separated by electrophoresis (1.8% agarose gel in 0.045 M Tris-borate/1 mM EDTA buffer) and visualized by ethidium bromide staining.

In a preliminary series of experiments, the use of RT-PCR for the identification of type I PACAP receptor isoforms was validated on the basis of the specificity of the primers and the size of the amplification products. In particular, the devised primers detected all five known PACAP receptor isoforms in total RNA from rat brain (data not shown).

Results

PACAP-induced cAMP and InsP formation in PC-12 cells. Exposure to 10 nM PACAP27 and PACAP38 failed to significantly affect intracellular cAMP and [^{3}H]InsP levels in undifferentiated PC-12 cells treated with vehicle (control), IGF-I (25 ng/ml), or EGF_{20-31} (100 μM) but greatly increased the levels after treatment with NGF (40 ng/ml) or FGF (40 ng/ml) for 4 days (Fig. 2). After NGF or FGF treatment, PC-12 cells exhibited a dramatically altered morphology, displaying neurites within 12 hr and prominent branching, growth cones, and enlarged perikarya within 48 hr. Exposure to IGF-I or EGF_{20-31} instead had no effect on PC-12 cell morphology and increased by 2–3-fold the incorporation of thymidine (data not shown).

The time course of the effect of neuronal differentiation on PACAP responsiveness in PC-12 cells is shown in Fig. 3. Stimulation by both PACAP27 and PACAP38 significantly increased cAMP (Fig. 3A) and [^{3}H]InsP (Fig. 3B) levels after 1 day of NGF treatment and maximally elevated the levels after 2 and 4 days, respectively.

In PC-12 cells differentiated with NGF (40 ng/ml) for 4 days, PACAP27 and PACAP38 stimulated cAMP and [^{3}H]InsP formation in a dose-dependent manner, displaying similar potencies but different efficacies (Fig. 4). Their minimal effective doses were 1 nM, whereas maximal stimulation (about 2- and 3-fold for PACAP27 and PACAP38, respectively) was obtained at 10–50 nM concentrations. The esti-

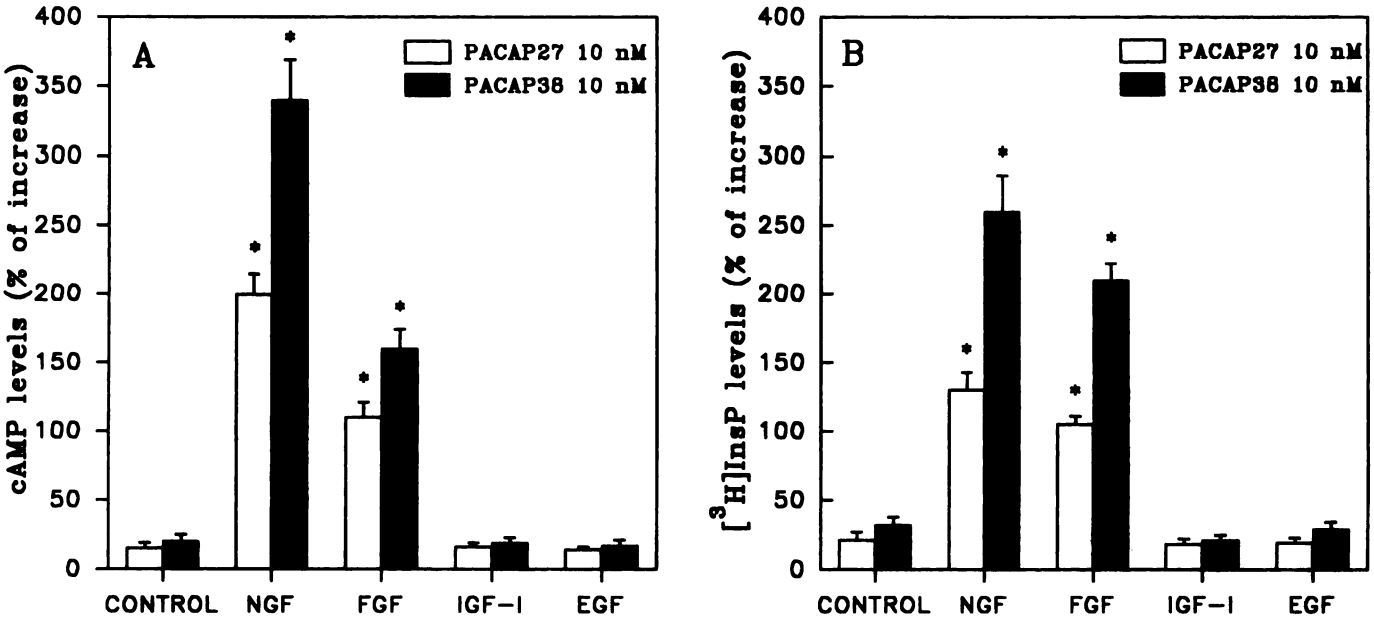

Fig. 2. PACAP-induced cAMP and InsP formation in undifferentiated and differentiated PC-12 cells. The effects of 10 nM PACAP27 or PACAP38 on cAMP (A) and [^{3}H]InsP (B) levels were determined in PC-12 cells that had been pretreated with vehicle (control), NGF (40 ng/ml), FGF (40 ng/ml), IGF-I (25 ng/ml), or EGF_{20-31} (100 μM) for 4 days. Basal levels of cAMP (549 ± 42 nmol/μg of protein) and [^{3}H]InsP (2458 ± 182 cpm/mg of protein) in undifferentiated PC-12 cells did not change after treatment with growth factors. Each value represents the mean ± standard error (four experiments). *, $p < 0.01$ (Student's *t* test).

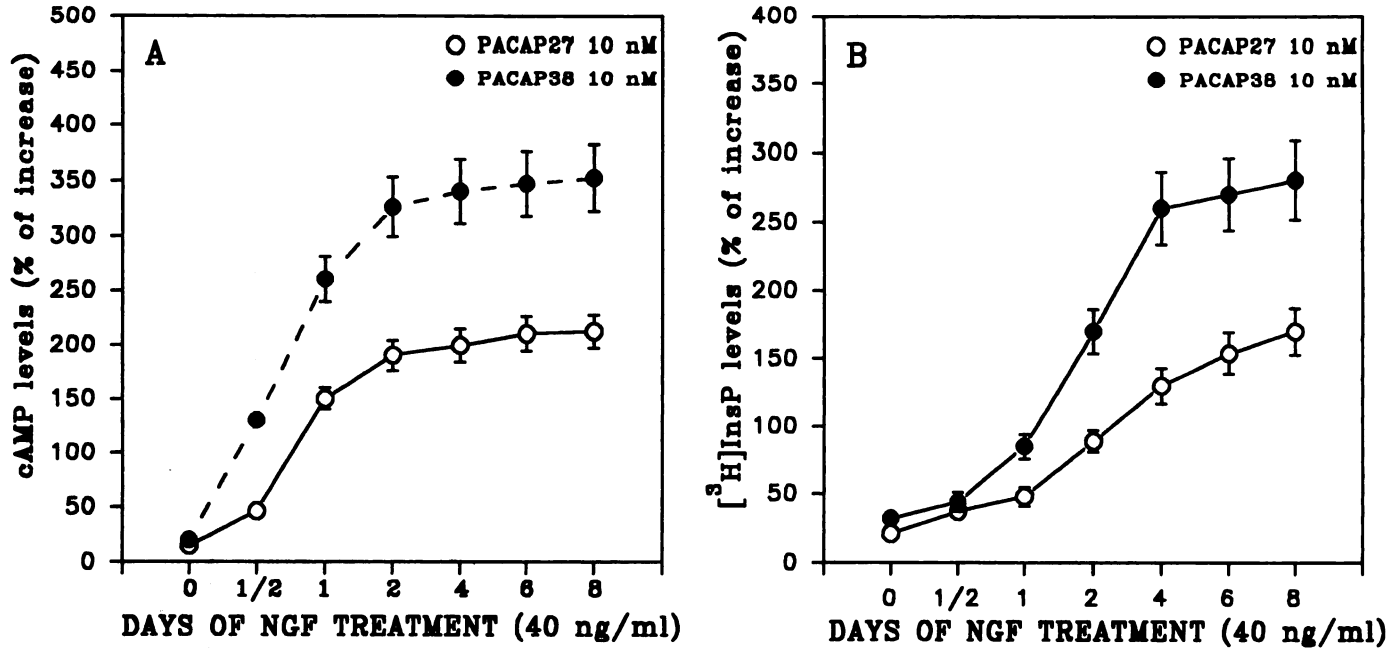

Fig. 3. Induction by NGF treatment of PACAP-stimulated cAMP and InsP formation in PC-12 cells. The effects of 10 nM PACAP27 or PACAP38 on cAMP (A) and [^{3}H]InsP (B) levels were determined in PC-12 cells before (day 0) and after pretreatment with NGF (40 ng/ml) for 0.5, 1, 2, 4, 6, and 8 days. Each value represents the mean ± standard error (five experiments).

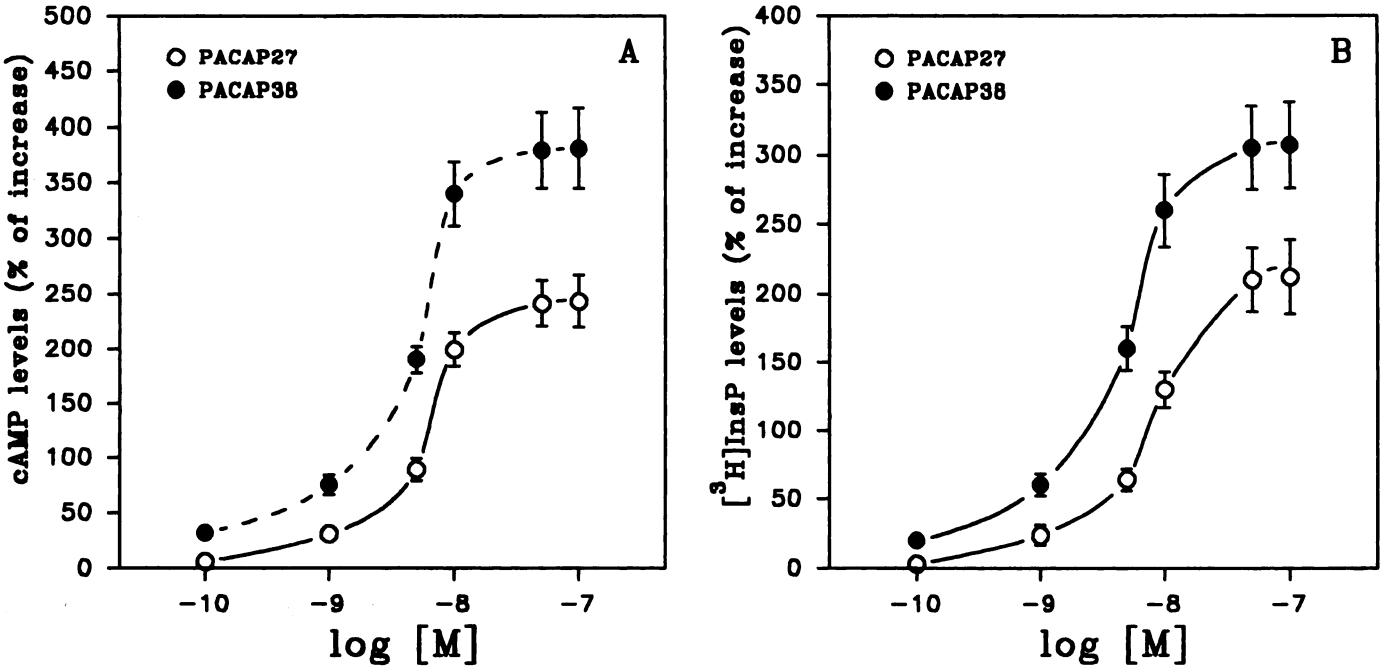

Fig. 4. Dose dependence of PACAP-stimulated cAMP and InsP levels in differentiated PC-12 cells. The effects of increasing concentrations of PACAP27 or PACAP38 on cAMP (A) and [^{3}H]InsP (B) levels were determined in PC-12 cells that had been differentiated with NGF (40 ng/ml) for 4 days. Each value represents the mean ± standard error (three experiments).

mated EC_{50} values for PACAP27 and PACAP38 were approximately 5 nM. Exposure of NGF-treated PC-12 cells to a maximal concentration (10 μM) of VIP slightly increased cAMP levels and failed to affect [^{3}H]InsP formation (Table 1).

To investigate the interrelationship between the cAMP and phosphoinositide signal transduction pathways in PC-12 cells, we studied the effects of forskolin and carbamylcholine on cAMP and [^{3}H]InsP levels (Table 1). Treatment with 10 μM forskolin greatly stimulated cAMP formation in vehicle- and NGF-treated PC-12 cells but had only negligible effects on [^{3}H]InsP levels. Stimulation with 1 mM carbamylcholine increased [^{3}H]InsP formation but not cAMP levels.

Characterization of ^{125}I-PACAP binding sites in PC-12 cells. To assess the binding characteristics of PACAP receptors in PC-12 cells, radioreceptor assays were performed using ^{125}I-PACAP27. The specific binding of ^{125}I-

TABLE 1

Effects of VIP, forskolin, and carbamylcholine on cAMP and [^{3}H]InsP levels in undifferentiated and differentiated PC-12 cells

Intracellular cAMP and [^{3}H]InsP levels were measured in undifferentiated (vehicle-treated) and differentiated (NGF-treated; 40 ng/ml for 4 days) PC-12 cells stimulated with VIP (10 μM), forskolin (10 μM), or carbamylcholine (1 mM). Each value is the mean of three independent determinations.

Stimulus	cAMP levels		[^{3}H]InsP levels	
	Vehicle	NGF	Vehicle	NGF
	% increase		*% increase*	
VIP (10 μM)	12	64[a]	8	19
Forskolin (10 μM)	415[b]	717[b]	24	44
Carbamylcholine (1 mM)	−15	−24	110[b]	549[b]

[a] $p < 0.05$, versus control (Student's *t* test).
[b] $p < 0.01$.

PACAP27 was virtually absent in undifferentiated PC-12 cells (data not shown) but was detected after differentiation with NGF (40 ng/ml) for 4 days. The competition between ^{125}I-PACAP27 and increasing concentrations of unlabeled PACAP27, PACAP38, or VIP in differentiated PC-12 cells is shown in Fig. 5. Scatchard analysis of competition data indicated the presence of a single PACAP binding site; no significant differences in the dissociation constant (K_d) or the maximal capacity (B_{max}) were found between PACAP27 and PACAP38 (Table 2). VIP, even at high concentrations, failed to displace the binding of ^{125}I-PACAP27 (Fig. 5).

Expression of type I PACAP receptor mRNAs in PC-12 cells. To identify the type I PACAP receptor isoform(s) expressed in PC-12 cells, we analyzed the expression of their transcripts by RT-PCR. To discriminate among the five known receptor splice variants, we used three pairs of primers (Fig. 1). The first primer pair (p1/p4) is external to the site of insertion of the hip/hop exons and produces a PCR

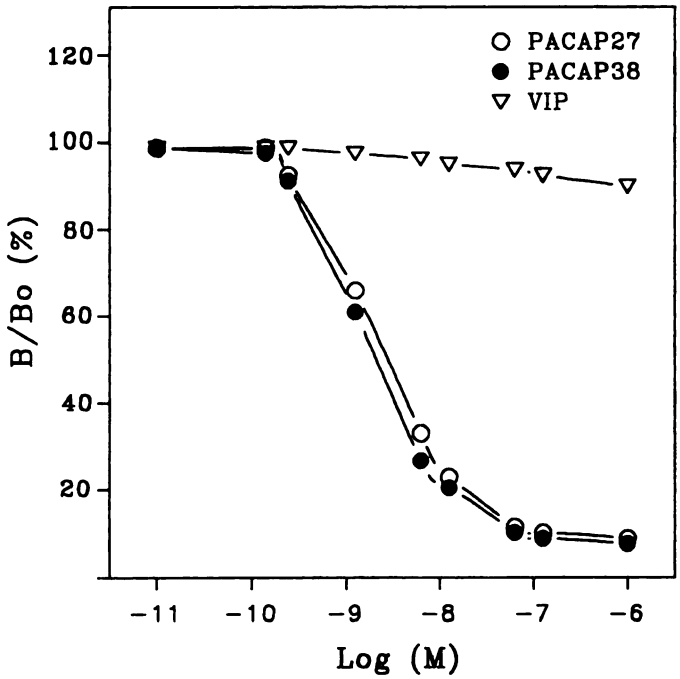

Fig. 5. Competition curves for PACAP27, PACAP38, and VIP competition with ^{125}I-PACAP27 binding in PC-12 cells that had been differentiated with NGF (40 ng/ml) for 4 days. Each value is the mean of four independent determinations. *B/Bo*, bound/free ratio.

TABLE 2

Properties of PACAP binding sites in differentiated PC-12 cells

Parameters were determined by Scatchard analysis of the displacement by unlabeled PACAP27 or PACAP38 of ^{125}I-PACAP27 binding.

Peptide	K_d	B_{max}
	nM	*sites/cell*
PACAP27	1.62 ± 0.37	21,430 ± 3,201
PACAP38	1.59 ± 0.41	22,609 ± 3,556

product for each of the PACAP receptor isoforms. The other two primer pairs, p2/p4 and p3/p4, were used to discriminate the PACAP-R-hip and PACAP-R-hop variants, respectively. To confirm RNA integrity and to control for differences attributable to errors in experimental manipulation from tube to tube, the levels of mRNA transcripts for rat PGK were simultaneously determined in each RT-PCR assay.

Fig. 6 depicts a representative agarose gel stained with ethidium bromide. Using primers p1/p4, no amplification product was observed in undifferentiated PC-12 cells, whereas a fragment of about 390 bp appeared in NGF- and FGF-treated PC-12 cells. The size of this PCR fragment is indicative of the PACAP-R-hip and/or PACAP-R-hop isoforms. Amplification with primers p2/p4 gave no amplification product in either undifferentiated or differentiated PC-12 cells, whereas primers p3/p4 produced a fragment of about 260 bp in NGF- and FGF-treated cells. Taken together, these data indicate the absence of type I PACAP receptor mRNAs in undifferentiated PC-12 cells and the induction of the PACAP-R-hop mRNA after NGF or FGF treatment.

Although no indication of PACAP receptors was found in undifferentiated PC-12 cells, we tested the ability of PACAP to induce the expression of its own receptors. Treatment of PC-12 cells with 10 nM PACAP38 for 4 days failed to induce the expression of type I PACAP receptor mRNA, as detected by RT-PCR (data not shown).

Discussion

In this study we have examined how neuronal differentiation of PC-12 cells affects PACAP receptor expression. Like normal precursor cells arising from the sympathoadrenal region of the neural crest, PC-12 cells, which display proper-

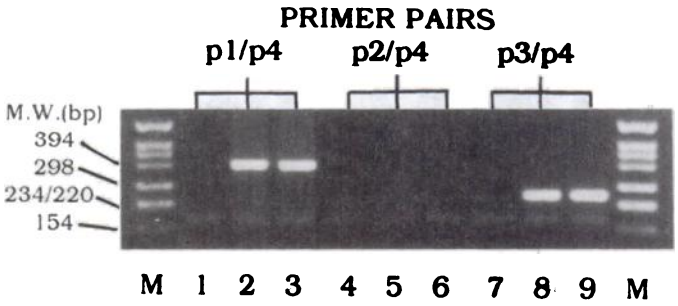

Fig. 6. RT-PCR analysis of type I PACAP receptor mRNAs in undifferentiated and differentiated PC-12 cells. To identify type I PACAP receptor mRNAs, total RNA from PC-12 cells treated with vehicle (*lanes 1, 4,* and *7*), NGF (*lanes 2, 5,* and *8*), or FGF (*lanes 3, 6,* and *9*) was reverse transcribed and amplified by PCR with three primer pairs (p1/p4, p2/p4, and p3/p4). Primers for PGK were used as an internal control in each PCR assay and generated a 182-bp fragment. Amplification products were electrophoresed on 1.8% agarose gels and visualized by ethidium bromide staining. *Lanes M*, DNA standards (DNA markers VI; Boehringer Mannheim) (labeled in bp). For experimental details, see Experimental Procedures.

ties of immature chromaffin cells, can be induced by soluble factors to give rise to a differentiated phenotype resembling postganglionic neurons (20–23). We have shown that differentiation of PC-12 cells by NGF or FGF treatment induces the functional expression of PACAP receptors positively coupled to adenylyl cyclase and phospholipase C. In contrast, treatment with IGF-I or EGF, which stimulates proliferation but not differentiation of PC-12 cells (24, 25), fails to increase PACAP responsiveness in PC-12 cells.

Pharmacological studies have indicated that there are at least two types of PACAP receptors (8–11). Type I PACAP receptors have high affinity only for PACAP and bind VIP with 1000-fold lower affinity. In contrast, type II PACAP receptors bind PACAP and VIP with similar affinities. The correlation between the abilities of PACAP27 and PACAP38, but not VIP, to compete with ^{125}I-PACAP27 binding and the stimulatory effects of these compounds on cAMP and InsP formation indicates that the effects of PACAP in differentiated PC-12 cells are consequent to activation of type I receptors.

Recently, the cDNAs for five type I PACAP receptors have been isolated and shown to originate from a common gene by alternative splicing (12–17). The absence or presence of either one or two exons, designated hip and hop, inserted in the carboxyl-terminal end of the third intracellular loop produces different splice variants that differentially activate adenylyl cyclase and phospholipase C (16). Because of the structural homology and pharmacological similarity of the type I PACAP receptors, the ligand binding probes presently available do not distinguish among the various isoforms. To investigate the expression of type I PACAP receptor isoforms in PC-12 cells, therefore, we analyzed their respective mRNAs by using RT-PCR methodology. This analysis revealed the absence of type I PACAP receptor mRNAs in undifferentiated PC-12 cells and the induction of the PACAP-R-hop variant after differentiation with NGF or FGF treatment. Additional studies are still needed to elucidate the mechanisms by which NGF and FGF regulate the expression of the PACAP receptor gene in PC-12 cells. The induction of the mRNA encoding the PACAP-R-hop variant after neuronal differentiation may reflect increased mRNA stability or, more likely, increased transcriptional activity.

One- or two-receptor models were previously envisioned to account for the activation of adenylyl cyclase and phospholipase C elicited by PACAP in PC-12 cells (11). Taken together, our data suggest that PACAP-induced cAMP and InsP formation in differentiated PC-12 cells is mediated by a single receptor, the PACAP-R-hop variant, coupled to different G proteins. Based on this interpretation, the different efficacies with which PACAP38 and PACAP27 activate the PACAP-R-hop variant may reflect changes in their biological activities and not their binding characteristics.

In time course experiments, the effect of differentiation on PACAP-induced cAMP levels preceded by a few hours that on [^{3}H]InsP formation. During differentiation (1–4 days after NGF treatment), we observed neither significant differences in PACAP potency nor the expression of other PACAP receptor mRNAs (data not shown). Based on these data, differences in the time of acquisition of cAMP and InsP responses probably do not reflect the presence of different type I PACAP receptors and may depend on changes in G protein levels and/or in the activity of the effector systems (adenylate cyclase and phospholipase C). In support of this hypothesis are the increased responsiveness of NGF-treated PC-12 cells to forskolin and previous investigations indicating changes in the G protein repertoire during differentiation (31).

In contrast to previous reports (11, 19), we have found no expression of type I PACAP receptors in undifferentiated cells. This discrepancy may be explained by the heterogeneity of PC-12 cell subclones used, the use of different culture conditions, or the phenotypic instability of these cells (32, 33). In particular, the demonstration of NGF and FGF in batches of FBS (34) implies that great care should be taken to ensure standardized culture conditions. With these considerations taken into account, PC-12 cells will remain an advantageous model system to clarify the role, mechanism, and consequences of PACAP actions.

Because the differentiating effects of NGF and FGF on PC-12 cells are thought to offer a system for investigation of the effects of these growth factors on neurons, the present study may suggest a physiological role for NGF and FGF in the induction and maintenance of specific PACAP receptors during development of the autonomic system. Previous investigations conducted in sympathetic neuroblasts (35) and PC-12 cells (11) have shown that PACAP promotes cell proliferation, neurite outgrowth, and cell survival. Although the neurotrophic effects of PACAP in PC-12 cells were beyond the scope of this study, the increased expression of PACAP receptors in differentiating PC-12 cells might underlie important neurotrophic activities.

The morphological differentiation of PC-12 cells is accompanied by a number of phenotypic modifications, including the increased expression of muscarinic acetylcholine receptors (36). The persistent expression of PACAP receptors in differentiated PC-12 cells suggests that PACAP, in concert with other neurotransmitters such as acetylcholine, may regulate the synaptic activity of mature sympathetic neurons.

References

1. Miyata, A., A. Arimura, R. R. Dahl, N. Minamino, A. Uehara, L. Jiang, M. D. Culler, and D. H. Coy. Isolation of a novel 38-residue hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. *Biochem. Biophys. Res. Commun.* **164**:567–574 (1989).
2. Miyata, A., L. Jiang, R. R. Dahl, C. Kitada, K. Kubo, M. Fujino, N. Minamino, and A. Arimura. Isolation of a neuropeptide corresponding to the N-terminal 27 residues of the pituitary adenylate cyclase activating polypeptide with 38 residues (PACAP38). *Biochem. Biophys. Res. Commun.* **170**:643–648 (1990).
3. Kimura, C., S. Ohkubo, K. Ogi, M. Hosoya, Y. Itoh, H. Onda, A. Miyata, L. Jiang, R. R. Dahl, H. H. Stibbs, A. Arimura, and M. Fujino. A novel peptide which stimulates adenylate cyclase: molecular cloning and characterization of the bovine and human cDNAs. *Biochem. Biophys. Res. Commun.* **166**:81–89 (1990).
4. Ogi, K., C. Kimura, H. Onda, A. Arimura, and M. Fujino. Molecular cloning and characterization of rat pituitary adenylate cyclase activating polypeptide (PACAP). *Biochem. Biophys. Res. Commun.* **173**:1271–1279 (1990).
5. Arimura, A., A. Somogyvári-Vigh, A. Miyata, K. Mizuno, D. H. Coy, and C. Kitada. Tissue distribution of PACAP as determined by RIA: highly abundant in the rat brain and testes. *Endocrinology* **129**:2787–2789 (1991).
6. Ghatei, M. A., K. Takahashi, Y. Suzuki, J. Gardiner, P. M. Jones, and S. R. Bloom. Distribution, molecular characterization of pituitary adenylate cyclase-activating polypeptide and its precursor encoding messenger RNA in human and rat tissues. *J. Endocrinol.* **136**:159–166 (1992).
7. Mauso, Y., N. Suzuki, H. Matsumoto, F. Tokito, Y. Matsumoto, M. Tsuda, and M. Fujino. Regional distribution of pituitary adenylate cyclase activating polypeptide (PACAP) in the rat central nervous system as determined by sandwich-enzyme immunoassay. *Brain Res.* **602**:57–63 (1993).
8. Buscail, L., P. Gourlet, A. Cauvin, P. De Neef, D. Gossen, A. Arimura, A. Miyata, D. H. Coy, P. Robberecht, and J. Christophe. Presence of highly selective receptors for PACAP (pituitary adenylate cyclase activating peptide) in membranes from the rat pancreatic acinar cell line AR 4–2j. *FEBS Lett.* **262**:77–81 (1990).
9. Shivers, B. D., T. J. Görcs, P. E. Gottschall, and A. Arimura. Two high

affinity binding sites for pituitary adenylate cyclase-activating polypeptide have different tissue distributions. *Endocrinology* **128**:3055–3065 (1991).
10. Arimura, A. Receptors for pituitary adenylate cyclase-activating polypeptide: comparison with vasoactive intestinal peptide receptors. *Trends Endocrinol. Metab.* **3**:288–294 (1992).
11. Deutsch, P. J., and Y. Sun. The 38-amino acid form of pituitary adenylate cyclase-activating polypeptide stimulates dual signaling cascades in PC-12 cells and promotes neurite outgrowth. *J. Biol. Chem.* **267**:5108–5113 (1992).
12. Pisegna, J. R., and S. A. Wank. Molecular cloning and functional expression of the pituitary adenylate cyclase-activating polypeptide type I receptor. *Proc. Natl. Acad. Sci. USA* **90**:6345–6349 (1993).
13. Hosoya, M., H. Onda, K. Ogi, Y. Masuda, Y. Miyamoto, T. Ohtaki, H. Okazaki, A. Arimura, and M. Fujino. Molecular cloning and functional expression of rat cDNAs encoding the receptor for pituitary adenylate cyclase activating polypeptide (PACAP). *Biochem. Biophys. Res. Commun.* **194**:133–143 (1993).
14. Hashimoto, H., T. Ishihara, R. Shigemoto, K. Mori, and S. Nagata. Molecular cloning and tissue distribution of a receptor for pituitary adenylate cyclase-activating polypeptide. *Neuron* **11**:333–342 (1993).
15. Morrow, J. A., E. M. Lutz, K. M. West, G. Fink, and A. J. Harmar. Molecular cloning and expression of a cDNA encoding a receptor for pituitary adenylate cyclase activating polypeptide (PACAP). *FEBS Lett.* **329**:99–105 (1993).
16. Spengler, D., C. Waeber, C. Pantaloni, F. Holsboer, J. Bockaert, P. H. Seeburg, and L. Journot. Differential signal transduction by five splice variants of the PACAP receptor. *Nature (Lond.)* **365**:170–175 (1993).
17. Svoboda, M., M. Tastenoy, E. Ciccarelli, M. Stiévenart, and J. Christophe. Cloning of a splice variant of the pituitary adenylate cyclase-activating polypeptide (PACAP) type I receptor. *Biochem. Biophys. Res. Commun.* **195**:881–888 (1993).
18. Kosugi, S., F. Okajima, T. Ban, A. Hidaka, A. Shenker, and L. D. Kohn. Mutation of alanine 623 in the third cytoplasmic loop of the rat thyrotropin (TSH) receptor results in a loss in the phosphoinositide but not cAMP signal induced by TSH and receptor autoantibodies. *J. Biol. Chem.* **267**: 24153–24156 (1992).
19. Watanabe, T., T. Ohtaki, C. Kitada, M. Tsuda, and M. Fujino. Adrenal pheochromocytoma PC-12H cells respond to pituitary adenylate cyclase activating polypeptide. *Biochem. Biophys. Res. Commun.* **173**:252–258 (1990).
20. Greene, L. A., and A. S. Tischler. PC-12 pheochromocytoma cultures in neurobiological research. *Adv. Cell. Neurobiol.* **3**:374–414 (1982).
21. Yankner, B. A., and E. M. Shooter. The biology and mechanism of action of nerve growth factor. *Annu. Rev. Biochem.* **51**:845–868 (1982).
22. Greene, L. A., and A. S. Tischler. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA* **73**:2424–2428 (1976).
23. Togari, A., D. Baker, G. Dickens, and G. Guroff. The neurite-promoting effect of fibroblast growth factor on PC-12 cells. *Biochem. Biophys. Res. Commun.* **114**:1189–1193 (1983).
24. Nielsen, F. C., and S. Gammeltoft. Insulin-like growth factors are mitogens for rat pheochromocytoma PC-12 cells. *Biochem. Biophys. Res. Commun.* **154**:1018–1023 (1988).
25. Huff, K., D. End, and G. Guroff. Nerve growth factor-induced alteration in the response of PC-12 pheochromocytoma cells to epidermal growth factor. *J. Cell Biol.* **88**:189–198 (1981).
26. Allison, J. H., M. E. Blisner, W. H. Holland, P. P. Hipps, and W. R. Sherman. Increased brain *myo*-inositol-1-phosphate in lithium-treated rats. *Biochem. Biophys. Res. Commun.* **71**:664–670 (1976).
27. Berridge, M. J., C. P. Downes, and M. R. Hanley. Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. *Biochem. J.* **206**:587–595 (1982).
28. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
29. Ciccarese, S., S. Tommasi, and G. Vonghia. Cloning and cDNA sequence of the rat X-chromosome linked phosphoglycerate kinase. *Biochem. Biophys. Res. Commun.* **165**:1337–1344 (1989).
30. Chomczynski, P., and N. Sacchi. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156–159 (1987).
31. Zubiaur, M., and E. J. Neer. Nerve growth factor changes G protein levels and localization in PC-12 cells. *J. Neurosci. Res.* **35**:207–217 (1993).
32. Greene, L. A., M. M. Sobeth, and K. K. Teng. Methodologies for the culture and experimental use of the PC-12 rat pheochromocytoma cell line, in *Culturing Nerve Cells* (G. Banker and K. Goslin, eds.). MIT Press, Cambridge, MA, 207–226 (1991).
33. Clementi, E., M. Raichman, and J. Meldolesi. Heterogeneity of NGF-induced differentiation in PC-12 cells investigated at a battery of isolated cell clones. *Funct. Neurol.* **8**:109–119 (1993).
34. Barde, Y. A., D. Edgar, and H. Thoenen. Sensory neurons in culture: changing requirements for survival factors during embryonic development. *Proc. Natl. Acad. Sci. USA* **77**:1199–1203 (1980).
35. DiCicco-Bloom, E., and P. J. Deutsch. Pituitary adenylate cyclase activating polypeptide (PACAP) potently stimulates mitosis, neuritogenesis and survival in cultured rat sympathetic neuroblasts. *Regul. Peptides* **37**:319 (1992).
36. Jumblatt, J. E., and A. S. Tischler. Regulation of muscarinic ligand binding sites by nerve growth factor in PC-12 pheochromocytoma cells. *Nature (Lond.)* **297**:152–154 (1982).

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