

The 38-Amino-Acid Form of Pituitary Adenylate Cyclase-Activating Polypeptide Induces Neurite Outgrowth in PC12 Cells that Is Dependent on Protein Kinase C and Extracellular Signal-Regulated Kinase but not on Protein Kinase A, Nerve Growth Factor Receptor Tyrosine Kinase, p21^{ras} G protein, and pp60^{c-src} Cytoplasmic Tyrosine Kinase

PHILIP LAZAROVICI, HAO JIANG, and DONALD FINK, JR.

Department of Pharmacology, School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem 91010, Israel (P.L.), Section on Growth Factors, National Institute of Child Health and Human Development, National Institutes of Health (H.J.), and Division of Cytokine Biology, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892 (D.F.)

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ABSTRACT

The 38-amino-acid isoform of pituitary adenylate cyclase-activating polypeptide (PACAP38) elicits a robust outgrowth of neurites in cultured PC12 cells. Initiation of neurite outgrowth occurs within 4–8 hr after the addition of PACAP38. Treatment with PACAP38 does not elicit collateral activation of p140^{trk} nerve growth factor receptor tyrosine kinase activity, nor is it associated with tyrosine phosphorylation of suc1-associated neurotrophic factor target, a selective target of neurotrophin tyrosine kinase receptors. Coadministration of epidermal growth factor with PACAP38 elicits an enhanced response. Induction of neurites is also observed on the addition of PACAP38 to dominant negative Src and Ras PC12 cell variants. PACAP38 stimulates extracellular signal-regulated kinase (Erk) activity >10-fold within 5 min, and the effect is augmented by cotreatment with epidermal growth factor. Pretreatment with

the cAMP-dependent protein kinase-selective inhibitor, H-89, is ineffective as an antagonist of PACAP38-induced neurite outgrowth, whereas down-regulation of protein kinase C (PKC) by phorbol ester or incubation with PKC-selective inhibitors GF109203X and calphostin C effectively blocks PACAP38-stimulated neurite formation. Stimulation of Erk activity is inhibited by incubation with PD90859, a pharmacological antagonist of the threonine/tyrosine dual-specificity Erk. Inhibition of ligand-stimulated Erk activation prevents PACAP38-induced neurite outgrowth. Collectively, these findings indicate that PACAP38-stimulated neuritogenesis requires PKC and Erk activation but is independent of cAMP-dependent protein kinase, nerve growth factor receptor tyrosine kinase, p21^{ras} G protein, and pp60^{c-src} cytoplasmic tyrosine kinase.

The PC12 cell line, derived from a rat adrenal medullary pheochromocytoma tumor, has been used extensively as a model for investigating biomolecular events involved in neuronal differentiation. In the presence of nanomolar concentrations of the neurotrophin NGF, replicating PC12 cells cease to divide and extend neuritic processes indicative of phenotypic transformation to a neuronal phenotype characteristic of sympathetic neurons (Fujita *et al.*, 1989). The neurodifferentiative effects of NGF in PC12 cells are elicited

by interaction of the neurotrophin with two distinct cell surface receptors: p75^{NGFR}, which binds all members of the neurotrophin family, and p140^{trk}, a prototypic receptor tyrosine kinase that selectively binds NGF and is activated by interaction with its cognate ligand (Kaplan and Miller, 1997). Many of the biological activities elicited by NGF are mediated by ligand-dependent activation p140^{trk} tyrosine kinase activity. Binding of NGF to p140^{trk} stimulates rapid tyrosine autophosphorylation of the receptor, as well as activation of

ABBREVIATIONS: NGF, nerve growth factor; PACAP38, pituitary adenylate cyclase-activating polypeptide with 38 residues and an amidated carboxyl terminus; EGF, epidermal growth factor; Src, pp60^{c-src} cytoplasmic tyrosine kinase; Trk, p140^{trk} nerve growth factor receptor tyrosine kinase; Ras, p21^{ras} G protein; Erk, extracellular signal-regulated kinase; MEK, threonine/tyrosine dual-specificity extracellular signal-regulated kinase; PVDF, polyvinylidene difluoride; SNT, suc1-associated neurotrophic factor target; PMA, phorbol-12-myristate-13-acetate; PKA, cAMP-dependent protein kinase; PKC, calcium- and phospholipid-dependent protein kinase C; MBP, myelin basic protein; PC12nnr5, a pheochromocytoma PC12 clone nonresponsive to NGF; PC12–6.24, a PC12 clone overexpressing human p140^{trk}; GsRasDN6, dexamethasone-inducible, dominant-negative Ras PC12 clone; M-M17–26, stable, dominant-negative Ras PC12 clone; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

several signal transduction cascades, including the Ras-Erk pathway, phospholipase-C γ 1, phosphatidylinositol-3 kinase, and SNT (Kaplan and Miller, 1997). Additionally, a role for pp60^{c-src} in the transduction of NGF biological signals has been proposed (Keegan and Halegoua, 1993).

PC12 cells also express cell surface receptors for various other peptides, including EGF (Lazarovici *et al.*, 1987), a mild mitogen for these cells (Huff *et al.*, 1981), as well as type I and type II receptors for the PACAP38 and PACAP27 (Deutsch and Sun, 1992; Cavallaro *et al.*, 1995). These two isoforms of PACAP represent alternatively processed forms of a precursor protein that share in common 27 amino-terminal amino acids. Both forms exhibit substantial amino acid sequence homology with vasoactive intestinal peptide (Miyata *et al.*, 1990). PACAP type I and II receptors are seven membrane-spanning, G protein-coupled moieties that mediate activation of adenylate cyclase and phospholipase C (Spengler *et al.*, 1993). In PC12 cells, both PACAP38 and PACAP27 activate these signaling pathways but with different efficiencies. PACAP38 is 200-fold more potent than PACAP27 in promoting phospholipase C-mediated inositol phospholipid metabolism (Deutsch and Sun, 1992), whereas both peptides are equally potent with respect to activation of adenylate cyclase and elevation of cAMP (Deutsch and Sun, 1992). The neurite-inducing activity of PACAP38 in PC12 cells is markedly more robust than that observed for PACAP27 (Deutsch and Sun, 1992). The vigorous neuritogenic activity of PACAP38 has been observed in a variety of systems, including numerous clones of PC12 (Deutsch and Sun, 1992; Hernandez *et al.*, 1995; Colbert *et al.*, 1994; Barrie *et al.*, 1997; Lazarovici *et al.*, 1997b), rat chromaffin cells (Wolf and Kriegstein, 1995), and human neuroblastoma cells (Deutsch *et al.*, 1993). The signal transduction pathway or pathways responsible for mediating PACAP-induced neurite outgrowth remain to be definitively identified, and their elucidation could reveal fundamentally novel mechanisms responsible for regulating processes associated with morphological neuronal development. The coexistence of receptors for NGF, EGF, and PACAP on the membrane surface of PC12 cells, coupled with recent progress in detailing the intracellular signaling pathways activated by these receptors in a ligand-dependent manner, demonstrates that the PC12 cell model is well suited for investigating cooperative, synergistic interactions between distinct families of peptide growth factors. Specifically, the issue of signal transduction cascade cross-talk between G protein-linked PACAP receptors and the tyrosine kinase activity of EGF-stimulated growth factor receptors resulting in neurotrophic activity may be investigated.

In the current study, a number of variant PC12 cell lines expressing different levels of p140^{trk} (Chao 1992), dominant-negative Ras (Green *et al.*, 1986), or Src (Hempstead *et al.*, 1992) PC12 transfectant cells and pharmacological inhibitors were used to elucidate the roles of Trk, Ras, Src, Erk, PKA, and PKC in promoting PACAP-stimulated neurite outgrowth. Results obtained indicate that PACAP-induced neuritogenesis in PC12 cells is Trk-, Ras-, Src-, and PKA-independent but PKC- and Erk-dependent.

Materials and Methods

Mouse NGF, EGF, and rat collagen type II were purchased from Collaborative Biomedical (Bedford, MA). Dexamethasone, poly-L-lysine,

myelin basic protein, and PMA were obtained from Sigma Chemical (St. Louis, MO). PD98059 was acquired from Research Biochemicals International (Natick, MA). U73122, GF109203X, calphostin C, and staurosporine were purchased from Calbiochem (La Jolla, CA). H-89 [N-[2-(p-bromocinnamyl)amino]ethyl]-5-isoquinolinesulfonamide, dihydrochloride] was obtained from Alexis (San Diego, CA). Rat PACAP38 and the antagonist PACAP(6–38) were acquired from Peninsula Laboratories (Belmont, CA). Monoclonal anti-phosphotyrosine antibody (clone 4G10) was procured from Upstate Biotechnology (Lake Placid, NY). Polyclonal antibodies directed against TrkA (C14), Src, Ras, and Erk were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-EGF receptor antibody was obtained from Medical and Biological Laboratories (Nagoya, Japan). Anti-phosphorylated Erk antibody was generously provided by Dr. Eric Schaeffer (Promega, Madison, WI). [³H]Thymidine and [γ -³²P]ATP were acquired from Amersham (Arlington Heights, IL). K-252a [8R*,9S*,11S*(-)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7 β ,11 α -triazadibenzo(a,g)cycloocta(c,d,e)trindene-1-one] and staurosporine were kindly given by Dr. Y. Matsuda (Kyowa Hakko Kogyo, Tokyo, Japan). Sp-cAMP was purchased from Boehringer-Mannheim (Mannheim, Germany).

Cell culture. PC12 cells were grown in Dulbecco's modified Eagle's medium supplemented with 7% fetal bovine serum, 7% horse serum, 100 μ g/ml streptomycin, and 100 units/ml penicillin (Life Technologies, Grand Island, NY) (Lazarovici *et al.*, 1987). In experiments involving extended treatment with growth factors, the medium was changed, and test agents replenished factors every 48 hr. PC12nnr cells were grown in collagen-coated tissue culture dishes in RPMI 1640 medium (Life Technologies) supplemented with 10% horse serum and 5% fetal bovine serum (Lazarovici *et al.*, 1997b). PC12–6.24 cells, a clone of PC12 manipulated to overexpress human p140^{trk}, were grown under culture conditions comparable to those for wild-type PC12 cells except for the inclusion of 200 μ g/ml G418 (Life Technologies) (Lazarovici *et al.*, 1997b). During treatment with test article, G418 was removed from the medium. PC12 cell variant cell line, GSrasDN6, which expresses a dominant-negative *ras* gene under the transcriptional control of the mouse mammary tumor virus promoter (Lazarovici *et al.*, 1997b); M-M17–26, expressing the *H-ras* Asn17 gene under the transcriptional control of the mouse metallothionein-I promoter (Lazarovici *et al.*, 1997b); and srcDN2, modified to express the K295R mutant (kinase inactive) form of chicken Src under transcriptional control of the cytomegalovirus promoter (Lazarovici *et al.*, 1997b), were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 5% horse serum. In experiments involving dexamethasone treatment, cells were switched to media supplemented with charcoal-stripped serum 24 hr before stimulation with 500 nM dexamethasone.

Immunoprecipitation and immunoblotting. Cells were plated in 10-cm tissue culture dishes 1 day before initiation of experiments. After treatment with selected growth factors, cultures were washed twice with Tris-buffered saline (20 mM Tris-HCl, pH 8.0, 137 mM NaCl) and subjected to lysis in 1 ml of 1% Nonidet P-40 lysis buffer (20 mM Tris-HCl, 137 mM NaCl, 0.5 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.15 unit/ml aprotinin, 20 mM leupeptin, 1 mM sodium vanadate) at 4° for 20 min. Insoluble material was removed by centrifugation for 10 min at 12,000 \times g. Lysate aliquots were diluted in SDS sample buffer (0.06 M Tris-HCl, pH 6.8, 12.5% glycerol, 1.25% SDS, 5% β -mercaptoethanol, 0.002% bromophenol blue), boiled for 5 min, and then subsequently electrophoresed through 7.5% polyacrylamide gels or lysates were subjected to immunoprecipitation with anti-TrkA or anti-Erk antibodies for 2 hr at 4° with continuous agitation, followed by an additional 2-hr incubation with Protein A-Sepharose. Precipitates were washed three times with lysis buffer and once with water and then boiled for 5 min in SDS sample buffer and subjected to SDS-PAGE on 7.5% polyacrylamide gels. After SDS-PAGE, resolved proteins were transferred to nitrocellulose. Blots were probed overnight at 4° with the primary antibodies and analyzed using enhanced

chemiluminescence systems with horseradish peroxidase-coupled secondary antibodies. After visualization on film, quantitation was performed by densitometry.

Erk kinase assay. Washed Protein A resin containing immunoprecipitated Erk was suspended in 30 μ l of kinase assay buffer [7.5 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 2.5 μ M protein kinase A inhibitor peptide PKI(6–22)-amide, 225 μ M cold ATP, 25 μ Ci of [γ - 32 P]ATP, 500 μ g/ml MBP as the kinase substrate] and incubated for 30 min at room temperature. The reaction was terminated by adding 30 μ l of 2 \times SDS sample buffer. Samples were heated for 5 min and then electrophoresed through a 4–20% Tris-glycine gradient gel (NOVEX). After electrophoretic resolution, radiolabeled proteins were transferred to Immobilon PVDF membranes. Phosphorylated MBP was visualized by exposure of the membranes to XAR film (Eastman Kodak). MBP-associated radioactivity was quantified by liquid scintillation counting of bands excised from the membrane. Dual threonine/tyrosine phosphorylation of activated Erk was demonstrated by Western blotting conducted as specified in a protocol developed by Promega.

PKA assay. Growth factor-stimulated activation of cAMP-dependent PKA was assessed in PC12 cells using a protein kinase assay system obtained from Life Technologies. The assay was conducted in accordance with the manufacturer's specifications. In brief, PC12 cells treated with specified test article were washed with ice-cold PBS containing 1 mM Na_3VO_4 and sonicated in extraction buffer (5 mM EDTA, 59 mM Tris, pH 7.5), and cellular debris was removed by centrifugation in a microfuge at 4 $^\circ$. Aliquots of cellular extract were incubated for 5 min at 30 $^\circ$ in an assay buffer composed of 50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 100 μ M [γ - 32 P]ATP, 0.25 mg/ml bovine serum albumin, and 50 μ M Kemptide as a substrate for PKA. Reactions were terminated by spotting an aliquot of the reaction mixture onto phosphocellulose discs. The spotted discs were washed twice with 1% (v/v) phosphoric acid followed by two washes with H_2O . Washed discs were placed into scintillation vials, and the peptide-incorporated 32 P was quantified by liquid scintillation counting.

Neurite outgrowth assay. To evaluate neurite outgrowth, cells were plated at low density (1000–5000 cells/dish) on collagen- and poly-L-lysine-coated tissue culture dishes in the appropriate medium. Cultures were treated for different periods of time with the indicated reagents, examined by light microscopy using an inverted Nikon-Diaphot microscope, and photographed at 320 \times magnification.

Results

PACAP38-induced neurite outgrowth in PC12 cells is p140^{trk} independent and is facilitated by coadministration with EGF. To demonstrate that PACAP38-elicited neuritogenesis does not involve collateral activation of high affinity, p140^{trk} NGF receptors, PC12 cell variants expressing variable levels of neurotrophin receptors were used (Fig. 1). Wild-type PC12 cells, which express both low affinity, p75 pan-neurotrophin binding receptors, and p140^{trk}, exhibit neurite outgrowth on treatment with NGF (Chao, 1992; see references). PC12nnr5 cells, a PC12 variant expressing levels of p75 comparable to wild-type cells but undetectable levels of p140^{trk}, do not elaborate neurites in response to NGF administration (Green *et al.*, 1986). Overexpression of p140^{trk} in wild-type PC12 cells results in accelerated neurite outgrowth when NGF is present (Hempstead *et al.*, 1992). Each of these PC12 cell lines expresses EGF receptors (Lazarovici *et al.*, 1997b). Uniformly, treatment of each PC12 cell line variant with EGF (10 ng/ml) fails to elicit neurite outgrowth under routine culture conditions (data not shown), indicating that stimulation of EGF receptor tyrosine kinase activity in and of itself is insufficient to

induce a neuronal morphological phenotype in any of these cell lines.

Treatment of PC12 variant cultures for 48 hr with 5 nM PACAP38 was observed to promote neurite outgrowth irrespective of the level of p140^{trk} receptors expressed (Fig. 1, Table 1). Inhibition of PACAP38-stimulated neurite outgrowth in the variant PC12 cell lines by the antagonist PACAP 6–38 (data not shown) indicates that the effects on PC12 cell morphology are mediated by PACAP-selective receptors. To determine whether coactivation of a non-neurotrophin, tyrosine kinase-initiated signal transduction cascade might amplify PACAP38-induced neurite outgrowth, each of the variant PC12 cell lines was treated with a combination of PACAP38 (5 nM) and 10 ng/ml EGF. The consequence of this coadministration in each of the cell lines tested was a more robust neuritogenic response than noted for PACAP38 alone (Fig. 1). Collateral activation of p140^{trk} was not elicited by PACAP38, EGF, or the combination of PACAP38 and EGF (Fig. 2). Similarly, these non-neurotrophin peptides did not elicit tyrosine phosphorylation of the nuclear protein SNT (Fig. 2), an event reported to be essential for the elaboration of neurites induced by NGF (Rabin *et al.*, 1993). Moreover, PACAP38-induced neurite outgrowth was not affected in any of the PC12 variant cell lines by pretreatment with 250 nM K-252a (data not shown), a highly

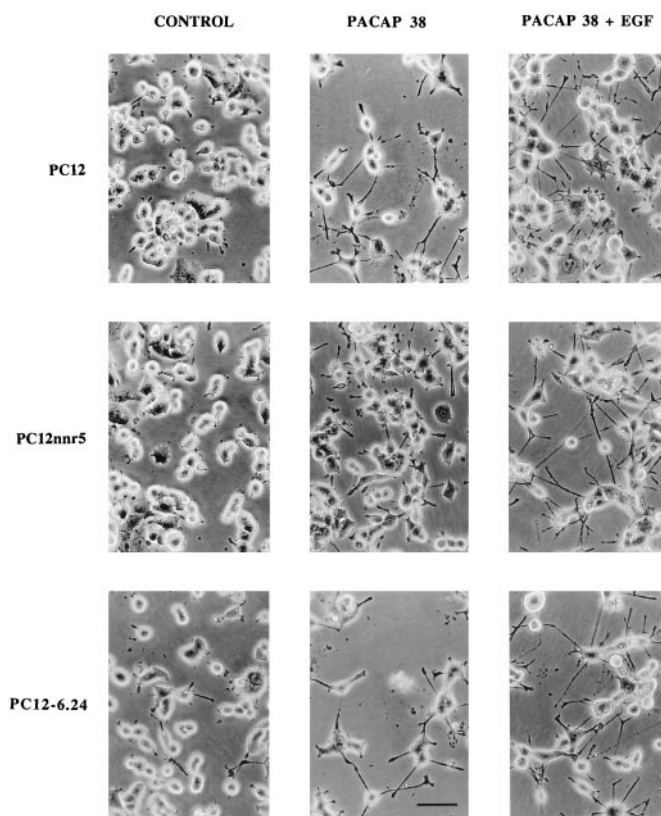


Fig. 1. PACAP-induced neurite outgrowth in wild-type PC12 cells and variant clones occurs independently from activation of p140^{trk}. Wild-type, PC12nnr5 (low level p140^{trk}), and PC12 6.24 (p140^{trk} overexpression) cells were seeded at low density (1000–5000 cells/dish) on collagen/poly-L-lysine-coated culture dishes. Cultures were maintained for 48 hr in the absence (CONTROL) or presence (PACAP38, PACAP38 + EGF) of PACAP38 (5 nM) and EGF (10 ng/ml). Cultures were evaluated for evidence of neurite outgrowth by light microscopy using an inverted Nikon-Diaphot microscope and photographed at 320 \times magnification. Scale bar, 25 μ m.

selective inhibitor of NGF-induced, p140^{trk} tyrosine kinase activity (Koizumi *et al.*, 1988; Berg *et al.*, 1992) and subsequent induction of neurites in PC12 cells. Collectively, these findings demonstrate that PACAP38 does not elicit neurite outgrowth in PC12 cell variants through coactivation of p140^{trk} NGF receptors.

PACAP38-induced neurite outgrowth in PC12 cells is Src and Ras independent

Variant PC12 cell lines were used to determine whether Src tyrosine kinase activity or Ras activation is involved in PACAP38-stimulated neurite outgrowth. Incubation of SrcDN2 variant PC12 cells with dexamethasone results in glucocorticoid-induced overexpression of a kinase-inactive dominant negative Src (Fig. 3). Similarly, the addition of dexamethasone to cultures of GSrasDN6 PC12 variants results in transient overexpression of a dominant negative mutant Ras (Fig. 3). The PC12 variant line M-M17-26, in which the Ras mutation is stably expressed without requiring glucocorticoid treatment of the cultures (not shown), was used as well. All of the cell lines demonstrate detectable levels of EGF receptor (Fig. 3). Each of these variant cell lines was treated with PACAP38 or PACAP38 plus EGF, and the extent of neurite outgrowth was evaluated (Fig. 4). In SrcDN2 PC12 cells, PACAP38 unambiguously induces the initiation of neuritic process outgrowth with coadministration of EGF amplifying the response (Fig. 4). Addition of PACAP38 or PACAP38/EGF to the Ras dominant negative-expressing PC12 variants M-M17-26 or GSrasDN6 cells pretreated 24 hr with dexamethasone also elicited the outgrowth of neurites. In each instance, the extent of neuritic outgrowth induced by the addition of PACAP38 was significant compared with untreated, control cultures (Table 1). Again, amplification of the PACAP38-induced neurite outgrowth response by simultaneous addition of EGF was observed, consistent with the noted expression of EGF receptor in each cell line. Collectively, these observations indicate that Src and Ras are unlikely to be involved in mediating PACAP38-stimulated neuritogenesis. In contrast, NGF failed to elicit a neurite outgrowth response in these variant, dominant negative Src/Ras cell lines (data not shown) as reported previously (Lazarovici *et al.*, 1997a). The decreased percentage of cells exhibiting neurite outgrowth in response to PACAP38 in nnr5, SrcDN, and RasDN variant lines compared with wild-type PC12 cells may be attributable, in part, to the clonal selection of colonies with lower levels of PACAP recep-

tor expression. Additional subcloning for PACAP receptors has not been performed.

Time-dependent, PACAP38-induced stimulation of Erk kinase activity: potentiation by coadministration with EGF. Addition of PACAP38 (5 nM) to wild-type PC12 cells elicits a rapid increase in Erk kinase activity, reaching a maximum of 12.1 ± 4.2 -fold above untreated control cultures within 5 min of treatment (Fig. 5). In the continuous presence of PACAP38, Erk activity declines to a level that remains ~2-fold above basal activity to 2 hr after the addition (Fig. 5). Increasing the concentration of PACAP38 failed to further elevate the level of Erk activation observed at 5 min. Considerably higher peaks in stimulated Erk activity were achieved by the addition of NGF (50 ng/ml) or EGF (10 ng/ml) reaching levels that were 22.5 ± 6.7 - and 19.5 ± 6.7 -fold above control values, respectively, at 5 min after treatment (Fig. 5). Simultaneous addition of PACAP38 (5 nM) and EGF (10 ng/ml) resulted in the maximal (24.5 ± 9.9 -fold) increase in Erk activity observed 5 min after treatment. The decline in PACAP38/EGF-stimulated Erk activity that occurs from the peak levels observed at 5 min is comparable to results obtained for NGF (Fig. 5). At 120 min after the addition, Erk phosphorylating activity remains at ~4-fold above basal levels for both NGF and PACAP38/EGF-treated cultures. Continued culturing of PC12 cells in the presence of PACAP38 alone, beyond 2 hr, and to 72 hr reveals a secondary stimulation of Erk activity occurring at 24 hr after PACAP38 addition (Fig. 5, *inset*).

PACAP38-stimulated Erk kinase activity is partially mediated by PKA. PACAP type II receptor activates adenylate cyclase through coupling to the guanine nucleotide binding protein G_s (Miyata *et al.*, 1990; Deutsch and Sun, 1992). To determine whether PKA is involved in PACAP38-induced Erk kinase activation, experiments were conducted using an analog of cAMP to activate PKA. Treatment of PC12 cells with the stable, cAMP analog sp-cAMP (25 μ M) for up to 72 hr was without effect on the level of Erk activity (Fig. 5, *inset*) as reported previously (Richter-Landsberg and Jastorff, 1986; Barrie *et al.*, 1997), although a transient neurite outgrowth response has been observed in PC12 cells as a consequence of elevating intracellular cAMP levels (Richter-Landsberg and Jastorff, 1986). As an alternative approach for testing the hypothesis that activation of PKA is required for PACAP38-mediated induction of Erk activity, H89, a potent, relatively selective inhibitor of PKA, was used (Chijiwa *et al.*, 1990). At a concentration of 20 μ M, the amount used in

TABLE 1

Quantification of neurite outgrowth induced by PACAP38 in variant PC12 cell lines

The following PC12 cell lines were seeded at low density (1000–5000 cells/dish) on collagen/poly-L-lysine-coated tissue culture dishes: wild-type, nnr5 (NGF-nonresponsive), 6.24 (p140^{trk} overexpressing), SrcDN2 (induced dominant negative Src), RasDN6 (induced dominant negative Ras), and M-M17-26 (constitutive dominant negative Ras). Cultures were incubated 48 hr in the absence or presence of PACAP38, examined by light microscopy, and photographed. Neurite length, measured directly from photographs, is expressed as a function of cell diameter. In untreated, control cultures for each cell line, <5% of the cells exhibit neuritic processes with an overall length less than one cell diameter.

PC12 cell line	Neurite length (\times cell diameter)	% Neurite-bearing cells (mean \pm standard error)	No. of fields evaluated	No. of cells/field
Wild-type	2–3	80.7 ± 7.5^a	12	600
nnr5	1–2	52.8 ± 8.9^a	9	160
6.24	2–4	85.1 ± 8.1^a	12	220
SrcDN2	1–2	64.6 ± 7.3^a	9	90
RasDN6	1–2	41.0 ± 8.6^a	15	140
M-M17-26	1–2	54.7 ± 10.6^a	12	170

^a Statistically significant ($p < 0.001$) compared with neurite outgrowth in untreated control cultures by analysis of variance.

experiments reported here, H89, an isoquinoline-sulfonamide, inhibits PKA but not calcium-calmodulin kinases, protein kinase C, casein kinase II, or cGMP-dependent protein kinases (Chijiwa *et al.*, 1990). By comparison, K-252a, a microbial alkaloid, when added to PC12 cells at a concentration of 200 nM, inhibits not only NGF-induced p140^{trk} tyrosine kinase activity but also a number of the previously mentioned protein kinases (Lazarovici *et al.*, 1997a). Pretreatment of PC12 cells for 30 min with 0.2% methanol (control), 20 μ M H89, or 200 nM K-252a was followed by the addition of NGF (50 ng/ml), EGF (10 ng/ml), PACAP38 (5 μ M), PACAP38 and EGF, or no peptides. In control cells, NGF, EGF, PACAP38, and PACAP38+EGF stimulated Erk kinase activity by 12.3-, 16.5-, 5.2-, and 20.0-fold respectively (Fig. 6). As was noted previously (Fig. 5), the combination of

PACAP38 and EGF exhibited an additive effect with respect to Erk activation. Pretreatment with H-89 (30 min) inhibited NGF, EGF, PACAP38, or PACAP38/EGF stimulation of Erk kinase activity by 76%, 74%, 65%, and 75%, respectively. As anticipated (Lazarovici *et al.*, 1997a), prior treatment with K-252a completely blocked NGF-stimulated Erk activity (>99% inhibition). In addition, Erk stimulation by EGF, PACAP, or PACAP38/EGF was inhibited by K-252a 69%, 48%, and 67%, respectively (Fig. 6). It should be noted that with the exception of the K-252a and NGF combination, the effect of inhibitor pretreatment on peptide-stimulated Erk activation was due, in large measure, to an inhibitor-mediated increase in basal Erk activity. Maximal peak stimulations of Erk elicited by acute (5-min) treatment with EGF, PACAP38, or PACAP38 plus EGF were not diminished by pretreatment with either K-252a or H-89. The peak level of Erk MBP phosphorylating activity induced by NGF was reduced to the base-line value observed in K-252a- but not H-89-pretreated control cultures.

Results for the activation of PKA by the same peptide additions as described above are presented in Table 2. Again, maximal stimulation was obtained by combined administra-

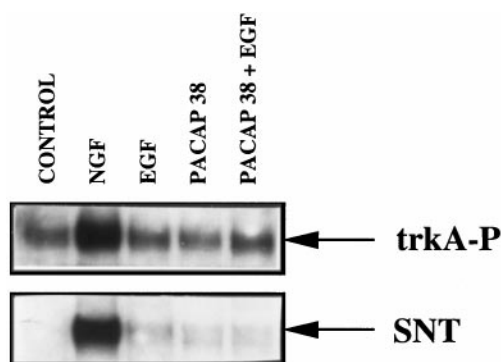


Fig. 2. PACAP38 does not induce tyrosine phosphorylation of p140^{trk} or the neurotrophin target nuclear protein SNT. Cultures of PC12 6.24 cells were incubated for 5 min with NGF (50 ng/ml), EGF (10 ng/ml), PACAP38 (5 nM), or PACAP38 + EGF or left untreated (*CONTROL*). Lysates prepared from the cultures were immunoprecipitated with anti-TrkA antibody or p13^{suc1}-agarose, proteins were resolved by SDS-PAGE, and blots were probed with 4G10 anti-phosphotyrosine antibody after transfer to PVDF membranes. The positions of tyrosine phosphorylated TrkA and SNT are indicated.

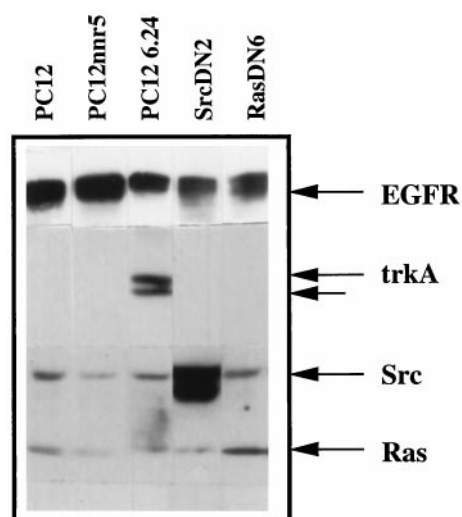


Fig. 3. Detection by Western blotting of dominant negative Src, Ras, and EGF receptor in PC12 cell lines. Protein-normalized lysates prepared from wild-type and variant PC12 cultures were electrophoresed through 7.5% polyacrylamide gels, and resolved proteins were transferred to nitrocellulose membranes and blotted with anti-Src, Ras, EGF receptor (*EGFR*), and TrkA antibodies. Arrows, positions. PC12, PC12nnr5, and PC126.24 cells were included for purposes of comparing protein expression levels across the spectrum of PC12 variant cell lines.

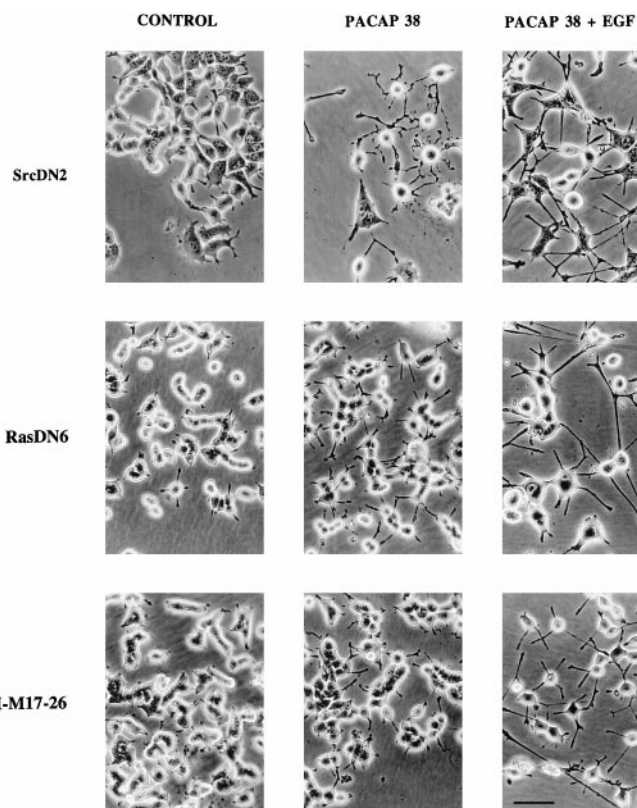


Fig. 4. Induction of neurite outgrowth by PACAP38 in PC12 cells is Src and Ras independent. The src dominant/negative PC12 cell line variant, SrcDN2, and ras dominant/negative PC12 variants RasDN6 (transient, dexamethasone-inducible) and M-M17-26 (stable) were cultured at low density (1000–5000 cells/dish) on collagen/poly-L-lysine-coated dishes. Cultures were maintained for 48 hr in the absence (*CONTROL*) or presence (*PACAP38*, *PACAP38 + EGF*) of PACAP38 (5 nM) and EGF (10 ng/ml). Cultures were evaluated for evidence of neurite outgrowth by light microscopy using an inverted Nikon-Diaphot microscope and photographed at 320 \times magnification. Scale bar, 25 μ m.

tion of PACAP38 and EGF. Pretreatment with 20 μM H-89 resulted in an 85–95% inhibition of PKA activity for all treatment groups evaluated.

PACAP38-induced neurite outgrowth and activation of Erk kinase activity is primarily PKC and MEK dependent. To affirm the involvement of PKA, PKC, and MEK in PACAP38-elicited neurite outgrowth in PC12 cells, cultures were incubated for 1 hr with various selective pharmacological inhibitors before the addition of PACAP38 (Figs. 7 and 8). Preincubation of wild-type and variant PC12 cells

with 20 μM H-89 (PKA antagonist) failed to significantly diminish PACAP38-induced neurite outgrowth (Figs. 7 and 8), suggesting that the contribution of PKA to promotion of PACAP38-stimulated neuritogenesis is minimal.

In addition to adenylate cyclase, PACAP stimulates PLC phospholipid hydrolytic activity. In PC12 cells, activation of PLC by PACAP results in the generation of diacylglycerol and stimulation of PKC. To assess the role of PKC in PACAP38 signal transduction, PC12 cells were incubated with 1 μM concentration of the PKC inhibitors GF10923X

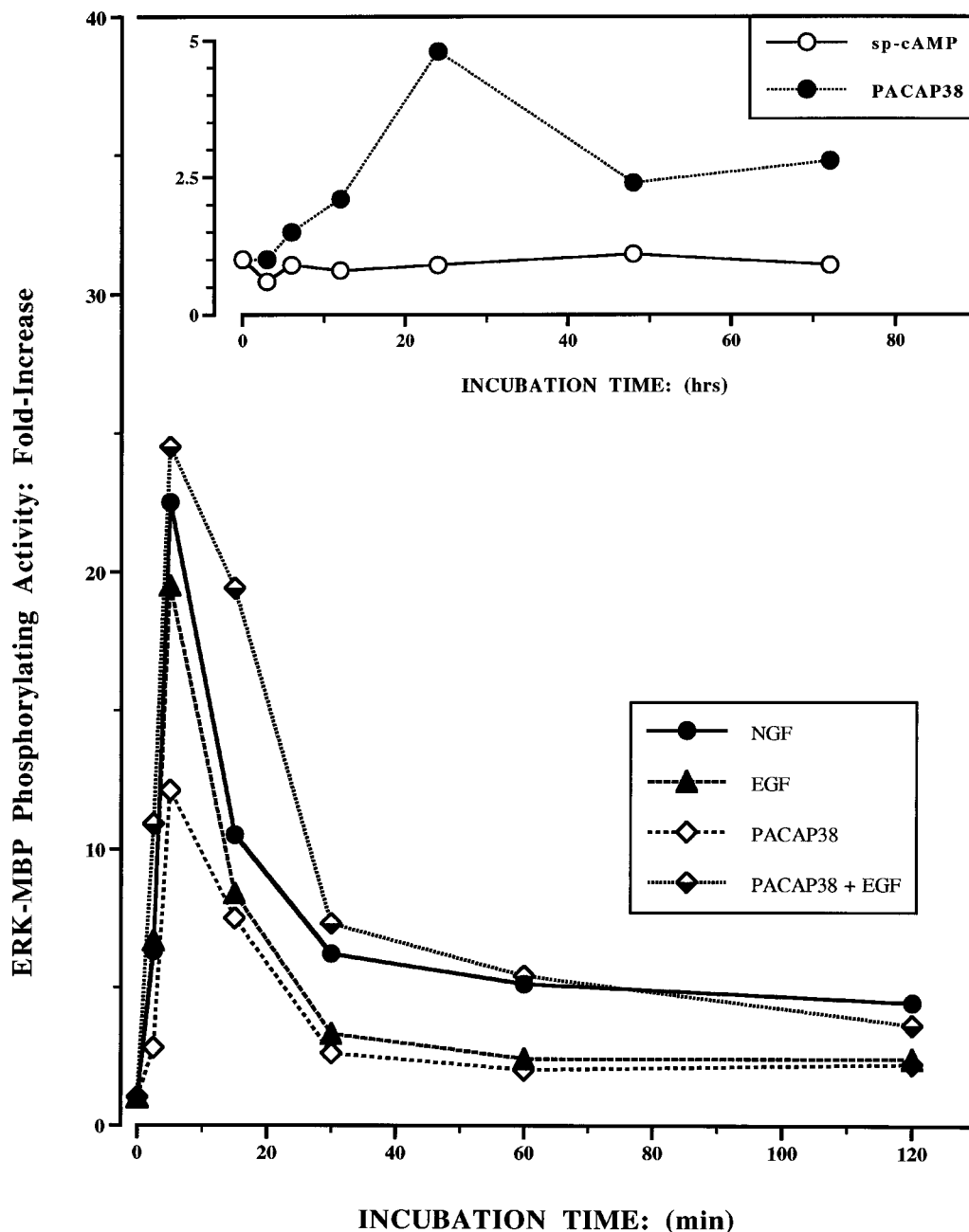


Fig. 5. Comparison of the time-dependent kinetics for activation of Erk in PC12 cells by peptide growth factors. Wild-type PC12 cells were treated with NGF (50 ng/ml), EGF (10 ng/ml), PACAP38 (5 nM), or PACAP38 + EGF for the specified periods of time (0–120 min or 0–72 hr, *inset*). Lysates were prepared and immunoprecipitated overnight with anti-Erk antibody. Erk phosphorylating activity was assessed using an immune complex *in vitro* kinase assay with MBP as the substrate. *Bottom*, mean values from three independent experiments. Error bars were omitted for purposes of clarity. In the majority of instances, with the exception of the highest fold-increases, standard errors were <10% of the mean. *Inset*, effect of PACAP38 and the cell-permeable PKA activator, sp-cAMP (25 μM), over an extended time course of 72 hr. Results are expressed as the mean of duplicate samples from a representative experiment and demonstrate the secondary, delayed activation of Erk activity by continuous PACAP38 treatment.

(Figs. 7 and 8) and calphostin C (data not shown) for 1 hr before the addition of PACAP38. PACAP38-induced neurite outgrowth in wild-type and dominant negative Ras PC12 cells was inhibited 70% and 88%, respectively, by pretreatment with GF10923X. Confirming the PKC-dependent character of this inhibition, down-regulation of phorbol-sensitive PKC isoforms by treatment with 1 μ M PMA (Zheng *et al.*, 1996) inhibited PACAP38-induced neurite outgrowth by 84% and 71% in wild-type and dominant negative Ras PC12 cells, respectively. Representative photomicrographs (Fig. 7) also illustrate the inhibitory effects of MEK inhibition on PACAP38-stimulated neurite formation. In wild-type and dominant negative Ras PC12 cells, pretreatment with 20 μ M PD98059, an inhibitor of the Erk kinase MEK, attenuated PACAP38-elicited neuritogenesis by $45 \pm 16\%$ and $87 \pm 12\%$,

respectively. Increasing the concentration of inhibitor to 50 μ M completely blocks PACAP38-induced neurite outgrowth; however, at this concentration, considerable toxicity was observed during the 3-day course of treatment.

To further establish that PACAP38-elicited stimulation of PKC is involved in mediating Erk activation associated with induction of neurite outgrowth, the effect of the PKC-selective antagonist GF10923X and down-regulation of phorbol-sensitive PKC by PMA on PACAP38-stimulated Erk activity was evaluated. Cultures of wild-type PC12 cells were incubated for 1 hr with 1 μ M GF10923X or overnight with 1 μ M PMA to down-regulate PKC before acute treatment (5 min) with PACAP38 (5 nM). Levels of activated Erk were assessed using an anti-phospho Erk antibody that detects the dual threonine/tyrosine phosphorylated form of the kinase (Fig.

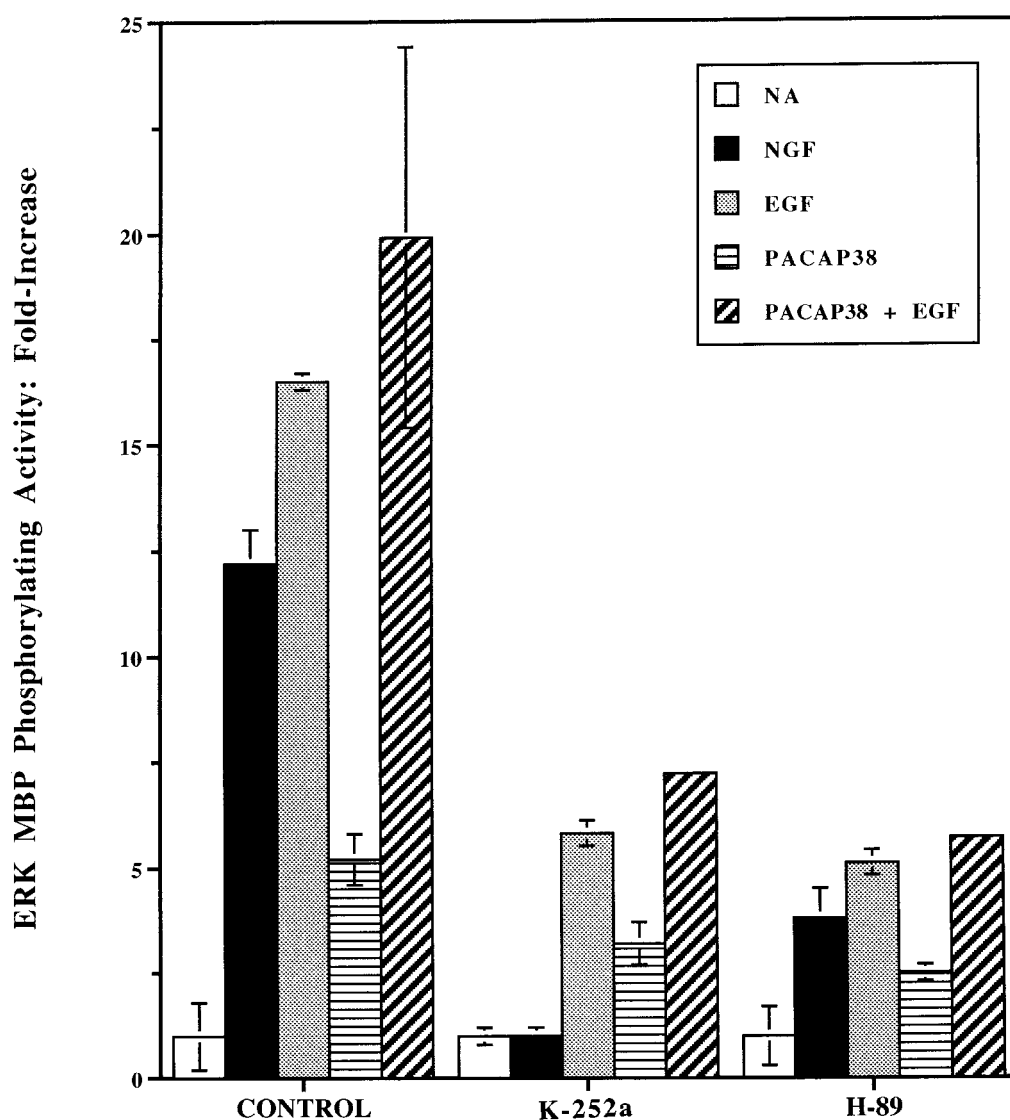


Fig. 6. Effect of the protein kinase inhibitors K-252a and H-89 on growth factor-stimulated Erk activity. Cultures of wild-type PC12 cells were incubated in the absence (CONTROL, vehicle) or presence of either K-252a (200 nM) or H-89 (20 μ M) 30 min before the addition of NGF (50 ng/ml), EGF (10 ng/ml), PACAP38 (5 μ M), or PACAP38 and EGF as indicated in the legend. Cultures were incubated an additional 5 min after treatment with peptide growth factors. Lysates were prepared and immunoprecipitated overnight with anti-Erk antibody. Erk phosphorylating activity was assessed using an immune complex *in vitro* kinase assay with MBP as the substrate. Results are expressed as the mean \pm standard error for determinations obtained from three independent experiments. Base-line Erk MBP phosphorylating activity in control cultures (cpm of 32 P incorporation, mean \pm standard error): no inhibitor (CONTROL), 1822 ± 536 ; K-252a pretreated, 5466 ± 697 ; H-89 pretreated, 7288 ± 2602 .

9). Treatment of PC12 cells with NGF (100 ng/ml) or PACAP38 triggered robust, MEK-dependent phosphorylation of Erk. Both pharmacological antagonism and down-regulation of PKC attenuated PACAP38-elicited Erk activation as indicated by decreases in the level of antibody-detected, dual threonine/tyrosine phosphorylated Erk (Fig. 9). These findings, when coupled with the noted effects of PKC inhibition on PACAP38-elicited neuritogenesis, implicate a signaling cascade that is PKC and Erk dependent.

Discussion

The neurotrophin family of polypeptides consists of nerve growth factor, brain-derived neurotrophic factor, and neurotrophins 3, 4, and 6. Through ligand-specific interaction with neurotrophin-selective Trk A, B, and C receptors, as well as the nonselective p75 neurotrophin receptor, neurotrophins assume significant physiological roles in the nervous system. They are essential for neuronal development, survival, main-

TABLE 2
Inhibition of growth factor-stimulated PKA activity by H-89 in PC12 cells

Cultures of PC12 cells were grown to high density (>70% confluency) in 10-cm-diameter dishes. Before the addition of test agents, cells were switched to a reduced-serum media (1% fetal bovine serum, 1% horse serum) and maintained overnight in this media. Cultures were incubated for 15 min in the absence or presence of 20 μM H-89 followed by an additional 5-min incubation with no addition (control), NGF (50 ng/ml), EGF (10 ng/ml), PACAP38 (5 nM), or PACAP38 + EGF. PKA activity was quantified using a protein kinase assay system (Life Technologies) as described in Materials and Methods.

Growth factor treatment	PKA activity		
	Control	H-89-pretreated cultures	
	PKA activated	PKA activated	Inhibition versus control
			%
None	20.0 ± 2.4	2.8 ± 1.0	86.2 ± 0.5
NGF	31.0 ± 2.4	2.2 ± 0.9	91.5 ± 2.5
EGF	29.0 ± 3.6	3.0 ± 0.7	89.6 ± 1.5
PACAP38	31.0 ± 2.5	3.8 ± 1.1	85.2 ± 1.4
PACAP38 + EGF	51.1 ± 7.4	2.6 ± 0.2	94.9 ± 0.5

Results are expressed as the mean ± standard error for data obtained from two independent experiments, with each sample was performed in triplicate.

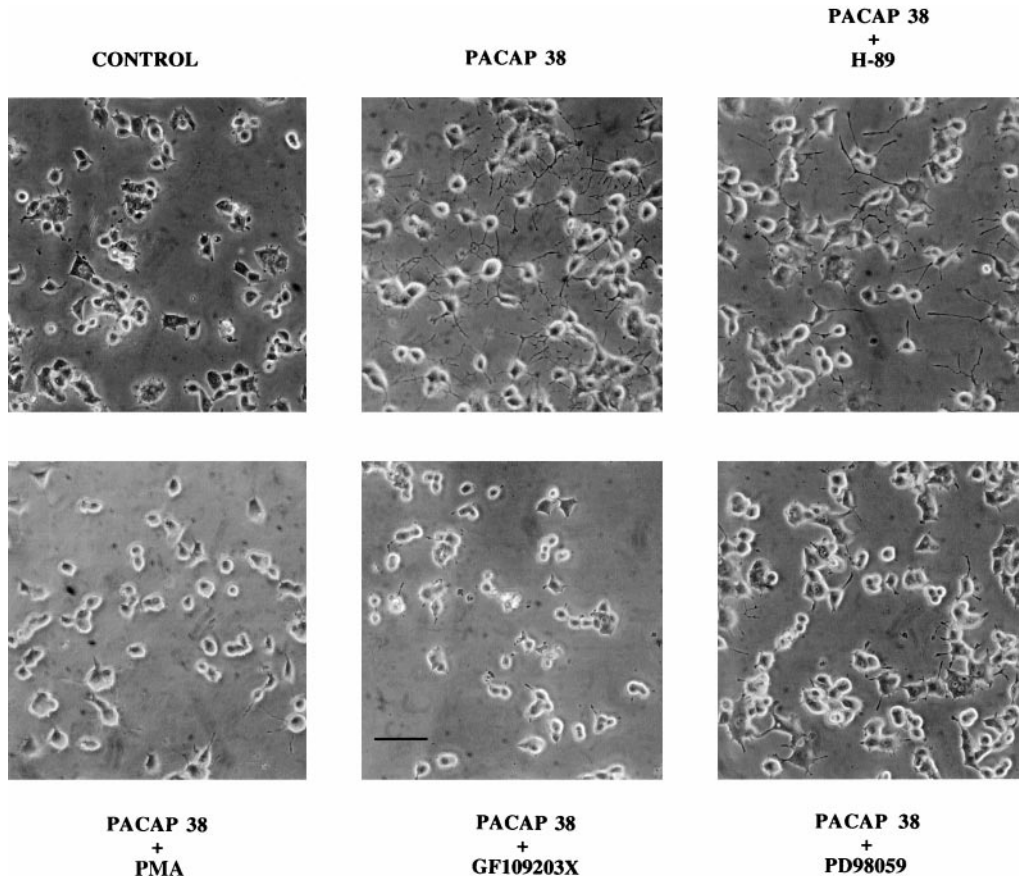


Fig. 7. Effect of PKA, PKC, and MEK pharmacological inhibitors on PACAP38-elicited neuritogenesis. Wild-type PC12 cell cultures were incubated for 1 hr in the absence (CONTROL) or presence of pharmacological inhibitors for PKA (H-89, 20 μM), PKC (GF10923X, 1 μM), or MEK (PD98059, 20 μM) kinases. Alternatively, phorbol-sensitive PKC isoforms were down-regulated by treatment overnight with PMA (1 μM). After pretreatment with the selected pharmacological agents, cultures were incubated in the absence (CONTROL) or presence of PACAP38 (5 nM) for 48 hr. Cultures were evaluated for evidence of neurite outgrowth by light microscopy using an inverted Nikon-Diaphot microscope and photographed at 320× magnification. Scale bar, 25 μm.

tenance of phenotype, and structural/functional repair after injury (Chao, 1992). Recent characterization of the PACAPs and the cloning of their cognate receptors (Hashimoto *et al.*, 1993) have lead to the suggestion that PACAP may serve as a neurotrophic factor for sympathetic neurons (Chang and Korolev, 1997).

Studies from numerous laboratories using PC12 cells as a model for nerve growth factor-induced neuronal morphological differentiation have served to elucidate signal transduction pathways used by this neurotrophin that are initiated by binding of NGF to the tyrosine kinase receptor p140^{trkA} (Kaplan and Miller, 1997). Mutagenesis studies involving TrkA indicate that the Ras/Raf/MEK/Erk cascade, acting in

conjunction with PLC- γ and tyrosine phosphorylation of the nuclear protein SNT, is involved in the initiation, elongation, and maintenance of neuritic processes (Kaplan and Miller, 1997). Nerve growth factor-evoked neuronal differentiation in PC12 cells is associated with prolonged Ras activity and, consequently, extended activation of Erk (Qui and Green, 1992), which has been demonstrated as necessary for the outgrowth of neurites (Fukuda *et al.*, 1995). PACAP also promotes neurite outgrowth in PC12 cells and has been reported to activate Erk; however, the molecular mechanisms involved in transducing signals from PACAP receptors to downstream targets such as Erk are less well defined than those for NGF. In the current study, a predominant role for

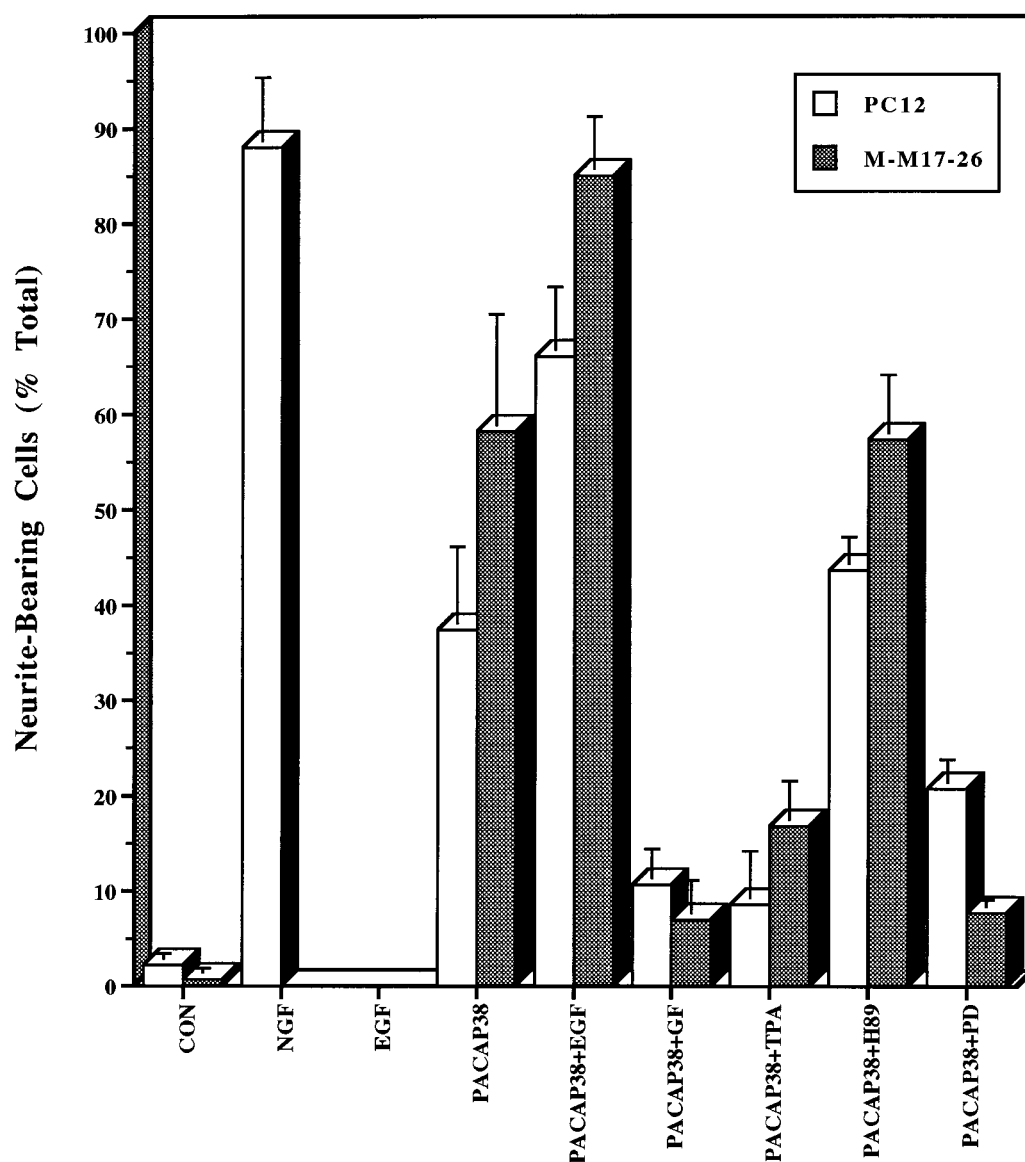


Fig. 8. Quantification of growth factor-stimulated neurite outgrowth and the effect of PKA, PKC, and MEK pharmacological inhibitors on the number of neurite-bearing cells. Wild-type and dominant negative Ras (M-M17-26) PC12 cell cultures were incubated for 1 hr in the absence (*CONTROL*) or presence of pharmacological inhibitors for PKA (H-89, 20 μ M), PKC (GF10923X, 1 μ M), or MEK (PD98059, 20 μ M) kinases. Alternatively, phorbol-sensitive PKC isoforms were down-regulated by treatment overnight with PMA (1 μ M). After pretreatment with the selected pharmacological agents, cultures were incubated in the absence (*CONTROL*) or presence of NGF (50 ng/ml), EGF (10 ng/ml), PACAP38 (5 nM), or PACAP38 + EGF for 48 hr. To quantify neurite outgrowth, cultures were examined by light microscopy using an inverted Nikon-Diaphot microscope and photographed at 320 \times magnification. Fields of cells (50–100 cells/field) in a series of photographs were scored for neurites by counting the number of cells bearing processes that were equal to or more than twice the diameter of the cell body. The data are expressed as the mean \pm standard error of the percentage of total cells possessing neurites.

PKC is proposed in mediating PACAP-elicited Erk activation. Stimulation of Erk activity by PACAP38 in PC12 cells occurs in a Ras-independent manner and promotes neurite outgrowth.

Pharmacological studies (Deutsch and Sun, 1992; Hernandez *et al.*, 1995) have indicated that PACAP38 stimulates both adenylate cyclase and phospholipase C enzymatic activ-

ity in PC12 cells (Fig. 10). Elevation of intracellular cAMP activates PKA, which, in turn, activates Erk through B-Raf (Fig. 10) involving a Ras-independent pathway (Vossler *et al.*, 1997). Indeed, results from experiments reported here using the PKA antagonist H89 reveal partial inhibition of PACAP38-stimulated Erk activity in wild-type and dominant negative PC12 cells; however, induction of neurite outgrowth is not prevented. The nature of this apparent inconsistency between inhibition of Erk activity and undiminished neurite outgrowth responsiveness can be most readily explained by fact that the majority of the inhibitory effect elicited by H-89 is due to an inhibitor-mediated increase in basal Erk activity

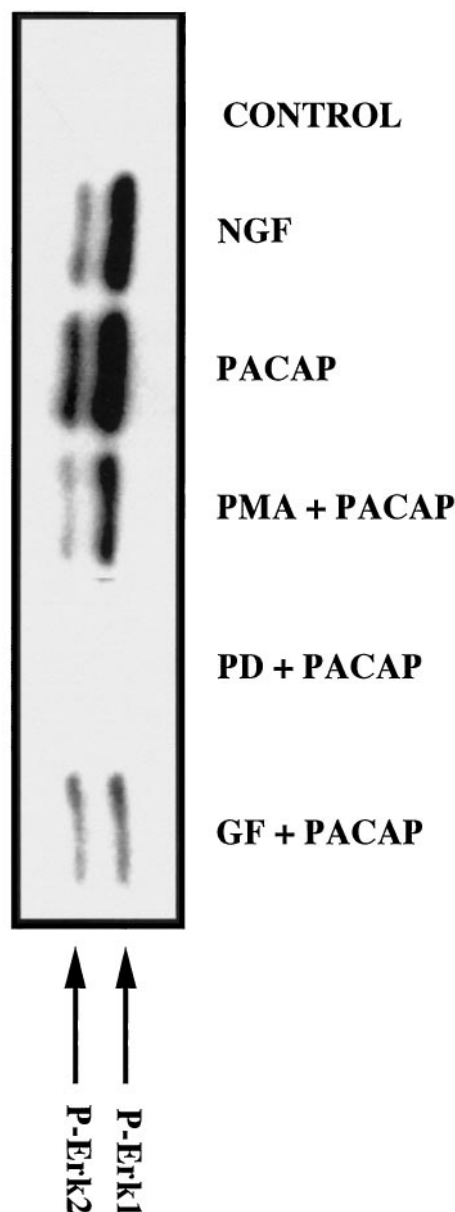


Fig. 9. Effect of PKC/MEK pharmacological inhibitors and phorbol-induced down-regulation of PKC on PACAP38-stimulated activation of Erk. Wild-type PC12 cell cultures were incubated for 1 hr in the absence (*CONTROL*, *NGF*, *PACAP*) or presence of pharmacological inhibitors for PKC (GF10923X, 1 μ M) or MEK (PD98059, 20 μ M) kinases. Alternatively, phorbol-sensitive PKC isoforms were down-regulated by treatment overnight with PMA (1 μ M). After pretreatment with the selected pharmacological agents, cultures were incubated in the absence (*CONTROL*) or presence of NGF (100 ng/ml) or PACAP38 (5 nM) for 5 min. Proteins in lysates prepared from the cultures were resolved by SDS-PAGE, and after transfer to PVDF membranes, blots were probed with an anti-phospho Erk antibody that recognizes the activated, threonine/tyrosine-phosphorylated form of Erk. Positions of phosphorylated Erk1 and Erk2 are indicated. Similar results were obtained in two independent experiments.

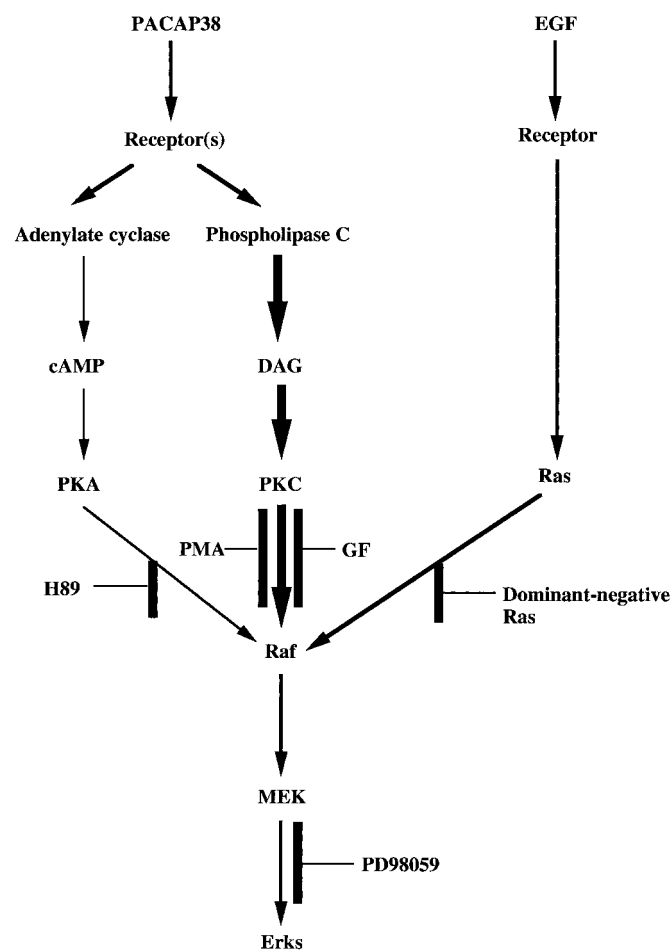


Fig. 10. Schematic representation of convergent signal transduction pathways activated by PACAP38 and EGF. Binding of PACAP38 to G protein-coupled PACAP receptors results in activation of adenylate cyclase and phospholipase C. Subsequent generation of the second messengers cAMP and diacylglycerol (DAG) evokes stimulation of PKA and PKC, respectively. These kinases, in turn, phosphorylate Raf, resulting in enhanced MEK phosphorylation and activation, which promotes dual threonine/tyrosine phosphorylation of Erk, an event critical for stimulation of Erk activity. Activation of EGF tyrosine kinase receptors results in the activation of a parallel, Ras-dependent signal transduction cascade that converges on Raf and promotes activation of Erk through stimulation of PKC. Pharmacological inhibition of PKA with H89 or expression of a dominant negative form of Ras did not prevent induction of neurite outgrowth in PC12 cells treated with PACAP38. Down-regulation of phorbol-sensitive PKC, inhibition of PKC activity with GF1093X (*GF*), or antagonism of Erk activation by the MEK kinase inhibitor PD98059 all substantially blocked PACAP38-induced neuritogenesis. The thickness of the arrows converging at Raf through PACAP receptor-mediated activation of PKA and PKC reflects the relative contribution of each cascade to stimulation of Erk as deduced from the pharmacological interventions.

and not a suppression of kinase activity level stimulated by PACAP38. The fact that chronic treatment with H-89 alone for up to 2 days fails to elicit a neurite outgrowth response suggests that this elevation in basal Erk activity observed on an acute basis as a consequence of H-89 treatment either is transient and short lived or the level of Erk activation is insufficient to support the induction of neurite formation. Similar findings have been obtained by others wherein H89 inhibits PACAP-stimulated neuropeptide Y gene expression in PC12 cells but not neurite outgrowth (Barrie *et al.*, 1997). Collectively, these data indicate that any direct contribution of PKA to PACAP38-induced neurite formation in PC12 cells is minimal (Lazarovici *et al.*, 1997b). In contrast, the cessation of cellular proliferation inferred from experiments demonstrating decreased thymidine incorporation on treatment with PACAP38 (data not shown) is consistent with the suggestion that PKA facilitates antiproliferative processes in PC12 cells (Mark and Storm, 1997). Moreover, the antiproliferative effect of PACAP38 was effectively abrogated by H-89 (data not shown), an observation that underscores further the featured role PKA subserves in regulation of PC12 cell proliferation. A hallmark feature of NGF-induced neuronal differentiation in PC12 cells is suppression of cellular replication; hence, inhibition of proliferation by PACAP38 may indicate activation of similar signal transduction cascades that involve PKA and are used by the neurotrophin NGF.

PACAP38 stimulation of phospholipase C in PC12 cells (Deutsch and Sun, 1992; Colbert *et al.*, 1994) produces an increase in intracellular calcium levels (Barrie *et al.*, 1997) and promotes diacylglycerol formation (Fig. 10). Together, these second messengers stimulate the activity of PKC isozymes, which, consequently, enhances the activity of Raf isoforms that, independent of Ras (Ueda *et al.*, 1996), lead to activation of Erk (Fig. 10). In the current study, depletion of PKC α , PKC δ , and PKC ϵ by prolonged phorbol treatment (Zheng *et al.*, 1996), inhibition of PKC α , PKC β , and PKC γ using the inhibitor GF109203X (Toullec *et al.*, 1991), or both, profoundly inhibited PACAP38-induced Erk activation (Fig. 9) and induction of neurite outgrowth (Fig. 7). The identity of PKC isoforms stimulated by PACAP38 in PC12 cells is under investigation in our laboratories. The role of calcium in promoting PACAP38-stimulated Erk activity and the commensurate elaboration of neurites was not addressed in this study due to the fact that Ras is required for calcium-mediated increases in Erk activity (Rosen *et al.*, 1994). In our hands, PACAP38 was capable of inducing neuritogenesis independent of the activities of either Ras or Src.

Consistent with features that characterize these signal transduction pathways (Fig. 10), we have shown that the MEK inhibitor PD98059, reported to block NGF-induced neurite outgrowth in PC12 cells (Pang *et al.*, 1995), significantly inhibited both PACAP38-stimulated Erk activity and neurite outgrowth. This finding supports the concept that stimulation of Erk activity in PC12 cells is sufficient for induction of neurite outgrowth, which, in turn, is extended by our current results suggesting PACAP38 effects are signaled through a PKC-Erk pathway.

In contrast to the long, robust, stable neurites elicited by NGF on p140^{trkA}-mediated activation of the Ras-Erk pathway, PACAP38-stimulated neurites resulting from activation of a PKC-Erk cascade are abbreviated in length, less rami-

fied, finer in diameter, and seem to be stable for only 3–4 days. However, on the concomitant administration of both PACAP38 and EGF, the latter at concentrations that do not elicit neurite outgrowth in PC12 cells, there is a pronounced amplification of both magnitude and duration of Erk activation accompanied by induction of neurites that more closely resemble in appearance those elicited by NGF with respect to length, complexity, and stability. These observations are reminiscent of the synergistic induction of neurite outgrowth reported for the combined treatment of PC12 cells with EGF and interleukin-6 (Wu and Bradshaw, 1996) or the addition of EGF in combination with depolarization or cAMP (Hilborn *et al.*, 1997). This amplification is correlated with a more robust and persistent stimulation of Erk activity, which is consistent with current interpretations regarding molecular mechanisms that mediate the actions of neurotrophins such as NGF. Consequently, the combination of PACAP38 with EGF in PC12 cells may promote synergistic interactions between PKC/Erk and Ras/Erk pathways that support morphological neuronal differentiation, whereas the role of PKA in this scheme is proposed to be one that involves inhibition of DNA synthesis and cellular replication. In circumstances of inhibited cell division, signals generated by the EGF receptor in PC12 cells are switched from growth promoting to supporting neuronal phenotypic differentiation (Mark and Storm, 1997).

Additional interactions occurring between neurotrophins and electrical activity under physiological conditions that serve to control neuronal development and plasticity are only now being systematically addressed at the pharmacological level (Hilborn *et al.*, 1997; Lu and Figurov, 1997). The principal outcomes of these events include neurite outgrowth, synapse formation, and the acquisition of electrical properties. It is tempting to suggest that depolarizing events, in combination with signals generated by mitogens (EGF) and neuropeptides (vasoactive intestinal peptide, PACAP) could promote morphological changes without altering physiological differentiation. The elaboration of neurites, synapse formation, and resulting development of neuron/neuron contact could evoke release of neurotrophins such as NGF, which, in turn, will be responsible for driving final differentiation and neuronal maturation.

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References

- Barrie AP, Clohessy AM, Buensuceso CS, Rogers MV, and Allen JM (1997) Pituitary adenyl cyclase-activating peptide stimulates extracellular signal-regulated kinase 1 or 2 (ERK 1/2) activity in a Ras-independent, mitogen-activated protein kinase/Erk kinase 1 or 2-dependent manner in PC12 cells. *J Biol Chem* **272**: 19666–19671.
- Berg MM, Sternberg DW, Parada LF, and Chao MV (1992) K-252a inhibits nerve growth factor-induced trk protooncogene tyrosine phosphorylation and kinase activity. *J Biol Chem* **267**:13–16.
- Cavallaro S, D'Agata V, Guardabasso V, Travalì S, Stivala F, and Canonico PL (1995) Differentiation induces pituitary adenylate cyclase-activating polypeptide receptor expression in PC12 cells. *Mol Pharmacol* **48**:56–62.
- Chang JY and Korolev VV (1997) Cyclic AMP and sympathetic neuronal programmed cell death. *Neurochem Int* **31**:161–167.
- Chao MV (1992) Neurotrophin receptors: a window into neuronal differentiation. *Neuron* **9**:583–593.
- Chijiwa T, Mishima A, Hagiwara M, Sano M, Hayashi K, Inoue T, Naito K, Toshioka T, and Hidaka H (1990) Inhibition of forskolin-induced neurite outgrowth and

- protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), in PC12D pheochromocytoma cells. *J Biol Chem* **265**:5267–5272.
- Colbert RA, Balbi D, Johnson A, Bailey JA, and Allen JM (1994) Vasoactive intestinal peptide stimulates neuropeptide Y gene expression and causes neurite extension in PC12 cells through independent mechanisms. *J Neurosci* **14**:7141–7147.
- Deutsch PJ, Schadlow VC, and Barzilai N (1993) 38 Amino acid form of pituitary adenylate cyclase-activating peptide induces process outgrowth in human neuroblastoma cells. *J Neurosci Res* **35**:312–320.
- Deutsch PJ and Sun Y (1992) The 38-amino acid form of pituitary adenylate cyclase-activating polypeptide stimulates dual signaling cascades in PC12 cells and promotes neurite outgrowth. *J Biol Chem* **267**:5108–5113.
- Fujita K, Lazarovici P, and Guroff G (1989) Regulation of the differentiation of PC12 pheochromocytoma cells. *Environ Health Perspect* **80**:127–142.
- Fukuda M, Gotoh Y, Tachibana T, Dell K, Hattori S, Yoneda Y, and Hishida E (1995) Induction of neurite outgrowth by MAP kinase in PC12 cells. *Oncogene* **11**:239–244.
- Green SH, Rydel RE, Connolly JL, and Greene LA (1986) PC12 cell mutants that possess low but not high-affinity nerve growth factor receptors neither respond to nor internalize nerve growth factor. *J Cell Biol* **102**:830–843.
- Hashimoto H, Ishihara T, Shigemoto R, Mori K, and Nagata S (1993) Molecular cloning and tissue distribution of a receptor for pituitary adenylate cyclase-activating polypeptide. *Neuron* **11**:333–342.
- Hempstead BL, Rabin SJ, Kaplan L, Reid S, Parada LF, and Kaplan DR (1992) Overexpression of the trk tyrosine kinase rapidly accelerates nerve growth factor-induced differentiation. *Neuron* **9**:883–896.
- Hernandez A, Kimball B, Romanchuk G, and Mulholland MW (1995) Pituitary adenylate cyclase-activating polypeptide stimulates neurite growth in PC12 cells. *Peptides* **16**:927–932.
- Hilborn M (1997) D, Rane SG, and Pollock JD. EGF in combination with depolarization or cAMP produce morphological but not physiological differentiation in PC12 cells. *J Neurosci Res* **47**:16–26.
- Huff K, End D, and Guroff G (1981) Nerve growth factor-induced alterations in the response of PC12 pheochromocytoma cells to epidermal growth factor. *J Cell Biol* **88**:189–198.
- Kaplan DR and Miller FD (1997) Signal transduction by the neurotrophin receptors. *Curr Opin Cell Biol* **9**:213–221.
- Keegan K and Halegoua S (1993) Signal transduction pathways in neuronal differentiation. *Curr Opin Neurobiol* **3**:14–19.
- Koizumi S, Contreras ML, Matsuda Y, Hama T, Lazarovici P, and Guroff G (1988) K-252a: a specific inhibitor of the action of nerve growth factor on PC12 cells. *J Neurosci* **8**:715–721.
- Lazarovici P, Dickens G, Kuzuya H, and Guroff G (1987) Long-term, heterologous down-regulation of EGF receptors in PC12 cells by NGF. *J Cell Biol* **104**:1611–1621.
- Lazarovici P, Matsuda Y, Kaplan D, and Guroff G (1997a) The protein kinase inhibitors K-252a and staurosporine as modifiers of neurotrophin receptor signal transduction, in *Cellular and Molecular Mechanisms of Toxin Action: Toxins and Signal Transduction* (Gutman Y and Lazarovici P, eds.) pp 69–94, Harwood Academy Publishers, Amsterdam.
- Lazarovici P, Oshima M, Shavit D, Shibutani M, Jiang H, Monshipouri M, Fink D, Movesesyan V, and Guroff G (1997b) Down regulation of epidermal growth factor

- receptors by nerve growth factor in PC12 cells is p140^{trkA}, Ras-, and Src-dependent. *J Biol Chem* **272**:11026–11034.
- Lu B and Figurov A (1997) Role of neurotrophins in synapse development and plasticity. *Rev Neurosci* **8**:1–12.
- Mark MD and Storm DR (1997) Coupling of epidermal growth factor (EGF) with anti-proliferative activity of cAMP induces neuronal differentiation. *J Biol Chem* **272**:17238–17244.
- Miyata A, Jiang L, and Dahl RR (1990) 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.
- Pang L, Sawada T, Decker SJ, and Saltiel AR (1995) Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by nerve growth factor. *J Biol Chem* **270**:13585–13588.
- Qui MS and Green SH (1992) PC12 cell neuronal differentiation is associated with prolonged p21^{ras} activity and consequent prolonged Erk activity. *Neuron* **9**:705–717.
- Rabin SJ, Cleghon V, and Kaplan DR (1993) SNT, a differentiation-specific target of neurotrophic factor-induced tyrosine kinase activity in neurons and PC12 cells. *Mol Cell Biol* **13**:2203–2213.
- Richter-Landsberg C, and Jastorff B (1986) The role of cAMP in nerve growth factor-promoted neurite outgrowth in PC12 cells. *J Cell Biol* **102**:821–829.
- Rosen LB, Ginty DD, Weber JJ, and Greenberg ME (1994) Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras. *Neuron* **12**:1207–1221.
- Spengler D, Waerber C, Pantaloni C, Holsboer F, Bockaert J, Seeburg PH, and Journot L (1993) Different signal transduction by five splice variants of the PACAP receptor. *Nature (Lond)* **365**:170–175.
- Toullec D, Pianetti P, Coote H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F, Duhamel L, Charon D, and Kirilovsky J (1991) The bisindolymaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J Biol Chem* **266**:15771–15781.
- Ueda Y, Hirai SI, Osada SI, Suzuki A, Mizuno K, and Ohno S (1996) Protein kinase C δ activates the MEK-Erk pathway in a manner independent of Ras and dependent on Raf. *J Biol Chem* **271**:23512–23519.
- Vossler MR, Yao H, York RD, Pan MG, Rim CS, and Stork PJS (1997) cAMP activates MAP kinase and Elk-1 through a B-Raf and Rap1-dependent pathway. *Cell* **89**:73–82.
- Wolf N and Krieglstein K (1995) Phenotypic development of neonatal rat chromaffin cells in response to adrenal growth factors and glucocorticoids: focus on pituitary adenylate cyclase-activating polypeptide. *Neurosci Lett* **200**:207–210.
- Wu YY and Bradshaw RA (1996) Synergistic induction of neurite outgrowth by nerve growth factor or epidermal growth factor and interleukin 6 in PC12 cells. *J Biol Chem* **271**:13033–13039.
- Zheng WH, Fink DW, and Guroff G (1996) Role of protein kinase C α in nerve growth factor-induced arachidonic acid release from PC12 cells. *J Neurochem* **66**:1868–1875.

Send reprint requests to: Donald W. Fink, Jr., Ph.D., FDA/CBER-Division of Cytokine Biology, 1401 Rockville Pike, Suite 200 N/HFM-505, Rockville, MD 20852-1448. E-mail: finkd@al.cber.fda.gov