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Staurosporine Induces Neurite Outgrowth in Neuronal Hybrids (PC12EN) Lacking NGF Receptors

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Abstract A novel neuronal model (PC12EN cells), obtained by somatic hybridization of rat adrenal medullary pheochromocytoma (PC12) and bovine adrenal medullary endothelial (BAME) cells, was developed. PC12EN cells maintained numerous neuronal characteristics: they expressed neuronal glycolipid conjugates, synthesized and secreted catecholamines, and responded to differentiative agents with neurite outgrowth. PC12EN lacked receptors for EGF and both the p75 and trk NGF receptors, while FGF receptor expression was maintained. Staurosporine (5–50 nM), but not other members of the K252a family of protein kinase inhibitors, rapidly induced neurite outgrowth in PC12EN, as also found in the parental PC12 cells, but not in BAME cells. Similarly, both acidic and basic FGF (1–100 ng/ml) were neurotropic in PC12EN. In contrast to the mechanism by which FGF promoted neurite outgrowth in PC12EN, the neurotropic effect of staurosporine did not involve activation of established signalling pathways, such as tyrosine phosphorylation of erk (ras pathway) or SNT (a specific target of neuronal differentiation). In addition, staurosporine induced the tyrosine phosphorylation of the focal adhesion kinase $p125^{FAK}$. However, since the latter effect was also observed with other protein kinase inhibitors of the K252a family, which induced PC12EN cells flattening but no neurite extension, we propose that FAK tyrosine phosphorylation may be related to ubiquitous changes in cell shape. We anticipate that PC12EN neuronal hybrids will become useful models in neuroscience research for evaluating unique cellular signalling mechanisms of novel neurotropic compounds.

Key words: staurosporine, neurotrophins, nerve growth factor (NGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), growth factor receptors, signal transduction, PC12 cells, endothelial cells, hybrids

Abbreviations: PC12, pheochromocytoma cells; NGF, nerve growth factor; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; NGFR, nerve growth factor receptor; EGFR, epidermal growth factor receptor; FGFR, fibroblast growth factor receptor; K-252a, (8R*, 9S*, 11S*-(−)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8, 11-epoxy-1H, 8H, 11H-2, 7b, lla-triazadibenzo (a,g) cycloocta(cde)trindene-1-one; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; DMSO, dimethylsulfoxide; EGTA, ethylene glycol bis(β-aminoethylether); N,N,N′,N′-tetraacetic acid; EDTA, ethylenediaamine tetraacetic acid; BSA, bovine serum albumin; PMSF, phenylmethylsulfonylfluoride; SDS, sodium dodecyl sulfate; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; DMEM, Dulbecco's modified Eagle's medium; LM, light microscopy; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; LB, lysis buffer; ILB, immunoprecipitation lysis buffer.

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Shortly after finishing this manuscript, David Rasouly, an extraordinarily gifted scientist, 23 years of age and in the final stages of his MD/PhD studies, was killed in a tragic car accident. During his short career he enriched science and all of us, who had the privilege to know him and to work with him. This paper is dedicated to David Rasouly's memory.

Somatic cell hybridization has been used as a cellular approach to obtain basic information on the mechanism underlying cell differentiation [Gottesman, 1985]. In general, hybrid cells exhibit a characteristic mixture of features derived from both parental cells [Davidson et al., 1963; Klebe et al., 1970; Schneider and Weiss, 1971; Peterson and Weiss, 1972; Amano et al., 1974; Köhler and Milstein, 1975]. In the past, numerous neuronal hybrid clonal cell lines have been used to study basic biological and pharmacological features of neurons. For example, neuroblastoma × L cell hybrids (NG108) are useful models for studying ion channels and neuronal receptors [Minna et al., 1971; Ogura et al., 1990; Hassan et al., 1989]. The dorsal root ganglion × neuroblastoma cell line F11 is used to analyze receptor-associated signal transduction mechanisms [Francel et al., 1987; Cruciani et al., 1993]. Cross-species hybrid cell lines derived from human neuroblastoma and thymidine auxotrophs of rat nerve-like cells serve as models for studying cAMP-dependent differentiation [Kazuhiro et al., 1990].

Rat PC12 pheochromocytoma cells, the transformed counterpart of adrenal medullary chromaffin cells, constitute a well-characterized neuronal model for investigating sympathoadrenal differentiation and function [Tischler and Greene, 1978]. For example, neurotrophins such as NGF induce differentiation of PC12 cells toward the mature, postmitotic sympathetic neuronal phenotype, while corticosteroids, such as dexamethasone, differentiate the cells toward the neuroendocrine chromaffinergic phenotype [Fujita et al., 1989].

During the course of our studies into the mechanisms of NGF-induced PC12 differentiation, we recently observed that staurosporine, a nonspecific, fungal kinase inhibitor of the K252a family, acted as a potent, partial NGF agonist that induced neurite outgrowth in PC12 cells [Rasouly et al., 1992]. In contrast to the well-characterized pathways of NGF-receptor signalization [Kaplan and Stephens, 1994], the mechanisms responsible for staurosporine-induced neurite extension are ill-defined [Rasouly et al., 1994; Rasouly and Lazarovici, 1994b; Rasouly et al., 1995]. Previous studies imply that the neurotropic action of staurosporine may not require the presence of functional NGF receptors [Rasouly and Lazarovici, 1994a]. However, because of the lack of neuronal systems devoid of NGF receptors, no conclusions as to specific roles of NGF receptors in staurosporine-induced neurite outgrowth could be drawn [Greene and Kaplan, 1995].

We recently developed a new model for organ-specific neuroendocrine differentiation in the adrenal medulla by co-culturing PC12 cells with adrenal medullary endothelial cells [Mizrachi et al., 1989; Mizrachi et al., 1990; Lelkes and Unsworth, 1992]. During the course of these co-cultures, we inadvertently generated a novel stable cross-species hybrid cell line, termed PC12EN cells. We previously reported that these heterokaryotypic hybrids, while maintaining numerous traits of both parental cell lines, show significant alterations in the expression of several growth factor receptors [Lelkes et al., 1992]. In this study, we provide evidence that PC12EN cells are unique neuronal hybrids which consistently fail to express the functional trk and p75 subtypes of NGF receptors. Nevertheless, they respond to the neurotropic action of staurosporine by neurite outgrowth, providing a new model for dissecting differentiative signal transduction pathways.

MATERIALS AND METHODS

Materials

NGF was prepared according to Bucchini and Angeletti [1969], and EGF by the method of Savage and Cohen [1972]. Bovine brain-derived acidic FGF (aFGF) was the kind gift of Dr. G. Neufeld (Technion, Haifa, Israel). Human recombinant basic FGF (bFGF) was obtained from Takeda Chemical Industries, Osaka, Japan. Heparin was purchased from Hepar (Franklin, OH). Rabbit polyclonal antiphosphotyrosine and anti-FAK monoclonal antibodies were from UBI (Lake Placid, NY). ^{125}I-NGF (2,000 Ci/mmol), ^{125}I-EGF (900 Ci/mmol), HRP-conjugated anti-rabbit and antimouse antibodies, hyperfilms, the ECL Western blot detection system, and rainbow molecular-weight markers were from Amersham (Buckinghamshire, UK). Biologically active ^{125}I-FGFs were prepared as previously described [Zimmer et al., 1993]. The protein kinase inhibitors K252a, and staurosporine were kindly provided by Dr. Y. Matsuda (Kyowa Hakko Kogyo Ltd., Tokyo, Japan). All other reagents were from Sigma (St. Louis, MO). Tissue culture reagents were from Kibbutz Bet-Haemek (Israel). Protein–A Sepharose was from Pharmacia (Uppsala, Sweden). Rabbit poly-

clonal anti-trk antiserum, and $p13^{suc1}$-agarose for isolating SNT protein were kindly provided by Dr. D.R. Kaplan (ABL, NCI, Frederick, MD). Polyclonal anti erk-1 and erk-2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-NGF antiserum was kindly provided by Alamone Laboratories (Jerusalem, Israel).

Cell Cultures

Rat PC12 pheochromocytoma cells were cultured as described previously [Mizrachi et al., 1989; Rasouly et al., 1992]. Bovine adrenal medullary cells (BAME) used for these studies were isolated from primary adrenal medullary chromaffin cell preparations by differential plating and purified by flow cytometry, based on the EC-specific uptake of dil ac-LDL [Voyta et al., 1984; Manolopoulos and Lelkes, 1993]. PC12 cells were originally obtained from Dr. G. Guroff (National Institutes of Health [NIH]). Co-culture conditions were as previously described [Mizrachi et al., 1989, 1990]. In December 1988, somatic fusion (presumably virus-mediated) occurred between BAME and PC12 in one of the co-cultures, resulting in the appearance of a novel morphological phenotype. Upon cloning by limited dilution, a stable adrenal medullary parenchymal–endothelial hybrid cell line, termed PC12EN, was established [Lelkes et al., 1992]. These cells are routinely grown in PC12 cell medium (DMEM supplemented with 7% fetal calf serum (FCS), 7% horse serum, 100 μg/ml streptomycin, and 100 U/ml penicillin). To date these cells have been passaged for more than 160 generations without apparent loss of the phenotype. Cultures were maintained at 37°C in a 5% CO_2 incubator, with medium changed twice a week and split at a 1:6 ratio once a week. The experiments described here were carried out with hybrids of generation numbers 20–50.

Proliferation Assays

Cell proliferation rates and population doubling times were evaluated by a fluorimetric assay based on the DNA binding, fluorescent dye, Hoechst 33258 [Papadimitriou and Lelkes, 1993]. Briefly, at various time points, cells, grown in 12-well tissue culture plates (in the case of PC12 cells, precoated with collagen/poly-*l*-lysine), were washed twice with PBS and fixed with absolute methanol for 10 min. Following extensive washings, monolayers were sequentially incubated with 0.5 ml Hoechst 33258 solution (10 μg/ml) for 30 min, washed with HEPES buffer and extracted for 5 min with 1 ml denatured ethanol. Fluorescence was measured in a Perkin-Elmer fluorescence spectrophotometer (model 250-10S) with excitation and emission wave lengths of 360 and 460 nm, respectively.

Chromosomal Analysis

PC12EN cells in the logarithmic phase of growth were treated with colcemide (0.4 μg/ml) for 40 min. The cells were then detached using trypsin–EDTA and subjected to hypotonic treatment with 0.005 M KCl, subjected to three changes of fixative (methanol–acetic acid 3:1), and applied to slides prepared by air drying. The chromosomes were G-banded using trypsin in PBS (pH 7.0) and stained with Giemsa at pH 7.0 [Jonasson, 1986]. The metaphases were analyzed and the chromosomes were identified by their morphological (length and centromere position) and banding characteristics [Benn and Perle, 1986]. The origin of the chromosomes, whether from cow or from rat, was identified by comparison with the normal karyotype for each animal [Hsu and Benirschke, 1967]. Photographic karyotypes from 100 cells from each of the cell types were evaluated for chromosome counts. Detailed analysis of the banding patterns was performed in 10 representative PC12 EN cells.

Light Microscopic Characterization

The morphology of the cells, grown in 4-well tissue culture chamber slides (Lab Tek), was examined by phase-contrast microscopy in an inverted Nikon Diaphot microscope. In addition, the cells were also characterized by immunocytochemistry, using established techniques [Papadimitriou et al., 1993]. The PC12 contribution was ascertained by staining the cells with a monoclonal IgG antibody, designated B2TT developed against PC12 plasma membranes. This antibody was generated, screened and amplified as ascites and purified by DEAE-cellulose and protein A chromatography, according to standard procedures [Yavin et al., 1981]. B2TT recognizes certain glycoproteins and polysialogangliosides of PC12 plasma membranes which are not present in cultured adrenal medullary endothelial cells (unpublished data). The endothelial cell contribution of the hybrids was assessed by the uptake of dil-labelled acetylated LDL [Voyta et al., 1984]. Preliminary studies indicated that the receptor for acetylated LDL was present on the parental endothelial cells, but not on the PC12 cells.

Catecholamine Assay

Cellular catecholamines were extracted by incubating 10^6 cells in 0.1 N $HClO_3$ (perchloric acid). The solutions were clarified by centrifugation (10,000*g* for 10 min). Aliquots of the supernatants were analyzed by reversed-phase HPLC followed by electrochemical detection, as previously described [Lelkes et al., 1994].

Assessment of Neurotropic Effects

Neurite outgrowth is one of the major criteria for neurotrophin-induced differentiation. To evaluate neurite outgrowth, the cells were plated at low densities (10^4 cells/well) onto 4-well tissue culture chamber slides (as above) coated with a mixture of collagen (0.1 mg/ml) and poly-*l*-lysine (0.01 mg/ml) in 0.1 M acetic acid. Cells cultures were treated for different time intervals and concentrations with the indicated compounds, washed once with PBS, fixed with 2% glutaraldehyde, and examined by phase-contrast microscopy, as previously described [Rasouly et al., 1992].

Growth Factor Binding Assays

For NGF and EGF binding assays [Lazarovici et al., 1987], the cells were plated on collagen/polylysine-coated 6-well plates (Costar) and cultured for 48 h. The medium was replaced with 1 ml fresh culture medium containing radiolabelled growth factors (150 pM) with or without a 100-fold excess of unlabeled growth factor. The cultures were incubated for 2 h at 4°C and, washed three times with serum-supplemented medium, followed by two washes with PBS. Cell-associated radioactivity was extracted in 0.1 N NaOH and measured in a γ-counter. FGF binding was assessed essentially as above, with the exception that (1) the cultures were used 24 h after plating the cells, and (2) the assay was performed in 200-μl binding buffer (DMEM containing 10 μg/ml heparin, 0.2% BSA, 25 mM HEPES, pH 7.4) with 0.4 ng of either ^{125}I-aFGF or ^{125}I-bFGF in the presence or absence of 50 ng of the respective unlabeled growth factors, as previously described [Zimmer et al., 1993]. Binding was terminated by two washings with binding buffer, followed by a third wash, for 5 min, with binding buffer pH 7.5 or pH 4 for low- and high-affinity FGF binding, respectively. Cell suspensions were then taken for γ-counting. All binding assays were done in sixtuplicates, and repeated at least three times using independent cell cultures.

^{125}I-NGF Cross-Linking

PC12 cells (3×10^6 cells per ml) and hybrids ($\leq 50 \times 10^6$ cells/ml), were incubated for 2 h at 4°C, with 0.5 nM ^{125}I-NGF in the presence or absence of 1 μM unlabeled NGF. Binding was terminated by three washings with DMEM containing 0.1% BSA, and sequentially followed by 30-min incubation at 4°C with 4 mM of the cross-linking reagent EDAC, and three washings with DMEM/BSA. Following centrifugation, cell pellets were lysed in immunoprecipitation lysis buffer (ILB: 20 mM Tris, 137 mM NaCl, 1% NP40, 10% glycerol, 1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 2 mM orthovanadate, pH 7.6), and spun (at 10,000*g*) for 10 min in a microfuge. Cell lysates were then incubated for 2 h at 4°C with anti-NGF antibody (1:100), followed by incubation with 50 μl protein–A Sepharose for additional 2 h. Protein A beads were spun, washed three times with immunoprecipitation lysis buffer, boiled for 5 min in SDS sample buffer, and subjected to SDS–PAGE and autoradiography for 2 weeks (Kodak X-OMAT).

Western Blotting

PC12 cells and hybrids were plated on collagen/polylysine-coated 20-cm dishes and treated with the various compounds for the indicated times. Following one wash with PBS, cells were collected and centrifuged at 1,000 rpm/min for 10 min, at 4°C. Cell pellets were suspended in lysis buffer (LB: 50 mM Tris–HCl pH 8.5, 1% NP40, 5 mM EDTA, 50 μg/ml PMSF and 10 μg/ml leupeptin, and 2 mM orthovanadate) homogenized on ice with a glass homogenizer. Following homogenization, lysates were centrifuged for 10 min in a microfuge. The supernatants were collected and stored at −70°C. For each sample, 100 μg lysate protein (as determined according to Lowry et al. [1951]) were solubilized in denaturing SDS sample buffer (containing β-mercaptoethanol), and boiled for 5 min at 100°C. Samples and rainbow-colored molecular-weight markers were separated on a 10% SDS–PAGE. Proteins were electrotransferred to nitrocellulose from Schleicher and Schuell (Keene, N.H.). The blots were rinsed once with TBS and incubated for 1 h with 3% gelatin in TBS for blocking of nonspecific binding. Following 3 washes with TBS–Tween 20 (0.1%), the blots were sequen-

tially incubated with primary antibodies (anti-phosphotyrosine, anti-EGFR or anti-Erk antibodies 1:5,000) in TBS–Tween 20 overnight at 4°C. Following three washes with TBS–Tween 20, the blots were incubated for one hour with HRP-conjugated secondary antibodies (anti-mouse or anti-rabbit antibodies (1:10,000 in TBS–Tween). Following three additional washes with TBS–Tween and twice with distilled water, the blots were incubated for 1 min with luminol (Amersham ECL detection reagents) and exposed for up to 1 min for autoradiographic detection using Amersham Hyperfilms. The immunoreactive bands were scanned by a laser densitometer.

Immunoprecipitation of trk, erk1, and FAK

Following treatment with the respective compounds, PC12 cells and hybrids were harvested and spun at 1,000 rpm for 10 min at 4°C. The pellets were lysed for 10 min on ice in 1 ml immunoprecipitation lysis buffer (ILB, see above). The lysates were spun in a microfuge for 15 min. The supernatants were collected and incubated, with continuous agitation, in the presence of anti-trk or anti-$p125^{FAK}$ antibodies (1:250) for 2 h at 4°C. For the immunoprecipitation of erk1, the antibody was used at a dilution of 1:50, and the samples were incubated overnight. Subsequently, 50 μl protein A–Sepharose was added to all samples for additional 2 h. Protein A beads were spun, washed three times with ILB, and boiled for 5 min as above. Precipitates were then subjected to SDS–PAGE and Western blotting as described above.

Affinity Purification of SNT

Following treatment, the hybrid cells were lysed for 10 min on ice, as above. The lysates were separated on $p13^{suc1}$ agarose, according to Rabin et al. [1993]. The SNT protein was desorbed from the agarose gel, electrophoresed, and detected by phosphotyrosine Western blotting, as described above.

RESULTS

Characterization of PC12EN Adrenomedullary Hybrids Cells

Morphological characterization of PC12EN cells. Figure 1 presents the morphology of PC12EN cells and that of their parental cells. As previously detailed, PC12 cells were rounded with a diameter of <16 μm (Fig. 1A), while confluent monolayers of BAME (Fig. 1B) attained the histiotypic cobblestone morphology [Mizachi et al., 1989]. As shown in Figure 1C,D, hybrid PC12EN cells, at confluence, were organized into monolayers that were morphologically more similar to BAME than to PC12 cells. As shown at higher magnification in Figure 1D, the shape of PC12EN cells appeared to have a more epithelium-like cuboidal, rather than the typical endothelium-like cobble stone morphology. Upon prolonged culture, the strict monolayer organization was abandoned, most probably because of the loss of contact inhibition and the high rate of cell proliferation (see below). Both these properties are characteristic features of transformed cell lines [van Heyningen, 1994]. Indeed, PC12EN are highly tumorigenic, causing widely disseminated neoplasms upon intraperitoneal injection of less than 10^3 cells into nude mice [Lelkes et al., 1992].

Expression of parental markers in PC12EN cells. As shown in Figure 2, PC12EN co-expressed typical markers of both parental cell types. For example, when stained with a monoclonal antibody (B2TT) developed against PC12-specific polysialoglycolipids (Yavin et al., 1981), PC12EN were strongly labelled both on the cell surface and intracellularly (Fig. 2A). A similar labelling pattern was observed for PC12 cells, while BAME were not stained with B2TT (not shown). In addition, PC12EN vigorously took up dil-labelled acetylated LDL, a typical endothelial cell marker (Figure 2 panel B), whereas PC12 cells were not stained with this marker (not shown). In addition, PC12EN and BAME, but not PC12 cells, stained with a monoclonal antibody against endothelial-cell specific angiotensin-converting enzyme [Auerbach et al., 1982] (not shown).

The expression of typical catecholaminergic features of the parental PC12 cells is further underscored by the fact that PC12EN were found to synthesize catecholamines. HPLC analysis (Fig. 3) suggests that the catecholamine contents of PC12EN was about one half of that of the parental PC12 cells and much smaller (by about three orders of magnitude) than that of nontransformed bovine adrenal chromaffin cells. PC12EN contained dopamine and norepinephrine, which was also present in the parental PC12 cells, but not in BAME. As previously noted, some strains of PC12 cells, including ours, express phenylethanolamine-N-methyltransferase (PNMT) activity and contain low

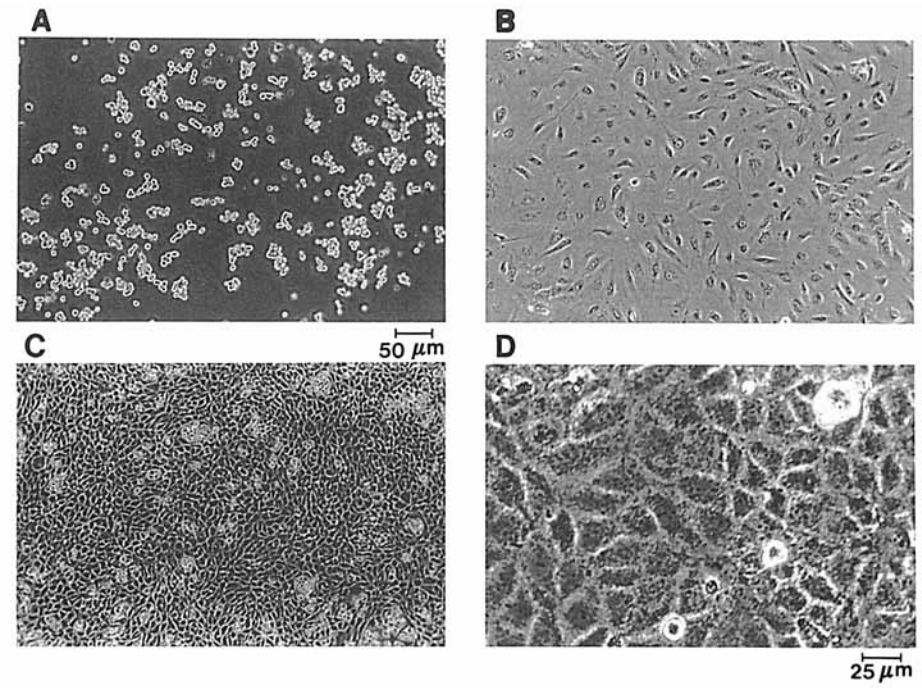

Fig. 1. Morphological characterization of confluent monolayers of PC12EN cells and their progenitors. PC12 cells were plated on collagen/poly-lysine coated 4-well tissue culture/chamber slides, BAME and PC12EN were plated on uncoated dishes. Upon confluence, the cultures were photographed in a phase contrast microscope. Note the high density of the hybrid cells and their epithelioid appearance at higher magnification. **A:** PC12 cells. **B:** BAME cells. **C,D:** PC12EN cells. Bars = 50 μm (A–C); 25 μm (D).

levels of epinephrine [Byrd et al., 1986; Lelkes, 1991; Kim et al., 1993; Galvan et al., 1995]. By contrast, no epinephrine was detected in PC12EN. Furthermore, the ability of carbamylcholine (as well as KCl and veratridine) to induce dopamine release [Lelkes et al., 1992], as well as the presence of cholinergic pirenzipine-sensitive muscarinic (M_2) receptors (Rasouly et al., unpublished observations), suggest that the hybrid cells maintained much of the exocytotic secretory mechanism characteristic of the parental PC12 cells. Ultrastructural studies by transmission electron microscopy indicated the presence of a small number of membrane bound electron-dense storage granules in PC12EN cells (data not shown). Based on these criteria, the cells may be regarded as neuronal hybrid cells.

Chromosomal analysis. The continued expression of both neuronal and endothelial markers suggested to us that PC12EN cells might represent true heterokaryotic hybrids. To test this hypothesis, we analyzed metaphase chromosomes in about 100 individual PC12, BAME, and PC12EN cells, respectively, and determined the chromosomal contribution of each of the parental cells to the hybrids population by means of banding, centromere location and chromosomal staining [Watt and Stephen, 1986; Harrison, 1986].

PC12 and BAME cells were diploid, containing on the average 42 and 66 chromosomes, respectively, typical for rat and bovine cells [Hsu and Benirschke, 1967]. PC12EN cells had a more complex chromosome pattern: a representative karyotype is shown in Figure 4A. As seen in this karyotype, a few chromosomes appear to be missing (e.g., 19, 22). Some chromosomes were represented as single copies (e.g., 1 and 2), while other chromosomes were present in 3–5 copies (e.g., 5, 6, 7). Moreover, in several instances, single copies of both the rat- and the cow-derived chromosome were expressed concomitantly (e.g., 3, 4, 18), confirming the truly heterokaryotypic nature of the hybrids. Rat chromosomes numbers 20 and upwards could not be detected in any of the hybrids examined.

Cumulative analysis of all PC12EN cells examined revealed chromosome counts in the range from 35 to 88, the modal number being 42 (Fig. 4B, panel 1). In spite of the initial cloning by limited dilution, all the cells exhibited varying numbers of cow and rat chromosomes. This

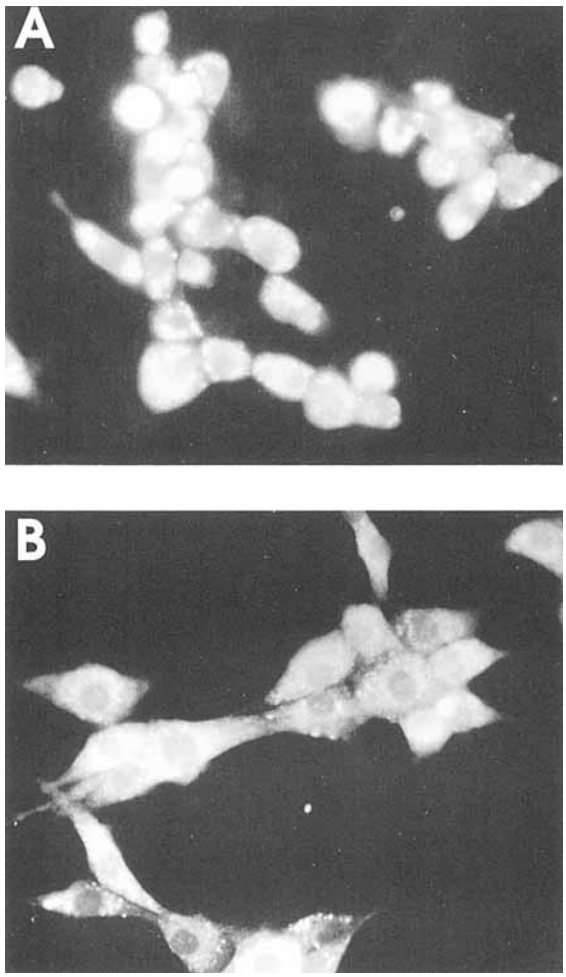

Fig. 2. PC12EN cells contain markers of both PC12 and BAME cells. **A:** Positive immunostaining of PC12EN with a monoclonal antibody (B2TT) against PC12 derived polysialogangliosides. **B:** Positive staining of PC12EN with DiI-acetylated LDL (DiI). BAME were also DiI positive, while PC12 did not take up this typical endothelial cell marker (not shown). ×450.

instability is quite typical for cross-species hybrid lines [van Heyningen, 1994]. If the hybrid cells had maintained all their (bovine and rat) chromosomes, 10 cells should have had 1,080 chromosomes: only 437 were found, i.e., 60% less than expected. At the normal ratio, the hybrid cells should have contained 39% of rat chromosomes and 61% of bovine chromosomes. Actually, we found 28% rat chromosomes and 72% bovine chromosomes, suggesting an underrepresentation of rat-derived chromosomes by ca. 25%. The combined karyotype (Fig. 4B, panel 2) shows that 3 chromosomes were lost; rat 12 and 21 and cow 28. Some chromosomes were specifically over-represented (rat 8 and 10, cow 3, 9, 12, 14, 16) and others underrepresented

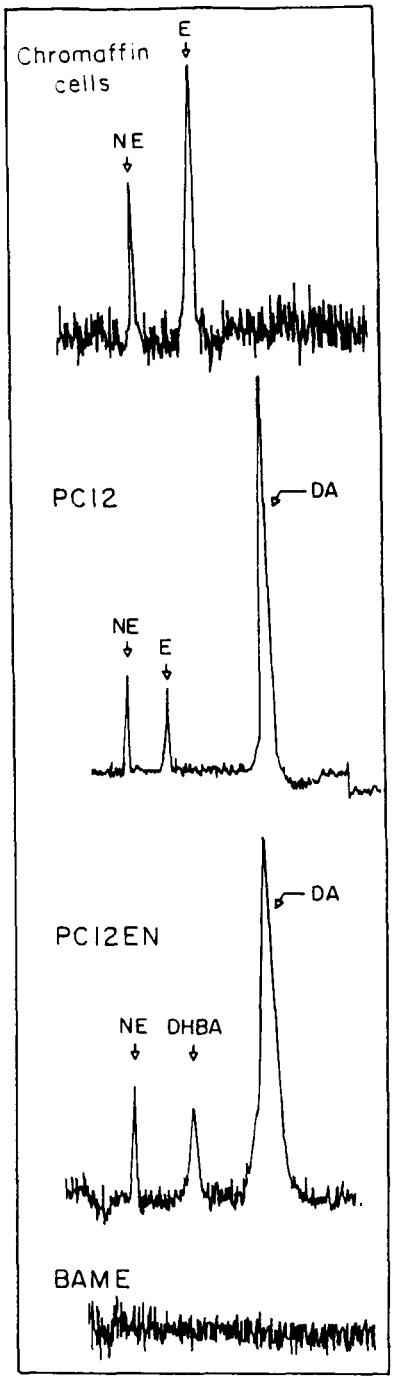

Fig. 3. Catecholamine contents of bovine adrenal medullary chromaffin cells, PC12 cells, PC12EN cells, and BAME by HPLC. Aliquots equivalent to total catecholamines from 20 chromaffin cells, 5×10^4 PC12 cells, 1×10^5 PC12EN and 5×10^6 BAME were analyzed by reversed phase HPLC with electrochemical detection (see Materials and Methods). The individual peaks were identified based on the elution times of known standards. NE, norepinephrine; E, epinephrine; DA, dopamine; DHBA, dihydroxybenzilamine (internal standard).

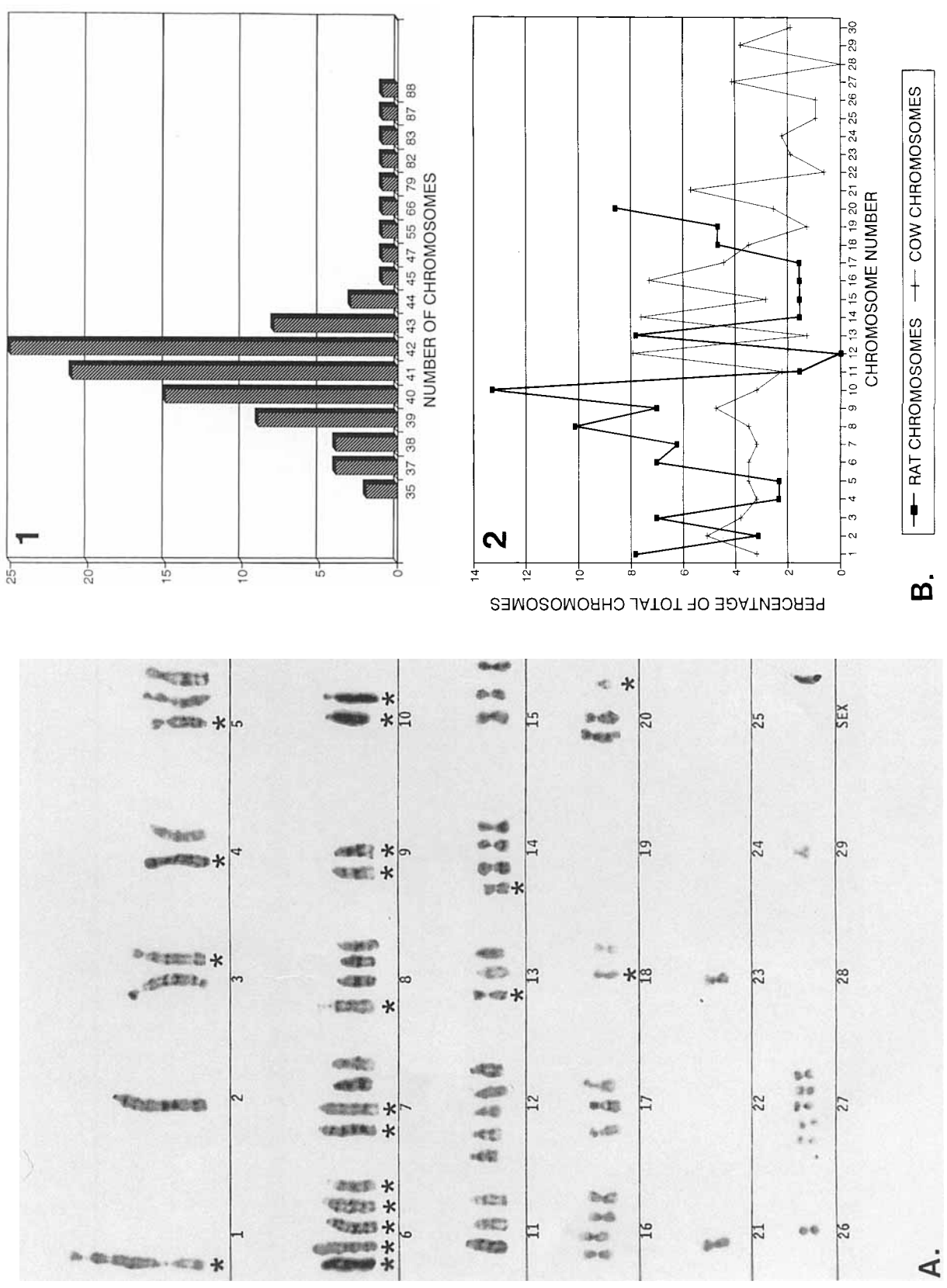
A.
B.
1
NUMBER OF CHROMOSOMES
2
PERCENTAGE OF TOTAL CHROMOSOMES
CHROMOSOME NUMBER
RAT CHROMOSOMES
COW CHROMOSOMES

Figure 4.

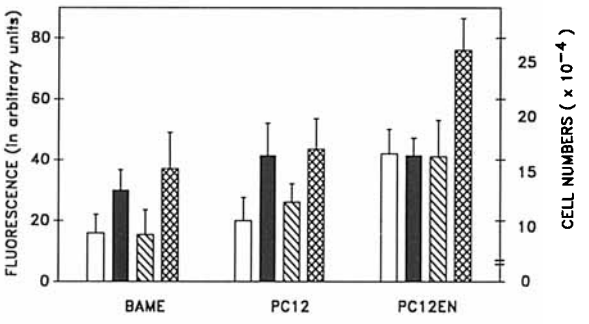

Fig. 5. Mitogenic effects of various growth factors on PC12EN cells and their progenitors. BAME, PC12, and PC12EN cells were plated on untreated or collagen/polylysine-coated 35-mm Tissue Culture-grade Petri dishes and treated for 48 hrs with, respectively, nothing (controls, open bars), 10 ng/ml EGF (filled bars), 50 ng/ml NGF (diagonally stripped bars), and 5 ng/ml bFGF (cross-hatched bars), as described in the text. Cell proliferation was assessed by both direct cell counts in a Coulter counter (left ordinate) and the DNA-directed, Hoechst 33258 fluorescence assay (right ordinate).

(rat 2, 4, 5, 11, 13, 15, 16, and 17, and cow 13, 19, 22, 25, and 26).

Responsiveness of PC12EN Cells to Various Mitogens and Differentiative Growth Factors

Mitogenic effects of growth factors on PC12EN cells and their parental cells. As shown in Figure 5, the rate of proliferation of the parental cells (PC12 and BAME) was similar, with a mean doubling time of approx. 35–42 h (Table I). By contrast, proliferation of PC12EN cells was significantly accelerated: the mean doubling time for PC12EN was about 26–28 h. The rate of PC12EN proliferation was not accelerated by EGF, in contrast to both parental cells: 10 ng/ml EGF increased the rate of proliferation of PC12 and BAME cells, by 2.3- and 1.9-fold, respectively (Fig. 5, filled bars). Fifty ng/ml NGF exerted a minor proliferative effect [Rudkin et al., 1989] on PC12 cells (approx 30% increase), but not on BAME or PC12EN cells (Fig. 5, diagonally striped bars). By contrast, 5 ng/ml bFGF significantly enhanced the proliferation of PC12EN cells (1.8-fold) and in both PC12 cells and BAME cells by 2.4- and 2.25-fold, respectively (Fig. 5, cross-hatched bars).

TABLE I. Population Doubling Times of PC12EN Cells and Their Parental Cells

Cell type	PD times (h)
BAME	35 ± 5
PC12 cells	42 ± 3
PC12EN-5	28 ± 2
PC12EN-50	26 ± 3
PC12EN-120	31 ± 3

Cells were plated on 24-well dishes and their proliferation rate was calculated by both direct cell counts in a Coulter counter and the DNA directed, Hoechst 33258 fluorescence assays, as described under Materials and Methods.

Differentiative effects of growth factors and staurosporine. Neurotropic growth factors such as FGF and NGF induce neurite outgrowth in PC12 cells, starting after 24 h of treatment [Fujita et al., 1989]. The limited ability of these conventional neurotrophins to induce neurite outgrowth in PC12EN is shown in Figure 6. NGF treatment of the hybrids for up to 7 days did not induce neurite extension (Fig. 6B), the cells remained morphologically indistinguishable from nontreated controls (Fig. 6A). Exposure of the hybrids to bFGF for 18 h resulted in cell elongation and incipient neurite outgrowth (Fig. 6C), which within 3–5 days developed into branched neurites (not shown). By contrast, exposure of PC12EN cells to 50 nM staurosporine for 18 h induced the extension of very long, thin processes, without drastic changes in the fusiform shape of the cell body (Fig 6D). Unlike the slower acting neurotropic effect of FGF, the onset of staurosporine-induced neurite extension was rapid, being clearly visible after only 4 h. EGF had no morphological effects in the hybrids or their parental PC12 cells. None of the tested growth factors had any morphological effects on BAME cells (data not shown).

Expression of Growth Factor Receptors in PC12EN Cells

EGF receptors. The presence of EGF receptors in PC12EN cells was examined by both radioreceptor assays and EGF-induced tyrosine phosphorylation. As shown in Table II, no specific EGF binding was detectable in PC12EN cells, although significant amounts of EGF bound to PC12 cells and to BAME cells [Lelkes et al., 1992]. Treatment of PC12EN cells for 5

Fig. 4. (On previous page.) Karyotyping of PC12EN cells. **A:** Representative karyotype of a PC12EN cell. Concomitant expression of both bovine and rat (*) chromosomes in a single cell confirms that these cells are heterokaryons. **B:** Cumulative analysis of PC12EN cell karyotype. 1, mean chromosome number in 100 PC12EN cells; 2, fractional representation of rat (■) and of bovine (+) chromosomes, based on the detailed analysis of banding patterns in 10 cells.

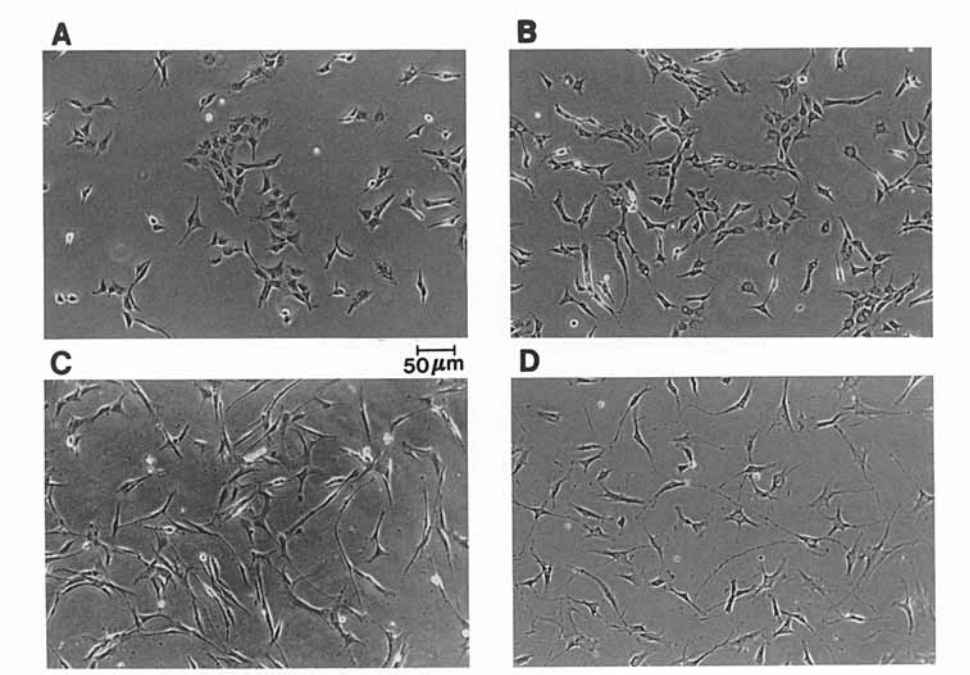

Fig. 6. Effects of neurotropic compounds on PC12EN neurite outgrowth. The cells were plated at low density (5×10^3/well) in collagen/polylysine-coated tissue culture chamber slides. After 24 h, the various neurotropic compounds were added, and the cells were cultured for additional 18 h. **A:** Control, untreated cells. **B:** NGF, 50 ng/ml. **C:** bFGF, 5 ng/ml. **D:** Staurosporine 50 nM. Bar = 50 μm.

min with EGF failed to induce the tyrosine autophosphorylation of the 170-kD EGF-receptor protein (Fig. 7). In contrast to the parental PC12 cells, the hybrids did also not express detectable basal levels of EGFR autophosphorylation. Thus, our data suggest that functional EGFR is not expressed in PC12EN cells.

NGF receptors. The presence of NGF receptors is routinely assessed by two different criteria: (1) binding and cross-linking of radiolabelled NGF, and (2) NGF-inducible trk tyrosine autophosphorylation. In contrast to PC12 cells, the hybrids expressed neither NGF binding (Table II), nor NGF cross-linking to either trk or p75 receptors (Fig. 8A) nor NGF-inducible trk tyrosine kinase activity (Fig. 8B). Previous binding studies, using ^{125}I-labeled NGF, suggested a low-level expression of NGF receptors on BAME cells, the nature of which remains to be elucidated [Lelkes et al., 1992]. Thus, our results suggest that PC12EN do not express functional NGF receptors.

FGF receptors. Table III presents the results of radioreceptor binding assays using ^{125}I-FGF binding to PC12EN cells and their parental cells. Both parental cells as well as the PC12EN hybrid cells expressed comparable levels of high-

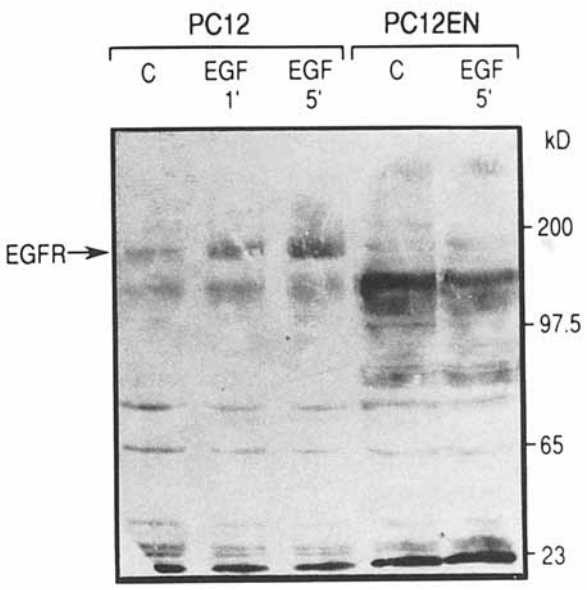

Fig. 7. Lack of EGF-induced tyrosine phosphorylations in PC12EN cells. PC12 and PC12EN cells were treated with 10 ng/ml EGF for the indicated times, and prepared for Western Blotting as described under Materials and Methods. Immunoblotting was performed using a polyclonal antiphosphotyrosine antibody. The positions of prestained molecular-weight markers are indicated.

TABLE II. Differential Binding of Radiolabeled NGF and EGF to PC12EN Hybrids and Their Parental Cells[a]

Growth factor	Cell type		
	PC12	BAME	PC12EN
^{125}I-NGF[b]	31,568 ± 3,541	2,075 ± 357	455 ± 50
^{125}I-EGF[c]	34,300 ± 3,288	44,180 ± 6,553	357 ± 64

[a]Binding of the various radiolabeled growth factors to confluent monolayers of the three cells types was determined as detailed under Materials and Methods. All data are given in cpm/mg protein and are the means ± SD from three independent determinations.
[b]Cells were incubated with 150 pM ^{125}I-NGF for 2 h in the presence or absence of unlabeled NGF. Radioligand binding was quantitated, as described under Materials and Methods. Values of ^{125}I-NGF binding represent specific NGF binding.
[c]Cells were incubated with 150 pM ^{125}I-EGF in the presence or absence of unlabeled EGF for 2 h. Radioligand binding was determined as described under Materials and Methods. ^{125}I-EGF binding values represent the specific EGF binding to the cells.

and low-affinity receptors for both acidic and basic FGF (Table III). The major difference noted from these binding data is an enhanced expression of high-affinity FGFRs in PC12EN, as compared to parental PC12 cells.

Differential Activation of Various Signal Transduction Pathways by FGF and Staurosporine in PC12EN Cells

Over the past few years, some of the common signal transduction pathways activated by different neurotrophins have been elucidated [Kaplan and Stephens, 1994]. The best characterized pathways include the ras, src, and SNT pathways [Greene and Kaplan, 1995]. Evaluation of specific downstream targets, such as erk (ras), FAK (src), and SNT (SNT), yield information on the activation of these particular pathways.

Induction of the tyrosine phosphorylation of erks and SNT. FGF was shown to induce the tyrosine phosphorylation and hence activation of the family of mitogen activated protein kinases (MAP kinases) also known as the erks (extracellular signal regulated kinases), specifically p44^{erk1}, and p42^{erk2} [Jaiswal et al., 1993]. Immunoprecipitation of cell lysates with an erk1-specific antibody (from Santa Cruz) indicated that treatment of PC12EN for 5 or 15 minutes with bFGF, but not with staurosporine (up to 60 min), induced the tyrosine phosphorylation of p44^{erk1} (Fig. 9A). The equal amounts of erk-1 protein in the different lanes in Figure 9A are evident from the Western blot evaluation (Fig. 9B), in which the immunoprecipitates were re-probed with the same anti-erk1 antibody. Similar results were obtained with a pan anti-erk antibody (from Promega) and also by an anti-erk1 antibody obtained from Dr. J. Blennis (not shown). Preliminary data indicate that bFGF, but not staurosporine, also activates erk2 (not shown).

Recently it was also shown that bFGF induces the tyrosine phosphorylation of a differentiation-related protein termed SNT in PC12 cells [Rabin et al., 1993]. As shown in Figure 9C, treatment for 5–15 min with bFGF, but not with staurosporine, induced the tyrosine phosphorylation of SNT in PC12EN cells.

Staurosporine and other protein kinase inhibitors activate p125^{FAK} in PC12EN cells. In view of the profound morphological changes induced by staurosporine in PC12EN, we hypothesized that staurosporine might activate p125^{FAK} (FAK), a ubiquitous cytosolic focal adhesion protein kinase, which is implicated in the signal transduction mechanisms associated with cell-substrate adhesion and cellular remodelling [Hanks et al., 1992; Schaller and Parsons, 1994; Maroney et al., 1995]. In support of this hypothesis we found that staurosporine treatment (50 nM) of PC12EN cells resulted in a rapid, transient tyrosine phosphorylation of FAK (Fig. 10A). FAK tyrosine phosphorylation occurred within 15 min after the addition of staurosporine, remained stable for 1 hr and then gradually decreased to basal levels at 2 h (Fig. 10A). However, as shown in Figure 10B, FAK tyrosine phosphorylation was not unique to staurosporine treatment, but was also induced by other, similar, protein kinase inhibitors with different ranges of selectivity, including K252a, K252b, (Koizumi et al., 1988)\calphostin C, and bisindolemaleimids (GF 109203X). Importantly, with the exception of staurosporine, none of these compounds exerted neurotropic effects in PC12EN; however they all induced visible changes in cell morphology, including flattening of the cell body (not shown).

DISCUSSION

The two main messages of this study are (1) the development and partial characterization of a novel neuronal hybrid (PC12EN), and (2) the ability of staurosporine and FGF to induce differ-

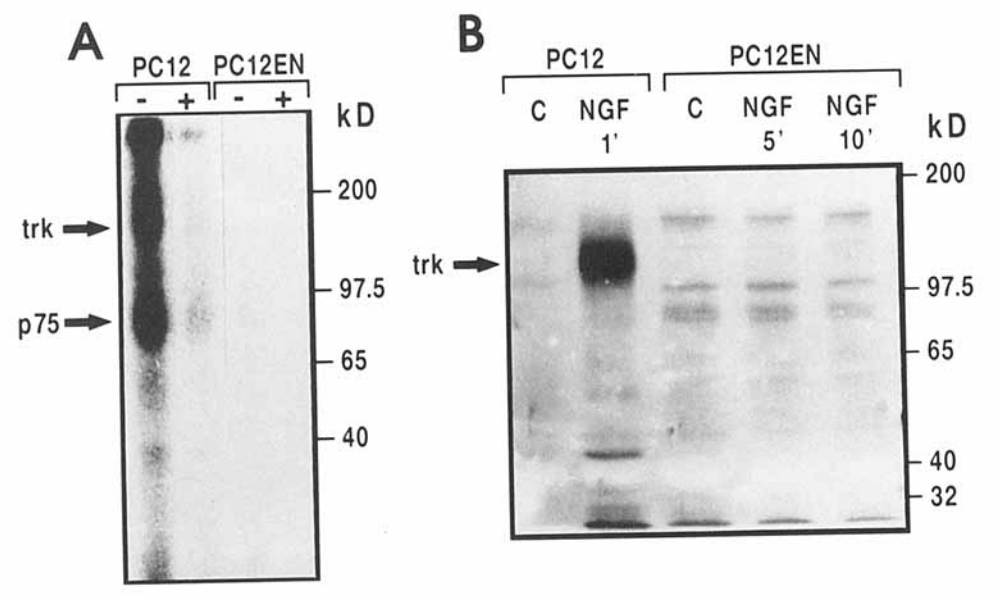

Fig. 8. Lack of p75 and trk NGFR in PC12EN cells. **A:** Absence of both trk and p75 in PC12EN cells. Cells were incubated with 0.5 nM iodinated NGF in the absence (−) or presence (+) of unlabeled NGF, followed by cross linking with EDAC and immunoprecipitation with anti-NGF antibody. Immunoprecipitates were subjected to SDS–PAGE and autoradiography as described under Materials and Methods. *Arrows,* approximate mobility of trk and p75. **B:** Absence of trk autophosphorylation in PC12EN cells. Cells were treated with 50 ng/ml NGF for the indicated times, harvested and lysed, as described under Materials and Methods. Lysates were subjected to trk immunoprecipitation and Western blotting using antiphosphotyrosine antibody as described. *Arrow (left ordinate),* approximate mobility of trk, positions of prestained molecular weights markers are indicated on the opposite ordinate.

TABLE III. Binding of Fibroblast Growth Factor to PC12 Cells and Hybrids*

	aFGF		bFGF	
Cell type	High	Low	High	Low
PC12	900 ± 250	1,192 ± 150	4,466 ± 200	6,255 ± 250
BAME	1,230 ± 420	1,428 ± 170	5,826 ± 380	4,295 ± 240
PC12EN	3,050 ± 300	1,899 ± 100	10,188 ± 500	7,858 ± 300

*PC12 cells and hybrids were plated on 24-well collagen/polylysine-coated dishes and incubated for 2 h with ^{125}I-FGFs in the presence or absence of excess unlabeled growth factors and were further processed as described under Materials and Methods. Values represent specific bindings (cpm/mg protein) of radiolabelled growth factor, as determined in three independent experiments.

entiative neurotropic effects in the absence of NGF receptors.

PC12EN cells maintain numerous neuronal traits, such as expression of PC12 cell-specific glycoconjugates, catecholamine synthesis, and release (Figs. 2, 3). In spite of the lack of EGF receptors, PC12EN cells maintain a constant rate of proliferation for over 150 generations, suggesting that the cells may have lost their competence for conventionally regulating the cell cycle. Alternatively, the hybrids might overexpress and/or constitutively release autocrine transforming/growth factors. This argument is in line with the enhanced rate of proliferation (Table I) and the tumorigenic properties of the cells [Lelkes et al., 1992]. Thus, PC12EN might also be useful as a model to study the mechanisms of tumorigenicity induced by hybridization of two non-tumorigenic cells. The tumorigenicity of PC12EN cells might be attributed to the shuffling of chromosomes, which in turn might result in inhibition of certain tumor suppressor genes and/or oncogene activation [Harrison, 1986; van Heyningen, 1994].

The PC12EN cell line exhibits some well-known characteristics of heterokaryotic hybrid cells: it has less chromosomes than if full integration of the genome had taken place [van Heyningen, 1994]. As shown in Figure 4, there is selective loss and gain of certain chromosomes, and one of the parental sources (cow) predominates by more than 3:1 over the other one (rat). In

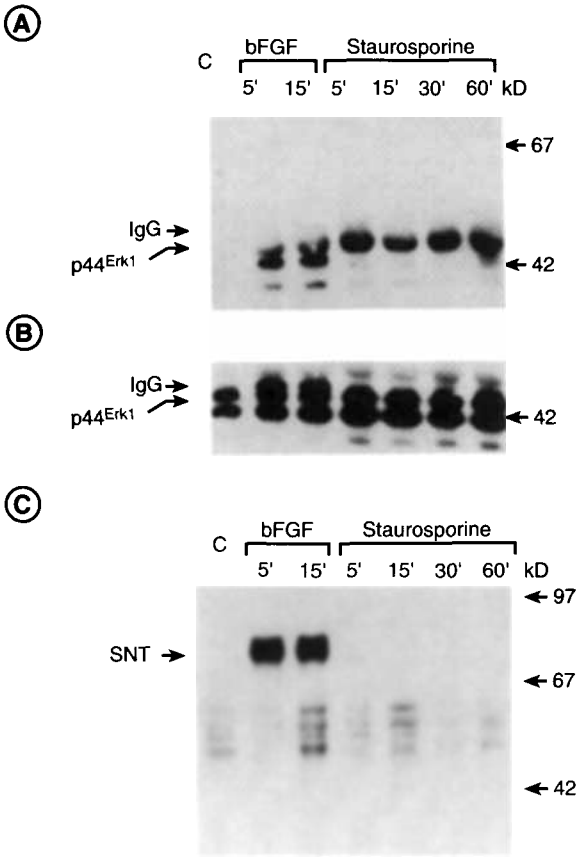

Fig. 9. FGF, but not staurosporine, activates the erk and SNT pathways in PC12EN cells. PC12EN cells were treated with 50 nM staurosporine or 2 ng/ml bFGF as detailed, lysed and subjected to erk1 immunoprecipitation (**A,B**) or SNT affinity chromatography (**C**). Subsequently, the samples were visualized by western blotting using anti phosphotyrosine antibodies (**A, C**) or anti-erk-1 antibodies (**B**). No phosphorylation was detected in untreated controls (**C**) *Arrows (right)*, apparent molecular masses in kD. *Arrows (left)*, identity of the erk and SNT substrates. IgG indicates the position of the co-eluted antibody, used for the immunoprecipitation.

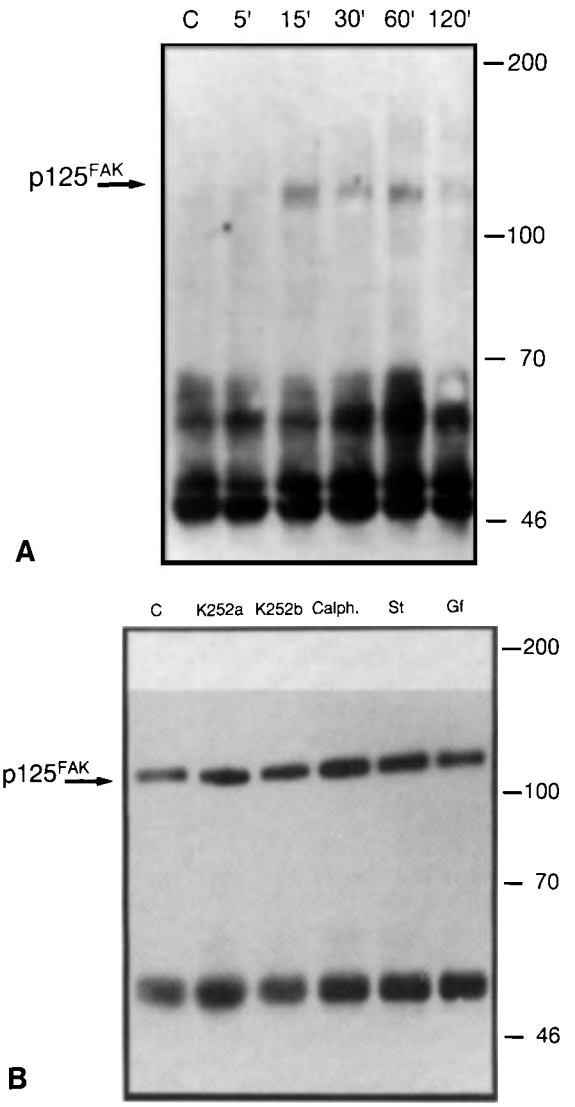

Fig. 10. Kinase inhibitors induce the tyrosine phosphorylation of p125^{FAK} in PC12EN cells. **A:** PC12EN cells were treated with 50 nM staurosporine for 5–120 min as indicated, lysed and subjected to p125^{FAK} immunoprecipitation followed by phosphotyrosine western blotting. **B:** PC12EN cells were treated for 60 min with 50 nM each of the different K252 compounds, lysed and subjected to p125^{FAK} immunoprecipitation followed by phosphotyrosine western blotting. Calph, calphostin C; St, staurosporine; Gf, GF109203X (bis-indole maleimide derivative of staurosporine, from Roche).

spite of the initial cloning step, karyotyping revealed a certain degree of genomic instability in individual cells. However, there was no evidence of deletions or duplications, nor was there evidence of structural abnormalities in which material from the cow was translocated into the rat chromosomes.

Chromosomal aberrations in the hybrids may be invoked to explain the lack of some of functional growth factor receptors (Figs. 7, 8). It is known that in the rat the gene for EGFR is located on chromosome 14 [Szpirer et al., 1991], while that for NGFR (trk) is on chromosome 10 [Hilbert et al., 1991]. The localization of these two genes is not known in the cow. According to the growth factor binding data (Table II) and the phosphorylation studies (Figs. 7, 8), PC12EN cells apparently lack functional receptors for both EGF and for NGF. Recent preliminary RT-PCR studies confirmed that the hybrid cells do not express any mRNA for trk, while the p75 neurotrophin receptor is present, albeit at very low abundance. This latter finding might explain the exceedingly low binding of ^{125}I-NGF to

the hybrids cells (approx 1% of that found in PC12 cells), which is practically at the detection limit of this radio-receptor binding assay. The lack of functional EGFR and NGFR in PC12EN cannot simply be explained by the loss of the chromosomes carrying these particular genes in the hybrids, because, as seen in Figures 4A,B, rat chromosome 10 (carrying the trk gene) is overexpressed, while at least one copy of rat chromosome 14 (harboring the EGFR gene) is present in all PC12EN cells. Also, as inferred from the banding analysis, there was no evidence for gross structural alterations such as translocations. Therefore, other mechanisms might explain the lack of functional EGFR and trk; for example, co-expression of bovine and rat genes in PC12EN may have altered the regulatory mechanisms which control the expression of these particular genes at the transcriptional level (e.g., for trk) or their efficient transcription into functional proteins. Current experiments are under way to test this hypothesis.

The lack of the receptors for neurotrophic growth factors in the hybrids offers a unique advantage over many of the existing neuronal models. For example, the fact that staurosporine can elicit neurite outgrowth in the absence of both the trk and the p75 NGF receptors establishes staurosporine as a unique neurotropic compound with a mechanism of action independent of NGF signalization. The interpretation of previous studies employing staurosporine as a neurotrophin have been blurred by the fact that most cellular models for neuronal differentiation express either trk or p75, or both [Meakin and Shooter, 1992].

Although PC12EN cells lack NGF receptors, certain downstream targets of trk receptor activation are preserved, such as erk and SNT. These targets, which are pivotal in the differentiative signalling cascade of neurotrophins [Kaplan and Stephens, 1994] or other growth factors, are activated by bFGF, but apparently not by staurosporine (Fig. 9), suggesting that the neurotropic action of staurosporine is independent of the erk or SNT pathways. This result confirms a previous observation by Miiyasaka et al (1991), that staurosporine at 100 nM concentration did not activate MAPK (erk) in GSRasDN1 cells, a ras-dominant negative PC12 cell variant. However, in those cells staurosporine failed also to induced neurite outgrowth, which sharply contrasts our findings in PC12EN cells. On the other hand, our results are in line with recent observations by Campbell and Neet (1995), who report the induction of neurite outgrowth in PC12 cells by both staurosporine and bFGF via a ras-independent pathway.

This study also demonstrates that certain microbial protein kinase inhibitors, such as staurosporine and K252a, can activate FAK in PC12EN cells. These results are in accordance with the finding that K252a induces FAK tyrosine phosphorylation and neurite outgrowth in SY5Y neuroblastoma but not in PC12 cells, suggesting a correlation between FAK tyrosine phosphorylation and neurite outgrowth [Maroney et al., 1995]. In PC12EN, K252a induces FAK tyrosine phosphorylation in the absence of neurite extension, thus placing some degree of doubt on a causal relationship between FAK tyrosine phosphorylation and neurite extension.

Alternatively, tyrosine phosphorylation of FAK may be indicative for the activation of distinct signalization pathways in PC12 cells and neuroblastoma cells, respectively. Since FAK localizes to focal adhesion sites [Schaller and Parsons, 1994], it is most probably activated by any agonist that may modulate transmembranal signaling via integrins, thus affecting cell shape and/or cytoskeletal organization [Shattil et al., 1994; Rankin and Rozengurt, 1994; Hanks et al., 1992]. Since K252a induces changes in the cell shape of PC12EN cells without causing visible neurite outgrowth in PC12EN cells, it is not surprising that K252a activates FAK in PC12EN. Indeed, spatial analysis by confocal fluorescence immunomicroscopy suggests that FAK is ubiquitously located throughout the cell body of PC12EN cells. When exposed to staurosporine, most of the FAK molecules remain in the cell body, and only a minor fraction is found in localized neuronal varicosities, where the neurites are in close contact with substratum [D. Rasouly, P. Lazarovici, P.I. Lelkes souly et al., unpublished observations]. Detailed mapping of FAK tyrosine phosphorylation sites and the integration of FAK in established signalization pathways responsible for morphological cell changes await further studies.

In summary, this study describes a novel cellular neuronal hybrid which is uniquely suited for studying the mechanisms of neuronal differentiation independent of NGF receptors. Thus, these cells might find a wide application in neurosciences to test novel neurotrophic agonists and to characterize their unique mechanism of action. In addition, we anticipate that this model

will also be useful for reconstituting wild type and mutant recombinant NGF and/or EGF receptors and studying their signal transduction targets.

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