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Staurosporine-Induced Neurite Outgrowth in PC12 Cells Is Independent of Protein Kinase C Inhibition

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SUMMARY

The protein kinase C (PKC) inhibitor staurosporine, a member of the K252a family of fungal alkaloids that are known as protein kinase inhibitors, induces neurite outgrowth in pheochromocytoma PC12 cells. The progressive staurosporine-induced neurotropic effect (EC_{50} = 50 nM) has the following characteristics: it is evident after 4 hr of incubation, requires the continuous presence of staurosporine, occurs at 37° but not at 4°, and is not blocked by K252a derivatives. Scanning electron micrographs showed long neurites, ruffling, and dense networks in nerve growth factor (NGF)-treated cells and short neurites, flattening, and smooth cell surface in staurosporine-treated cells. [^{3}H]Staurosporine binding, which was time, temperature, and dose dependent, saturated at 5–10 nM. Other kinase inhibitors were poor competitors. The [^{3}H]staurosporine bound over 20 hr at 37° was poorly dissociated by acetic acid wash or unlabeled staurosporine. These results suggest an uptake process occurring at 37° that is required for the neurotropic effect of staurosporine. NGF did not interfere with staurosporine binding, and staurosporine did not affect NGF receptor binding. At neurotropic concentrations of staurosporine, PKC in PC12 cells was completely inhibited. When PKC activity was down-regulated by prolonged exposure to phorbol myristate acetate, PC12 cells responded to staurosporine with neurite outgrowth similar to that of untreated cells. Although the target and mechanism of the neurotropic effects of staurosporine remain to be determined, the observed effects on PKC-deficient cells indicate that PKC may not be required for the neurotropic effect of this compound in PC12 cells. These results suggest that caution should be taken in the interpretation of staurosporine action *in vivo*, and they provide a pharmacological tool for the development of potential neurotropic drugs.

NGF is a polypeptide required for normal differentiation, maturation, and survival of sensory and sympathetic neurons (1). In PC12 cells, a clone derived from a pheochromocytoma tumor of the rat adrenal medulla, NGF induces differentiation (2, 3). This process is expressed morphologically by extensive outgrowth of neurites and represented physiologically by a series of membranal, cytoplasmic, and nuclear actions, including post-translational modifications, such as protein phosphorylation (4), and transcription-dependent systems (3). However, the precise mechanisms by which NGF induces the mature sympathetic neuron phenotype are still relatively unknown.

In order to define the pharmacological mechanisms underlying NGF-induced differentiation, two approaches might be considered, i.e., to develop potential agonists of NGF action or to search for specific antagonists of NGF action. This second approach was provided by a new family of protein kinase inhibitors (5, 6). K252a, a member of this family, is a fungal alkaloid (Fig. 1), isolated from the culture broth of *Nocardiopsis sp.*, that was found to inhibit both the morphological and physiological changes induced by NGF (7, 8). It has been shown that K252a does not interfere with the binding of NGF to its receptor in PC12 cells (7), and it was suggested that the site of K252a action is on an intracellular kinase closely associated with the NGF receptor (7, 8). Therefore, K252a might be considered as a functional NGF antagonist. It has been recently reported that staurosporine (Fig. 1), another alkaloid of the aforementioned kinase inhibitor family (5, 6), similar to K252a, antagonizes NGF actions in PC12 cells (9, 10). Staurosporine, an indole carbazole produced by *Streptomyces sp.*, is widely

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ABBREVIATIONS: NGF, nerve growth factor; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; 4α-PMA, 4α-phorbol-12-myristate-13-acetate; K-252a, (8*R**, 9*S**, 11*S**)-(−)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7b,11a-triazadibenzo(*a*,*g*)cycloocta(*c*,*d*,*e*)trindene-1-one; K-252b, 9-carboxylic acid derivative of K-252a; KT-5720, 9-*n*-hexyl derivative of K-252a; KT-5822, 9-methoxy derivative of K-252a; GF109203X, 2-[1-(3-dimethylaminopropyl)-indol-3-yl]-3-(indol-3-yl)maleimide; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethylsulfoxide; PBS, phosphate-buffered saline; EGTA, ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*′*N*′-tetraacetic acid; SEM, scanning electron microscopy.

used in pharmacological research as a PKC inhibitor (11, 12). Staurosporine is proposed to interact with the ATP-binding region of the catalytic domain of PKC (13). It does not prevent the association of PKC with its lipid activators but inhibits substrate phosphorylation and enzyme autophosphorylation (14). However, it is clear that, although staurosporine selectively inhibits PKC at nanomolar concentrations, it is not specific for this kinase (12). Inhibition of different serine/threonine kinases (14, 15) and tyrosine kinases (16, 17), as well as different receptor tyrosine kinases (16–18), has also been reported. Consistent with this lack of selectivity, different cellular effects of staurosporine, which may not be related to inhibition of PKC, have also been measured (19–23). Surprisingly, it was recently found that staurosporine mimics NGF in promoting outgrowth of short neurites from certain PC12 subclones (9, 24) and neonatal and adult rat chromaffin cells (9). We have independently made similar observations with other PC12 cell clones. Therefore, because staurosporine mimics NGF neurotropic effects at low concentrations and blocks them at higher concentrations, it could be considered as a mixed-function agonist/antagonist neurotropic compound.

The present study was undertaken with the primary objectives being to characterize quantitatively the NGF agonistic neurotropic effects and binding of staurosporine in PC12 cells and to consider whether PKC plays a role in such effects.

Materials and Methods

NGF was isolated from mouse submaxillary glands by liquid chromatography, according to the method of Bocchini and Angeletti (25), and was purified to homogeneity by high performance liquid chromatography (26).

(3-[^{125}I]Iodotyrosyl)-murine 2.5 S NGF (1500 Ci/mmol) was purchased from Amersham (Buckinghamshire, England). *N,N*-Dimethyl-[*dimethyl*-^{3}H(N)]staurosporine, (0.1 mCi/ml) was purchased from NEN DuPont. The radioactive compounds were stored at −20° in the dark. K-252a, K-252b, KT-5720, KT-5822, calphostin C, and staurosporine were all prepared at Kyowa Hakko Kogyo, Ltd., Tokyo Research Laboratory (Tokyo, Japan).

GF109203X was provided by Dr. Herve Coste, Laboratoires GLAXO (Centre de Recherches, France). PMA and 4α-PMA were purchased from LC Services Corporation (Wilburn, MA). Collagen and poly-L-lysine were from Sigma (St. Louis, MO), and all tissue culture reagents were obtained from Kibbutz Bet-Haemek (Israel).

PC12 cell culture. PC12 cells [sublines originated by Drs. L. Green (New York University) and G. Guroff (National Institutes of Health, Bethesda, MD)] were grown as monolayers in DMEM with 7.5% fetal calf serum, 7.5% horse serum, 100 mg/ml streptomycin, and 100 units/ml penicillin. Cultures were maintained at 37° in a 5% CO_2 incubator, with medium being changed twice per week, and were split at a 1:6 ratio once per week. For binding experiments, we used 6- or 12-well dishes coated with collagen (0.1 mg/ml collagen in 0.1 M acetic acid) and poly-L-lysine (0.01 mg/ml), at a 1:1 (v/v) ratio, as previously described (27).

Addition of compounds. Stock solutions of the lipophilic compounds K-252a, K-252b, KT-5720, KT-5822, calphostin C, GF109203X, PMA, 4α-PMA, and staurosporine were prepared in DMSO, at a final concentration of 2 mM, and were stored at −20° in the dark. NGF was prepared in DMEM, at a final concentration of 10 μg/ml, and was stored at −70°. Compounds were added directly from the stock solutions to the culture medium or were further diluted in DMEM, keeping DMSO concentrations below 0.1%, a concentration that was not effective on the parameters measured in this study. DMSO at the same concentration was used as a control.

Binding assays. PC12 cells were plated at high densities, in 6- or 12-well coated dishes, 1 day before the experiment. The cells were incubated with [^{3}H]staurosporine at 4° or 37° for different periods of time, in the presence or absence of unlabeled staurosporine (for nonspecific binding) or other inhibitors (for competition experiments). The incubation was ended by three washings with serum-supplemented medium, followed by DMEM and PBS. Cell monolayers were dissolved overnight with 0.5 N NaOH (0.5 ml). Aliquots (0.2 ml) were then transferred to scintillation liquid and β counted. Aliquots (0.1 ml) were used for protein determination, according to the method of Lowry *et al.* (28) or Smith *et al.* (29). When the acetic acid assay (30) was carried out, cells were washed as previously described and then incubated with 0.2 M acetic acid for 5 min at room temperature. Aliquots (0.1 ml) were dissolved in scintillation liquid and counted, and the cell monolayers were further processed in 0.5 N NaOH for protein determination and β counting.

For [^{3}H]staurosporine dissociation experiments, cells were incubated with 1 nM [^{3}H]staurosporine at 37° or 4° for the periods of time indicated, washed twice with serum-supplemented medium, and incubated with 1 μM unlabeled staurosporine at the aforementioned temperatures. Aliquots of medium were taken at the different time intervals, the monolayers were solubilized with 0.5 N NaOH, and radioactivity and protein were determined.

In growth factor binding assays, the cells were incubated for 1 hr at 37° with labeled growth factor, in the presence or absence of unlabeled growth factor (26). The monolayers were washed and dissolved as described above, and aliquots were measured in a γ counter. All binding assays were done in sextuplicates.

SEM. For electron microscopy, monolayers of PC12 cells were used. The monolayers were rinsed twice with PBS and then fixed for 1 hr with 2% glutaraldehyde in PBS, pH 7.4. After several rinses with PBS, they were postfixed in 1% osmium tetroxide. Dehydration in graded ethanol was followed by drying in graded Freon-113, prepared in absolute ethanol. After triple rinsing in 100% Freon, the specimens were vigorously shaken for a few seconds to dry. The samples were introduced into a sputter coater (Polaron ES100), coated with gold, and examined with a Philips 505 SEM at an accelerating voltage of 30 kV. Photographs of selected fields have been taken at different magnifications.

Neurite outgrowth assessment. A neurotropic factor is a compound that induces the cells to extend neurite outgrowths (cytoplasmic processes). PC12 cells were grown as monolayers in 24-well dishes, as previously described, and were exposed to the tested compounds. Phase-contrast photographs were taken at different time intervals. Neurite outgrowth units represent the ratio between the mean length of the cell neurites and the cell diameter. Neurite outgrowth values represent the mean ratio of about 100 cells from each experimental group. Poor neurite outgrowths were scored in the range of 0–2 units; maximal neurotropic effect were scored as 7–10 units.

PC12 subcellular preparations. Control and experimentally treated PC12 cells were harvested and washed with Ca^{2+}/Mg^{2+}-free PBS. The cell pellets were disrupted by sonication in a homogenization buffer containing Tris (20 mM), chelators EGTA (10 nM) and EDTA (2 mM), and protease inhibitors phenylmethylsulfonyl fluoride (0.5 mM), leupeptin (1 mg/ml), and aprotinin (5 mg/ml) (31). The cell homogenate was centrifuged for 15 min at 3000 × *g*, and the unbroken pellet was discarded. The supernatant was centrifuged for 1 hr at 30,000 × *g* at 4°. The pellet (particulate fraction) and supernatant (cytosol) were separated, and aliquots were taken for protein determination or submitted immediately for phosphorylation experiments. The experiments were repeated three times under similar conditions.

Measurements of PKC activity. PKC phosphorylation activity was assayed by using histone 1 as an exogenous substrate, as previously described (32), with the following protocol. PKC activity was assayed by measuring the incorporation of γ-^{32}P into histone 1, based on the technique of Lester (32, 33). Multilamellar vesicles (20% phosphatidylserine, 80% phosphatidylcholine, with or without 5% diacylglycerol) were prepared in a Ca^{2+}-free buffer (0.5 mM EGTA). The concentration

of free Ca^{2+} (10 μM) in assays was determined using defined concentrations of Ca^{2+} and EGTA (33). All other conditions were as described by Lester (32, 33). To measure PKC activity with the particulate fractions, the membrane pellets from the 30,000 × *g* centrifugation step were suspended in homogenization buffer containing 0.5% Nonidet P-40 detergent. The suspensions were stirred at 4° for 30 min, followed by recentrifugation at 30,000 × *g* for 60 min at 4°. The supernatant represented the membrane extracts (31).

Results

Dual neurotropic effects of staurosporine. K252a derivatives (Fig. 1), which are known to inhibit protein kinases *in vitro* with different selectivities (Table 1), have been also found to inhibit, with different potencies, NGF-induced neurite outgrowth (Table 1). K252a was the most potent (7, 8). Staurosporine was the only compound to promote, at certain concentrations, neurite outgrowth (Table 1), therefore partially mimicking NGF neurotropic effects. Because staurosporine partially mimics NGF and also inhibits its actions, it could be considered a mixed-function neurotropic tool.

Morphological characteristics of staurosporine neurotropic effects. The neurite outgrowths induced by staurosporine and NGF were visualized by SEM (Fig. 2). Upon comparison between staurosporine-treated, NGF-treated, and control PC12 cells, the following differences have been noticed. (a) The number and size of neurite outgrowths were larger in NGF-treated (Fig. 2, C and D) than in staurosporine-treated (Fig. 2, E and F) cells, and outgrowths were absent in untreated cultures (Fig. 2, A and B). (b) Cell bodies of staurosporine-treated cultures became flattened and had a smoother surface appearance (Fig. 2F) than NGF-treated (Fig. 2D) or control cells (Fig. 2B). (c) Upon treatment with NGF for 5 days or more, PC12 cells reached a differentiation stage characterized by a dense neuronal network (Fig. 3A); in contrast, staurosporine lacked such effects on the PC12 cells (Fig. 3B). Two dose-response curves for staurosporine-induced neurite outgrowth are presented in Fig. 4A. The first curve presents the percentage of responsive cells, and the second expresses the ratio between neurite lengths and cell diameter, both indexes of the potency of the neurotropic effect. Staurosporine neurotropic effect occurs in the range of 10–1000 nM, with an ED_{50} of about 50 nM. At staurosporine concentrations exceeding 200 nM, both dead cells and living cells with neurite outgrowths were observed. Thus, in the following experiments 10–50 nM concentrations were used, in order to achieve maximal neurite outgrowth without interfering cytotoxic effects. Continuous exposure of PC12 cells to staurosporine at 37° resulted in neurite outgrowth that was evident at 4–6 hr and reached maximum by the second day of treatment (Fig. 4B). Exposure of PC12 cell cultures to 100 nM staurosporine at 4° from 1 to 24 hr resulted in a very poor neurotropic effect (4% responsive cells). Single exposure of the cells to staurosporine (10^{-7} M) at 37° resulted in transient, weak, neurite outgrowth (data not shown), suggestive of a

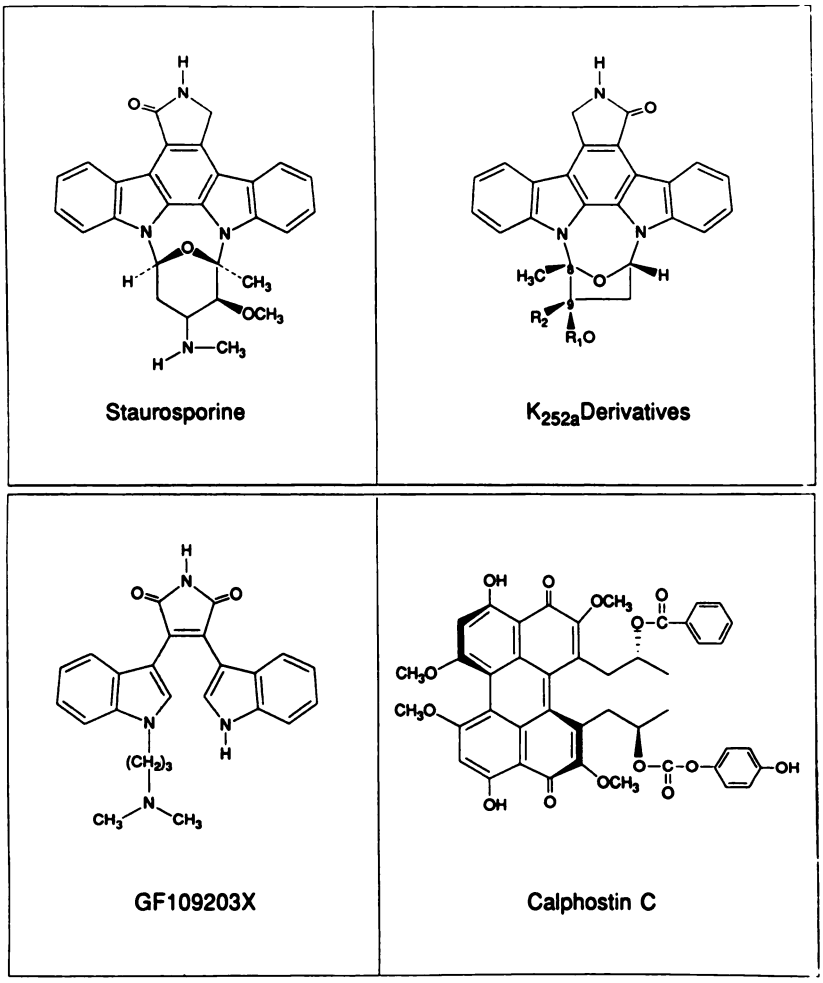

Fig. 1. Chemical structures of K252a derivatives and calphostin C. R1 and R2 are listed in Table 1.

TABLE 1

Relationship between protein kinase specificity, inhibition of NGF neurotropic effects, and promotion of neurite outgrowths for the K252a family of inhibitors

Compound	R1	R2	*In vitro* relative protein kinase specificity[a]	K_i for PKC	Concentration for inhibition of NGF-induced neurite outgrowth[b]	Concentration for promotion of neurite outgrowth[c]
				μM	μM	μM
K252a	H	$COOCH_3$	PKC = PKA = PKG	0.025	0.1–1	—
K252b	H	COOH	PKC > PKA > PKG	0.020	0.5–10	—
KT-5720	H	$COO(CH_3)_5CH_3$	PKA ≫ PKC ≫ PKG	>2.00	0.25–1	—
KT-5823	CH_3	$COOCH_3$	PKG > PKA > PKC	4.00	0.25–1	—
GF109203X			PKC ≫ PKA	0.010[d]	0.1–0.8	—
Calphostin C			PKC ≫ PKG > PKA	0.013[e]	0.1–0.5	—
Staurosporine			PKC ≫ PKA > PKG	0.0007	>0.05	0.01–0.5

[a] PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PKG, cGMP-dependent protein kinase. Taken from Ref. 40.

[b] K252a derivatives were incubated with PC12 cell cultures in the presence of 50 ng/ml NGF. Neurite outgrowth evaluation was performed after 4 days and compared with control untreated or NGF (50 ng/ml)-treated cultures. Values represent the range of concentrations of the inhibitors needed for maximal inhibition of NGF-induced neurite outgrowth.

[c] The cells were treated with up to 200 nM levels of the derivatives for 2 days, and the neurotropic effect was estimated as described in Materials and Methods. Values indicate the range of concentrations needed for maximal effect. —, No effect at any concentration.

[d] Taken from Ref. 34.

[e] Taken from Ref. 41.

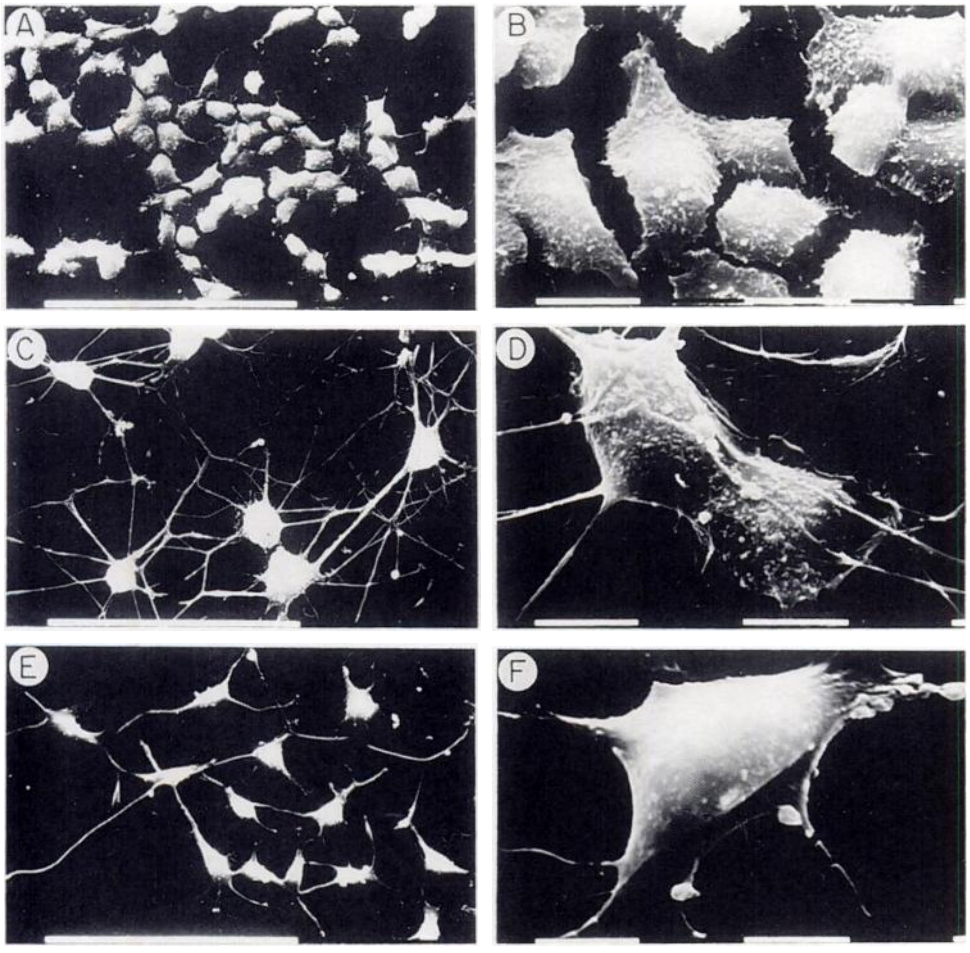

Fig. 2. SEM visualization of PC12 cells and the neurotropic effect induced by staurosporine or NGF. PC12 cells, grown on Petri dishes coated with collagen-polylysine, were exposed for 4 days to NGF (50 ng/ml) (C and D) or staurosporine (50 nM) (E and F), fixed, and processed for SEM, as indicated in Materials and Methods. A and B, Untreated controls. A, C, and E, Lower magnifications; B, D, and F, higher magnifications. *White bar*, 10 μm.

degradation process and/or a requirement for persistent stimulation. Withdrawal of staurosporine from the culture medium when maximal neurite outgrowth was reached (5 days, 37°) was followed by a gradual loss of outgrowths, indicative of reversibility of the staurosporine-induced neurotropic effects.

Suggestion of an uptake process for [^{3}H]staurosporine-PC12 cell association. To gain insights into the process required for staurosporine induction of the neurotropic effects, [^{3}H]staurosporine binding experiments with PC12 cells were performed. [^{3}H]Staurosporine binding at 37° reached saturation at 5–10 nM (Fig. 5A, *inset*). To characterize the specificity of [^{3}H]staurosporine binding, competition experiments with structurally related K252a derivatives were performed (Table 2). It is evident that staurosporine was the strongest competitor (72%) for the radioactive derivative. K252a and calphostin C were very poor competitors (5 and 9%, respectively), and the synthetic staurosporine derivative GF1092003X (34) showed partial competition (37%). The saturation and competition

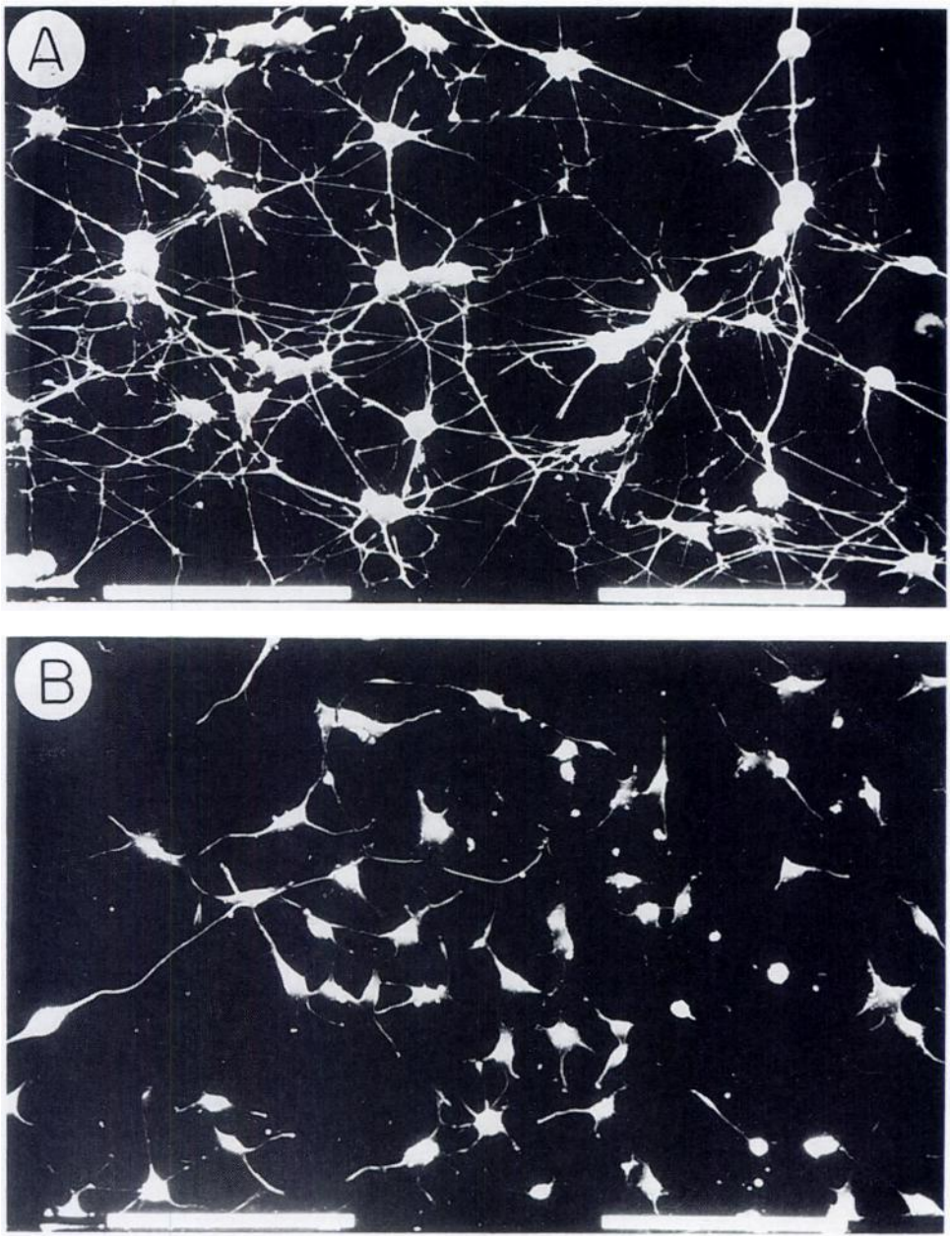

Fig. 3. Representative SEM of PC12 cell cultures treated for 5 days with NGF (50 ng/ml) (A) or staurosporine (50 nM) (B). Notice the neural outgrowth branching and network present in A and their absence in B. *Bar*, 10 μm.

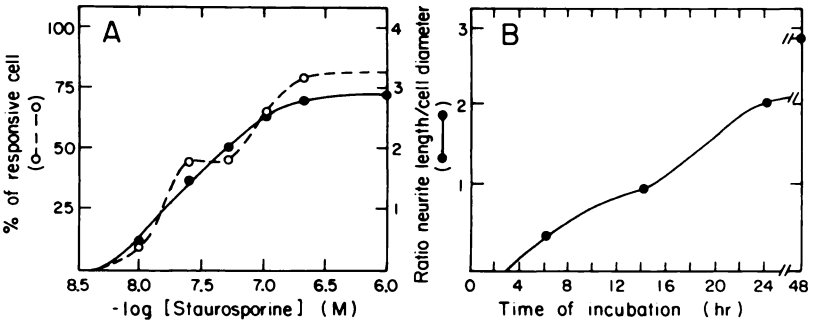

Fig. 4. Dose-response (A) and kinetics (B) of staurosporine neurotropic effects in PC12 cells. Sextuplicate cultures of PC12 cells were incubated for 6 hr (A) or different periods of time (B) with either different concentrations (A) or 50 nM (B) staurosporine. Thereafter, the neurotropic evaluations were performed as described in Materials and Methods. Standard deviations were 10% (A) or 30% (B).

binding data demonstrate the specificity of [^{3}H]staurosporine association to PC12 cells. Several binding approaches used here suggest that [^{3}H]staurosporine incorporation into PC12 cells is via an uptake process. (a) The binding was temperature dependent (Fig. 5A), gradually increasing only at 37° and reaching a maximum after 20 hr of incubation (Fig. 5A). (b) Acetic acid treatment of receptor-bound ligands is an established method (30) to separate cell surface-bound from internalized ligands. Using this method, we have separated [^{3}H]staurosporine bound at 37° into two cell-associated fractions (Fig. 5B). The first, acid-released, fraction did not change with increased time of incubation of [^{3}H]staurosporine with PC12 cells and represents cell surface interaction; the second, acetic acid wash-resistant, fraction increased gradually with incubation time, reaching a

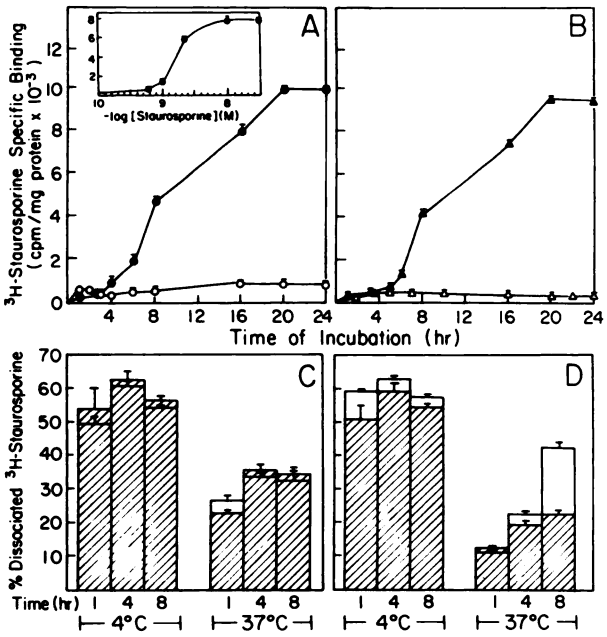

Fig. 5. Characteristics of [^{3}H]staurosporine binding to PC12 cells. A, Time course of binding at 4° (○) and 37° (●). *Inset*, saturation curve of [^{3}H]staurosporine binding. B, Time course for cell surface-bound (△) and residual cell-associated (▲) [^{3}H]staurosporine at 37°. C and D, Effects of temperature and time of exposure on dissociation of [^{3}H]staurosporine. Six-well dishes of confluent PC12 monolayers were incubated up to 24 hr with 5 nM [^{3}H]staurosporine, in the presence (nonspecific binding) or absence (total binding) of 2 μM staurosporine (A). The 37° exposed cultures were washed twice with serum-supplemented medium, and a portion was dissolved in 0.5 N NaOH and subjected to β counting (A); other replicates (B) were incubated for an additional 5 min at room temperature with 1 ml of 0.2 M acetic acid (pH 2.5) in PBS, and 0.2-ml aliquots were β counted (△). The remaining associated radioactivity (▲) was removed with 0.5 N NaOH and separately β counted. C and D, PC12 cells were incubated with 5 nM [^{3}H]staurosporine for 4 hr (C) or 26 hr (D), at 4° or 37°. The incubations were terminated by two washings and used for dissociation experiments of 1, 4, and 8 hr, in the presence (□) or absence (▨) of 2 μM staurosporine. Substraction of values obtained without staurosporine from those obtained staurosporine yields the specifically [^{3}H]staurosporine-dissociated fraction.

TABLE 2

Competition between different PKC inhibitors and [^{3}H]staurosporine binding to PC12 cells

PC12 cells were incubated with 5 nM [^{3}H]staurosporine in the presence or absence of 2 μM tested compound (toxicity was below 15%) for 8 hr. Cells were washed three times, harvested, and β counted, as described in Materials and Methods.

Compound added	Staurosporine binding	
	cpm/mg of protein	*% of control*
None	8737 ± 1490	100
Staurosporine	2468 ± 320	28
K252a	8300 ± 1130	95
GF109203X	5455 ± 1290	63
Calphostin C	7970 ± 1600	91

maximum around 20 hr of incubation (Fig. 5B) and is thought to represent "internalized" (30) [^{3}H]staurosporine. (c) At 4°, under all conditions (Fig. 5, C and D), 50–60% of cell-bound [^{3}H]staurosporine was dissociated, compared with 15–40% at 37° (Fig. 5, C and D). These data show that a larger fraction of [^{3}H]staurosporine is bound at the cell surface and available for dissociation at 4° than at 37°. [^{3}H]Staurosporine bound after 26 hr at 37° (Fig. 5D) was gradually dissociated in a 1–8-hr incubation with the unlabeled inhibitor. At 8 hr of incubation (Fig. 5D, last bar), 20% of [^{3}H]staurosporine was specifically dissociated, representing inhibitor that had been taken up.

These intensive analyses were made in order to determine the specificity of staurosporine uptake and its intracellular distribution. The data support the proposal that staurosporine is specifically taken up into PC12 cells.

Possible targets of staurosporine action on PC12 cells. The unique ability of staurosporine to mimic partially the actions of NGF in PC12 cells, compared with other PKC inhibitors (Table 1), can be interpreted as binding either to the NGF receptor or to one of its postreceptor targets and activation of NGF signal transduction, leading to mimicry of NGF actions. Alternatively, staurosporine can bind to another cellular system, which is unrelated to NGF signal transduction. The lack of competition between NGF and staurosporine on living PC12 cells (Table 3) excludes the possibility of some interactions between staurosporine and the NGF receptor binding domain. Other possible interactions between staurosporine and other domains of the NGF receptor (35) cannot be excluded and have to be examined. In line with these data, K252a, an inhibitor of NGF neurotropic effects, does not compete with NGF receptor binding (7), thereby suggesting another mechanism of action for these inhibitors.

Because staurosporine was found to be an effective PKC inhibitor *in vitro* and in some cases *in vivo*, we considered PKC to be the possible target of staurosporine in PC12 cells. To verify this possibility, two questions have been considered. (a) Do staurosporine-treated cells express PKC activity?. (b) Does phorbol ester-induced down-regulation of PKC affect staurosporine neurotropic effects? The PKC activity in PC12 cells, treated with staurosporine or with micromolar concentrations of PMA, which down-regulates the enzyme (36, 37), is presented in Table 4.

In PC12 cells, either undifferentiated or treated for 24 hr with NGF, the PKC phosphorylation activity was relatively low, compared with brain neurons (38), and the specific activity was 8–10-fold higher in the cytosol, compared with the particulate fraction (Table 4). Six-hour treatment of PC12 cells with 50 nM staurosporine fully inhibited PKC activity in the cytosol and in the particulate fractions. Therefore, under these experimental conditions, which are optimal for the initiation of staurosporine-induced neurotropic effects, *in vivo* translocation of PKC activity (39) from the cytosol to membranes could not be detected (Table 4). PKC in many systems, as well as in PC12 cells (20, 36, 37), can be down-regulated with micromolar concentrations of phorbol esters, such that within 24 hr the PKC activity and protein are lost. We have observed the same down-regulation of PKC under the experimental conditions described above (Table 4). In addition, when PKC down-regulated cells were incubated with 50 nM staurosporine or 50 ng/

TABLE 3

Effect of staurosporine on NGF binding to PC12 cells

PC12 cells were incubated with 100 pM ^{125}I-NGF, in the presence or absence of unlabeled NGF (1 μM) or staurosporine (1 μM), for 1 hr. After incubation, the cells were washed three times, harvested, and γ counted.

Compound added	^{125}I-NGF binding
	cpm/mg of protein
None	1790 ± 250
Staurosporine	1855 ± 140
NGF	50 ± 13

TABLE 4

PKC activity and neurotropic effects in PC12 cells treated with staurosporine, PMA, and NGF

PC12 cell cultures were treated for 24 hr with NGF (50 ng/ml), 6 hr with 50 nM staurosporine, 24 hr with 500 nM PMA, or 24 hr with 500 nM PMA followed by 6 hr with 50 nM staurosporine. Cells were then harvested and washed, and cytosol and particulate were prepared and subjected to PKC assay, as described in Materials and Methods.

Compound added	PKC activity		Neurotropic effects[a]
	Cytosol[b]	Particulate[c]	
	nmol of P_i/mg/min		%
None	0.080	0.010	0
NGF	0.093	0.008	13 ± 1.4
Staurosporine	ND[d]	ND	53 ± 8.0
PMA	ND	ND	0
PMA + staurosporine	ND	ND	58 ± 9.5

[a] Neurotropic effect, expressed as percentage of responsive cells with neurites (scored 2–5 units), was estimated for the indicated time intervals, as described in Materials and Methods.
[b] Values represent mean ± 10% standard deviation.
[c] Values represent mean ± 20% standard deviation.
[d] ND, not detected.

ml NGF for 6 hr, there was no significant recovery of PKC activity (Table 4), indicating that staurosporine or NGF (data not shown) did not modulate the kinase synthesis in PKC-deficient cells.

Effect of down-regulation of PKC on staurosporine-induced neurite outgrowth. Because the effect of down-regulation of PKC activity by PMA gave rather clear results, we used the same paradigm to investigate staurosporine neurotropic effects. PC12 cell cultures, plated on collagen/polylysine-coated dishes, were incubated for 24 hr with PMA to down-regulate PKC, treated with staurosporine or NGF, and analyzed for neurite outgrowth at different times. Staurosporine-treated cells showed considerable neurite outgrowth (Table 4; Fig. 6D), and the number of neurite outgrowth-bearing cells increased after 2 days to about 75% of that observed with untreated PC12 cells (Fig. 6) or 4α-PMA-treated cells (data not shown). In addition to the lack of effect on the length of neurite outgrowths formed or the percentage of responsive cells, down-regulation of PKC did not apparently affect the morphology of PC12 cells and neurite outgrowths, as evaluated by SEM and described in Figs. 2 and 3. In summary, the PKC-down-regulated PC12 cells did not lose their neurite-extending response to staurosporine, suggesting that staurosporine-induced neurite outgrowth can occur in the absence of PKC activity.

Discussion

Staurosporine is a pleiotropic compound (12, 42) that induces neurite outgrowth from PC12 cells at nanomolar concentrations, as previously reported for other cellular subclones (9, 24), normal rat chromaffin cells (9), and human neuroblastoma cell lines NB-1 (43), SK-N-SH (44), and SH-SY-5Y (45). Unlike the neurite outgrowths induced by NGF, those formed in response to staurosporine are reduced in length, not blocked by K252a, reach a terminal length within 5 days, and do not form a neurite network typical of the advanced stage of NGF-induced differentiation. Staurosporine (20 nM) causes dissolution of actin microfilament bundles in some cells (20), inhibits the induction of actin/gelsolin (46) or the phosphorylation of pleckstrin and myosin light chain (47), and inhibits cell motility (48). Although the role of protein kinases in cytoskeleton control and neurite outgrowth of PC12 cells is far from being completely understood (49, 50), staurosporine neurotropic effects might be related to some kinase signals required to organize neurite cytoskeleton elements. Another property of staurosporine is its ability to inhibit PKC and, therefore, to promote a variety of pharmacological processes (42). The relevance of staurosporine inhibition of PKC to the pharmacological effect is still a matter of debate. The majority of studies favor PKC-dependent staurosporine effects, based on the antagonism between phorbol esters and staurosporine (20, 46, 51, 52) or the similarity between the effects of staurosporine and those of other protein kinase inhibitors, such as H7 (45). This aspect, however, is underscored by reports indicating similar actions of phorbol esters and staurosporine on epidermal cells (51) and the tumor promotion activity of staurosporine itself in mouse skin (53). Therefore, the effects of staurosporine on PKC-mediated systems may vary, depending on the signal transduction pathway involved, the cell type investigated, and the biophysical properties of the cell membranes (54).

In this study, we have shown that PKC phosphorylation activity in PC12 cells treated with staurosporine is completely blocked. Therefore, it may be reasonable to assume that PKC inhibition plays a role in the neurotropic effects of staurosporine. However, based on the identical neurotropic effects of staurosporine on cells containing active PKC and cells whose PKC had been down-regulated by phorbol ester, we conclude that the neurotropic effects of staurosporine occur by a PKC-independent mechanism. This conclusion is supported by recent findings indicating the ability of NGF to induce neurite outgrowths and some phosphorylations by PKC-independent mechanisms (37, 55, 56). A recent report indicates that H7, another protein kinase inhibitor, induces outgrowths and flattening and potentiates the neurogenic properties of NGF in PC12 cells, with a concomitant change in the accumulation of the β_2 PKC isoform (57). Although the enzymatic activity was not tested in that study, it might suggest a different neurotropic mechanism of action for H7, compared with staurosporine. The identity of staurosporine targets in PC12 cells is unknown, but they might be related to the [^{3}H]staurosporine uptake process. Although the biochemical characteristics of this uptake process require further elucidation, the temperature dependency of staurosporine-induced neurotropic effects might suggest a correlation with the uptake process. This possibility is presently under investigation. The cellular complexity of phosphorylation pathways makes the elucidation of the precise mechanism of staurosporine neurotropic action difficult. Although the possibility that inhibition of some other kinase might be involved remains open, PKC was essentially ruled out as a mediator of staurosporine neurotropic effects. We have also excluded the possibility of a direct interaction between staurosporine and the NGF-binding domain. Recent findings indicate that the human *trk* proto-oncogene encodes a tyrosine kinase with the structural characteristics of high affinity NGF receptors (35). Therefore, possible stimulatory and/or inhibitory actions of staurosporine on NGF receptor tyrosine kinase activity have to be tested, and positive findings could provide an explanation for the mixed neurotropic functions of staurosporine.

Research on the neurotropic mechanism of action of staurosporine might facilitate the design of future neurotropic analogues that are less toxic and more selective, such as UCN 01 (41) and others (11). Elucidation of the mechanisms by which staurosporine induces its neurotropic actions also has clinical

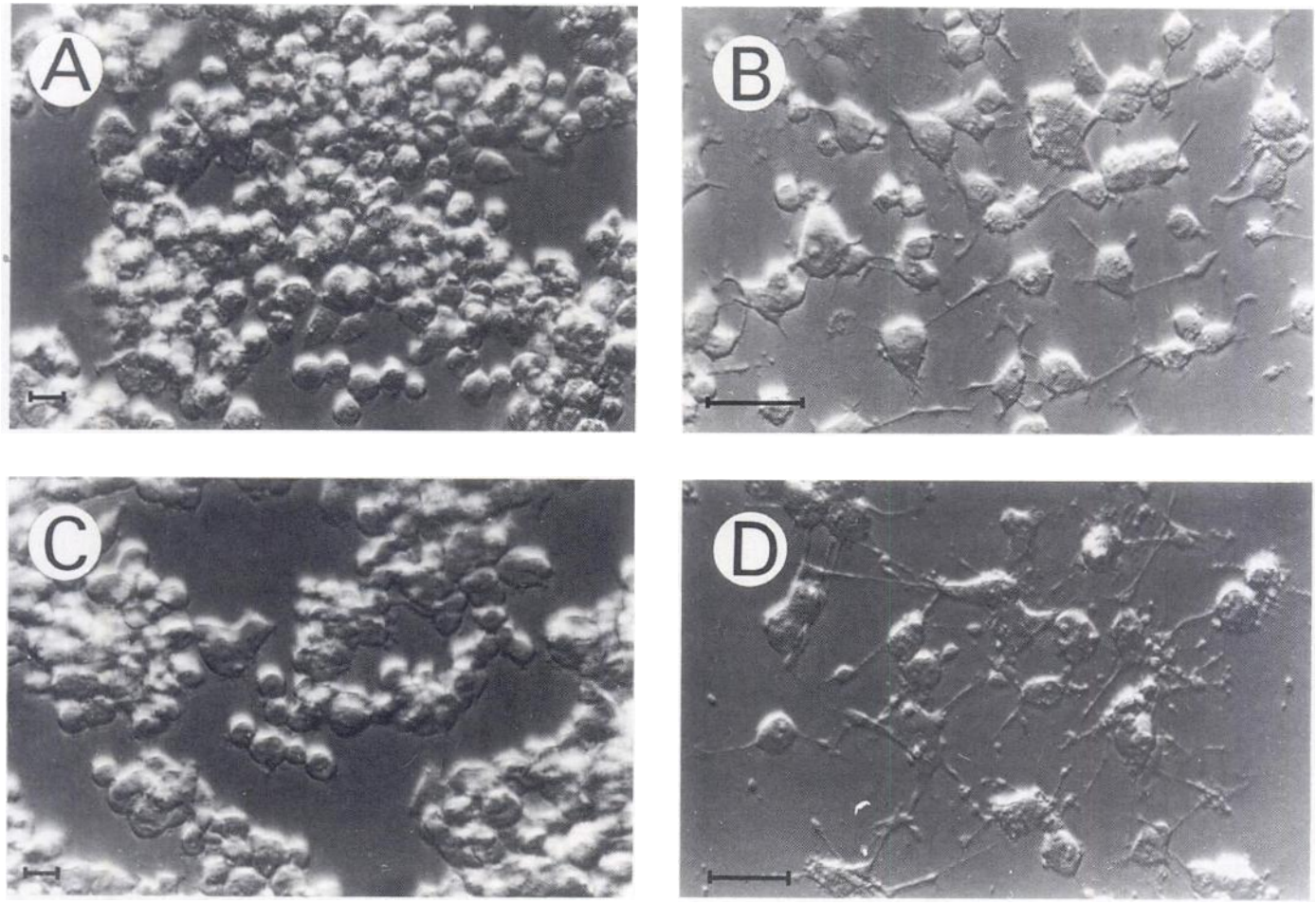

Fig. 6. Morphology of neurite outgrowths in PKC-down-regulated and staurosporine-treated PC12 cells. The experiment was performed as described in Table 4, and phase contrast photographs were taken after 6 hr. A, Cells that were not down-regulated and were treated with buffer. B, Cells that were not down-regulated and were treated for 6 hr with 50 nM staurosporine. C, Cells that were down-regulated with 1 mM PMA and then treated for 6 hr with buffer. D, Cells that were down-regulated with 1 mM PMA and then treated with 50 nM staurosporine for 6 hr. *Black bar*, 30 μm.

implications, because no neurotropic drugs are available yet, and will contribute toward the understanding of the pharmacological mechanisms underlying neuronal growth, regeneration, and plasticity.

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