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Neurites Induced by Staurosporine in PC12 Cells are Resistant to Colchicine and Express High Levels of *Tau* Proteins

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SUMMARY

Staurosporine, a protein kinase inhibitor, induces neurite outgrowth in pheochromocytoma cells and, therefore, may serve as a potential prototype for neurotropic drugs. The principal aim of the present study was to characterize the cytoskeletal properties of neurites induced in pheochromocytoma cells by staurosporine, in comparison to those induced by nerve growth factor, with emphasis on tubulin and *tau* proteins. Two major findings are described: a) staurosporine rapidly induces outgrowth of neurites that are resistant to colchicine treatment; and b) staurosporine treatment causes a rapid increase in *tau* protein levels, with a time course similar to the initiation of its neurotropic effects. The following observations exclude tubulin as the cellular target for staurosporine action: a) the level, cellular distribution, and assembly properties of tubulin are not affected by staurosporine treatment; and b) colchicine uptake, its binding to tubulin, and its interference with tubulin polymerization are not changed by staurosporine. On the other hand, staurosporine treatment causes a transient, dose-dependent increase in *tau* protein levels. This increase, which is already evident after 1 hr, reaches a maximum of 2 to 3 fold after 5 hr of treatment and declines to basal level within the next 10 to 15 hr. The rapid, transient increase of *tau* protein levels induced by staurosporine is reminiscent of its neurotropic properties. Here we characterize and compare the cytoskeletal properties of neurites induced by treatment with staurosporine and with nerve growth factor, and we offer a mechanistic explanation for the rapid stabilization of staurosporine induced neurites.

Neurite outgrowth is a morphological expression of neuronal plasticity and differentiation (1). Cells of rat PC12 cell line (2, 3) are induced to differentiate into sympathetic neurons in response to NGF (3, 4). In the course of their differentiation, PC12 cells cease dividing and extend neurites that elongate, sprout, and form synaptic connections, resulting in a dense neuronal network (3, 5, 6). These differentiated neurons serve as an ideal model system to investigate the dynamics and mechanisms of neurite outgrowth and to screen for potential neurotropic drugs (7, 8, 9).

Neurite outgrowth involves a number of biochemical steps directed toward promotion of the assembly of tubulin monomers, thus, creating cytoskeletal polymers that support the growing neurites (7, 9, 10). The mechanisms governing microtubule polymerization are largely unknown and are being explored intensively (1). Pharmacological tools that modulate cytoskeletal polymerization, such as the cytoskeletal interactive drugs colchicine, vinblastine, and taxol (11), are being used in some of these studies. In PC12 cultures, it was found that NGF-induced neurites undergo changes in colchicine susceptibility. Acute NGF treatment (1–3 days) induces short neurites that retract upon exposure to colchicine (7). However, upon chronic treatment with NGF (>7 days), the neurites mature and become colchicine-resistant (7). Accordingly, colchicine resistance and/or susceptibility may be considered as one of the pharmacological criteria of the differentiation and/or maturation of the neurites (7). Several mechanisms have been suggested to underlie colchicine resistance, such as posttranscriptional modifications of cytoskeletal proteins by phosphorylation or acetylation (12–15), and induction of high molecular weight MAPs (16, 17) and *tau* proteins (18, 19).

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ABBREVIATIONS: PC12, pheochromocytoma; NGF, nerve growth factor; MAP, microtubule associated proteins; PKC, protein kinase C; MTP, microtubules; DMSO, dimethylsulfoxide; EGTA, ethylene glycol bis (*beta*-aminoethylether),*N,N,N′,N′*-tetraacetic acid; EDTA, ethylenediamine tetraacetic acid; BSA, bovine serum albumin; PMSF, phenyl methyl sulfonyl fluoride; GTP, guanosine trisphosphate; SDS, sodium dodecyl sulphate; HRP, horseradish peroxidase; PIPES, piperazine-*N,N′*-bis[2-ethanesulfonic acid]; MES, 2(*N*-morpholino) ethanesulfonic acid; DEAE, diethylaminoethyl; PBS, phosphate-buffered saline; TBS, tris-buffered saline; PEM, PIPES-EGTA-$MgCl_2$ buffer; DMEM, Dulbecco's modified Eagle's medium; SEM, scanning electron microscopy; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence.

Staurosporine, a fungal alkaloid known to inhibit several protein kinases with relatively high specificity for PKC (20–22), was shown recently to induce neurite outgrowth in PC12 cells (23). This specific neurotropic effect of staurosporine has a rapid onset as compared to induction by NGF, and is independent of PKC inhibition (23). In the present study we further characterized the mechanism of staurosporine action and investigated the properties of neurites induced by staurosporine treatment, with special reference to their colchicine sensitivity. Because colchicine binds to monomeric tubulin and, thereby, prevents its polymerization (24, 25), we characterized the interactions between colchicine and staurosporine in an attempt to explain the colchicine resistance of staurosporine-induced neurites. Our findings indicated that, although staurosporine treatment does not affect tubulin properties and levels, it induces a substantial increase in levels of *tau* proteins. We suggest, therefore, that the rapid increase in *tau* levels, which correlates with the rapid maturation of staurosporine-induced neurites, may underlie their resistance to colchicine.

Materials and Methods

Materials. NGF was isolated from mouse submaxillary gland by liquid chromatography and further purified to homogeneity by HPLC (26). Staurosporine was synthesized at Kyowa Hakko Kogyo Ltd., Tokyo Research Laboratories (Tokyo, Japan). Colchicine was of reagent grade. [^{3}H]Colchicine (5–15 Ci/mmol) was purchased from Amersham (Buckinghamshire, England). Collagen and poly(L-lysine) were from Sigma Chemical Co. (St. Louis, MO). All tissue culture reagents were from Kibbutz Beit-Ha-emek, Israel. Anti-*beta*-tubulin antibody, anti-mouse HRP, rainbow molecular weight markers, and the ECL detection system were purchased from Amersham. Anti-*tau*-1 antibody was kindly provided by Dr. L. Binder (Birmingham, AL). Rhodamine-labeled anti-mouse antibody was purchased from Cooper-Biomedical (Malvern, PA). Nitrocellulose papers were from Schleicher and Schuell (Keene, NH).

PC12 cell cultures. PC12 cells, a subline originally produced by Dr. L. Greene (New York University, NY) and Dr. G. Guroff (National Institutes of Health, Bethesda, MD), were grown in DMEM with 7% fetal calf serum, 7% horse serum, 100 μg/ml streptomycin, and 100 units/ml of penicillin. The cell cultures were maintained in an incubator at 37° and 5% CO_2. Medium was changed twice per week and splitted at a 1:6 ratio once per week. Binding experiments were performed in 6- or 12-well dishes coated with equal parts of collagen (0.1 mg/ml collagen in 0.1 M acetic acid) and poly(L-lysine) (0.01 mg/ml) as previously described (23).

Colchicine treatment and neurite outgrowth assessment. One day before the experiment, PC12 cells were plated on 12-well dishes coated with collagen/polylysine. After treatments of the cells, colchicine or DMSO (control) was added for 45 min. The cells then were washed with PBS and fixed with 2% glutaraldehyde for 1 hr. Phase contrast photographs were taken and analyzed for neurotropic effect (23). Cells bearing neurites at least one cell diameter in length were considered to have responded positively and were expressed as a percentage of the number of cells counted. At least 200 cells from each experimental group were analyzed.

[^{3}H]Colchicine uptake. [^{3}H]Colchicine binding experiments were performed according to Black and Greene (7). PC12 cells were plated on 6-well dishes coated with collagen/polylysine and treated with NGF (50 ng/ml) or staurosporine (50 nM) for 2 days. After being washed once with DMEM, cells were incubated with 1.3 μM [^{3}H]colchicine for different time intervals and then washed twice with DMEM and once with PBS. Cell monolayers were dissolved with 0.5 N NaOH, and aliquots were taken for radioactivity and protein determinations. Experiments were performed in triplicate, and values were expressed as cpm/mg of protein. Nonspecific binding was defined as the radioactivity associated with cell-free collagen/polylysine-coated dishes.

Competition with [^{3}H]colchicine for binding to tubulin preparations. Competition with [^{3}H]colchicine for binding to tubulin preparations was assessed as described by Ringel *et al.* (27). Briefly, 100 μl of tubulin (1 mg/ml) was mixed with 8 μM [^{3}H]colchicine in the presence or absence of competing compounds. After incubation at 37° for 45 min, aliquots of the mixtures were applied to DEAE-cellulose filter paper disks (Whatman, DE81, 2.5-cm diameter), washed 3 times with MES buffer (0.1 M MES, 2 mM EGTA, 0.5 mM $MgCl_2$, pH 6.7), and dissolved in scintillation fluid (Lumax-toluene, 1:3 v/v). The radioactivity retained in the filters was measured by a scintillation liquid *beta* counter.

Preparation of microtubule proteins and measurement of tubulin polymerization. Calf brain MTPs were purified by two cycles of temperature-dependent assembly-disassembly by a procedure modified from Shelanski *et al.* (28) as previously described (29). MTP preparations were stored at −20° in MES buffer containing 5 M glycerol, pH 6.7. Stock aliquots were diluted in glycerol-free MES buffer (1:1 v/v) and centrifuged at 30,000 rpm for 15 min. MTP samples were stabilized for 30 min before the addition of polymerizing agents (taxol, GTP) or drugs. To allow drug-MTP interactions, the compounds were added at intervals of at least 5 min. Microtubule assembly was performed in glass cuvettes, was kept in a thermostatic device at a constant temperature of 35°, and was monitored spectrophotometrically (Uvicon 930, Kontron, Switzerland). Changes in turbidity, reflecting changes in microtubule mass concentration, were monitored as changes in absorbance with time at 350 nm. Traces of continuous recordings of the polymerization kinetics are presented.

Immunofluorescence. PC12 cells were plated on collagen/polylysine-coated coverslips and treated with NGF or staurosporine for the indicated time intervals. The cells then were washed twice with PBS and exposed to microtubule-stabilizing extraction buffer (80 mM PIPES, 1 mM $MgCl_2$, 1 mM EGTA, 30% glycerol, 1 mM GTP, pH 6.6) for 2 min at room temperature and fixed with 2% glutaraldehyde in PEM buffer (80 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$) for 15 min at 37° (30). Excess glutaraldehyde was washed out with PBS containing 5% fetal calf serum. Permeabilized cells were exposed to antitubulin antibody (diluted in PBS with 5% fetal calf serum at 1:100 dilution) for 30 min at room temperature, then washed 4 times with PBS. Rhodamine-labeled anti-mouse antibody (diluted 1:200 in PBS with serum) was added for 30 min at room temperature and then washed twice with serum-supplemented PBS and 3 times with PBS. Coverslips then were photographed (Kodak T-Max 2000) at identical exposure times under a fluorescence microscope (Olympus, BH-2, Japan) fitted with a differential interference contrast attachment. Representative fields are presented.

Extraction of PC12 proteins. PC12 cells were plated on 20-cm dishes coated with collagen/polylysine and treated with staurosporine or NGF for the indicated times. After being washed once with PBS, cells were collected and centrifuged at 1000 rpm/min for 10 min at 4°. Pellets of PC12 cells were suspended in lysis buffer (50 mM Tris-HCl, pH 8.5, 1% Triton X100, 5 mM EDTA, 0.15 M NaCl, 50 μg/ml of PMSF, and 10 μg/ml of leupeptin) and gently homogenized on ice with a glass homogenizer. The lysates were then centrifuged in Eppendorf tubes (15,000 rpm) for 15 min, and the supernatants were collected and stored at −70°.

Western blotting. Samples of cells lysates (100 μg of protein measured according to Lowry *et al.* (31) and verified by Coomassie blue staining of SDS-PAGE gels) from the different treatment groups were dissolved in denaturating sample buffer (0.06 mM Tris-HCl, pH 6.8, 12.5% glycerol, 1.25% SDS, 5% β-mercaptoethanol, 0.002% bromophenol blue), boiled for 5 min at 100°, and separated at 100 V by a 10% SDS-PAGE. Proteins were electrotransferred to nitrocellulose papers overnight at 30 V, were rinsed once with TBS (20 mM Tris, 137 mM NaCl, pH 7.6), and were blocked with 3% gelatin in TBS for 1 hr. After three washes with TBS-Tween 20 (0.1%), filters were incubated with either antitubulin or anti-*tau* antibody for 1 hr at room temperature, followed by three washes with TBS-Tween 20 and incubation with HRP-conjugated anti-mouse antibody for 30 min. After three additional

washes with TBS-Tween and two with distilled water, the filters were exposed to luminol (ECL detection reagents, Amersham) for 1 min and autoradiographed on Amersham Hyperfilms, which provide very low background levels. The immunoreactive bands were scanned by laser densitometry (Helena Laboratories Quick Scan R&D, CA, laser densitometer) and expressed in arbitrary units (cm^2 of peak area). Fold increase is defined as the ratio between the intensity of immunopositive bands of NGF/staurosporine-stimulated cells divided by the intensity of immunopositive bands of control, untreated cells.

Results

Staurosporine-induced neurites are resistant to colchicine treatment. Exposure of PC12 cells to nontoxic concentrations of staurosporine (10–100 nM) resulted in rapid neurite outgrowth (Fig. 1B, Ref. 23). Unlike NGF, staurosporine induced short neurites that did not form complex networks (Fig. 1B, Ref. 23). The induction of neurites by staurosporine was already evident after 6 hr of staurosporine treatment, and the neurites reached maximum length after 2 days.

To characterize the microtubule properties of the neurites induced by short term exposure to staurosporine, we challenged the cells with drugs known to disrupt microtubule structures. Fig. 1 presents SEM photographs of PC12 cells treated for 3 days with 50 ng/ml NGF (Fig. 1 A,C) or for 1 day with 50 nM staurosporine (Fig. 2 B,D), followed by treatment for 45 min with 50 μM colchicine (Fig. 1 C,D). The NGF-treated cultures possess neurites 1 to 3 cell diameters in length (Fig. 1A). Colchicine treatment led to complete retraction of these neurites, resulting in rounded cells with convex surfaces and prominent spheric protrusions (Fig. 1C). Cells treated with staurosporine exhibited neurites 1 to 2 cell diameters in length (Fig. 1B), and the cells were flattened. However, in contrast to NGF-treated cells, exposure of staurosporine-treated cells to 50 μM colchicine for 45 min did not result in neurite retraction (Fig. 1D). These results were quantitated by the statistical evaluation of large populations of PC12 cells treated with either staurosporine or NGF, followed by colchicine, as described above. As shown in Fig. 2, the staurosporine-induced neurites are resistant to colchicine, whereas 90% of the NGF-induced neurites are colchicine-sensitive. After 7 days of NGF treatment the neurites became colchicine-resistant (data not shown) as previously reported (18). Treatment with vinblastine (1 μM for 45 min), another microtubule-disrupting alkaloid, similarly caused the retraction of neurites induced by NGF, but did not affect neurites induced by staurosporine (data not shown).

Colchicine uptake by PC12 cells is not affected by staurosporine treatment. We considered the possibility that staurosporine, because of its lipophilic nature, accumulates in the plasma membranes of treated PC12 cells and changes their properties, resulting in altered cellular uptake mechanisms. In a previous study we showed that during the first few hours of exposure to staurosporine most of the bound compound is membrane-associated, and uptake is initiated only after prolonged exposure (23). We examined, therefore, whether the staurosporine-treated cells take up colchicine at a reduced rate. As shown in Fig. 3, treatment of PC12 cells for 1 day with staurosporine did not affect the uptake of [^{3}H]colchicine into the cells. Likewise, colchicine uptake by PC12 cells is not affected by treatment with NGF (7).

Immunofluorescence analysis of tubulin in staurosporine-induced neurites. Tubulin is an abundant protein in axons and dendrites of the nervous system and is directly associated with neurite outgrowth (1, 8). Because tubulin is the target of colchicine (25), we examined staurosporine-induced neurites for their tubulin content.

Microtubule organization in staurosporine-treated, NGF-treated, and control PC12 cells was followed by immunofluorescence, with the addition of detergent-permeable PC12 and antibodies to tubulin. As shown in Fig. 4, intense and specific immunostaining was detected in the neurites and in the perikaryon periphery of both NGF-treated and staurosporine-treated cells, indicating that the microtubule organization induced by staurosporine was not different from that induced by NGF.

Staurosporine does not affect cellular tubulin content. Because neurite outgrowth requires considerable amounts of tubulin (10), we examined the levels of tubulin in PC12 cells

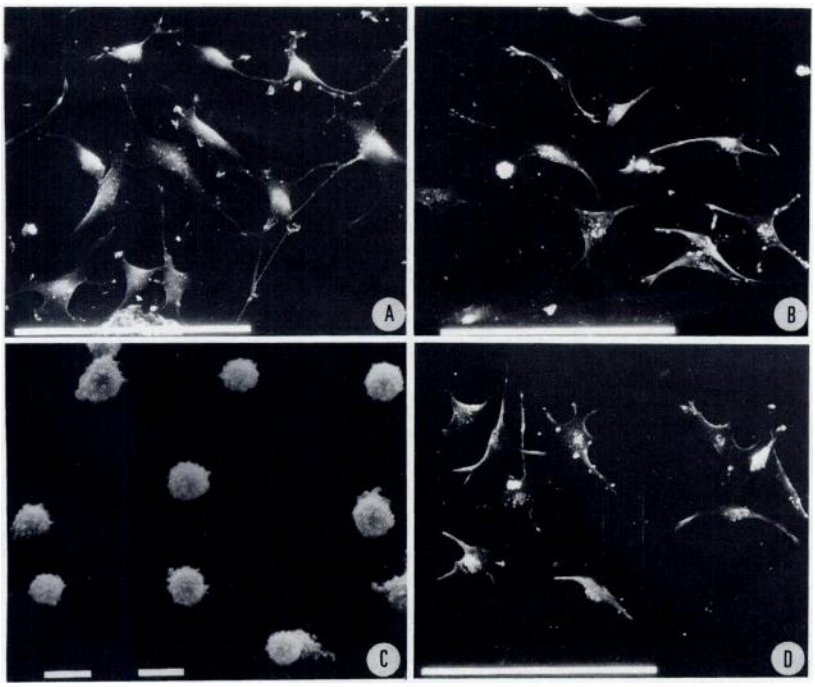

Fig. 1. SEM visualization of PC12 cells treated with NGF or staurosporine and then exposed to colchicine. PC12 cells grown on petri dishes coated with collagen/polylysine were treated with 50 ng/ml NGF for 3 days (*A, C*) or 50 nM staurosporine for 1 day (*B, D*), then were washed and exposed to PBS in the absence (*A, B*) or presence (*C, D*) of 50 μM colchicine for 45 min, and were processed for SEM as described in Materials and Methods. In *A, B,* and *D*, *bar* = 100 μm; in *C*, *bar* = 10 μM.

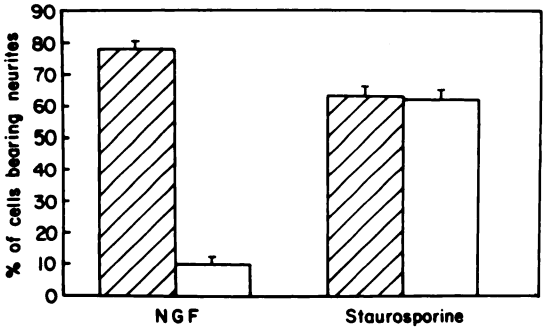

Fig. 2. Effects of colchicine treatment on PC12 cells pretreated with NGF or staurosporine. PC12 cells were grown on 24-well dishes coated with collagen/polylysine in the presence of 50 ng/ml NGF for 3 days or 50 nM staurosporine for 1 day. Cultures then were exposed for 45 min at 37°C to 50 μM colchicine (*open bars*) or 0.1% DMSO (*hatched bars*). Monolayers were washed and fixed for light microscopy as described in Materials and Methods. Phase contrast micrographs were taken for each experimental group and analyzed for the neurotropic effect (mean ± standard error).

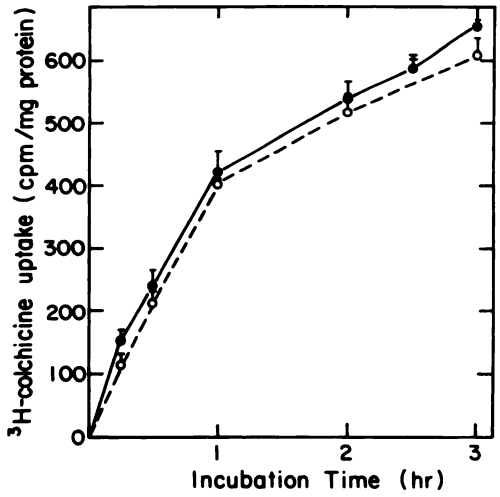

Fig. 3. Uptake of [^{3}H]colchicine by control (●——●) and staurosporine-treated (○——○) PC12 cells. PC12 cells were plated on 6-well dishes coated with collagen/polylysine and treated with 50 nM staurosporine or control medium for 2 days. After being washed with fresh medium, the cells were incubated with 1.3 nM colchicine (including [^{3}H]colchicine) for different time periods. The monolayers were washed and processed for *beta* counting, as described in Materials and Methods. Nonspecific binding, assessed by association of colchicine to cell-free dishes coated with collagen polylysine, was deducted from each experimental group. The experiment was performed in triplicate and the mean ± standard error is presented.

treated by staurosporine and compared them to those of NGF-treated cells. As shown in Fig. 5, Western blots revealed no change in the total cellular tubulin content even after 2 days of staurosporine treatment. We also have observed that prolonged treatment with NGF (≥5 days) causes a multi-fold increase in tubulin levels (data not shown) as previously reported (9).

Staurosporine does not compete with [^{3}H]colchicine for binding to tubulin. We also considered the possibility that *in vivo* competition between staurosporine and colchicine for binding to tubulin interferes with colchicine-induced microtubule depolymerization. To determine whether such competition occurs, *in vitro* [^{3}H]colchicine binding experiments were performed in the presence of staurosporine. As controls we used unlabeled colchicine and its inactive derivative colchiceine, which does not bind to the colchicine-binding site on tubulin (27). The results presented in Table 1 indicate that staurosporine, up to 80 μM, does not compete with [^{3}H]colchicine for binding to tubulin. Because these *in vitro* concentrations of staurosporine are 1000 fold higher than the neurotropic dose, the results suggest that there is no competition between staurosporine and colchicine in intact cells.

Staurosporine does not affect tubulin polymerization properties. Because staurosporine does not compete with colchicine for binding to tubulin, we wondered whether it has any other functional effects on the tubulin structure that might alter its polymerization properties. We examined this possibility by studying the effect of staurosporine on tubulin polymerization induced by GTP or taxol (28, 29). Staurosporine did not affect the polymerization induced by either GTP (Fig. 6, *trace a* and *b*) or taxol (Fig. 6, *trace c* and *d*) when added either before or after the polymerizing agent. It is well known that colchicine blocks GTP-induced tubulin polymerization but has no effect on taxol-induced tubulin assembly (Fig. 6, *trace e*) (29). From Fig. 6 it is clear that staurosporine does not interfere with the action of GTP, colchicine, or taxol. These results exclude the possibility that staurosporine treatment causes changes in tubulin properties that might inhibit colchicine-induced tubulin depolymerization.

Staurosporine increases *tau* protein levels. The maturation of neurites induced by NGF was shown to be accompanied by induction of *tau* proteins and appearance of new, mature *tau* isoforms, which are involved in microtubule stabilization (18). We were interested, therefore, in examining whether staurosporine affects *tau* protein levels. As shown in Fig. 7, staurosporine induced an increase in cellular *tau* protein levels. The transient elevation in *tau* levels was evident within 2 hr, reached its maximum (2- to 3-fold increase) at 5 hr, and decreased thereafter, returning to basal levels after another 10 hr of staurosporine treatment. Levels of both the low molecular mass (50–60 kDa) and the high molecular mass *tau* proteins (100–110 kDa) were increased similarly by staurosporine (Fig. 7).

Staurosporine at concentrations of 10 nM or less, at which neurite outgrowth is not promoted (23), did not increase *tau* protein levels. However, at a concentration of 10–70 nM, staurosporine elicited a dose-dependent increase in levels of *tau* proteins. Staurosporine concentrations exceeding 100 nM are cytotoxic to PC12 cells on chronic exposure (23), and, therefore, were not tested.

Discussion

Staurosporine was shown previously to induce rapid neurite outgrowth in PC12 cells (23) and, therefore, is potentially useful as a drug that promotes sprouting and regeneration of neurons. In the present work, we have extended our study of the neurotropic action of staurosporine in an attempt to better understand its mechanism of action and further substantiate its clinical potential.

NGF-induced neurite outgrowth appears to involve a series of biochemical changes of cytoskeletal components (9, 10), which underlies the initiation, elongation, and stabilization of the neurites (18). Upon long-term NGF treatment (more than 5 days), the neurites acquire colchicine resistance and, unlike the early neurites, do not retract upon exposure to colchicine (7). In the present study we found that, in contrast to NGF-induced neurites, staurosporine-induced neurites acquire colchicine resistance after exposure for only 1 day. To substantiate

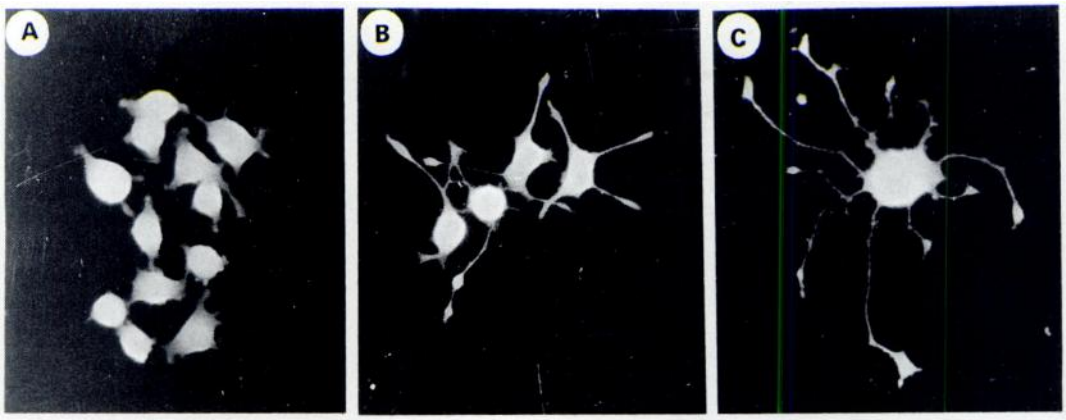

Fig. 4. Tubulin immunostaining of PC12 cells treated with NGF or staurosporine. PC12 cells were grown on glass coverslips and treated with 50 nM staurosporine for 2 days or 50 ng/ml NGF for 4 days. After being washed, the cells were permeabilized and processed for the immunofluorescence reaction as described in Materials and Methods. The coverslips were examined under a fluorescence microscope, and typical fields were photographed. A, control PC12 cells; B, cells treated with staurosporine; C, cells treated with NGF. Magnification ×350.

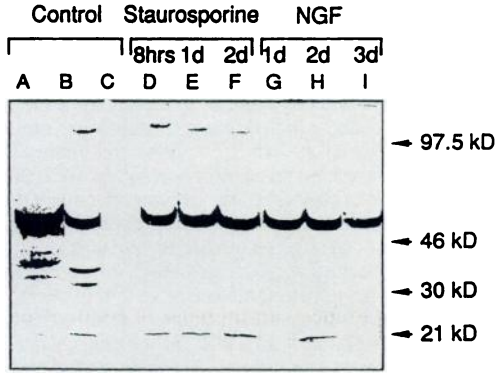

Fig. 5. Western blots analyses of tubulin in PC12 cells after treatment with staurosporine or NGF. PC12 cells were grown on 20-cm petri dishes coated with collagen/polylysine and were treated with 50 ng/ml NGF or 50 nM staurosporine for different time periods. Monolayers then were prepared for Western blotting, as described in Materials and Methods. Equal amounts of cellular proteins of different experimental groups were subjected to electrophoresis on 10% SDS-PAGE, electrotransferred to nitran papers, and reacted with antitubulin antibody as described in the text. Visualization of immune complexes was achieved by ECL detection system and fluorography. *Arrows* indicate the mobility of rainbow-colored molecular weight markers. A, Control tubulin preparation as reference; B, control, unstimulated cells; C, control, unstimulated cells in competition with tubulin preparation; D, E, F, after treatment with staurosporine for the indicated time periods; G, H, I, after treatment with NGF for the indicated time periods. Specificity of the tubulin immune reaction is indicated by the strong reaction of isolated tubulin (*A*) and the disappearance of the tubulin immunoreactive band in (*C*) caused by antibody competition between the soluble tubulin preparation and the immobilized cellular tubulin on the filter.

this observation, we performed experiments aimed at characterizing colchicine-resistant neurites and suggesting a mechanistic explanation for the colchicine resistance.

Resistance of neurites to colchicine treatment could result from a wide range of cytological changes, including altered uptake and degradation mechanisms or changes in the cytoskeletal components of the cells (32). We showed that colchicine uptake is not affected by staurosporine treatment. Besides, because staurosporine-induced neurites are resistant to both colchicine and vinblastine, which differ in their chemical structures, the observed resistance is unlikely to be the result of a specific degradation system. Because tubulin is the target of colchicine, we examined whether the staurosporine-induced neurites have a different cytoskeletal organization from that of

TABLE 1

Competition with [^{3}H]colchicine for binding to tubulin *in vitro*

Tubulin (1 mg/ml) was mixed with 8 μM [^{3}H]colchicine. Increasing concentrations of the competing compounds were added for 45 min at 37°. Tubulin-associated radioactivity was measured as described in Materials and Methods. The experiment was performed in triplicate. Values in parentheses are percentages of total binding (9270 ± 500).

Compound tested	Concentration (μM)		
	4	20	80
Colchicine	8871 ± 454 (95%)	5111 ± 496 (55%)	3098 ± 311 (33%)
Colchiceine	9096 ± 646 (98%)	9484 ± 1023 (102%)	6120 ± 200 (66%)
Staurosporine	9261 ± 572 (100%)	8523 ± 294 (92%)	9560 ± 865 (103%)

NGF-induced neurites, or contain cytoskeletal proteins that are less affected by colchicine. Such a candidate protein considered was actin, which is known to participate in cellular movement and cellular extension (33). We found that tubulin immunostaining in staurosporine-treated PC12 cells is similar to that in NGF-treated cells. Moreover, staurosporine did not bind to colchicine-binding site on tubulin or to any other site that would alter its colchicine-binding properties. Finally, we excluded a number of theoretical functional interactions between staurosporine and drugs affecting tubulin assembly properties.

Microtubule-associated proteins include a set of high molecular weight members, termed MAP1 to MAP5, and low molecular weight members, including *tau* and chartins (34, 35). MAP proteins are known to promote tubulin polymerization *in vitro* (36). *Tau* proteins appear to be essential components of neurite outgrowth (37). Chronic NGF treatment, which induces colchicine-resistant neurites, was found to be correlated with increased synthesis of *tau* proteins (9, 18). Increased *tau* protein levels and changes in their isoforms were observed in the developing central nervous system (38, 39) as well as in differentiating neuroblastoma (40) and PC12 cells (9, 18). Because *tau* proteins promote the stabilization of tubulin polymers and, therefore, might antagonize colchicine actions, we examined the levels of *tau* proteins after staurosporine treatment. We found a transient, but substantial, increase in *tau* proteins in PC12 cells within 5 hr of staurosporine treatment; this increase was already evident at 1 hr, reached a maximum (2- to 3-fold increase) at 5 hr, and returned to basal levels during the next

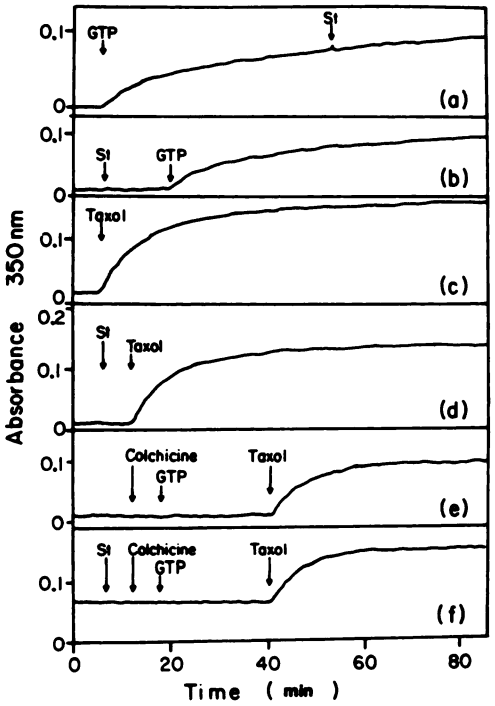

Fig. 6. Effects of staurosporine on tubulin polymerization by GTP and taxol *in vitro*. Tubulin polymerization was assessed spectrophotometrically as described in Materials and Methods and detailed elsewhere (23). Tubulin samples (1 mg/ml) in 1 ml of MES buffer with 3M glycerol were incubated at 35° in the presence or absence of staurosporine (St., 1 μM) for 15 min before the addition of colchicine (5 μM), GTP (1 mM), or taxol (20 μM). Alternatively, polymerization of tubulin was initiated by GTP or taxol, and staurosporine was added at the indicated time. *Arrows* indicate the addition of compounds to the cuvette.

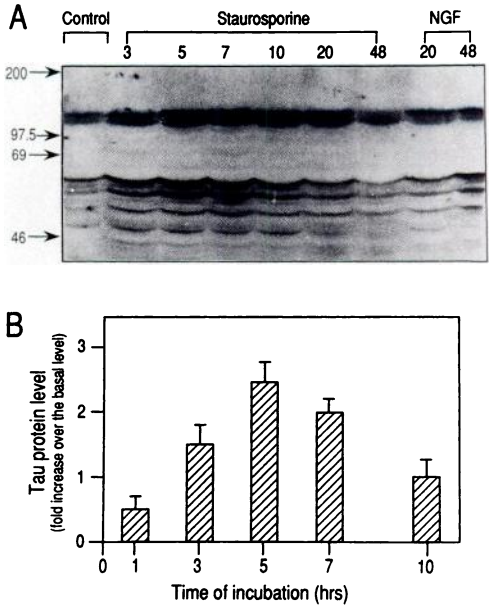

Fig. 7. Kinetics of the staurosporine-induced increase in *tau* protein levels. One day before the experiment, PC12 cells were plated on 20-cm dishes coated with collagen/polylysine and treated with staurosporine (50 nM) or NGF (50 ng/ml) as indicated. After treatment, cells were harvested, lysed and subjected to electrophoresis on 10% SDS-PAGE, electrotransferred to nitrocellulose filters, and immunostained with anti-*tau* antibody, an ECL detection system. A, a representative Western blot autoradiograph of PC12 cell cultures treated with staurosporine or NGF; *numbers* on top of the autoradiograph indicate hr of treatment. *Arrows* indicate mobility of rainbow-colored molecular weight markers. B, quantitation of *tau* protein levels by laser densitometry in four different kinetic experiments. Levels of 55- and 110-kDa *tau* proteins were analyzed separately. Values (mean ± standard error) represent *tau* proteins level in the treated cells relative to their level in unstimulated control cells.

10 to 15 hr. The *tau* proteins that showed increased levels included all isoforms ranging between 55 and 65 kDa as well as the high molecular mass *tau* of 110 kDa. This latter *tau* form was described recently in the peripheral nervous system as well as in chromaffin cells (41), the normal counterpart of PC12 cells. The kinetics of the staurosporine-induced increase in *tau* levels in PC12 cells correlated well with those of the staurosporine-induced neurite outgrowth previously documented by us (23), and might point to a causal relationship. The observed increase in *tau* levels also could be explained partly by changes in its post-translational modification (i.e., state of phosphorylation). This is based on the information that the reactivity of *tau*-1 antibody used in this study may be affected by the state of *tau* phosphorylation (45). As previously documented, the difference caused by phosphorylation, which is reflected in the antibody's reactivity, is much less than could account for the 2- to 3-fold increase in *tau* levels observed in this study. However, because *tau* is a phosphoprotein (45), and because staurosporine is a PKC inhibitor (21) known to be involved in *tau* phosphorylation, this consideration cannot be excluded completely. Either outcome, i.e., an increase in *tau* protein levels and/or a decrease in its phosphorylation, will induce microtubule stabilization.

Tubulin antisense oligonucleotide blocks the induction of neurites by NGF early in PC12 cells differentiation (42). In contrast, addition of *tau* antisense does not inhibit the initial phase of neurite induction, but blocks the stabilization of these neurites, resulting in their regression after 2 to 3 days of NGF treatment (18). These findings suggest that tubulin underlies the initiation and elongation of the neurites, and that the late induction of *tau* promotes their stabilization (18). In the present study, we could not detect any changes in tubulin levels during the first 2 days of staurosporine treatment. However, we did observe a transient increase of *tau* proteins. We assume that the rapid induction of *tau* proteins by staurosporine, but not by NGF, may explain the observed early stabilization of the neurites to colchicine treatment. It is tempting, therefore, to propose that the early increase in *tau* protein levels, which is correlated with the staurosporine-induced neurotropic effect, is required for the rapid induction of neurite outgrowth by staurosporine.

In conclusion, we suggest that the ability of staurosporine to induce rapid increase in *tau* protein levels and/or affect its post-translational modification promotes neurite outgrowth *in situ* and, therefore, that staurosporine is potentially useful as a drug to enhance sprouting. It seems, however, that staurosporine will not be effective in the early phases of regeneration, when the newly injured neuron has to reorganize the cytoskeletal elements of its recovering neurites (47); an increase in microtubule-stabilizing proteins, like *tau*, might inhibit these processes by changing the dynamic features of the microtubule system to more rigid polymeric structures. Structure-function studies with staurosporine analogs (48) might result in the discovery of a neurotropic drug that could be applied also during the first phases of neurites regeneration.

Further understanding of staurosporine's mechanism of action and the properties of staurosporine-induced neurite outgrowth will provide the necessary pharmacological background for the development of adequate neurotropic drugs.

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