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Protein Kinase- and Staurosporine-Dependent Induction of Neurite Outgrowth and Plasminogen Activator Activity in PC12 Cells

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ABSTRACT. We analysed how interactions between protein kinase-dependent intracellular signalling pathways were implicated in the control of the production of tissue-type plasminogen activator (tPA) and the generation of neurite outgrowth by PC12 cells. To that aim, cells were treated with agents that interact with the *trk* receptor and with protein kinases A and C. Nerve growth factor induced only the formation of large neurites. The release of the protease and the production of short neurite outgrowth were found to be protein-kinase-A-dependent events that could be enhanced by simultaneous activation of protein kinase C with phorbol ester. At high concentration, staurosporine, a nonselective inhibitor of protein kinases, induced the production of short neurites and mimicked the protein-kinase-A-dependent effect on tPA release. Such a response was not observed with K-252a, an analogue of staurosporine devoid of neurite-outgrowth-promoting activity. The responses to protein kinase A stimulation and the addition of staurosporine, although similar, seemed to occur through an activation of distinct, yet interacting, signalling pathways. In conclusion, tPA release and large neurite outgrowth from PC12 cells are controlled by parallel, albeit interacting, pathways, suggesting that these two potentially antagonistic events in PC12 cell differentiation can be modulated in a concerted way or independently of each other, depending on the activity of several protein kinases. BIOCHEM PHARMACOL 52;9:1399–1405, 1996. *Copyright © 1996 Elsevier Science Inc.*

KEY WORDS. proteases; plasminogen activator inhibitor; nerve growth factor; K-252 compounds

PC12 pheochromocytoma cells, the transformed counterparts of chromaffin cells, have been used extensively for the study of the biochemical events underlying neuronal differentiation induced by NGF.† Upon treatment with NGF, the proliferating PC12 cells stop dividing and undergo the morphological and biochemical changes associated with sympathetic neuronal differentiation [1]. Inhibition of NGF-induced differentiation by K-252 microbial protein kinase inhibitors led to the conclusion that NGF action was dependent on the activity of a protein kinase closely associated with the NGF receptor [2–4]. This protein kinase was recently shown to be the high-affinity NGF receptor $gp140^{trk}$ itself [5, 6]. This receptor (the *trk* receptor) has an intracellular tyrosine kinase domain that is ligand activated [7, 8].

The intracellular signalling pathways involving Ca^{2+}/phospholipid-dependent protein kinase (or PKC) and cAMP-dependent protein kinase (PKA) have been implicated in many aspects of NGF actions. Some of the morphological and biochemical effects of NGF can be mimicked by the direct activation of either PKA or PKC [9–12]. However, the morphological changes induced by NGF and many associated biochemical responses have also been obtained in PC12 cells treated with inhibitors of PKA [13] or made deficient in PKA [14], in PKC [15, 16] or in PKA and PKC [17, 18]. Thus, PKA- and PKC-dependent events appear to be part of parallel pathways modulating the specifically NGF-dependent intracellular transducing pathway.

Neurite outgrowth, a morphological process used as a criterion of neuronal differentiation, can be induced in PC12 cells by treatment with NGF and mimicked in part by activation of PKA with cAMP analogues. The number, stability and length of the neurites are all higher when NGF is the inducing agent, suggesting that *trk*-mediated and PKA-mediated responses involve different signalling pathways. These pathways, however, interact because the simultaneous stimulation of *trk* and PKA induces a synergistic response [19, 20]. PKC activation is also known to stimulate neuritic outgrowth in PC12 cells but only when the differentiation of these cells is induced by simultaneous

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† *Abbreviations:* CM, conditioned medium; dibutyryl-cAMP, dibutyryl-cyclic-AMP; DME, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; NGF, nerve growth factor; PA, plasminogen activator; PAI, plasminogen activator inhibitor; PBS, phosphate-buffered saline; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RPMI, RPMI 1640 medium; tPA, tissue-type PA.

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PKA activation [21]. Increased PKC activity has no effect by itself on the morphology of PC12 cells, but it does shift the dose–response curve of NGF in initiating neurite outgrowth [22].

The importance of these intracellular pathways in controlling the neuronal differentiation of PC12 has been further studied in experiments where inhibitors were used to modulate the activity of the different protein kinases. One unexpected result of these investigations was the finding that staurosporine, a member of the K-252 family of alkaloid inhibitors of protein kinases, induced a limited neurite outgrowth when used by itself at high concentration [4, 23].

During NGF-induced differentiation, PC12 cells extend neurites that adhere to ECM proteins. Neurite outgrowth and adhesion to ECM components is under the control of local proteolytic activity provided by the serine protease PAs [24–27]. In the course of our study of the role of extracellular proteolytic activity in neuronal differentiation, we found that the release of tPA was stimulated by activation of the PKA-dependent pathway in PC12 cells but was independent of activation of *trk* [24]. In the present study, we report how the release of tPA and the induction of neurite outgrowth by PC12 cells are affected by modulation of PKA and PKC activity and by treatment with staurosporine. This finding allows a more precise understanding of some of the interactions that can occur between these protein-kinase-dependent pathways during the neuronal differentiation of PC12 cells.

MATERIALS AND METHODS

Materials

DME, RPMI and fetal calf serum were obtained from Gibco (Ghent, Belgium). Donor horse serum was obtained from Flow (Brussels, Belgium). A sterile solution of bovine serum albumin (Path-O-Cyte-4) was obtained from Miles (Elkhart, IN, USA). All other cell culture nutrients, dibutyryl-cAMP, and staurosporine were obtained from Sigma (St Louis, MO, USA), PMA was obtained from Pharmacia and K-252a from ICN (Costa Mesa, CA). NGF was prepared by the method of Mobley *et al.* [28]. tPA purified from human melanoma cells was a gift of Dr. D. Collen (KUL, Leuven, Belgium). Human plasminogen and bovine fibrinogen fragments were prepared as described [29]. Protein concentrations were determined by the method of Lowry *et al.* [30].

Cell Culture and Conditioning

PC12 cells were grown in RPMI medium supplemented with 5% fetal calf serum and 10% donor horse serum in 10-cm Petri dishes (Nunc, Roskilde, Denmark). The cells were passaged every 6 days, with a complete medium change after 3 days. For experiments, the cells were plated at 100,000 cells per well in 24-well multi dishes (Nunc) in 0.5 mL of serum-supplemented growth medium. After 16 hr, the cultures were rinsed once with serum-free RPMI and fed again with DME-SATO+ medium composed of DME supplemented with the following nutrients: progesterone, 2×10^{-7} M; putrescine, 2×10^{-4} M; L-thyroxine, 5×10^{-7} M; selenium, 2.25×10^{-7} M; tri-iodo-thyronine, 5×10^{-7} M; transferrin 1.25×10^{-9} M; bovine insulin, 10 μg/mL; and 1% (vol/vol) bovine serum albumin (Path-O-Cyte 4). After 24 hr, NGF (usually at 50 ng/mL), dibutyryl-cAMP (usually at 10^{-3} M), PMA (usually at 10^{-8} M) or staurosporine (between 10^{-10} and 10^{-7} M, higher concentrations induced some cell toxicity) were added in DME-SATO medium, which is similar to DME-SATO+ medium but lacking bovine serum albumin. Conditioning was usually for 24 hr in the presence of the growth factors. The media were collected and centrifuged at 2000*g* for 5 min.

Measurement of Neurite Outgrowth

Neurite outgrowth from PC12 cells was measured after fixation of the cells with 4% paraformaldehyde. Cultures were observed with a phase-contrast microscope (magnification 125×) and cellular processes longer than 1 cell diameter were counted in 1 × 1-mm fields. The neuritic index was calculated by dividing the number of these processes by the number of cell bodies (at least 200 per count) in these fields. Results are expressed as mean ± SD. *P* values were calculated by the Bonferroni *t* test.

PA Measurement and Analysis

PAs in the conditioned media were measured by a colorimetric assay [29] that allows discrimination of Urokinase and tPA activities. PA species were visualized by direct zymography on casein-agar gels [24]. The zymography technique applied to PC12 CMs showed the presence of two bands at 75 and 110 kDa joined by a continuous smear of proteolytic activity (Fig. 4). The lower mol wt species was previously identified as tPA and the higher mol wt species as a complex between tPA and PAI. Thus, part of the tPA protein in the extracellular medium was complexed with PAI released by PC12 cells to form the partially dissociating tPA–PAI complex.

RESULTS

Effect of Dibutyryl-cAMP and NGF on tPA Release and Neuritic Outgrowth

The release of tPA by PC12 cells was not stimulated by treatment of the cells with NGF alone (up to 50 ng/mL; Fig. 1). When added with 1 mM dibutyryl-cAMP, NGF at concentrations from 0.5 to 50 ng/mL did not increase the concentration of total tPA in the conditioned media (Fig. 1). Because NGF enhances the release of PAI activity, the tPA released by dibutyryl-cAMP plus NGF-treated cells is rapidly involved in formation of tPA–PAI complexes [24]. Accordingly, the measurement of tPA activity in such CMs showed a progressive inhibition of tPA due to an increased release of PAI and subsequent formation of enzymatically inactive tPA–PAI complexes (Fig. 1).

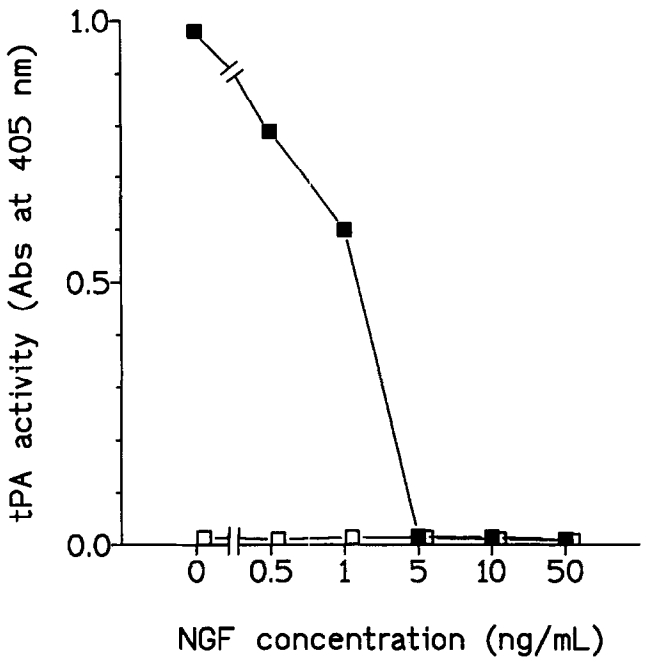

FIG. 1. tPA activity released by PC12 cells treated with different concentrations of NGF in the absence (open squares) or the presence (closed squares) of 1 mM dibutyryl-cAMP. Media conditioned by 5 × 10^4 cells per well in the presence of the different concentrations of NGF shown were analyzed for tPA activity by a colorimetric assay as described in Materials and Methods.

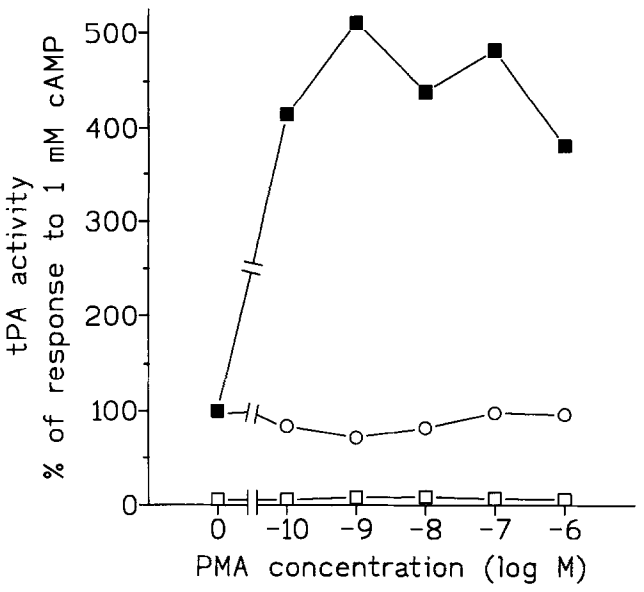

FIG. 2. tPA activity released by PC12 cells treated with different concentrations of PMA in the absence or the presence of 1 mM dibutyryl-cAMP. Media were conditioned and analysed as described in Fig. 1. Cells were treated with PMA (open squares) or PMA plus dibutyryl-cAMP without (closed squares) or with (open circles) a 24-hr pretreatment with 10^{-6} M PMA to downregulate PKC activity. tPA activity is represented as the percentage of the tPA activity released in the presence of 1 mM dibutyryl-cAMP alone (1.0–1.2 optical density at 405 nm for each experiment).

Effects of PKC and PKA Stimulation on the Release of tPA Activity and Neurite Outgrowth from PC12 Cells

PMA was used as a stimulating agent for PKC. By itself, PMA had no significant effect on tPA release (Fig. 2). To investigate whether the simultaneous activation of PKC- and PKA-dependent pathways would modulate the release of tPA differently, we added 1 mM dibutyryl-cAMP with PMA at concentrations ranging from 10^{-10} to 10^{-6} M. An increase in the release of tPA (Fig. 2) and the subsequent increased formation of tPA-PAI complexes were confirmed by zymography (Fig. 3). This effect was dependent on PKC activity because it disappeared after preincubation with 10^{-6} M PMA for 24 hr, a procedure commonly used to downregulate PKC (Fig. 2). Measurement of tPA activity in the CMs of cells in which both PKA and PKC were stimulated showed a 4- to 5-fold increase in the release of tPA activity as opposed to the level seen when only PKA was activated (Fig. 2).

PMA had no significant neurite promoting activity on PC12 cells (Table 1). However, PMA has been shown to act synergistically with 1 mM dibutyryl-cAMP, inducing a neurite production that is quantitatively similar to that observed after treatment with NGF [21, 31]. We found that the neuritic index was similar after treatment with a combination of dibutyryl-cAMP and PMA or after treatment with 50 ng/mL NGF. Approximately 60% of cells showed neurites after each of these treatments as opposed to 35%for dibutyryl-cAMP alone and 13% for PMA alone (Table 1).

Effect of Staurosporine on tPA Release and Neurite Outgrowth

Staurosporine was used to analyse further the role of protein-kinase-dependent intracellular pathways in the control of tPA release and neurite outgrowth from PC12 cells. Conditioned media of cells induced to grow neurites by treatment with different concentrations (10^{-10}–10^{-7} M) of staurosporine were analysed for the presence of tPA by zymography and enzymatic activity assay. These cells were found to release large amounts of tPA in their conditioned media (Figs. 4 and 5). As with the formation of neurites (Fig. 4), production of tPA is maximally elicited between 10^{-8} and 10^{-7} M staurosporine. Comparison with the response obtained after treatment with cAMP analogue showed that staurosporine could induce 10 times as much tPA activity as that released after PKA activation with 1 mM dibutyryl-cAMP (Fig. 5). To determine whether this effect of staurosporine is due to an interaction of this compound at some point of the cAMP-dependent pathway or involves a direct action on tPA release, we looked for a possible additivity of the effects of both agents. Instead of such an additivity, we found that the concentrations of staurosporine able to induce the production of tPA were lower when the cells were simultaneously treated with 1 mM dibutyryl-cAMP. Together with this shift to lower active staurosporine concentrations, a synergistic effect of dibutyryl-cAMP and staurosporine was apparent when this latter compound was used at concentrations between 1 and 30 × 10^{-9} M (Fig. 5).

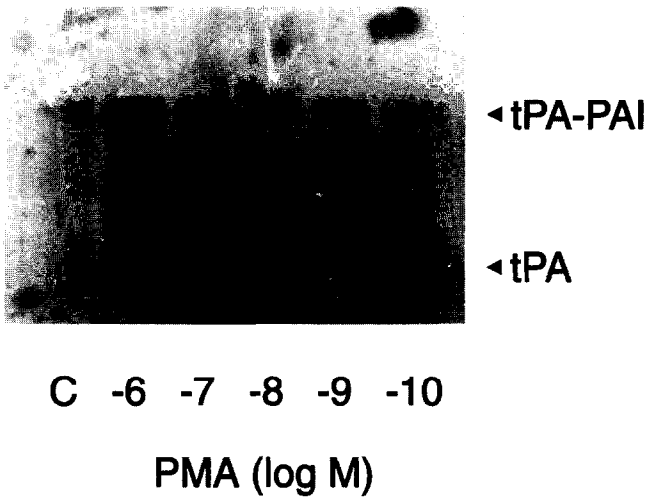

FIG. 3. PA species released by PC12 cells treated with different concentrations of PMA and 1 mM dibutyryl-cAMP. Media were conditioned as described in Fig. 1. The positions of tPA and tPA–PAI complexes are shown on the right.

To determine whether the effect of staurosporine on tPA release is closely associated to its neurite-promoting activity, we tested whether K-252a, a closely related analogue of staurosporine that does not induce neurites in PC12 cells, was also able to increase the release of tPA by PC12 cells. Figure 5 shows that, unlike staurosporine, K-252a had no effect on tPA release.

DISCUSSION

In the present study, the relationship between intracellular signalling pathways that control neurite outgrowth and the production of tPA in PC12 cells has been investigated by using a relatively selective protein kinase inhibitor and PKA and PKC agonists as pharmacological tools. The differentiation of these cells, expressed in neurite outgrowth, is induced by *trk*- and PKA-dependent pathways, each of which elicits the appearance of processes that differ in size and stability [19]. Neurite outgrowth is also seen after treatment with high concentrations of staurosporine [4, 23]. How these three types of process formation depend on interactions between distinct intracellular pathways has been studied in this work by comparing the morphological responses with the induction of tPA release by the cells.

The interaction between PKA- and *trk*-dependent signalling pathways appears in our study to be restricted to a positive effect of cAMP analogue on NGF-induced neuritic outgrowth. No effect of NGF was found on tPA release, a PKA-dependent process [24], thus confirming the findings of Pittman and DiBenedetto [27]. An interaction between PKA-dependent and mitogen-activated protein kinase pathway (controlled by the *trk* receptor) has been described in the PC12 cells. It has been recently shown that stimulation of PKA in these cells by different agents directly activates the MAP kinase cascade by increasing the activity of the MAP kinase kinase [32]. This cAMP-dependent stimulation of the MAP kinase cascade is increased by treatment with NGF or PMA, thereby providing a molecular framework that can be used to explain the synergistic action on the differentiation of PC12 cells of PKA and *trk* activation, on the one hand, and PKA and PKC activation, on the other.

When tPA release was measured from cells subjected to activation of both PKA and PKC, another interaction between signalling pathways became apparent. Such a positive interaction between the two protein kinase pathways has been documented in studies of neurite outgrowth [21, 31]. The absence of effect of PMA alone on tPA release and neurite outgrowth suggests that the increase in these two

TABLE 1. The neurite outgrowth effect of dibutyryl-cAMP, PMA and NGF on PC12 cells*

Conditions	% Cells with neurites†
Control	8.09 ± 1.12
1 mM dibutyryl-cAMP	35.3 ± 3.41
10^{-8} M PMA	13.1 ± 2.19‡
1 mM dibutyryl-cAMP + 10^{-8} M PMA	60.1 ± 7.54
50 ng/mL NGF	54.0 ± 4.52#

* Cells were treated with dibutyryl-cAMP, PMA, PMA plus dibutyryl-cAMP or NGF. Cells were fixed after 48 hr and the neurites counted as described in Materials and Methods.

† The cellular processes with a length longer than 1 cell diameter were counted in four 1- × 1-mm fields. The neuritic index was calculated by dividing the number of these processes by the number of cell bodies (at least 200/count) in these fields. Results are expressed as mean ± SD.

‡ Not significantly different from control by Bonferroni *t*-test.

Not significantly different from dibutyryl-cAMP + PMA treatment by Bonferroni *t*-test.

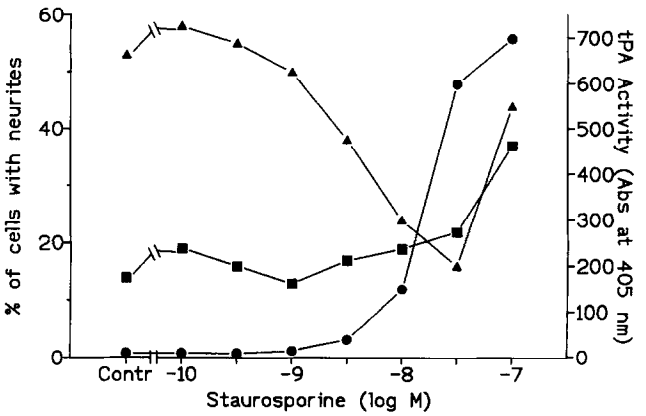

FIG. 4. Effect of staurosporine on tPA release and neurite outgrowth from PC12 cells. Cells were treated with increasing concentrations of staurosporine for 48 hr, after which media were collected for tPA activity measurement (circles, right scale). The inhibition by increasing concentrations of staurosporine of the neurite outgrowth induced by treatment with 50 ng/mL NGF (triangles), and the neurite-promoting effect of increasing concentrations of staurosporine (squares) are plotted relative to the left scale. Cells were fixed after 48 hr and the neurites counted as described in Materials and Methods.

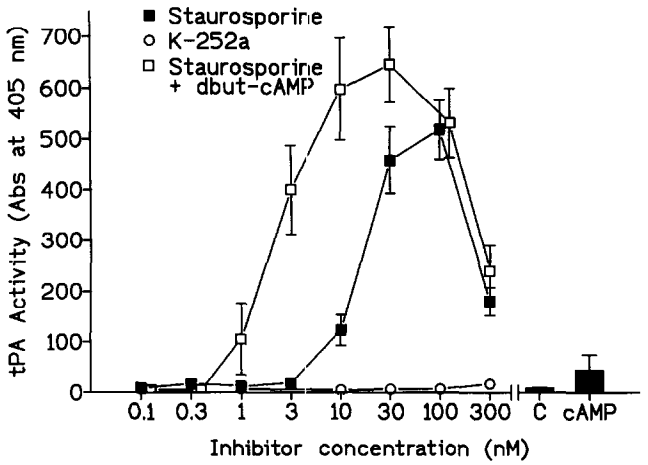

FIG. 5. Quantitative measurements of the effect of staurosporine and K-252 on tPA release from PC12 cells. Cells were treated with increasing concentrations of staurosporine (closed squares) or K-252a (open circles) for 48 hr, after which media were collected for tPA activity measurement. Cells were also treated with 1 mM dibutyryl-cAMP and increasing concentrations of staurosporine for 48 hr, after which media were collected for tPA activity measurement (open squares). tPA activity released by untreated (C) and 1 mM dibutyryl-cAMP-treated cells (cAMP) is shown by the closed bars.

responses when both PKA and PKC are activated is due to a positive effect of a PKC-dependent process on the signalling pathway controlled by PKA. A positive effect of PMA on the accumulation of cAMP in PC12 cells has been shown to occur [33]. Thus, a positive interaction between the PKA and PKC pathways might be involved in a modulation of some of the cellular responses associated with neuronal differentiation. The local production of protease activity is possibly such a response, one that could be triggered by local environmental cues acting on specific receptors signalling to PKA or PKC pathways. Because it is controlled by PKA activity independently of the *trk*-dependent pathway, the release of tPA will lead to increased protease activity only when an appropriate stimulus is presented to the cell, even if this cell is at the same time actively growing neurites following NGF stimulation. This effect and the simultaneous release of PAI activity controlled by the *trk*-dependent pathway [24] ensure that the extracellular protease activity in differentiating PC12 cells remains highly regulated to allow efficient neurite outgrowth.

Staurosporine at high concentration was found to increase neurite outgrowth [4, 23, this study] and the release of tPA by PC12 cells. These two effects are elicited by similar concentrations (10–100 nM) of staurosporine, suggesting that they could be induced by a similar mechanism. The concentrations of staurosporine that stimulate the release of tPA are shifted to lower values (1–10 nM) when the cells are simultaneously subjected to PKA activation. This phenomenon is indicative of an increased affinity for staurosporine of a putative binding site in the transduction chain, and it could be studied by binding experiments with ^{3}H-staurosporine [34]. A question addressed in view of these results is that of the relationship between the PKA signal transduction pathway and intracellular mechanisms triggered by high concentrations of staurosporine. The similarity of the cellular responses induced by the two treatments suggests that a single transduction chain might be the target of both cAMP analogues and staurosporine. However, earlier work with staurosporine had led to the conclusion that the mechanism of formation of neurites elicited by staurosporine was different from that elicited by NGF or dibutyryl-cAMP [23]. Thus, it is more likely that the two agents produce different signals that converge upstream of the mechanisms of induction of neuritogenesis and tPA release. Several investigators have also examined the molecular mechanism by which staurosporine exerts its effects at high concentration. The positive effect on tPA release and short neurite production could result from an inhibition of a kinase acting negatively downstream from the PKA-dependent pathway. Supporting this hypothesis is the observation that staurosporine inhibits the phosphorylation of tyrosine on several proteins in hippocampal neurons [35] and of tau, a microtubule-associated protein, in PC12 cells [36]. However, because the maximal effect on tPA release by PC12 cells is more pronounced after staurosporine addition than after PKA activation, staurosporine may directly activate this response. It is interesting to note in this context that staurosporine induces the tyrosine phosphorylation of a 145-kDa protein distinct from gp140^{trk} [37] and at high concentration stimulates a 60-kDa protein kinase in chromaffin cells [38]. K-252a, which does not have a neurite-promoting activity in PC12 cells, is also unable to increase the release of tPA. This finding suggests that the differentiating effect of staurosporine results from a specific interaction with (a) target kinase(s) not recognized by other protein kinase inhibitors of the K-252a type. The different members of this inhibitor family show a spectrum of toxic and trophic activities directed toward distinct subsets of neurons [39]. It is likely that this variety of effects and target cells is the consequence of the interaction with different protein kinases by increasing concentrations of each of the inhibitors, thus explaining the complex dose–response relationships seen at the cellular level.

By at least three different mechanisms staurosporine can influence the neuronal phenotype of PC12 cells: (1) PKC, which is inhibited at subnanomolar concentrations; (2) *trk*, which is inhibited at nanomolar concentrations; and (3) a third unknown target that is more closely related to the PKA-dependent pathway and is activated at submicromolar concentrations. These possibilities are featured on Figure 6, which is a tentative representation of the interactions between the intracellular pathways found to be involved in our study of PC12 cell differentiation. It is based on the findings that treatment with NGF and PKA activation induce distinct events (in this study, long neurites for NGF and tPA release and short neurites for PKA activation) through parallel signalling pathways. The *trk*-dependent

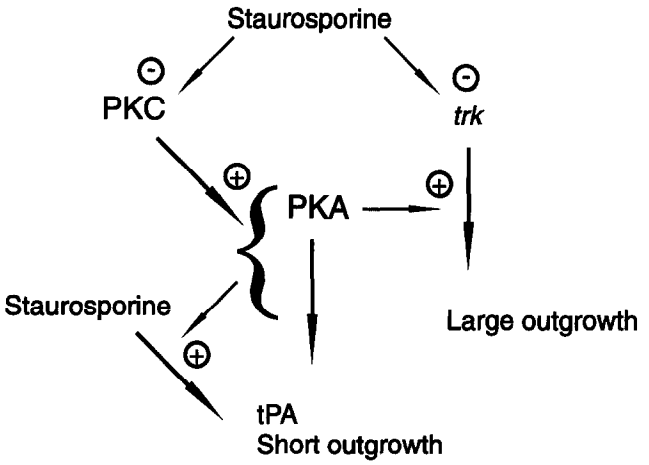

FIG. 6. Schematic representation of the putative intracellular pathways controlling the production of tPA and neuritic outgrowth in PC12 cells. Positive interactions between pathways are represented by a plus sign within a circle. The localization where staurosporine exerts its positive and negative effects are also shown.

pathway can be positively activated by PKA activity, and the PKA-dependent responses are themselves enhanced by increased PKC activity. Functional consequences of these different interactions are that increased protein kinases activity will be able to modulate positively the NGF-induced response of PC12 cells. Such positive effects could be used to convey cellular environment information modulating neuronal differentiation. The transduction of such information originating from the extracellular space has recently been shown to occur through the binding of specific cell adhesion molecules to their receptor in the PC12 cell membrane [40] and to involve the specific activation of second messenger systems [41].

The positive effects of staurosporine on the PC12 cell responses that we have studied are seen at concentrations that will inhibit these different pathways, thus making it unlikely that such a molecule could be used as a neurotrophic agent [42] without its target specificity first being greatly improved. Clearly, progress still has to be made in the design of staurosporine analogues and in the study of the interactions between the intracellular pathways that control neuronal differentiation if new drugs aimed at improving neuronal survival and regeneration are to be derived from K-252a-type protein kinase inhibitors.

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