

Neurite Outgrowth and Protein Phosphorylation in Chick Embryonic Sensory Ganglia Induced by a Brief Exposure to 12-*O*-Tetradecanoylphorbol 13-Acetate

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Abstract: An exposure to 12-*O*-tetradecanoylphorbol 13-acetate (TPA) at 20 nM for as short as 30 min was sufficient to elicit neurite outgrowth from explanted chick embryonic sensory ganglia. Attachment of the ganglia to the collagen-coated substratum during exposure to TPA was essential for subsequent neurite outgrowth. Pulse-labeling with [³⁵S]-methionine indicated no significant difference in protein synthesis between control and TPA-treated ganglia. In vitro phosphorylation assay revealed a prominent protein kinase C substrate with an apparent molecular mass of 66,000 dalton (66 kDa) in chick embryo ganglia extracts. Treatment of intact ganglia with TPA for 30 min also specifically stimulated the phosphorylation of the same protein. When staurosporine, a potent inhibitor of protein kinase C, was present during TPA treatment, both neurite outgrowth and the

phosphorylation of the 66-kDa protein were blocked. Biochemical analysis of the phosphorylated 66-kDa protein indicated that (1) phosphorylation was only in serine residue, (2) the pI value was 4.5, (3) after V8 protease digestion, two phosphorylated peptide fragments, 6.0 and 7.5 kDa in size, were produced, and (4) it cross-reacted with an antiserum raised against a 66-kDa neurofilament subunit from rat spinal cord. These results suggest that early activation of protein kinase C and the phosphorylation of the 66-kDa protein may be involved in neuritogenesis. **Key Words:** Neurite outgrowth—Phosphorylation—Protein kinase C—Sensory ganglia—Phorbol esters. Mehta S. et al. Neurite outgrowth and protein phosphorylation in chick embryonic sensory ganglia induced by a brief exposure to 12-*O*-tetradecanoylphorbol 13-acetate. *J. Neurochem.* 60, 972–981 (1993).

Phorbol esters can bind and activate calcium-sensitive and phospholipid-dependent protein kinase C, (PKC) (Castagna et al., 1982; Kikkawa et al., 1982) due to their structural similarity to diacylglycerols, the endogenous activators of PKC (Sharkey et al., 1984). Activation of PKC has been correlated with neuronal functions including regulation of neurotransmitter release (Malenka et al., 1986), ionic conductance (Baraban et al., 1985), as well as modification of neuronal plasticity (Routtenberg et al., 1985) and outgrowth from both central and peripheral neurons (Burgess et al., 1986; Meiri et al., 1988). Using explanted sensory ganglia from chick embryos, we have shown that the phorbol ester, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), is a potent neurite-promot-

ing agent (Hsu et al., 1984; Hsu, 1989). The effects of TPA on neurite outgrowth are dose dependent. After 48 h of incubation at low concentrations of TPA (20 nM), long, thin radial fibers developed from the ganglia explant. At higher concentrations (160–480 nM), TPA produced highly fasciculated and shorter neurites. Other activators of PKC, such as synthetic diacylglycerols and phospholipase C, also elicit neurite development (Hsu et al., 1989), suggesting that activation of PKC is involved in axonal elongation.

The action of TPA is pleiotropic and complicated (Yamasaki, 1984). To further dissect and analyze the biochemical events underlying neurite outgrowth in TPA-treated ganglia, we have defined the minimal exposure time to TPA required for eliciting neurite out-

Received June 10, 1992; revised manuscript received August 5, 1992; accepted August 7, 1992.

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Abbreviations used: dBcAMP, dibutyryl cyclic AMP; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EBSS, Earle's balanced salt

solution; NF, neurofilament; NGF, nerve growth factor; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; PS, phosphatidylserine; SAC, protein A-bearing *Staphylococcus aureus*; SDS, sodium dodecyl sulfate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

growth. With the effective exposure time defined, the biochemical events immediately after TPA treatment can be analyzed without complications from the chronic effects of TPA. We have examined protein synthesis by pulse-labeling with [³⁵S]methionine and protein phosphorylation pattern by *in vivo* phosphorylation assay in ganglia explants after TPA treatment. The role of PKC in neurite outgrowth in chick embryonic ganglia explants was determined through (1) the use of the inhibitor staurosporine in both morphological and phosphorylation studies, (2) an identification of the 66-kDa protein to be a specific PKC substrate both *in vitro* and *in vivo*, and (3) further biochemical analysis of the phosphorylated 66-kDa protein.

MATERIALS AND METHODS

Materials

Ten-day-old chick embryos were reared from fertile eggs obtained from Avian Services (Frenchtown, NJ, U.S.A.). Culture media and Earle's balanced salt solution (EBSS) were obtained from GIBCO (Grand Island, NY, U.S.A.). ITS Premix and collagen-coated 96-well cluster dishes were from Collaborative Research (Bedford, MA, U.S.A.). Progesterone, TPA, *N*⁶,*O*²-dibutyryl adenosine 3',5'-cyclic monophosphate (dBcAMP), 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), phosphatidylserine (PS), and staurosporine were obtained from Sigma (St. Louis, MO, U.S.A.). Radioisotopes including [^γ-³²P]ATP, [³²P]orthophosphoric acid, and [³⁵S]methionine were from ICN (Irvine, CA, U.S.A.). X-ray films were from Kodak (Rochester, NY, U.S.A.).

Tissue culture and TPA treatment

Sensory ganglia from 10-day-old chick embryos were explanted and maintained in control medium consisting of Ham's F12 supplemented with defined growth factors including 5 μg/ml of insulin, 5 μg/ml of transferrin, 5 ng/ml of selenium (ITS Premix), and 6.28 ng/ml of progesterone for 18 h. To test the effects of TPA, these explants were treated with TPA for various time periods. After treatment with TPA, the explants were thoroughly rinsed three times with Ham's F12 medium and maintained in the fresh control medium until the end of the culture period of 48 h. The explants were then fixed and silver-stained to demonstrate the extent of neurite outgrowth (Hsu et al., 1984). Neurite length and density based on positive silver staining for nerve fibers were measured and ranked (Hsu et al., 1984). Differences in the mean neurite length and density of control and TPA-treated groups were subjected to analysis of variance by the Mann-Whitney *U* test. For each time point and dose concentration, duplicate samples were tested. These experiments were repeated at least four times.

Protein synthesis

Explanted ganglia (four per well) were treated with 0, 20, 200, or 800 nM TPA in control medium for specific time periods. The medium was removed and the ganglia were pulse-labeled with [³⁵S]methionine (5 μCi/100 μl, specific activity 1,066 Ci/mmol) in methionine-free EBSS for 30 min. After washing with cold methionine (5 mM), ganglia were resuspended and homogenized in a buffer containing 5 mM Tris-HCl (pH 7.5), 5 mM NaCl, 10 mM EGTA, 1 mM MgCl₂, 2 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF). The ganglia homogenate

was mixed with one-fifth volume of sodium dodecyl sulfate (SDS) stop solution containing 50 mM Tris-HCl, pH 7.2, 10% β-mercaptoethanol, 50% sucrose, 15% SDS, and 0.005% pyronin Y. Samples were boiled for 5 min and equal amounts of protein as determined by the Bio-Rad microassay (Bradford, 1976) were applied onto a 12% polyacrylamide gel. Autoradiograms were made by exposing the dried gels to Kodak x-ray film.

In vitro phosphorylation of proteins

Cell free phosphorylation was performed in ganglia or brain homogenates using exogenously added PKC. The ganglia and brain homogenates were subjected to mild heat treatment (60°C, 5 min) to inactivate endogenous kinases (Verma and Chen, 1986). The heat-treated homogenates were added to the assay mixture (75 μl) containing 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 5 mM MgCl₂, 100 mM NaCl, and 50 μM [^γ-³²P]ATP (4,500 Ci/mmol). PKC purified from embryonic chick brain (Jeng et al., 1986) was added to the mixture to initiate phosphorylation. About 0.5 unit (1 unit = 1 nmol/min) of PKC was used in each assay. Incubation was performed at 30°C for 15 min and terminated by adding one-fifth volume of SDS stop solution. The pattern of protein phosphorylation was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography.

Intact ganglia phosphorylation

In vivo phosphorylation was performed by treating the ganglia explants (four per well) with 0, 20, 200 nM TPA, or 1 mM dBcAMP in control medium for 1 h followed by labeling with ³²P_i (1 mCi/ml) for 30 min. Subsequently, ganglia were washed with phosphate-buffered saline and resuspended and homogenized in a buffer containing 5 mM Tris-HCl (pH 7.5), 5 mM NaCl, 10 mM EGTA, 1 mM MgCl₂, 1 mM PMSF, 50 μg/ml of leupeptin, 2 mM DTT, 0.5 mM Na₃VO₄, and 20 mM NaF. Equal amounts of protein (2–4 μg) harvested from control and TPA-treated ganglia were analyzed by SDS-PAGE and autoradiography.

Protein kinase inhibitor studies

Staurosporine, a potent inhibitor of PKC (Tamaoki et al., 1986), was dissolved in dimethyl sulfoxide (DMSO) and further diluted in F12 medium. DMSO alone at the concentrations used had no effect on neurite outgrowth. The effects of staurosporine were tested alone, or together with 200 nM TPA for the duration of the short exposure period or for the entire 48 h in culture. In some experiments, the inhibitor was added to ganglia culture before or after the exposure to TPA. Morphometric analysis of neurite outgrowth was performed to determine the inhibitory effect.

Biochemical analysis of the phosphorylated 66-kDa protein

The pI of the phosphorylated protein was determined by using the Phast System IEF electrophoresis (Pharmacia) on a premade IEF 3-9 Phast gel. Peptide mapping after limited proteolysis using *Staphylococcus aureus* V8 protease was performed using the phosphorylated 66-kDa band excised from SDS gels according to the procedure described by Cleveland et al. (1977). The phosphoamino acid residues of the protein were examined by hydrolyzing the phosphorylated protein band excised from the gel in 6 M HCl and analyzing the hydrolysates by electrophoresis on a cellulose

thin layer plate. The position of the radioactive spot on the autoradiogram was compared with that of standard phosphoamino acids (P-Ser, P-Thr, and P-Tyr) visualized by ninhydrin staining.

Immunoprecipitation

Immunoprecipitation was performed according to the procedure described by Nestler and Greengard (1980). In brief, ganglia extracts were phosphorylated *in vitro* and mixed with a 1% suspension of protein A-bearing *Staphylococcus aureus* (SAC). After 15 min of mixing, 10 μ l of supernatant was mixed with antiserum for 30 min at room temperature followed by an incubation with an equal volume of SAC suspension (1%) for another 30 min. The mixture was briefly centrifuged and the pellet was washed five times. The washed SAC pellet was subjected to SDS-PAGE. Antisera used in the present study included anti-rat NF66 polyclonal antibody (Chiu et al., 1989); anti-bovine brain 87-kDa PKC substrate polyclonal antibody (Aderem et al., 1988), anti-68-kDa antibody (Sigal and Tatum, 1988), and anti-GAP43 antibody (Meiri et al., 1988). Anti-rat NF66 antibody recognizes a unique neuronal intermediate filament protein, but does not cross-react with neurofilament (NF) triplet proteins including NF-L. Anti-68-kDa antibody, a monoclonal antibody developed against *B. burgdorferi* flagellin, can cross-react with a 68-kDa protein in human neuroblastoma cells, but not with NF-L (L. H. Sigal, personal communication).

RESULTS

Minimal exposure time to TPA for morphological differentiation

Ganglia explants in control medium developed few or no neurites after 48 h of incubation. The outgrowth from the core of these explants consisted mostly of nonneuronal cells (Fig. 1A). In contrast, ganglia explants treated with 20 nM TPA for a duration of only 30–60 min exhibited long and extensive neurites, comparable with that produced by incubating explants with TPA for the entire culture period (Fig. 1B and C vs. D). Fig. 1E shows that such ganglia produced distinctly enhanced neurite outgrowth even after an exposure to TPA (200 nM) for as short a time as 10 min. Such neurite development was comparable, but not as extensive as that caused by exposure to TPA for the entire 48-h period (Fig. 1E vs. H). Brief exposure to TPA at higher concentrations (100–600 nM) for 30 min produced denser outgrowths and obvious fasciculation (Fig. 1F; also see Table 1). But beyond the 30-min exposure period, continuous presence of high concentrations of TPA in culture did not appear to significantly affect neurite density (Fig. 1F vs. G and H). The addition of TPA at concentrations of >800 nM reduced neurite density (Table 1) even though the exposure time was only 30 min.

The attachment of ganglia to the substratum during the 30-min period of exposure to TPA appeared to be essential for neurite outgrowth. Unattached ganglia that were exposed to TPA for 30 min and then explanted on collagen-coated wells did not develop any detectable neurite outgrowth (Fig. 2A). The lack of

response to TPA in these ganglia explants appeared to be solely related to their unattached state during the exposure period. When these same ganglia explants were reexposed to TPA, neurite outgrowth was subsequently observed (Fig. 2B).

Effect of staurosporine on TPA-induced ganglia differentiation

The addition of staurosporine, an inhibitor of PKC (Tamaoki et al., 1986), to control medium for 30 min or for 48 h had no effect on neurite outgrowth (Fig. 3A and B). The morphology of the staurosporine-treated ganglia explants was essentially identical to control explants (Fig. 3A vs. Fig. 1A). However, when staurosporine was added together with TPA for a duration of either 30 min or 48 h, neurite outgrowth induced by TPA was significantly reduced (Fig. 3C and D vs. Fig. 1F and H). If staurosporine was added after TPA was removed from the incubation medium, neurite outgrowth induced by brief exposure to TPA was unaffected (Fig. 3E). The inhibitory effect of staurosporine was specific for TPA-induced neurite outgrowth. Neurite outgrowth induced by 1 mM dBcAMP was unaffected by treatment with staurosporine (Fig. 3F).

Protein synthesis

By using radioactive precursors as metabolic labeling agents in as few as two ganglia explants per sample, we were able to examine protein synthesis by pulse-labeling in control and TPA-treated explants. Figure 4 showed that there was no qualitative difference in radiolabeled protein patterns between the control and TPA-treated samples, nor was there any difference in ganglia that were treated with low (20 nM) or high (200 nM) concentrations of TPA. Even samples from explants exposed to inhibitory doses of TPA (800 nM) exhibited the same pattern of newly synthesized proteins. Furthermore, the protein pattern remained the same whether the treatment of TPA was for 1 or 6 h before pulse-labeling with [³⁵S]-methionine (Fig. 4, lanes 2–4 vs. lanes 6–8). Garrels and Schubert (1979) have reported that no significant qualitative change in protein synthesis occurred in pheochromocytoma (PC12) cells that were induced to differentiate neurites by treatment with NGF. Bloom and Black (1979) have also shown that superior cervical ganglia explants generated neurites despite inhibition of both RNA and protein synthesis. In light of these studies, we did not pursue further possible minor differences in protein synthesis using two-dimensional gels.

PKC protein substrate identified by *in vitro* phosphorylation

Partially purified PKC devoid of all other kinase activities was used as the sole source of kinase to identify specific substrate proteins in chick embryo ganglia extracts. We have previously reported that a brief, mild heat treatment (60°C, for 5 min) inactivates all

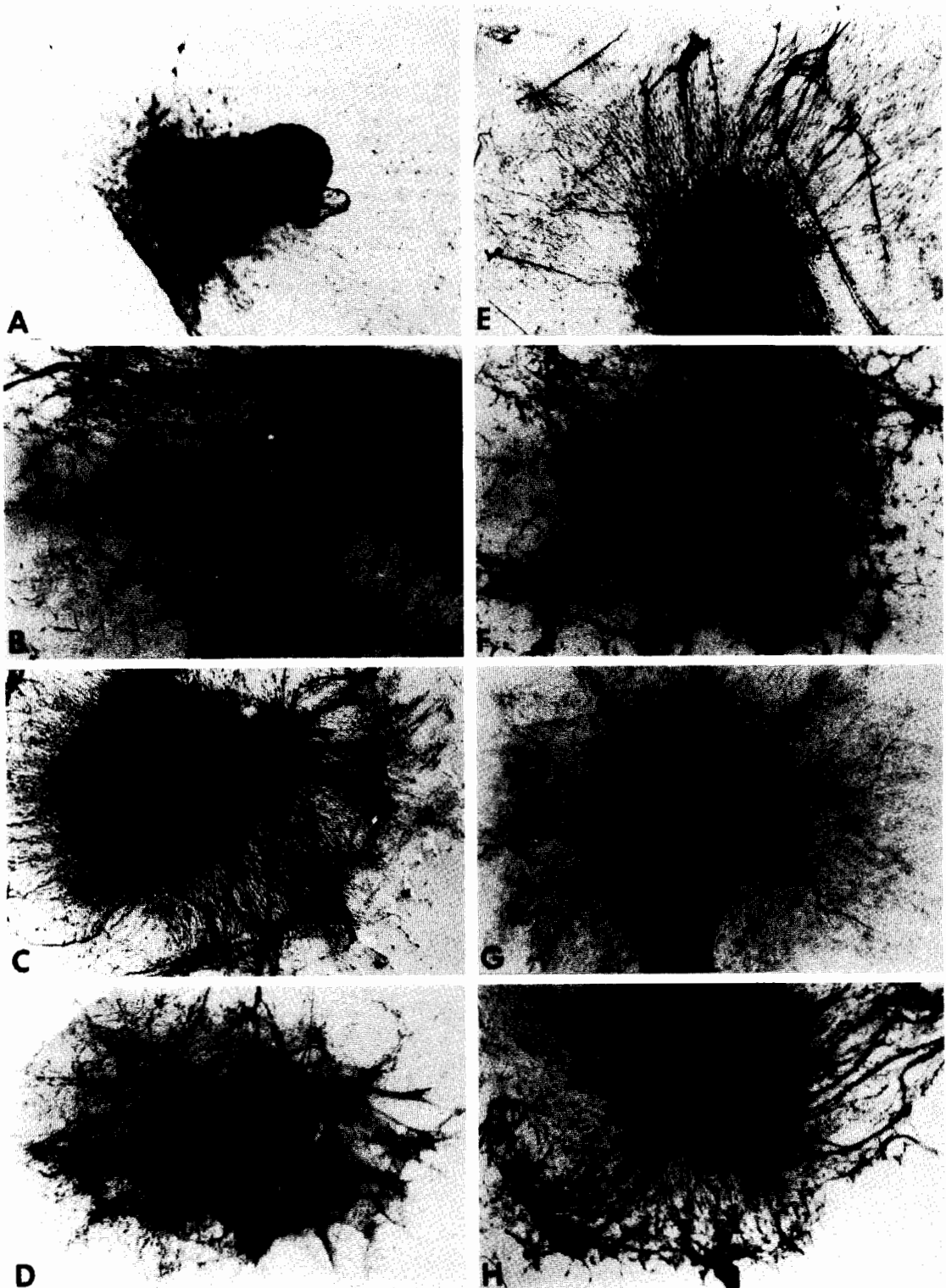


FIG. 1. Effect of exposure time to TPA on neurite outgrowth in chick embryonic sensory ganglia explants. Ganglia were exposed to TPA at 20 or 200 nM for various times and then incubated in the control medium for a 48-h culture period. At the end of the 48-h culture period, neurite outgrowth was examined morphologically. **A:** Ganglia in control medium without TPA. **B:** Ganglia exposed to 20 nM TPA for 30 min. **C:** Ganglia exposed to TPA (20 nM) for 60 min. **D:** Ganglia exposed to TPA (20 nM) for 48 h. **E:** Ganglia exposed to TPA (200 nM) for 10 min. **F:** Ganglia exposed to TPA (200 nM) for 30 min. **G:** Ganglia exposed to TPA (200 nM) for 60 min. **H:** Ganglia exposed to TPA (200 nM) for 48 h.

TABLE 1. Effect of dose concentration of TPA on neurite density^a

TPA (nM)	Mean neurite density (Ranking \pm SE)
0	1.00 \pm 0
20	2.25 \pm 0.17
100	2.30 \pm 0.12
200	2.20 \pm 0.20
400	2.08 \pm 0.15
600	2.00 \pm 0.35
800	1.67 \pm 0.24
1,000	1.60 \pm 0.29

^a Chick embryonic ganglia explants were treated with TPA at indicated concentrations for 30 min and then assessed for morphological differentiation 48 h later as described in Materials and Methods. Mean neurite density is based on ranking of 10 ganglia explants per treatment group after 48 h in control medium. Neurite density is ranked as follows: 0 = no neurite, 1 = sparse, 2 = moderate, 3 = dense, as previously described (Hsu et al., 1984; Hsu, 1989). All TPA-treated groups were significantly different from the control group in neurite density ($p < 0.01$) as analyzed by the Mann-Whitney *U* test. The TPA-treated groups from 20 to 600 nM were not significantly different from each other, but were significantly different from the high TPA-treated groups (800–1,000 nM) as analyzed by the Mann-Whitney *U* test.

endogenous kinase activities without affecting substrate activities (Verma and Chen, 1986). When PKC was added to heat-treated ganglia homogenate, a major protein band with an apparent molecular mass of 66 kDa was phosphorylated (Fig. 5a, lane 2 vs. lane 1). TPA enhanced the phosphorylation of this protein band by more than fivefold (Fig. 5a, lane 4 vs. lane 2). Without added PKC, no phosphorylation was observed whether TPA was present or not (Fig. 5a, lanes 1 and 3). Other PKC activators such as OAG with or

without PS also significantly stimulated the PKC activity (Fig. 5a, lanes 6 and 8 vs. lane 5). In contrast, Ca^{2+} or PS alone added to PKC was not stimulatory (Fig. 5a, lanes 7 and 9). Unlike TPA, OAG/PS also stimulated the phosphorylation of several protein bands with molecular masses of <20 kDa (Fig. 5a, lane 8). Fig. 5b shows that the 66-kDa protein band also appeared to be a major PKC substrate protein in embryonic chick brain whose phosphorylation was prominently stimulated by TPA, OAG, or OAG/PS.

TPA stimulated the phosphorylation of a 66-kDa protein in intact ganglia

The *in vivo* phosphorylation of the 66-kDa protein was also demonstrated in intact ganglia. Figure 6 shows that a brief exposure of ganglia to TPA (20 or 200 nM) caused a fivefold stimulation of the phosphorylation of a 66-kDa protein in intact ganglia (lanes 2 and 3 vs. lane 1). In contrast, exposure of intact ganglia to dBcAMP, which activates PKA, did not lead to any stimulation of the phosphorylation of the 66-kDa protein (Fig. 6, lane 4 vs. lane 1). To further demonstrate that this 66-kDa protein is a substrate protein of endogenous PKC and that its phosphorylation may be involved in TPA-induced neurite outgrowth, we have examined effects of staurosporine. Figure 6 shows that staurosporine, when added at 50 nM together with TPA to the ganglia culture, reduced the *in vivo* phosphorylation of the 66-kDa protein (Fig. 6, lane 7 vs. lane 6), suggesting that the effect of staurosporine on the phosphorylation of the 66-kDa protein may be related to its inhibitory effect on neurite outgrowth in ganglia explants.

Biochemical characterization of the 66-kDa protein

To ensure that both the *in vitro* and *in vivo* phosphorylated 66-kDa proteins are indeed identical, we

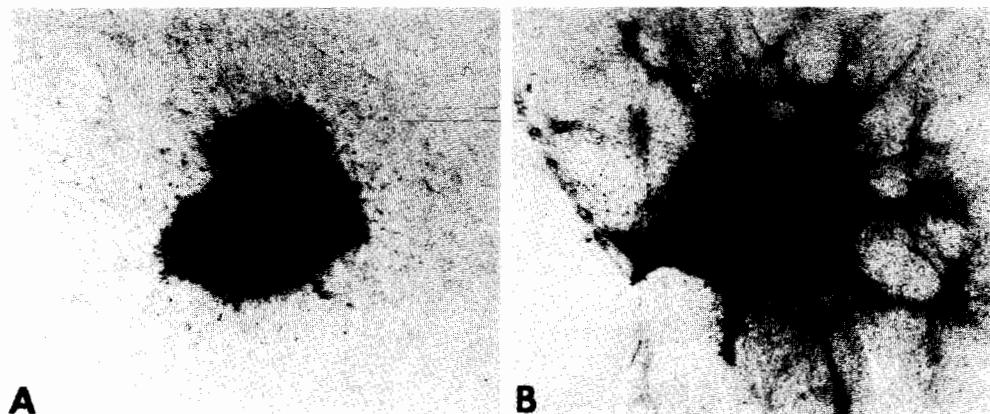


FIG. 2. Effect of attachment during TPA exposure on neurite outgrowth in chick embryonic sensory ganglia explants. **A:** Ganglia were unattached during 30 min of TPA (200 nM) exposure, followed by explantation on collagen substratum in control medium for 48 h. **B:** Ganglia were unattached during 30 min of TPA (200 nM) exposure, followed by explantation on collagen substratum and reexposure to TPA (200 nM) for 48 h. Ganglia were silver-stained for detection of neurite outgrowth.

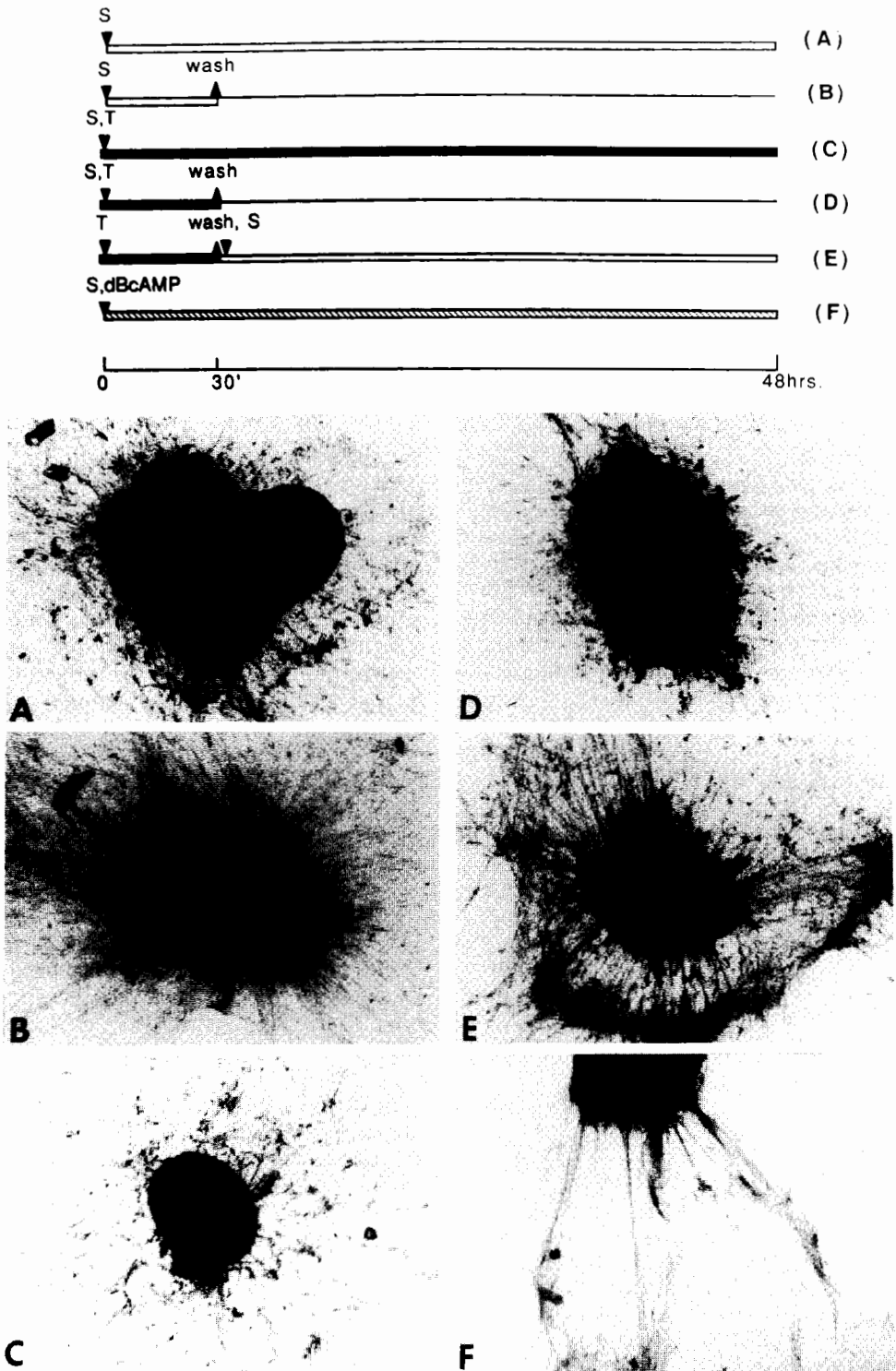


FIG. 3. Effect of staurosporine on TPA-induced neurite outgrowth in chick embryonic sensory ganglia explants. Ganglia explants treated with 50 nM staurosporine (S) in the presence or absence of 200 nM TPA (T) during or after the 30-min exposure period as shown in the schematic representation (A-E). Additional control included ganglia explants treated with a combination of 50 nM staurosporine and 1 mM dBCAMP for 48 h (F). Ganglia explants were fixed and silver-stained, representative photomicrographs corresponding to each treatment were shown.

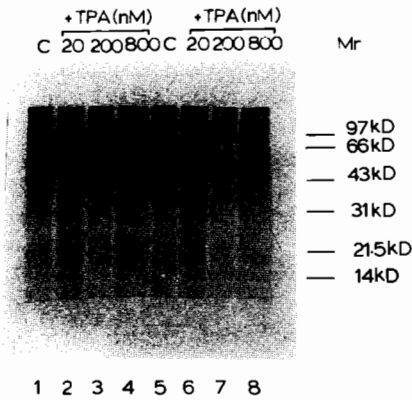


FIG. 4. Effect of TPA treatment on protein synthesis in chick embryonic sensory ganglia explants. Autoradiogram showing the pattern of protein synthesis in ganglia treated with varying concentrations of TPA for 1 h (lanes 1–4) or 6 h (lanes 5–8) and pulse-labeled with [³⁵S]methionine in EBSS for 30 min. After washing, ganglia were processed for SDS-PAGE as described in Materials and Methods.

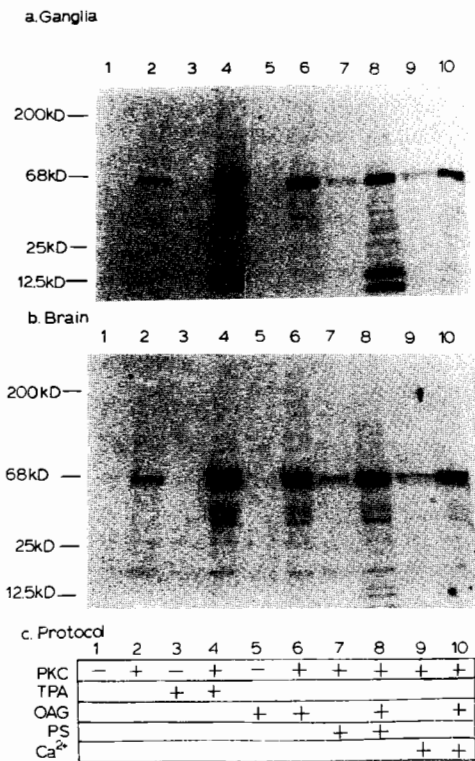


FIG. 5. In vitro phosphorylation of PKC substrate proteins in chick embryonic ganglia (a) and in chick brain homogenates (b). Homogenates of ganglia and brain tissues from 10-day chick embryos were prepared and processed for in vitro phosphorylation as described in Materials and Methods. Various additions were made as indicated in Protocol (c). When present (+), the additions were TPA (200 nM), OAG (1 mM), PS (0.3 μg/ml), and CaCl₂ (0.5 mM). Presence (+) or absence (-) of exogenously added PKC (0.5 unit) was also indicated.

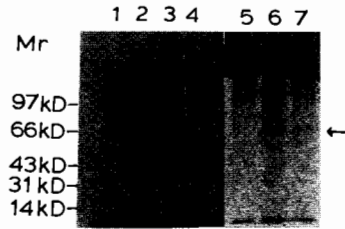


FIG. 6. In vivo phosphorylation of intact ganglia. Phosphorylation of proteins in intact ganglia incubated in the presence or absence of TPA or dBcAMP as described in Materials and Methods. Lane 1, control; lane 2, 20 nM TPA; lane 3, 200 nM TPA; lane 4, 1 mM dBcAMP; lane 5, control; lane 6, 200 nM TPA; lane 7, 200 nM TPA plus 50 nM staurosporine.

eluted both proteins from excised gel slices and found that both proteins comigrated on 7 and 12% SDS-polyacrylamide gel (data not shown). When both the in vitro and in vivo ³²P-labeled 66-kDa proteins were subjected to partial proteolytic digestion with *Staphylococcus aureus* V8 protease, identical ³²P-labeled peptide fragments with estimated sizes of 6.0 and 7.5 kDa were obtained (Fig. 7A), strongly suggesting that both proteins are the same. Phosphoamino acid analysis indicated that only the serine residue was phosphorylated in the 66-kDa protein from ganglia tissue (Fig. 7B). The phosphorylated 66-kDa protein was quite acidic with a pI value of 4.5 (Fig. 7C). Interestingly, the pI of many major phosphoproteins phosphorylated by PKC are also acidic, ranging from 4.5 to 4.8 (Woodgett et al., 1987).

As an initial approach to identify the 66-kDa protein, we have attempted to immunoprecipitate the 66-kDa protein with antisera against known PKC substrate proteins. We could not detect any cross-reactivity between the 66-kDa protein and the antisera directed against GAP43 (Meiri et al., 1988), or an 87-kDa bovine brain protein (Aderem et al., 1988), or a 68-kDa neuronal protein (Fig. 7D, lanes 2–4). However, we found that antiserum against NF from rat spinal cord (Chiu et al., 1989) appeared to cross-react with the 66-kDa protein (Fig. 7D, lane 1).

DISCUSSION

In the present study, we have shown that a brief exposure to TPA for 30 min was sufficient to elicit morphological differentiation of neurites in chick embryo ganglia. Beyond this critical period, increasing the duration of exposure merely affected the fasciculation of the outgrowths, presumably through the promotion of side-to-side adhesive interactions between neighboring neurites (Hsu, 1989).

With the identification of this critical period of exposure to TPA, we have examined specific biochemical events underlying this early phase of neurite out-

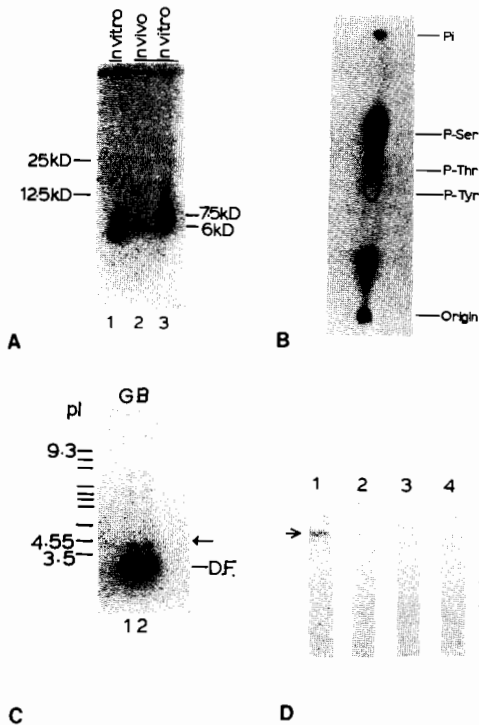


FIG. 7. Biochemical analysis of the ^{32}P -labeled 66-kDa protein in ganglia explants phosphorylated in vitro or in vivo. **A:** Peptide fragments obtained on limited proteolysis of the in vitro ^{32}P -labeled (lanes 1 and 3 were two different samples) and in vivo ^{32}P -labeled (lane 2) 66-kDa protein with V8 protease. **B:** Phosphoamino acid analysis of ^{32}P -labeled 66-kDa protein. **C:** Isoelectric focusing of the ^{32}P -labeled 66-kDa protein from ganglia (lane 1) and from brain (lane 2). **D:** Immunoprecipitation of the ^{32}P -labeled 66-kDa protein with antibodies against a rat NF66 protein (lane 1), GAP43 (lane 2), an 87-kDa bovine brain PKC substrate protein (lane 3), and a 68-kDa neuronal protein from mouse neuroblastoma cells (lane 4).

growth. Our results indicate that treatment with phorbol ester alone does not guarantee the induction of neurite outgrowth. When attached and unattached ganglia were exposed to TPA for the same period, only the attached ganglia were responsive. This would imply that attachment of the ganglia was required for the neurite-promoting effects of TPA. Several studies have shown the importance of enhanced cell-to-substratum adhesion in promoting axonal elongation. For example, sensory neurons treated with nerve growth factor (NGF) extended further on the more adhesive polylysine (Letourneau, 1975) or laminin-coated substratum (Rogers et al., 1983). More recently, Bixby (1989) has shown that in chick ciliary ganglionic neurons, TPA potentiated process outgrowth that was initiated by the laminin substratum. In that study, ganglionic neurons that were not plated on laminin did not respond to TPA. These results suggest that substratum attachment may be an integral component in the activation of PKC that subse-

quently leads to morphological differentiation in neuronal cells.

There appears to be as much evidence supporting the notion that neurite outgrowth is the consequence of PKC activation (Burgess et al., 1986; Hall et al., 1988; Hashimoto and Hagino, 1989) as there is evidence that down-regulation of the PKC is responsible for neurite outgrowth (Morrison et al., 1988; Reinhold and Neet, 1989; Girard and Kuo, 1990). Moreover, activation of PKC can produce adverse effects on hippocampal neurons, causing reduction in neuronal survival and regression of both axons and dendrites (Mattson et al., 1988). The fact that PKC exists in at least nine isoforms differing in their biochemical properties and localization has made the task of defining the functions of PKC difficult (Schulman, 1991). In addition, the existence of multiple intracellular signaling pathways within a neuron further complicates analysis of individual kinase systems (Mattson et al., 1988).

To evaluate the role of PKC activation in TPA-induced neuritogenesis, we have examined both the in vitro and in vivo phosphorylation pattern of substrate proteins of PKC. The significant findings are (1) that a brief treatment with TPA resulted in a three- to five-fold stimulation in the phosphorylation of a protein with an apparent molecular mass of 66 kDa in intact explanted ganglia (Fig. 6, lanes 2 and 3), and (2) that this 66-kDa protein was found to be a major PKC substrate protein in ganglia and brain extracts (Fig. 5). That the 66-kDa protein is of physiological significance in neurite outgrowth is also supported by the finding that inhibition of PKC by staurosporine not only blocked neurite outgrowth (Fig. 3), but also reduced the phosphorylation of the 66-kDa protein (Fig. 6, lane 7 vs. lane 6). It can be noted that the inhibitory action of staurosporine did not appear to be mediated by blocking PKA activity because the induction of neurites by dBcAMP was not disturbed by the presence of staurosporine (Fig. 3F). Previous studies have also shown that staurosporine inhibited neurite outgrowth in other neuronal systems. For example, at concentrations of ≥ 100 nM, staurosporine inhibited NGF induction of neurite formation and phosphorylation of specific proteins in PC12H cells (Hashimoto and Hagino, 1989). Campenot et al. (1991) have recently reported that staurosporine at 100 nM to 1 μM significantly slowed the outgrowth of distal neurites of neonatal sympathetic neurons treated with NGF.

Clarification of the role of PKC in neurite outgrowth in chick embryo ganglia can be gained through identification of its substrate proteins. Biochemical characterization of the 66-kDa protein indicates that it is acidic in nature and phosphorylated at the serine residue (Fig. 7). Positive immunoprecipitation with antiserum raised against a novel rat NF subunit (Fig. 7D) is alluring, although more direct evi-

dence, including purification and amino acid sequencing of the 66-kDa protein, will be needed to establish the identity of this major PKC substrate protein in chick embryo ganglia as an NF protein. NFs represent a major constituent of neuronal cells (Wuerker and Palay, 1969). In fact, increase in NFs in TPA-treated neurons was observed in a previous ultrastructural study (Hsu, 1989). Moreover, phosphorylation of NFs is implicated in numerous activities including signal transduction and axonal growth (Nixon and Sihag, 1991). In summary, our study shows that (1) a brief exposure (~30 min) of explanted chick embryonic ganglia can cause an increase in the phosphorylation of the 66-kDa protein, a major PKC substrate protein identified *in vitro*, and lead to subsequent neurite outgrowth, and (2) staurosporine, a potent PKC inhibitor, inhibits both the phosphorylation of the 66-kDa protein and the morphological differentiation in explanted ganglia. These results suggest the 66-kDa protein, possibly a NF-like protein, may be involved in TPA-induced neurogenesis.

Acknowledgment: We are grateful to Drs. F.-C. Chiu, Albert Einstein College of Medicine, Paul Greengard, Rockefeller University, L. H. Sigal, Robert Wood Johnson Medical School, and K. F. Meiri, SUNY Health Science Center, for kindly giving us, respectively, anti-NF66, anti-87-kDa, anti-68-kDa, and anti-GAP43 antisera. We thank Drs. R. Poretz, K. Reuhl, and P. Yurchenko for useful discussions. This work was supported by grant NS 21262 from the National Institute of Neurological and Communicative Disorders and Stroke, NIH, and by a grant from the Charles and Johanna Busch Memorial Fund. A preliminary account of this work has been presented [Mehta S., Hsu L., Jeng A. Y., and Chen K. Y. (1990) *Soc. Neurosci. Abstr.* **16**, 1009].

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