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Induction of neurite outgrowth from chick embryonic ganglia explants by activators of protein kinase C

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Neurite outgrowth from chick embryonic sensory ganglia explants was induced by activators of protein kinase C and other compounds known to stimulate the hydrolysis of inositol phospholipids. The addition of diacylglycerols, phospholipase C and muscarine chloride to a defined growth medium promoted the outgrowth of dense neurites from ganglia explants which were morphologically distinct from those induced by the phorbol ester TPA. Moreover, these neurite-promoting agents did not enhance non-neuronal cell proliferation and were ineffective in the absence of insulin and/or progesterone.

Phorbol esters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) have been extensively studied as tumor-promoting agents. These plant-derived diterpene compounds have been shown to be potent inducers of differentiation in a wide variety of normal and transformed cell types [3]. We have found TPA to be extremely effective in inducing the outgrowth of neurites from peripheral ganglia neurons of the chick embryo [7, 8]. While the mechanisms by which TPA promotes neurite development are not currently understood, there is considerable evidence that the major phorbol ester receptor is the calcium-sensitive, phospholipid-dependent protein kinase or protein kinase C (PKC) [2, 11, 13, 14]. PKC is believed to play a role in signal transduction across the cell membrane with subsequent phosphorylation of cytosolic proteins [12]. Diacylglycerols (DAGs), which are products of inositol phospholipid breakdown, compete specifically for phorbol ester binding and are shown to be the endogenous activator for PKC [2, 11, 13, 14]. In our continuing effort to understand how TPA or PKC is involved in the molecular mechanisms underlying neuritogenesis, we have attempted to correlate the induction of neurite outgrowth with activation of PKC by DAGs as well as by compounds known to stimulate the hydrolysis of inositol phospholipids.

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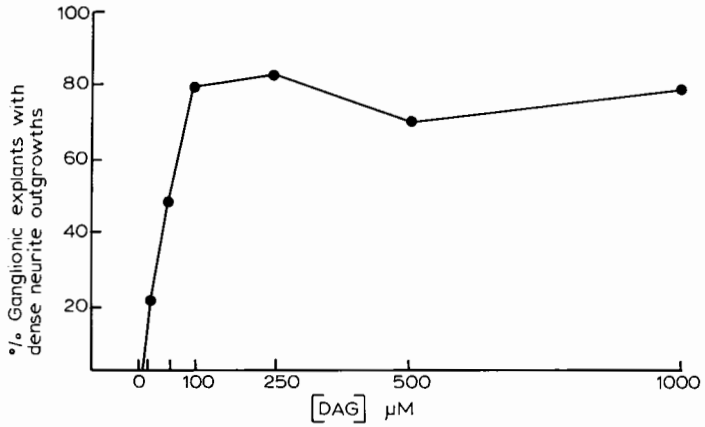


Fig. 1. Neurite-promoting effects of 1-oleoyl-2-acetyl-glycerol. Ganglionic explants were maintained in complete growth medium supplemented with increasing concentrations of the diacylglycerol (DAG). Each point represents the percent of explants with dense neurite outgrowth (over 200 neurites/explant) in control or DAG-treated groups.

Ganglia explants from chick embryos (9 d) were maintained on collagen coated wells in a defined medium of Ham's F12 supplemented with insulin (5 μg/ml), progesterone (6.28 ng/ml), transferrin (5 μg/ml) and selenium (5 μg/ml). This formulation was modified after Bottenstein's N_1 medium [1]. This complete medium supported the survival of the ganglia explants (Fig. 2) and served as the control vehicle for testing the neurite-promoting effect of TPA and other activators of PKC. Neurite-promoting effects were also evaluated in unsupplemented F12 medium. The reagents tested included synthetic DAGs (1-oleoyl-2-acetyl-sn-glycerol, 1,2[dioleoyl-rac-glycerol, 1,2-dicapryloyl-rac-glycerol), phospholipase C (Type IX from C. perfringens), phospholipase A_2 (from *Crotalus adamanteus*) and muscarine chloride (all reagents from Sigma). Synthetic DAGs were incorporated into control growth medium at concentrations ranging from 10 μM to 1 mM. Repeated freeze-thaw cycles facilitated the dispersal of DAG in the growth medium and maximized the solubility of these compounds. Control and treated cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. After 2 days in culture, the explants were fixed and silver-stained [6]. To measure neurite density, each explant was divided into 4 quad-

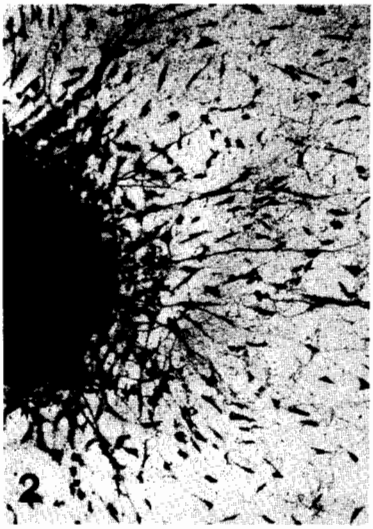
Fig. 2. Control explant maintained in complete growth medium developed a background of non-neuronal cells and a sparse outgrowth of fine neurites after 2 days in vitro. Silver stain, ×264.

Fig. 3. Treatment with high concentrations of TPA (200 ng/ml) for 2 days produced an outgrowth of dense neurites which formed short, thick fascicles over a background of non-neuronal cells. Silver stain, ×277.

Fig. 4. Explant treated with 200 μM of DAG for 2 days developed a dense outgrowth of long, fine neurites. Non-neuronal cell outgrowth was limited. Silver stain, ×288.

Fig. 5. Long, thin neurites extend from one side of the explant which was treated with PLC (0.02 units/ml). Non-neuronal cell outgrowth was limited. Silver stain, ×262.

Fig. 6. Explant treated with muscarine chloride (200 μM) developed a dense outgrowth of fine, short fibers. Silver stain, ×242.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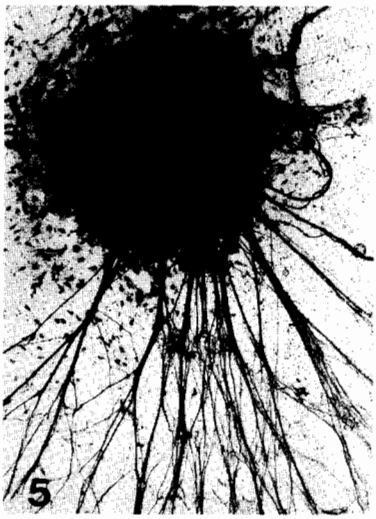
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rants, each quadrant was visually estimated and ranked. No growth was scored as 0, sparse growth with less than 10 neurites/quadrant was scored as 1, moderate growth of 20–30 neurites/quadrant was scored as 2 and dense growth of 50 and more neurite/quadrant was scored as 3. All cultures were scored blind and the mean density was obtained from over 30 explants/treatment group. To compare the extent of non-neuronal cell outgrowth, photographs of the regions peripheral to the explant core measuring approximately 40% of the total outgrowth area were examined. Non-neuronal cells in the photographed areas from 4 cultures/treatment group were counted and compared. Differences between control and treatment groups were subjected to statistical analysis [7, 8].

The addition of DAGs to complete control medium elicited a dense outgrowth of neurites from the ganglia explants in a dose-dependent manner (Fig. 1). At concentrations greater than 100 μM, more than 70% of the treated explants exhibited long, thin, radial neurites after 2 days in culture (Fig. 4). The morphology of DAG-induced neurite development was similar to the classic 'halo' effect observed in peripheral ganglia in response to Nerve Growth Factor [10]. It was also comparable to the neurite outgrowth typical of treatment with low concentrations of TPA (10 ng/ml) [7]. All 3 synthetic DAGs were similarly effective. At the highest concentration tested (1 mM), no inhibitory or cytotoxic effects were observed. Maintenance of explants in high concentrations of DAG did not affect the fasciculation pattern of neurite outgrowth. This is distinctly different from explants treated with high concentrations of TPA (200 ng/ml) which typically developed a dense outgrowth of shorter and thicker fascicles (Fig. 3). Within the DAG-treated cultures the outward migration of non-neuronal cells from the explant core was limited. Non-neuronal cells which accompanied the neurite extensions were sparsely distributed (compare Figs. 3 and 4) and far fewer in DAG-treated explants (mean = 178 ± 12) than in control (mean = 368 ± 26) or TPA-treated explants (mean = $632 + 17$).

The effects of phospholipase C (PLC) and muscarine chloride, which were known to increase hydrolysis of phosphatidylinositol were next examined to determine if the subsequent increase in the intracellular concentrations of DAG was correlated with induction of neurite outgrowth. When explants were maintained in control growth medium containing PLC (0.01–0.05 enzyme U/ml), significantly more neurites developed. The neurites were long and thin but were not uniform in their distribution around the circumference of the explant (Fig. 5). Non-neuronal cell outgrowth was usually limited and did not accompany the neurites distally. Furthermore, treatment with PLC supplemented medium was also correlated with detachment of explants from the underlying substrate after 2 days. Treatment of ganglia explants with PLA_2 (0.2 enzyme U/ml), which does not generate intracellular DAG, did not enhance neurite outgrowth.

Activation of muscarinic receptors in the superior cervical ganglia has been shown to increase phospholipid turnover [4, 9]. More recently, the addition of muscarine (at 27°C) was found to significantly increase the intracellular levels of diacylglycerol in PC12 cells [5]. In our study, the addition of muscarine chloride to the growth medium at concentrations ranging from 100 to 400 μM significantly enhanced the

outgrowth of neurites. As depicted in Fig. 6, the morphology of such muscarine-induced neurite outgrowth was distinctly different. Neurites were short, fine and densely packed but not organized as thick bundles. Muscarine as a neurite-promoting agent was temperature-sensitive since the enhanced neurite outgrowth was only observed when the cultures were incubated at temperatures ranging from 27 to 30°C but not at 37°C.

Our results suggest that exogenous DAGs or agents which increased intracellular DAGs, such as PLC or muscarine chloride can mimick the neurite-promoting effects of TPA. However, there were distinct differences between these activators of PKC. Neurite outgrowth was not induced by DAGs, PLC or muscarine chloride if insulin and/or progesterone were lacking in the growth medium (Table I). Furthermore, cultures maintained in F12 medium alone for 1 or 2 days were unresponsive to the subsequent additions of activators of PKC. This suggests that hormonal requirement may be necessary for the survival of specific ganglionic neurons which respond to exogenous or intracellular DAGs. In contrast, the inclusion of high concentrations of TPA effectively induced neurite outgrowth from explants which were maintained in unsupplemented medium. This difference in the potency between TPA and the other agents may be related to their stability. DAGs are highly labile while phorbol esters metabolize slowly thereby exerting a long-lasting effect on the activation of PKC. The effects of TPA on ganglionic development also appear to be more pleiotropic than those of DAGs. Thus in addition to promoting neurite outgrowth, TPA also affected the fasciculation of the neurites and the outgrowth of the non-neuronal cells.

TABLE I

GROWTH FACTORS AND HORMONAL REQUIREMENTS FOR PROMOTION OF NEURITE OUTGROWTH

Extent of neurite density was rated as described in text. I (insulin) = 5 μg/ml; S (selenium) = 5 μg/ml; T (transferrin) = 5 μg/ml; P (progesterone) = 6.28 ng/ml.

Growth medium composition	Mean neurite density
F12	0
F12 + 200 μM DAG	0
F12 + 0.02 units/ml PLC	0
F12 + 200 μM muscarine	0
F12 + 1 or 20 ng/ml TPA	0
F12 + 200 ng/ml TPA	2.0 ± .06
F12 + I	1.3 ± .04*
F12 + I, P, S, T	1.4 ± .02
F12 + 1 ng/ml TPA + I	1.5 ± .06
F12 + 20 ng/ml TPA + I	2.2 ± .03
F12 + 200 ng/ml TPA + I	2.9 ± .02
F12 + I + 200 μM DAG	2.5 ± .04
F12 + S or T	0
F12 + S or T + 200 μM DAG	0

*Value not significantly different if progesterone (6.28 ng/ml) was added instead.

Treatment of ganglionic explants with DAGs only elicited neurite development. The cellular mechanisms underlying neuritogenesis induced by activators of PKC remains to be defined.

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