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Dopamine induces neurite retraction in retinal horizontal cells via diacylglycerol and protein kinase C

(cell culture/D1 receptors/phospholipase C/phorbol esters)

PAULO DOS SANTOS RODRIGUES* AND JOHN E. DOWLING†

Department of Cellular and Developmental Biology, The Biological Laboratories, Harvard University, Cambridge, MA 02138

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ABSTRACT **Dopamine causes a significant retraction of neurites of bull-head catfish horizontal cells maintained in culture. The effects of dopamine are blocked by haloperidol and SCH 23390, a D1 antagonist, but not by sulpiride, a D2 antagonist. The dopamine-induced morphological changes were mimicked by SKF 38393, a D1 agonist, but not by quinpirole, a D2 agonist. Kainate also caused process retraction, but other neuroactive substances tested including glutamate, 5-hydroxytryptamine, *N*-methyl-D-aspartate, γ-aminobutyric acid, and glycine caused only minor changes in neurite length. Cyclic AMP analogues do not induce neurite retraction in horizontal cells, indicating that this effect of dopamine is not mediated by cyclic AMP. However, a protein kinase C activator (phorbol 12-myristate 13-acetate) and synthetic diacylglycerol analogs (1-oleoyl-2-acetyl-*sn*-glycerol and dioctanoglycerol) caused marked neurite retraction. Their effects, as well as the dopamine-induced changes, were blocked by staurosporine, a potent protein kinase antagonist. The results suggest that dopamine causes neurite retraction by the activation of protein kinase C via diacylglycerol.**

Dopamine exerts multiple effects on teleost horizontal cells (reviewed in ref. 1). It decreases light responsiveness of the cells by modifying a glutamate conductance (2); it reduces electrical coupling between the cells by decreasing gap junctional conductance (3), and it depresses γ-aminobutyric acid (GABA) release from the cells by an unknown mechanism (4). All of these effects are mediated by cyclic AMP, which activates cyclic AMP-dependent kinases (protein kinase A) in teleost horizontal cells.

We have been studying the effects of neuroactive substances on process outgrowth of teleost horizontal cells maintained in culture and have found that dopamine causes a significant and reversible retraction of their neurites. Cyclic AMP analogues do not affect neurite retraction in these cells, indicating that this effect of dopamine is not mediated by cyclic AMP. Following on a recent report that phorbol esters stimulate spinule formation in teleost horizontal cells (5), we have studied the effects of a phorbol ester, diacylglycerol analogues, and protein kinase inhibitors on process outgrowth in cultured catfish horizontal cells. The results suggest that dopamine causes retraction of processes in horizontal cells by activating phospholipase C, resulting in the formation of diacylglycerol and the activation of protein kinase C (PKC).

MATERIALS AND METHODS

Cell Culture. Methods for the isolation and culture of teleost horizontal cells have been described (6). Dark-adapted eyes from young bull-head catfish (*Ictalurus nebulosus*) were dissected and quickly rinsed in ethanol (70%). The retinas were isolated under red dim light and incubated for 40 min at room temperature (20°C) in Leibowitz's medium (L-15) (GIBCO) containing 60–70 units of papain per ml (Worthington) activated with L-cysteine. The retinas were washed with fresh L-15 and triturated with disposable glass pipettes until they were broken up completely. After trituration, samples (400 μl) of supernatant containing isolated horizontal cells were distributed into 35-mm sterile plastic dishes (Corning). The cells were allowed to settle and adhere to the bottom of the dishes for 2–4 hr; 3 ml of L-15 containing penicillin/streptomycin solution (1000 units/ml) (GIBCO) was added to each dish. The medium was changed every 2–3 days or on the day before an experiment.

Experimental Procedure. Horizontal cells maintained in culture for up to 10 days were used. Most experiments, however, were carried out on cells kept in culture for 2–5 days. For assessment of morphological changes, photomicrographs of isolated horizontal cells were taken in control medium and at regular time intervals after test agents were added to the culture dishes. Usually 5–20 cells from each culture dish were monitored. Drug effects were assayed by measuring the perimeter of cells 2 and 4 hr after drug addition. In some experiments, pictures were taken at regular intervals after removal of the test drug from the bathing medium to document recovery. Cells were located in relation to a set of coordinates applied to the bottom of the culture dish. Quantitative measurements were made by determining the perimeter of individual cells from projected photographic negatives. Perimeter measurements were done with Sigma-Scan software and related equipment (Jandel, Corte Madera, CA). Values are expressed as means ± SEM and statistical comparisons were done by Student's *t* test.

Chemicals. Dopamine, 8-Br-cAMP, 5-hydroxytryptamine, isoproterenol, glutamate, kainate, *N*-methyl-D-aspartate, GABA, and glycine (Sigma) were dissolved in L-15 at relatively high concentrations just before an experiment. Sulpiride, SKF 38393, SCH 23390, phorbol 12-myristate 13-acetate (PMA), 4-phorbol 12,13-didecanoate, and staurosporine (Research Biochemicals, Natick, MA) were dissolved in ethanol, whereas 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) (Sigma) and dioctanoylglycerol (Boehringer Mannheim) were dissolved in dimethyl sulfoxide. 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) (Sigma) and quinpirole (Research Biochemicals, Natick, MA) were dissolved in L-15 acidified to pH 4–5. These stock solutions were stored as recommended by the manufacturers. Adjusted volumes (3–300 μl) of stock solutions were added to the cultures to obtain the desired dilution of drug to be tested in the bathing medium. The vehicles (ethanol, dimethyl sulfoxide, and acidified L-15) were tested

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Abbreviations: GABA, γ-aminobutyric acid; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol.

*Present address: Departmento de Neurobiologia, Instituto de Biologia-UFF, Outeiro S. J. Baptista, 24210 Niteroi-RJ, Brazil.
†To whom reprint requests should be addressed.

in control cultures, and they did not cause major changes in cell perimeter. For experiments in which treatment with antagonists and agonists were combined, antagonists were added to the cultures 30 min (receptor antagonists) or 60 min (kinase antagonists) prior to addition of the agonists.

RESULTS

At least two types of horizontal cells have been identified in cultures of the bull-head catfish retina. We have confined our observations to the larger of these cells, an example of which is shown in Fig. 1. These cells, like cone horizontal cells in other teleost species, appear to receive input from dopaminergic interplexiform cells. That is, immunohistochemical preparations of the intact retina show numerous tyrosine hydroxylase-staining processes surrounding the horizontal cells (L. Young, personal communication).

Immediately after the dissociation procedure, many of these large horizontal cells present long processes. Most cells attach to the bottom surface of the culture dish in 2–4 hr, but a significant proportion die during the first 24 hr. From the 2nd to the 5th day in culture, the number of large horizontal cells usually remains stable, and changes in perikaryal shape and neurite length occur only very slowly thereafter.

Dopamine typically caused significant retraction of horizontal cell processes and changes in the shape of cell perikarya within 2 hr of its addition to the culture medium. By 4 hr the cell perimeters were reduced an average by 30–40%. Fig. 1*b* shows typical changes induced after 4 hr in a horizontal cell by 100 μM dopamine. Neurites showing particularly marked retraction are indicated by solid arrows in Fig. 1*a*. Fig. 1*c* shows that substantial recovery occurred within 24 hr after dopamine was removed from the bathing solution. The saturating concentration of dopamine for these effects was 100 μM, and significant effects of dopamine (>10% perimeter decrease) were observed with concentrations as low as 1 μM. The effects were generally maximal after 4 hr of exposure to the drug, and cell death was seldom observed even when horizontal cells were exposed to dopamine concentrations of 500 μM.

Fig. 2 shows that haloperidol (10 μM) and SCH 23390 (5 μM), a D1 specific antagonist (7), were able to block substantially the effects of dopamine, whereas sulpiride (50 μM), a selective D2 antagonist (8), was not effective in blocking dopamine effects. Sulpiride did appear to slow the action of dopamine on neurite retraction. That is, in the presence of sulpiride, dopamine had only minimal effects for ≈2 hr, although the dopamine effects were normal by 4 hr in the presence of sulpiride (Fig. 2). The antagonists, when added alone, caused only minor changes in the cell perimeter (i.e., changes of <10%).

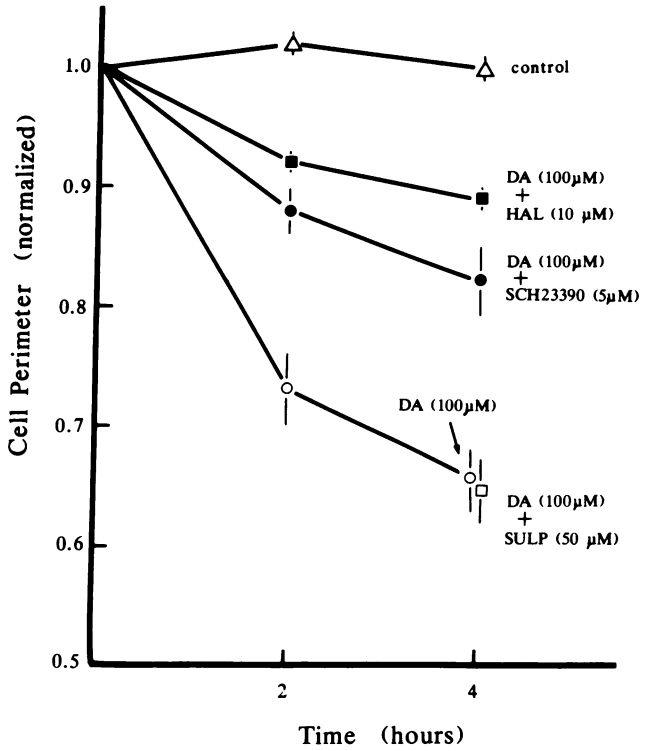

FIG. 2. Effects of 100 μM dopamine (DA; n = 26) on the cell perimeter of large horizontal cells (○). In the presence of 50 μM sulpiride (SULP; n = 10), a D2 antagonist, the effects of DA at 4 hr were about the same as observed in the absence of sulpiride (□). SCH 23390 (5 μM; n = 18) and haloperidol (HAL; 100 μM; n = 19) both significantly blocked the DA effects (●, ■). △, Measurements obtained from cells not exposed to pharmacological agents (control). Ordinate values are posttreatment perimeters expressed as a proportion of the pretreatment cell perimeter (mean ± SEM). Abscissa indicates time of exposure to the pharmacological agents.

Fig. 3 shows that the dopamine effects were partially mimicked by the D1 agonist SKF 38393 (100 μM) but not at all by the D2 agonist, quinpirole (100 μM) (9). A membrane permeable analogue of cyclic AMP, 8-Br-cAMP, at a concentration of 5 mM did not cause significant neurite retrac-

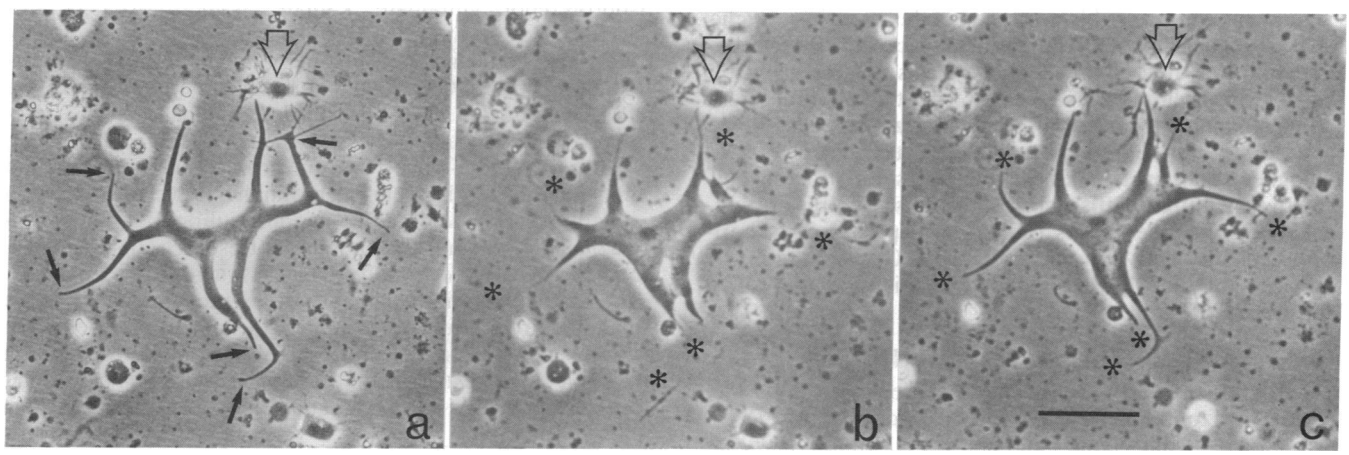

FIG. 1. Phase-contrast light micrographs of bull-head catfish retinal cells. Two morphologically distinct types of horizontal cells are observed in the cultures. The effects of dopamine on the morphology of horizontal cells are more easily seen on the large horizontal cells as compared to the small ones (open arrow). The micrographs show a large horizontal cell just before adding dopamine (100 μM) to the culture (*a*), marked neurite retraction 4 hr after adding dopamine to the bathing medium (*b*), and the recovery that occurred 24 hr after removing dopamine from the bathing solution (*c*). Asterisks in *b* and *c* indicate the extent of the processes marked by solid arrows in *a*. (Bar = 15 μm.)

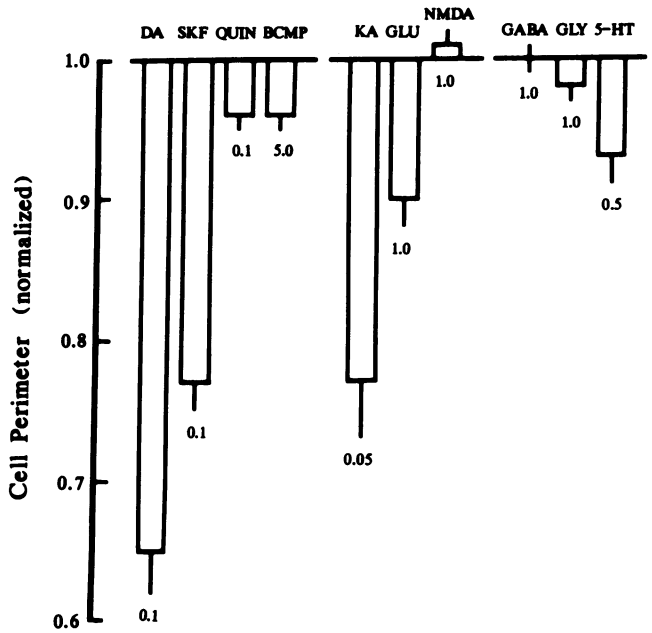

FIG. 3. Effects of dopamine (DA; n = 26), SKF 38393 (SKF; n = 28), quinpirole (QUIN; n = 22), 8-Br-cAMP (BCMP; n = 18), kainate (KA; n = 21), glutamate (GLU; n = 18), N-methyl-D-aspartate (NMDA; n = 13), GABA (n = 25), glycine (GLY; n = 20), and 5-hydroxytryptamine (5-HT; n = 23) on the cell perimeter of large horizontal cells. Each bar represents the posttreatment perimeter (ordinate) expressed as a proportion of the pretreatment cell perimeter (mean ± SEM). Numbers below bars indicate the concentration (mM) of the substance used.

tion, nor did forskolin (data not shown). Isoproterenol (100 μM), like dopamine, caused a marked decrease of cell perimeter (data not shown), but the effects of isoproterenol were blocked by 10 μM haloperidol, indicating that it was most likely activating dopamine receptors.

The only other neuroactive substance that caused neurite retraction comparable to that of dopamine was kainate (50 μM), but the effect of kainate may reflect a toxic action of this agent on the cells. In the presence of kainate, horizontal cells often died, especially after exposure to higher concentrations of the drug (50–500 μM). Glutamate had much less effect than kainate, and other substances tested including N-methyl-D-aspartate (1 mM), GABA (1 mM), glycine (1 mM), and 5-hydroxytryptamine (0.5 mM) were essentially without effect on the cell perimeter.

Effects of Phorbol Esters. As shown in Fig. 4*a*, the phorbol ester PMA caused marked neurite retraction in concentrations as low as 1 nM. This effect was not mimicked by the inactive isomer of PMA, 4-phorbol 12,13-didecanoate, presented at the same concentration (1 nM). Staurosporine (1 μM), an inhibitor of kinase activity (10), effectively blocked the PMA effects on horizontal cells.

As shown in Fig. 4*b*, staurosporine also blocked the effects of dopamine on neurite retraction in horizontal cells. H7 (100 μM), another kinase inhibitor (11), also substantially blocked the effects of dopamine, but it was not as effective as was staurosporine.

Effects of Diacylglycerol Analogs. To investigate further the possible linkage between dopamine and the activation of PKC in mediating neurite retraction in horizontal cells, we next tested the effects of synthetic diacylglycerol analogs (12, 13). Both dioctanoylglycerol (5 μM) and OAG (5 and 30 μM) caused marked neurite retraction. Fig. 4*c* illustrates results with OAG, whose effects were maximal after only 2 hr of exposure. Indeed, by 4 hr, the cells typically showed some recovery from the effects of the drug. As shown in Fig. 4*c*,

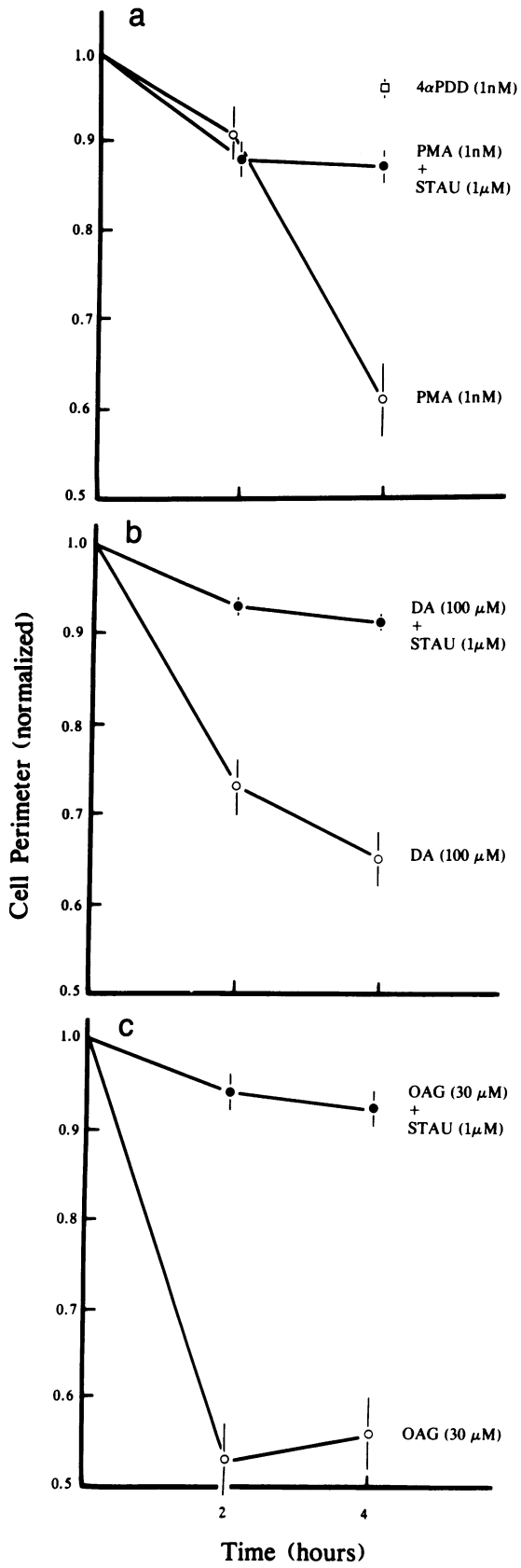

FIG. 4. The phorbol ester PMA (n = 25) (*a*), dopamine (DA; n = 26) (*b*), and the diacylglycerol analogue (OAG; n = 21) (*c*) all cause a marked reduction of the cell perimeter of large horizontal cells (○). The effects of all three drugs are blocked by staurosporine (STAU) (●) (*a*, n = 16; *b*, n = 20; *c*, n = 17). The inactive isomer of PMA, 4-phorbol 12,13-didecanoate (4αPDD), had virtually no effect on horizontal cell perimeter (*a*, □; n = 19).

the effects of OAG were almost completely blocked by staurosporine (1 μM).

DISCUSSION

Our results show that dopamine causes substantial neurite retraction in teleost horizontal cells maintained in culture. Furthermore, this effect is reversible; substantial process outgrowth occurs when dopamine is withdrawn from the bathing medium. Our results further indicate that dopamine mediates this structural effect via diacylglycerol and the activation of PKC. This suggests that dopamine receptors in retinal horizontal cells are linked to phospholipase C, the enzyme that leads to the formation of diacylglycerol and inositol trisphosphate in cells (14, 15).

Numerous studies have demonstrated that dopamine receptors in many neurons are linked to adenylate cyclase, including the dopamine receptors found on teleost horizontal cells (16). Two dopamine receptor types, D1 and D2, are recognized. Activation of D1 receptors increases cyclic AMP production (17, 18), whereas activation of D2 receptors often decreases levels of cyclic AMP (19, 20). Horizontal cell dopamine receptors are of the D1 type (16). Recently, evidence was provided that stimulation of D1 receptors enhances inositol phosphate formation in the rat brain (21, 22), whereas activation of D2 receptors causes a decrease in the basal levels of inositol phosphates (23). In renal tissue also, stimulation of D1 receptors increases inositol phosphate formation (24, 25). The data presented here suggest that in retinal horizontal cells dopamine initiates inositol phospholipid breakdown through interaction with a D1, or D1-like, receptor. However, it is important to note that sulpiride altered the time course of the dopamine effects on neurite retraction, suggesting that D2 antagonists may interact with these receptors on horizontal cells. Further experiments are necessary to characterize better the properties of the receptor involved in the dopamine-mediated effects on neurite retraction. It is also important to determine whether adenylate cyclase and phospholipase C are activated by the same dopamine receptor in horizontal cells via, for example, the same or different guanine nucleotide-binding regulatory proteins. A recent report suggests that dopamine receptors linked to phospholipase C are distinct from dopamine receptors linked to adenylate cyclase (26).

Glutamate, 5-hydroxytryptamine, *N*-methyl-D-aspartate, GABA, and glycine caused only minor changes in cell perimeters, indicating that the dopamine-induced neurite retraction in horizontal cells is specifically related to dopamine action. Isoproterenol was the only neuroactive substance tested that completely reproduced the neurite retraction caused by dopamine. However, its effects were blocked by haloperidol, indicating that isoproterenol was acting via dopamine receptors. Although kainate also caused substantial neurite retraction, its effects seemed to be qualitatively different from the dopamine effects. Kainate in the bathing medium was often associated with cell death, especially when concentrations >50 μM were used.

Dopamine has been shown to inhibit neurite outgrowth in some chicken retinal neurons via cyclic AMP (27). However, neither 8-Br-cAMP nor forskolin mimicked the effects of dopamine on catfish horizontal cells, indicating that neurite retraction caused by dopamine in this system is not mediated by cyclic AMP. This result led us to look for alternative mechanisms that could explain how dopamine induces neurite retraction. Our findings show that the phorbol ester PMA, like dopamine, caused marked neurite retraction in horizontal cells. Furthermore, the effective concentration range of PMA (0.1–1.0 nM) and the lack of effect of the inactive isomer (4-phorbol 12,13-didecanoate) indicate that PKC was being specifically activated (28, 29). Furthermore, both PMA and dopamine-induced neurite retractions were blocked by staurosporine, a potent kinase inhibitor (10). Synthetic diacylglycerol analogs, which are known to activate PKC (12, 13), also caused neurite retraction, and the effects of these substances were also blocked by staurosporine. It is interesting to note that recovery from OAG effects could be observed even without removing the drug from the medium, which probably indicates that OAG is rapidly metabolized in retinal horizontal cells. This has been observed in other biological preparations (13).

During the last few years increasing evidence has been provided showing that neuroactive substances can influence neuronal morphology and structural–functional plasticity by the activation of PKC (30–34). The present work provides evidence that dopamine-induced neurite retraction in isolated horizontal cells results from phospholipase C activation, causing the formation of diacylglycerol and activation of PKC. It should be kept in mind that activation of phospholipase C also results in the formation of inositol 1,4,5-trisphosphate, which is usually involved with the mobilization of calcium from intracellular pools (14, 15, 35). Thus, dopamine-induced morphological changes may result both from the interaction of PKC-catalyzed phosphorylation of specific protein substrates as well as by calcium-mediated processes that affect cytoskeleton structure.

The significance of the retraction of neurites by dopamine in cultured horizontal cells remains to be established. Horizontal cells are known to undergo structural changes between light- and dark-adapted states of the retina [i.e., spinule formation and dissolution (36, 37)], but dopamine does not appear to be the primary agent responsible for these morphological changes (38). On the other hand, it is well established that dopamine modulates several physiological properties of horizontal cells via activation of protein kinase A (see Introduction). We have demonstrated here that dopamine also activates PKC in horizontal cells, resulting in morphological changes in the cells. Thus, dopamine, via the same or a similar receptor, is capable of altering both the structure and function of horizontal cells by the activation of different second messenger systems.

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