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Protein kinase C mediates transient spinule-type neurite outgrowth in the retina during light adaptation

(horizontal cells/synaptic plasticity/phorbol esters/dopamine/neural phosphoproteins)

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ABSTRACT **Light and dark adaptation of the teleost retina is accompanied by a remarkable morphological rearrangement of the synaptic connections between photoreceptors and second-order neurons: during light adaptation, numerous new neurites, the so-called spinules, arise from the terminal dendrites of horizontal cells invaginating the cone pedicle, and during dark adaptation, these spinules are retracted. The formation of these spinules is paralleled by the appearance of color opponency in horizontal and ganglion cells, which led to the suggestion that these spinules are the site of the inhibitory synapses in the negative feedback loop between cones and horizontal cells. The formation of the spinules in the light and their disappearance in darkness have a time course of minutes and are modulated by the neurotransmitters dopamine and glutamate, respectively. Neurotransmitters can modulate neuronal processing through a variety of second messengers that activate protein kinases, resulting most commonly in protein phosphorylation. Herein we report that activation of protein kinase C by phorbol esters promotes the formation of new horizontal-cell spinules in animals kept in the dark. Partial inhibition of protein kinase C activation with sphingosines prevents the formation of new spinules during light adaptation but does not affect established spinules. The spinule-forming effect of phorbol esters is not mediated by dopaminergic neurons, since the effect is also seen in retinas depleted of dopaminergic neurons. Phorbol esters also initiate the formation of spinules in synaptically isolated horizontal cells, demonstrating that they have a direct action on these cells. In addition, isolated horizontal cells have substrate proteins that are phosphorylated in a protein kinase C-dependent manner.**

Horizontal cells are second-order neurons in the vertebrate retina that mediate lateral inhibition in the outer plexiform layer through a negative feedback onto photoreceptors (1). This inhibitory interaction contributes in an essential way to the spatial resolution and luminosity sensitivity of the retina and, in the teleost retina, also to the formation of color opponency (2). Moreover, the extent and the efficacy of this inhibitory interaction is modulated by light and dark adaptations, which affect the electrical coupling between horizontal cells (3), the pattern of their synaptic connections with photoreceptors (4), the sensitivity of their glutamate-gated channels (5), and the balance of rod and cone input in mixed horizontal cells (6).

In the teleost retina, axon-bearing horizontal cells are connected exclusively with cones. Their dendrites invaginate the cone pedicle where they terminate in apposition to the synaptic ribbon (7). During light adaptation, these terminal dendrites form numerous new neurites with membrane densities at their tips, the so-called spinules (4). About 30 min after the onset of light adaptation, their formation is completed and about 20 spinules per dendrite are formed (8). During dark adaptation, these spinules are retracted and disappear on about the same time scale. The formation of these spinules is paralleled by the appearance of color opponency in horizontal and ganglion cells (8–11), making it very likely that these spinules are the site of the inhibitory synapses in the negative feedback circuit.

Spinule-type neurite formation is affected by agonists and antagonists of the neurotransmitters glutamate and dopamine (12–14), which are present in cones and interplexiform cells, respectively; both of these cell types make synaptic contacts onto horizontal cells (2). Glutamate agonists of the kainate/quisqualate-type, but not of the *N*-methyl-D-aspartate type, induce the degradation of established spinules in a light-adapted retina (12). Spinules are not formed during light adaptation in a retina depleted of dopaminergic neurons or in the presence of the dopamine antagonist haloperidol. Exogenously applied dopamine, however, only weakly stimulates the formation of spinules (12, 13, 15). There are conflicting reports about the involvement of cAMP. Isolated retinas incubated with forskolin and isobutylmethylxanthine showed an increased spinule formation (16). Injection of these two substances into the vitreous body of dark-adapted animals, however, did not induce any spinule formation although the retinal cAMP levels were more than doubled (15).

Neurotransmitters can modulate cellular activity through a variety of second messengers and we have recently concentrated on the analysis of a possible contribution of Ca^{2+}/phospholipid-stimulated activation of protein kinase C (PKC). We find that the initiation of spinule formation strongly depends on activation of PKC in horizontal cells whereas the persistence of the spinules does not. Moreover, horizontal cells have substrate proteins that are phosphorylated in a PKC-dependent manner.

MATERIALS AND METHODS

All experiments were done with carp of body length 10–20 cm, which were kept under a controlled light/dark cycle of 12 hr. Fish were light or dark adapted for 4 hr. All surgery with dark-adapted animals were done under dim red light.

Application and Concentration of Drugs. After anaesthesia with MS 222, 10 μl of the vehicle alone [2% (vol/vol) dimethyl sulfoxide at the retinal level for phorbol esters and diacylglycerol, 0.1% ethanol at the retinal level for sphingosine, and fish Ringer's solution (15) for all other substances] was injected into the vitreous body of the left eye. None of the vehicles affected neurite formation in control experiments. The vitreous body of the right eye was injected with the vehicle containing the drug. Drug concentrations were chosen in accordance with the size of the eyeball to reach an

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Abbreviations: PKC, protein kinase C; PDB, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; PDP, 4α-phorbol 12,13-didecanoyl; spr, spinules per synaptic ribbon.

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estimated drug concentration at retinal levels comparable to drug concentrations used in other parts of the central nervous system, which were as follows: phorbol 12,13-dibutyrate (PDB), 2 nM to 500 μM; phorbol 12-myristate 13-acetate (PMA), 100 μM; 4α-phorbol 12,13-didecanoyl (PDP), 100 μM; 1-oleoyl-2-acetyl-*rac*-glycerol, 100 μM; D-sphingosine sulfate, 15 μM; cobalt chloride, 10 mM. The fish were then allowed to swim for 45 min in complete darkness or under room light, after which they were sacrificed. The depletion of dopaminergic neurons was achieved by injection of 50 μg of 6-hydroxydopamine at two consecutive days, 10–14 days prior to the experiments (13, 15).

Electron Microscopy. Both eyes were enucleated either in darkness or under room light. The eyes were hemisected, immediately immersed into ice-cold fixative [2.5% (wt/vol) glutaraldehyde/1.0% paraformaldehyde/3% (wt/vol) sucrose/0.05 M sodium phosphate, pH 7.3], and fixed overnight at 4°C. They were then embedded in resin (TAAB Laboratories) and sections were cut. Central parts of the retina dorsal to the optic nerve were used for analysis.

A quantitative analysis of spinule formation was achieved by counting the number of spinules, identified by their typical shape and membrane densities at their tips, and the number of synaptic ribbons in cross sections of cone pedicles. This was carried out in a minimum of 30 cone pedicles per retina, and three to nine retinas were analyzed for each series. The number of spinules per synaptic ribbon (spr) was calculated (4) and normalized to the control value within a series and the standard deviation was calculated.

Isolation of Horizontal Cells, Phosphorylation, and Gel Electrophoresis. Fractions of horizontal cells were obtained using a modified protocol after Van Buskirk and Dowling (17). The modifications mainly concerned the shape of the glass tube containing the Ficoll gradient and volumes and times that were all adapted to fractionate 10 retinas at the same time. Cell counts of horizontal cell fractions revealed a purity of 65–75%. Protein (10 μg) of these fractions was heat-inactivated (5 min/95°C) and subsequently phosphorylated by added PKC (0.12 ng/10 μg of protein; a gift from A. B. Oestreicher, Utrecht, The Netherlands) in a buffer containing 10 mM Tris·HCl, 10 mM $MgCl_2$, 0.5 mM EGTA, 0.6 mM $CaCl_2$, 2 μM [γ-^{32}P]ATP (2–4 μCi; 1 Ci = 37 GBq; specific activity, 3000 Ci/mmol; NEN), and phosphatidylserine (50 μg/ml) at pH 7.4 in a final incubation volume of 30 μl. The reaction was carried out for 30 min at room temperature and stopped by adding 15 μl of SDS buffer [3% (wt/vol) SDS/30% (vol/vol) glycerol/3% (vol/vol) 2-mercaptoethanol/187 mM Tris·HCl, pH 6.8] and immediately boiling for 1 min. Proteins were separated by SDS/PAGE on a 5–15% polyacrylamide gradient slab gel. The gel was stained with Coomassie brilliant blue then dried on Whatman 3mm filter paper and exposed to Kodak XR-5 x-ray film for 2 days.

RESULTS

After 30 min of light adaptation, about 20 spinules were formed per horizontal cell dendrite (4, 8). In a cross section at the level of the cone pedicle two or three spr could be seen at this stage (Fig. 1*b*). Such spinules are not detectable in dark-adapted control retinas (Fig. 1*a*). The same spinule-type neurite outgrowth could be achieved without any light adaptation by injection of the phorbol esters PDB or PMA into the vitreous of the eye of a dark-adapted fish 45 min prior to enucleation (Figs. 1*d* and 2*a*). The ultrastructure of the cone pedicle including the number of synaptic ribbons and its synaptic connections was identical with that of an animal kept in the light, except that the membrane densities at the tips of the spinules were more pronounced in the phorbol ester-treated retinas. The effect of PDB on spinule formation was dose-dependent and increased over the range 20 nM to 200 μM. Horizontal-cell dendrites of the retina of the control eye, which received an injection of only the vehicle, did not exhibit any neurite outgrowth (Figs. 1*c* and 2*a*) and had an appearance typical of dark-adapted retinas. Injection of the inactive phorbol ester PDP was without any effect on neurite outgrowth (Fig. 2*a*).

To test whether phorbol esters exert their activity through the assumed activation of PKC, we injected the synthetic diacylglycerol 1-oleoyl-2-acetyl-*rac*-glycerol, a known activator of PKC, or D-sphingosine, which competes with diacylglycerol for PKC activation (18), into the vitreous body. Injection of the diacylglycerol initiated the formation of spinule-type neurites in dark-adapted retinas, but to a lesser extent than the injection of phorbol esters (Fig. 2*b*). Injection of sphingosine into either a light- or dark-adapted retina did not reduce the number of spinules already present. However,

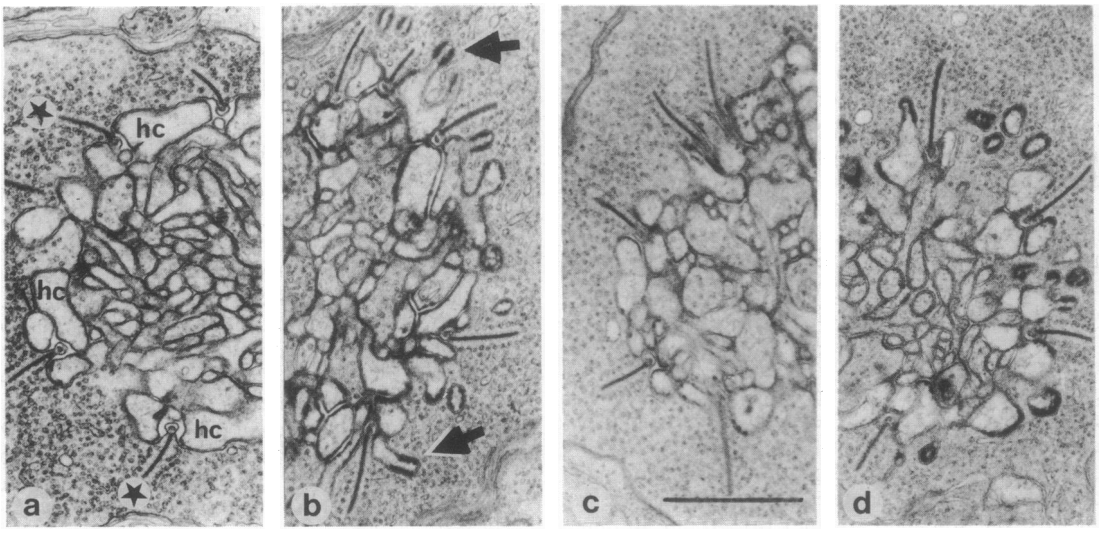

FIG. 1. Electron micrographs of tangential sections through the outer plexiform layer of the carp retina. Each micrograph shows the central part of a cone pedicle with its synaptic ribbons (stars) flanked by the profiles of the horizontal cells (hc). (*a*) Dark-adapted control retina. (*b*) Light-adapted control retina. Numerous spinules (arrows) arise from the horizontal cell dendrites. (*c* and *d*) Left and right retina from a dark-adapted fish. Dendrites from the control retina (*c*) do not have spinules. Dendrites from the retina that received an injection of 100 μM PDB have formed numerous spinules (*d*). (Bar = 1 μm.)

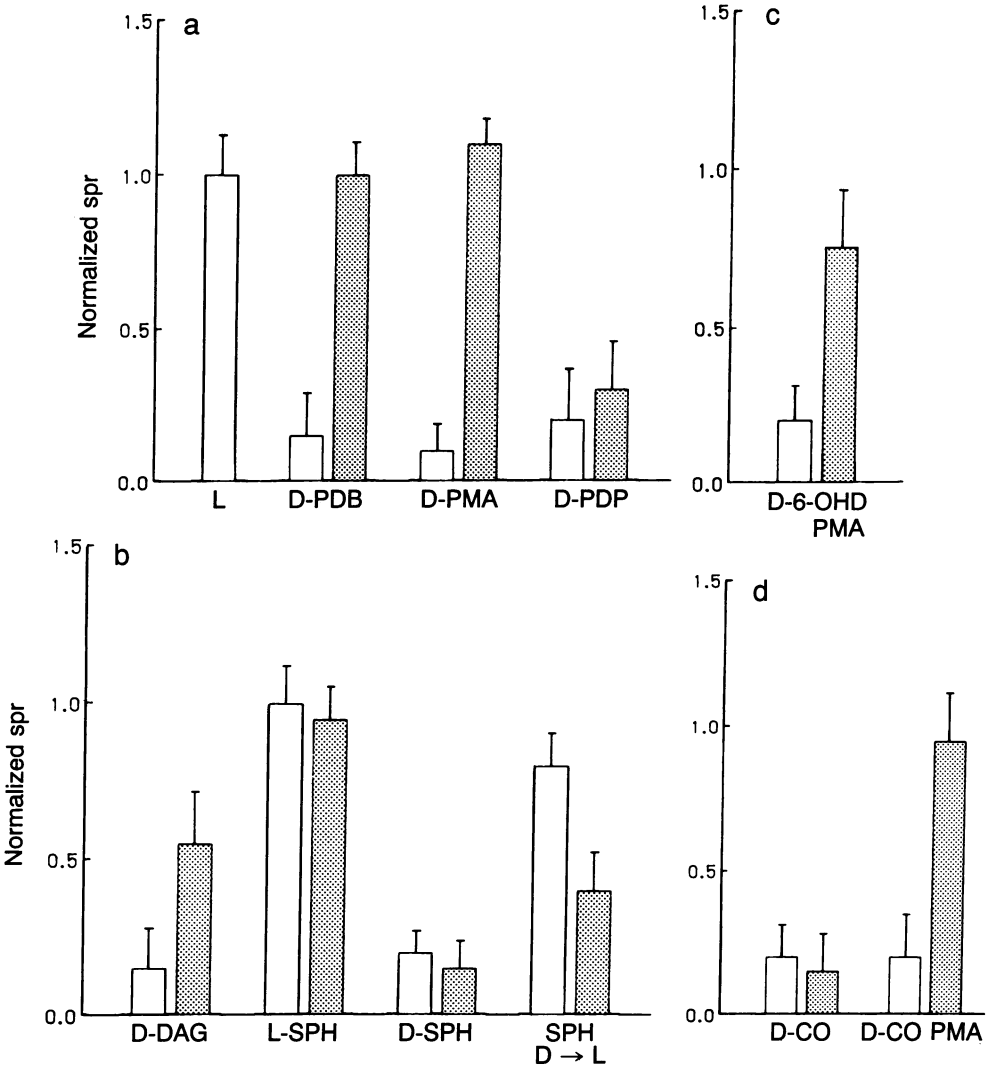

FIG. 2. Normalized spr values. The number of spinules was counted in a minimum of 30 cone pedicles per retina and three to nine retinas were analyzed for each series. Light-adapted control is bar L. This value was set to 1 for all the presented histograms. Open bars represent control retinas; stippled bars represent drug-injected retinas. The first letter indicates the mode of adaptation prior to and during the experiment. L, light; D, dark. (*a*) Effects of active and inactive phorbol esters (100 μM). (*b*) Effects of diacylglycerol and sphingosine. DAG, 1-oleoyl-2-acetyl-*rac*-glycerol; SPH, D-sphingosine sulfate. For the last histogram sphingosine was injected 10 min prior to light adaptation into one eye of a dark-adapted animal. The bars represent the normalized spr value found after 30 min of light adaptation in the control eye and in the injected eye. (*c*) Effect of PMA in dopamine-depleted retinas. 6-OHD, 6-hydroxydopamine. (*d*) Effects of PMA on synaptically isolated horizontal cells. CO, cobalt chloride.

if sphingosine was injected into one eye of a dark-adapted animal 10 min prior to light adaptation, it inhibited the formation of neurites during subsequent light adaptation. After 30 min of light adaptation the number of spinules in the sphingosine-injected eye was significantly lower than that in the control eye (Fig. 2*b*). These results demonstrate that activation of PKC is needed for the formation of spinule-type neurites but not for their persistence since it affected only the process of spinule formation but not existing spinules in a light-adapted retina.

Does the spinule formation result from activation of PKC within horizontal cells themselves or does it result from a modulation of the synaptic input? Two recent papers have localized PKC immunoreactivity in bipolar cells and dopaminergic interplexiform cells and it was reported that phorbol esters increased the release of dopamine (19, 20), suggesting that the action of phorbol esters may be through the dopaminergic system. However, PMA was capable of initiating spinule formation in a retina depleted of dopaminergic neurons (Fig. 2*c*), ruling out the involvement of this system. The depletion was controlled by measuring the total dopamine content of the retina using HPLC-EC techniques (21) and only data from retinas where the dopamine level was less than 10% of an untreated retina were included.

To rule out effects on other presynaptic inputs, we tried to isolate horizontal cells synaptically in the eye cup preparations by superfusion with Ringer's solution where Ca^{2+} was replaced by Co^{2+}. Whereas electrophysiological experiments have demonstrated the synaptic isolation of horizontal cells in such preparations, the number of spinules in both dark- and light-adapted retinas remained unaffected by this treatment (12). Addition of PMA to this superfusate, however, initiated the outgrowth of spinules in synaptically isolated horizontal cells of a dark-adapted retina (Fig. 2*d*). This is in favor of a direct activation of PKC within horizontal cells.

To test whether horizontal cells are capable of a PKC-mediated phosphorylation of proteins, we prepared enriched fractions of isolated horizontal cells. Protein phosphorylation after heat inactivation by added PKC in this horizontal cell fraction was compared with that in an enriched photoreceptor fraction to unravel putative horizontal-cell-specific substrate proteins for PKC. This revealed that PKC has four substrates

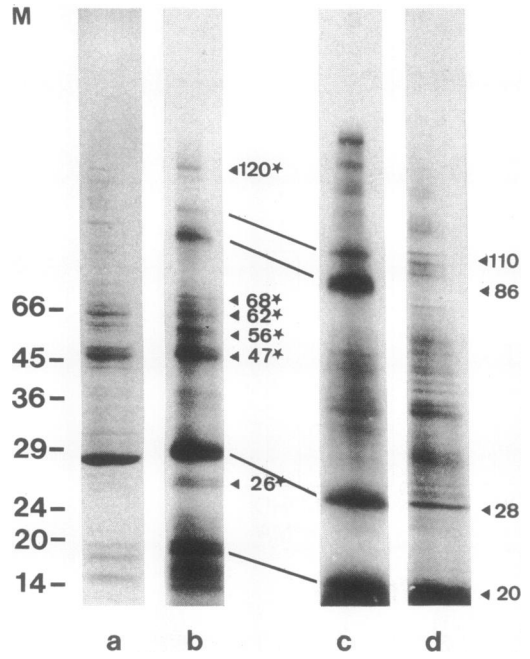

FIG. 3. PKC substrates in isolated and heat-inactivated horizontal cell and photoreceptor fractions of the carp retina. Coomassie-stained protein pattern of horizontal cell fraction (lane a) and photoreceptor cell fraction (lane d). Molecular mass markers are shown in lane M and values in kDa are indicated. Corresponding autoradiographs of exogenous PKC-mediated phosphorylation of the horizontal cell fraction (lane b) and the photoreceptor cell fraction (lane c) are shown. Stars indicate the phosphoproteins prominent in the horizontal cell fraction.

of 20, 28, 86, and 110 kDa, which appear in both fractions. However, the appearance of six other phosphoproteins of 26, 47, 56, 62, 68, and 120 kDa was prominent only in the horizontal-cell fraction (Fig. 3). Since the photoreceptor fraction and the horizontal-cell fraction were obtained during the same fractioning procedure, it is very unlikely that these six phosphoproteins are due to contaminations that would be expected in both fractions. This further supports the idea that specific PKC-mediated substrate phosphorylation within horizontal cells is part of the molecular events underlying the observed transient spinule-type neurite outgrowth from horizontal-cell terminal dendrites during light adaptation.

DISCUSSION

We conclude from the results of this study that activation of PKC is an important step during the initiation of spinule formation from horizontal cell dendrites during light adaptation of the retina. The persistence of established spinules, however, seems not to depend on continuous activation of PKC since it is not sensitive to competitors for this activation. This aspect of light-induced spinule formation is thus similar to the induction vs. maintenance of long-term potentiation in the hippocampus (22, 23).

Activation of PKC has been reported to stimulate or suppress neurite outgrowth during development (24). Its activation is sensitive to a number of substances known to play a role during developmental processes such as nerve growth factor (25) and excitatory amino acid neurotransmitters (26). Our results demonstrate that activation of PKC is also involved in a transient short-term reversible neurite outgrowth during adaptation in a fully developed part of the central nervous system.

Like others (19, 20), we were not able to localize PKC immunocytochemically in horizontal cells by using a monoclonal antibody (Amersham). Since PKC exists in numerous isomeric forms (27), this negative result may be the consequence of using the wrong antibody. On the other hand, horizontal cells of the frog retina increased their incorporation of [^{3}H]inositol into inositol phospholipids when incubated in the light, indicating the possible existence of PKC-mediated effects in these cells (28). Our demonstration that phorbol esters can induce spinule formation even in synaptically isolated horizontal cells and the demonstration of specific PKC-sensitive substrate proteins in horizontal cells strongly suggest the existence of a PKC in these neurons.

Phorbol esters have been reported to increase dopamine release in the fish retina (20) and one might argue that spinule-type neurite formation is a consequence of increased dopaminergic action onto horizontal cells that have D1 receptors (29). However, our data show that phorbol esters can induce spinule formation in synaptically isolated cells and even in retinas that lack dopaminergic neurons. Thus with the observation that spinule formation *in vivo* does not depend on cAMP (15), these data exclude an action of phorbol esters through dopaminergic neurons.

The neuronal circuit underlying this plasticity in the retina involves both glutamate and dopamine (12, 13). We do not yet know how the activity of these neurotransmitters is linked to the cellular regulation of PKC activation in horizontal cells. However, from the observation that glutamate inhibits the formation of spinules (12) one might conclude that glutamate acts through an inhibition of phosphatidylinositol metabolism (30).

Phorbol esters produce a long-lasting enhancement of synaptic responses similar to long-term potentiation in hippocampus neurons (31, 32). The underlying mechanisms are not yet fully understood and several possibilities are discussed. Our observations that phorbol esters can stimulate morphological changes at synaptic sites on a similar time scale reveal aspects of synaptic enhancement and may contribute to a further understanding of this important aspect of synaptic plasticity.

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