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Characterization of Protein Kinase C in Photoreceptor Outer Segments

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Abstract: Protein kinase C (PKC) has been implicated in regulating several proteins involved in phototransduction. This contribution characterizes the biochemical and immunological properties of PKC isozyme(s) in the photoreceptor outer segment. Activity measurements revealed that at least 85% of the PKC in this specialized compartment belongs to the subfamily of Ca^{2+}-regulated (conventional) PKCs. Of the known Ca^{2+}-dependent PKCs, only PKCα was immunodetected by western blot analysis of rod outer segment proteins. However, the ratio of immunoreactivity to enzyme activity for rod outer segment PKC was no more than 40% of that for brain PKC, using antibodies against conventional PKCs. Therefore, at least half the Ca^{2+}/lipid-stimulated activity in rod outer segment preparations cannot be accounted for by the known isozymes, suggesting the presence of a previously uncharacterized isozyme. Despite extensive tests using a variety of antibodies against different domains of PKCα, PKCα could not be detected in rod outer segments by immunofluorescence of retinal sections. In summary, our data reveal that most of the PKC in photoreceptor outer segments is of the conventional type and that most, if not all, of this conventional PKC activity comes from a novel isozyme(s). **Key Words:** Retina—Photoreceptor—Outer segment—Protein kinase C—Isozymes—Phorbol ester.
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Protein kinase Cs (PKCs) make up a family of serine/threonine kinases. The first-discovered members were found to be activated by Ca^{2+}, phospholipid, and diacylglycerol (Nishizuka, 1992; Newton, 1995), although of the dozen mammalian isozymes now known, only the α, βI, βII, and γ isozymes (conventional PKCs) are regulated by Ca^{2+} (Dekker et al., 1995). PKCs have important roles in the brain, where they function in a wide variety of cellular events, from short-term changes, such as membrane permeability, to long-term changes, such as cell growth (Nishizuka, 1986, 1992).

In the neural retina, a number of different PKC isozymes have been detected, and immunocytochemical studies show isozyme-specific distributions among the different types of cells in the inner retina. Indeed, the selective labelling by antibodies against PKCα and PKCβ, for example, has been exploited extensively by electrophysiologists and anatomists interested in the classification of inner retinal cells (e.g., de la Villa et al., 1995; Euler and Wassle, 1995; Gillette and Dacheux, 1995; Strettoi and Masland, 1995). The functions of PKCs in the inner retina are unclear, although one identified substrate is vimentin (Williams et al., 1994), and indicated roles include regulation of $GABA_c$ receptors in bipolar cells (Feigenspan and Bormann, 1994) and mediation of synaptic plasticity between horizontal cells and photoreceptor cells (Weiler et al., 1991).

In photoreceptor outer segments, PKC activity has been detected (Kelleher and Johnson, 1985, 1986; Wolbring and Cook, 1991), and a number of proteins that are important in phototransduction have been reported to be phosphorylated by PKC: the α subunit of transducin (Sagi-Eisenberg et al., 1989); the γ subunits of cyclic GMP-phosphodiesterase (Udovichenko et al., 1994); the rod cyclic GMP-gated channel (Liu et al., 1994); arrestin (Weyand and Kühn, 1990); and rhodopsin (Kelleher and Johnson, 1986; Newton and Williams, 1991, 1993; Greene et al., 1995). In addition, outer segment guanylate cyclase activity is increased by conditions that promote PKC activation (Wolbring and Schnetkamp, 1995). However, despite a variety of possible roles for PKC in outer segments, little is known about the PKC isozyme (or isozymes)

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Abbreviations used: PDBu, phorbol dibutyrate; PKC, protein kinase C.

TABLE 1. *Antibodies used to detect PKCα in retinal sections; none labelled the photoreceptor outer segments*

Antibody	Type	Epitope location	Source	Reference
PKCα and β	pAb against peptide	Pseudosubstrate	Makowske (gift)	1
	pAb against peptide	Pseudosubstrate	D.S.W.'s laboratory (two different antibodies)	
	mAb	Catalytic domain	S.J.'s laboratory (M7)	4
PKCα only	mAb	Hinge domain	Amersham (MC5)	2
	mAb	Regulatory domain	UBI	3
	mAb	Catalytic domain	S.J.'s laboratory (M6)	4
	mAb	Regulatory domain	S.J.'s laboratory (M9)	4
	pAb against peptide	Hinge domain	Stramm, Lilly (gift)	
	mAb	Catalytic domain	Stramm, Lilly (gift)	
	pAb against peptide	α carboxyl tail	Fabbro, Ciba (gift)	
	pAb against peptide	Hinge domain	GIBCO	

Antibodies were specific for either PKCα only (lower group) or for PKCα and β (upper three). pAb, polyclonal antibody; mAb, monoclonal antibody. References are as follows: (1) Makowske and Rosen (1989); (2) Walker et al. (1990); (3) Usuda et al. (1991); (4) Leach et al. (1988). The pseudosubstrate antibodies were made against peptides, either RFARKGALRQKNVHEVKN (Makowske and Rosen, 1989) or RFARKGALRQKNVH (D.S.W.'s lab). The PKCα antibody from Dr. Stramm was made against the peptide, LGPAGNKVISPSEDRKQPSNN-LDRV. The PKCα antibody from Dr. Fabbro was made against the peptide, SYVNPQFVHPILQSAV. The PKCα antibody from GIBCO was made against the peptide, AGNKVISPSEDRRQ. The monoclonal antibody, M7, recognizes PKCα and β.

in this compartment, including its identity. In the first immunocytochemical study on PKC in the retina, Wood et al. (1988) showed that a polyclonal antibody against brain PKC labelled the outer segments intensely. But since then, studies using isozyme-specific antibodies have been notable for the *lack* of labelling of outer segments (Greferath et al., 1990; Osborne et al., 1991, 1992, 1994; Usuda et al., 1991; Zhang and Yeh, 1991; Kolb et al., 1993; Ghalayini et al., 1994; Koistinaho and Sagar, 1994). In the present study, we have explored the characteristics and identity of the PKC isozyme (or isozymes) in outer segments.

MATERIALS AND METHODS

Materials

Bovine brain L-α-phosphatidylserine and L-α-dioleoylglycerol were purchased from Avanti Polar Lipids. ATP, 5-bromo-4-chloro-3-indoyl phosphate, histone H1, leupeptin, and phorbol dibutyrate (PDBu) were supplied by Sigma Chemical Company. The use and supply of Ep-475 were described by Williams et al. (1989). [γ-^{32}P]ATP (3.0 Ci mmol^{-1}) was from Du Pont–New England Nuclear, and [^{3}H]PDBu (10–20 Ci mmol^{-1}) was from Amersham. Nitrocellulose was purchased from Schleicher and Schuell. Alkaline phosphatase-conjugated goat anti-rabbit IgG was supplied by Boehringer Mannheim Biochemicals. The PKC-selective peptide Ac-FKKSFKL-NH_2 (Chakravarthy et al., 1991) was synthesized by the Biochemistry Biotechnology Facility at Indiana University. The antibodies against PKCα and PKC consensus sequences are listed in Table 1. They were either made in our labs, given to us by Drs. Stramm (Eli Lilly, Indianapolis, IN, U.S.A.), Mochly-Rosen (Stanford, CA, U.S.A.), Makowske and Rosen (Makowske and Rosen, 1989), or Fabbro (Ciba–Geigy, Basel, Switzerland), or purchased from Amersham, Calbiochem, UBI, or GIBCO. Antibodies against PKCβI, βII, and γ were obtained from Drs. Stramm or Fabbro, or purchased from GIBCO. The antibodies against the pseudosubstrate domain of conventional PKCs were made against the peptide, RFARKGALRQKNVH or RFARKGALRQKNVHEVKN, which correspond to the sequence found in rat PKCα and β. A mixture of PKC isozymes was partially purified from rat brain by Q-Sepharose chromatography (Orr and Newton, 1992).

Isolation of photoreceptor outer segments

Photoreceptor outer segments were isolated from dark-adapted rats (as described by Williams et al., 1989) or, for the [^{3}H]PDBu binding experiments, from light-adapted bovine eyes (on sucrose gradients as described by Papermaster and Dreyer, 1974). Bovine retinas yield a population of photoreceptor outer segments that is predominantly from rod photoreceptors, but it does contain some cone outer segments. Rat retinas contain very few cone photoreceptors, so that the rat preparation is highly enriched in rod photoreceptor outer segments.

In brief, the procedure for obtaining rat rod outer segments is as follows. Retinas were removed and collected in 120 m*M* NaCl, 1 m*M* EGTA, 1 m*M* EDTA, 10 μ*M* Ep-475, 20 m*M* HEPES, pH 7.3, at 4°C (10 retinas/3 ml). They were then vortexed to break off the rod outer segments, and the resulting suspension of crude rod outer segments was centrifuged at 800 *g* for 3 min to remove nuclei, and then layered over a Percoll gradient. Centrifugation at 9,400 *g* for 45 min at 4°C, resulted in the separation of purified sealed and unsealed rod outer segments as two distinct bands. The sealed and unsealed rod outer segments were collected separately and centrifuged at 100,000 *g* for 60 min at 4°C, to remove the Percoll. The loose pellet of rod outer segments (on top of the solid Percoll pellet) was removed. To effect osmotic lysis, the outer segments were resuspended in 10 m*M* EDTA, 1 m*M* dithiothreitol, 5 m*M* KCl, 21 μ*M* leupeptin, 0.2 m*M* phenylmethylsulfonyl fluoride, 20 m*M* Tris, pH 7.4, at 4°C, except for one experiment in which the lysis buffer also contained 0.5% Triton X-100. The cytosolic and particulate fractions were then separated by centrifugation of the lysate at 500,000 *g* for 20 min at 4°C. In some experiments, sealed and unsealed rod outer segments were combined prior to lysis. All procedures were carried out under dim red light unless otherwise stated.

PKC activity assay

The initial rate of phosphorylation of the PKC-selective peptide Ac-FKKSFKL-NH_2 catalyzed by PKC from the cytosolic fraction or detergent-soluble fraction of sealed, or combined sealed and unsealed, rod outer segments was measured as described (Orr et al., 1992). In brief, rod outer segment samples were incubated in an 80-μl reaction volume containing phosphatidylserine (0–12 mol%)/diacylglycerol (5 mol%)/Triton X-100 (0.1%) mixed micelles, 0–1 m*M* $CaCl_2$, 20 μM ATP, 15 m*M* $MgCl_2$, 50 μg ml^{-1} peptide substrate, 0.6–1.0 m*M* EGTA, 0–0.1 m*M* EDTA, 0.1 m*M* leupeptin, 1 m*M* dithiothreitol, 0.01 m*M* phenylmethylsulfonyl fluoride, 15 m*M* Tris, pH 7.4, at 30°C. Lipid-independent activity was assayed by omitting phosphatidylserine and diacylglycerol from Triton X-100 micelles; Ca^{2+}-independent activity was measured in the presence of 0.7 m*M* EGTA, 0.2 m*M* EDTA, and no Ca^{2+}. Reactions were quenched after incubation at 30°C for 10 min, and phosphate incorporation was determined as described (Orr et al., 1992). In some experiments, the activity of baculovirus-expressed isozymes or partially purified PKC from brain was assayed, and in others, the synthetic peptide substrate was replaced with histone H1 (157 μM). Concentrations of free Ca^{2+} were calculated according to Klee (Jean and Klee, 1986).

^{3}H-Phorbol ester binding

Binding of [^{3}H]PDBu to bovine photoreceptor outer segments, prepared by sucrose density centrifugation (Papermaster and Dreyer, 1974), was performed as described (Jaken, 1987). In brief, the amount of [^{3}H]PDBu binding to aliquots (50 μg of protein) of outer segments was measured over a concentration range of 1–80 n*M* [^{3}H]PDBu in the presence or absence of 1 μM cold PDBu. Bound and free ligand were separated by centrifugation of rod outer segments at 10,000 *g* for 15 min at 4°C, and radioactivity was quantified by liquid scintillation counting. Data were analyzed by the method of Scatchard using the program LIGAND (Munson and Rodbard, 1980).

Western blotting

The PKC activity in the cytosolic fraction from freshly isolated rod outer segments was assayed using histone as substrate. Cytosol containing a defined amount of PKC activity was prepared for sodium dodecyl sulfate–polyacrylamide gel electrophoresis, as were samples with the same amount of PKC activity from a preparation of PKC purified from rat brain (~90% of the lipid-stimulated activity from the rat brain preparation was Ca^{2+}-dependent). Samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10% acrylamide), transferred to nitrocellulose or Immobilon-P, and probed with primary and secondary antibodies. The secondary antibodies were conjugated to alkaline phosphatase, so that labelling was detected by the formation of the insoluble product of 5-bromo-4-chloro-3-indoyl phosphate hydrolysis.

Immunocytochemistry

Bovine and rat eyecups were fixed in 4% paraformaldehyde in 0.1 *M* phosphate buffer, pH 7.4. Retinas were infiltrated in OCT compound for thick cryosections (10 μm) and sucrose for semithin (0.5 μm) cryosections. Immunofluorescence labelling was carried out as described previously (Williams et al., 1990; Azarian et al., 1993).

RESULTS

PKC activity in photoreceptor outer segments

For assays of photoreceptor outer segment PKC activity, we attempted to obtain outer segments that were as pure as possible. Thus, we measured the PKC activity in lysates of sealed rat rod outer segments that had been isolated on Percoll gradients. Despite a considerably lower yield of material, this preparation was preferable to the more commonly used bovine photoreceptor outer segment preparations. First, it contains less contamination from other parts of the photoreceptor cells and from other retinal cells; it contains the highest concentration of rhodopsin and the lowest concentration of non–outer segment proteins (e.g., vimentin) (Williams et al., 1994, and unpublished observations). Second, in this procedure, most of the outer segments have been shown to reseal and retain a proportion of their soluble proteins (Shuster and Farber, 1984). The sealed outer segments are well separated in the Percoll gradient from the lighter outer segments that have not resealed. Still, it should be noted that this preparation is far from perfect. Cytosolic proteins were released from the outer segments during breakage of the connecting cilium, and some proteins were undoubtedly gained following leakage from other retinal cells or other photoreceptor cell compartments and as a result of contamination. For example, after centrifugation to sediment the sealed rod outer segments, 98% of rhodopsin kinase activity was recovered from the supernatant. Feraudi (1983) noted previously that most of the rhodopsin kinase was lost from bovine photoreceptor outer segment preparations.

To test whether PKC in photoreceptor outer segments belongs to the subtype of conventional (Ca^{2+}- and lipid-regulated), novel (lipid-regulated), or atypical (activity requires phosphatidylserine) isozymes, the cofactor dependence for its phosphorylation of a PKC-selective peptide (Chakravarthy et al., 1991) was measured. Figure 1 shows that phosphorylation of the peptide was stimulated by both Ca^{2+} and lipid, indicating that the activity in rod outer segments is catalyzed by a conventional PKC. Allowing for the limits of accuracy of the assays, we conclude that Ca^{2+}-independent, but lipid-dependent, activity accounted for <15% of the measured activity.

Under the conditions of the assay, the apparent K_m for Ca^{2+} for rod outer segment PKC was 21 ± 5 μM Ca^{2+} (Fig. 2a). For comparison, under the same assay conditions, PKCβII had a K_m for Ca^{2+} that was ~10-fold higher. Because the affinity of PKC for Ca^{2+} is regulated allosterically by phosphatidylserine, it is important to note that this K_m is specific for these experimental conditions and serves only as an indication that this isozyme is dependent on Ca^{2+} for activity, with a 10-fold higher affinity for this cation than another Ca^{2+}-regulated isozyme, PKCβII. The affinity of PKCα for Ca^{2+} is also ~10-fold higher than that for PKCβII (L. Keranen and A. Newton, unpub-

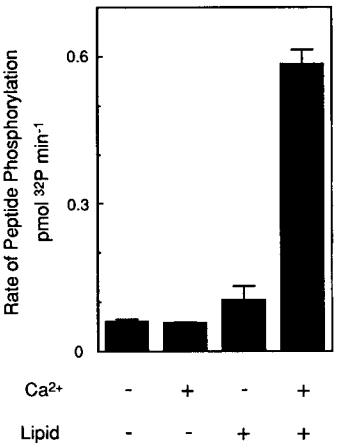

FIG. 1. The PKC activity in the detergent-soluble fraction from sealed rod outer segments is Ca^{2+}-dependent. Sealed rat rod outer segments isolated by Percoll gradient centrifugation from dark-adapted retinas were lysed as described in Materials and Methods, and PKC activity in the cytosolic fraction was measured in the presence of Triton X-100 (0.1%) micelles in the presence or absence of lipid [10 mol% phosphatidylserine (173 μM) and 5 mol% diacylglycerol (86 μM)] and in the presence or absence of 0.4 mM free Ca^{2+}. Phosphorylation of a PKC-selective peptide was measured as described in Materials and Methods. The rate is expressed as picomoles of phosphate incorporated into saturating amounts of peptide catalyzed by PKC in rod outer segments recovered per retina; data represent the means ± SEM of triplicate measurements.

lished observations), revealing that both PKCα and rod outer segment PKC have similar Ca^{2+} dependencies.

The phosphatidylserine dependency for activity of rod outer segment PKC, using a Triton X-100 mixed micelle assay, revealed an apparent K_m for phosphatidylserine of 5.8 ± 0.1 mol% (Fig. 2b, ○). For comparison, that of PKCβII, measured under the same conditions, was 7.2 ± 0.2 mol% (△). Previously published values for the apparent K_m values for phosphatidylserine, under these assay conditions, for the conventional PKCs are 6.3 ± 0.1 mol% for PKCα, 7.1 ± 0.1 mol% for PKCβII, and 6.1 ± 0.1 mol% for PKCγ (Burns et al., 1990). Similar to the dependence of PKCα and PKCβII, the activity of rod outer segment PKC in Triton micelles was absolutely dependent on phosphatidylserine (PKCγ has significant basal activity). Thus, the affinity of rod outer segment PKC for phosphatidylserine is relatively high and most similar to that of PKCα.

In an attempt to quantify the number of molecules of PKC in photoreceptor outer segments, binding of the PKC ligand, PDBu, was measured. Due to limitations in the amount of material available, this experiment was performed with bovine photoreceptor outer segments rather than Percoll gradient-purified rat rod outer segments. Figure 3 shows a Scatchard analysis of data from the binding of [^{3}H]PDBu to light-adapted bovine photoreceptor outer segments, measured in the presence of Ca^{2+} (~0.2 mM free Ca^{2+}), from a typical experiment: compilation of the data from six experiments revealed a K_D of 9.4 ± 1.9 nM and binding capacity of 10.5 pmol of phorbol ester per milligram of protein. Assuming 95% of the protein in outer segment membranes is rhodopsin (Krebs and Kühn, 1977), then the binding capacity suggests 10.5 pmol of phorbol ester-binding protein per 25 nmol of rhodopsin (MW, 38,000), equivalent to one PKC per 2,000 rhodopsin [the stoichiometry of phorbol ester binding to PKC is 1:1 (Sharkey et al., 1984)]. The high-affinity binding to PDBu was sensitive to Ca^{2+}: in the absence of Ca^{2+}, the affinity decreased to 25 nM, consistent with the presence of a Ca^{2+}-regulated PKC. Note that Ca^{2+}-regulated PKCs bind membranes in the absence of Ca^{2+} if membranes contain sufficiently high concentrations of either of its other cofactors, phorbol esters or phosphatidylserine (Newton, 1995). No significant differences in PDBu binding to dark-adapted or light-adapted outer segments were observed. The amount of binding increased fourfold between crude outer segments (before sucrose gradient purification) and puri-

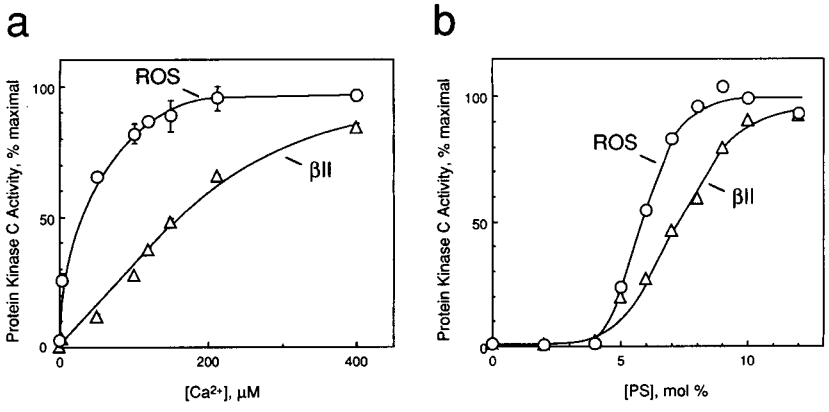

FIG. 2. Cofactor dependence of the PKC isolated from rod outer segments (ROS). **a:** The detergent-soluble fraction of combined sealed and unsealed rod outer segments was incubated in the presence of Triton X-100 (0.1%) mixed micelles containing 10 mol% phosphatidylserine and 5 mol% diacylglycerol, and the indicated concentrations of free Ca^{2+} (○). For comparison, the Ca^{2+} dependence of rat PKCβII assayed under identical conditions is shown (△). Data represent the means ± SEM of triplicate measurements. **b:** The cytosolic fraction of combined sealed and unsealed rod outer segments was incubated with 0.4 mM free Ca^{2+} and Triton X-100 (0.1 mol%) mixed micelles containing 5 mol% diacylglycerol and 0–12 mol% diacylglycerol (○). For comparison, the phosphatidylserine (PS) dependence of rat PKCβII is indicated (△). Curves are those predicted by fitting the data to a modified Hill equation, as described (Orr and Newton, 1992).

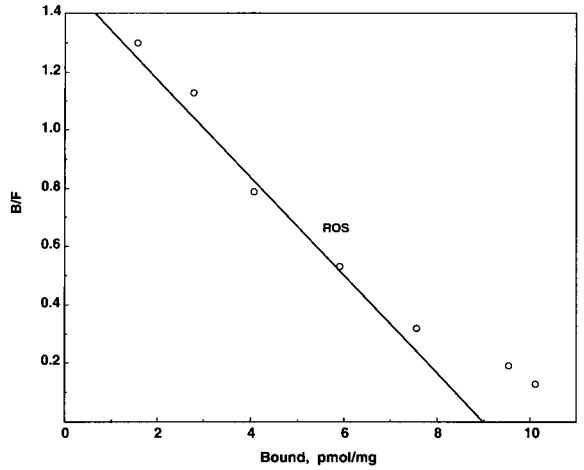

FIG. 3. Scatchard analysis of ^{3}H-phorbol ester binding to rod outer segments (ROS). Rod outer segments were prepared as described in Materials and Methods. Aliquots (50 μg) of protein were assayed for [^{3}H]PDBu binding over the concentration range of 1–80 n*M* [^{3}H]PDBu. Nonspecific binding was measured in the presence of 1 μ*M* cold PDBu. Bound and free PDBu were separated by centrifugation of treated rod outer segments, as described in Materials and Methods. Data are plotted as bound/free ligand versus bound ligand per milligram of protein; the line is the best fit to the Scatchard equation.

fied outer segments, consistent with enrichment of PKC in photoreceptor outer segments.

Identity of PKC in photoreceptor outer segments

As the above tests indicated that outer segment PKC is dependent on Ca^{2+}, we explored whether it was immunoreactive with antibodies against the known conventional PKCs on western blots of the cytosolic fraction of sealed rat rod outer segments. PKC activity was measured in preparations of rod outer segments, partially purified rat brain PKC, and purified recombinant rat PKCβII. Samples of each, with equivalent activities (0.012 unit using histone as the substrate), were prepared for western blot analysis. As all samples originated from cytosolic fractions, they would all contain PKC that was quantitatively phosphorylated at the two C-terminal sites that regulate activity. No immunoreactivity of rod outer segments was observed when blots were probed with antibodies against peptides corresponding to the C-terminus of PKCβI (data not shown), PKCβII, or PKCγ (Fig. 4b and c). However, faint immunoreactivity of the rod outer segment sample was observed when blots were probed with antibodies against PKCα; we used antibodies against the hinge domain (from GIBCO) or the regulatory domain (from UBI) (Fig. 4a). This immunoreactivity with rod outer segment PKC was considerably less intense than that observed for the rat brain isozymes, of which approximately one third of the PKC was PKCα as assessed by isozyme separation on hydroxylapatite chromatography (data not shown). These results indicate that rod outer segments contain PKCα plus another PKC isozyme that is not recognized by antibodies against the known Ca^{2+}-dependent isozymes. Given the paucity of cones in a rat retina, this unidentifiable PKC activity is unlikely to have originated from cone outer segments in the outer segment preparation.

In an additional test, we probed western blots with antibodies against the pseudosubstrate domain, which is highly conserved among the Ca^{2+}-dependent PKC isozymes. The activity from the cytosolic fraction of sealed rat rod outer segments was measured, with the concentration adjusted so that this sample and a sample of rat brain cytosolic PKC had the same activity. When both rod outer segment and brain samples were combined, the activity doubled. This linearity revealed that inhibitors or activators were not influencing the activity of PKC in these samples. Because the specific activity of PKC isozymes is similar [~10 reactions catalyzed per second per PKC (Newton, 1995)], both samples should contain the same concentration of PKC. Aliquots containing equal units of PKC were then analyzed. Once again, the PKCα immunoreactivity of the rod outer segment PKC was less than half that for a comparable amount of rat brain PKC (Fig. 5). Compilation of data from three experiments indicated that 40% of rod outer segment protein is immunoreactive with antibodies against PKCα or the pseudosubstrate domain, suggesting that 40% of the sample is PKCα and 60% is a previously undescribed Ca^{2+}-dependent PKC. When the experiment was done with crude rod outer segments instead of purified sealed rod outer segments, this ratio changed from 40:60 to 80:20, indicating that the outer segments are the source of the unknown Ca^{2+}-dependent PKC.

Next, retinal sections were labelled with antibodies

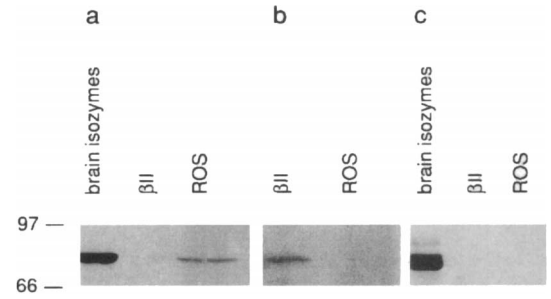

FIG. 4. Western blot analysis of rod outer segments (ROS) probed with antibodies against conventional PKCs. The cytosolic extract from dark-adapted, sealed rat rod outer segments was assayed using histone as the substrate. Samples of this extract, each containing 0.012 unit of activity (corresponding to nine retinas), were loaded in gels. Also loaded, in adjacent lanes, was purified PKCβII, containing 0.012 unit of activity, and partially purified rat brain PKC, containing 0.012 unit of activity (this brain PKC is a mixture of different isozymes, of which one third of the activity was from PKCα). Western blots of the gels were probed with antibodies against PKCα (GIBCO) (**a**), PKCβII (Lilly) (**b**), or PKCγ (GIBCO) (**c**). Labels above the blots indicate the PKC isozyme(s) in each lane.

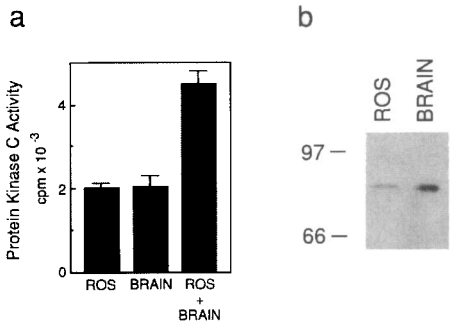

FIG. 5. The pseudosubstrate domain of rod outer segment (ROS) PKC differs from that of other Ca^{2+}-dependent PKCs. **a:** Aliquots of cytosolic PKC from sealed rat rod outer segments (light-adapted) or from rat brain were assayed using concentrations so that the activity toward phosphorylating the PKC-selective peptide would be identical. When rod outer segment and brain aliquots were combined, the total activity increased by a factor of 2, indicating that inhibitors or activators were not influencing the activity in either fraction. **b:** Aliquots of cytosolic PKC from rod outer segments or from brain, each containing 0.007 unit of PKC activity, were analyzed by western blot analysis and probed with antibodies against the conserved pseudosubstrate of conventional PKCs.

against the known conventional PKCs. Because immunoreactivity was detected on western blots of rod outer segment proteins with antibodies against PKCα, we focused on antibodies that would recognize this isozyme. A summary of the antibodies used is given in Table 1. Western blot analysis to test the immunoreactivity of the antibodies is shown for some in Fig. 6; all antibodies recognized pure rat PKCα (lane 2) and a polypeptide of the same mobility among rat retinal proteins (lane 1); most, such as those used in Figs. 6 and 7, did not recognize other rat retinal polypeptides. Invariably, none of the antibodies—neither those specifically against PKCα nor those against the pseudosubstrate domain of PKCα, β, and γ, or other PKC consensus sequences—labelled the outer segments in sections of rat or bovine retinas. Both light- and dark-adapted retinas were examined, and no difference was noted in the labelling pattern between the two conditions. We used tissue that had been fixed in formaldehyde for different lengths of time (from just a few minutes to weeks) and prepared cryosections, as they typically give superior antigen preservation. Both thick and semithin (0.5 μm) sections were used; the latter were preferred, for they are less than the diameter of a single photoreceptor outer segment, and thus permit better penetration of antibody into the tissue. These procedures have been used by us previously to immunolabel outer segment proteins, including another Ca^{2+}-dependent enzyme that associates with membranes, calpain II (Azarian et al., 1993).

Examples of semithin (0.5 μm) sections of rat retinas labelled with antibodies against PKCα are shown in Fig. 7. It is interesting that the retinal labelling pattern differed somewhat among the different antibodies. The most commonly used antibody against PKCα (MC5 from Amersham) labelled bipolar cells entirely, as well as amacrine cell bodies in the rat retina (data not shown here, but see the following: Greferath et al., 1990; Osborne et al., 1991, 1992; Zhang and Yeh, 1991). Another antibody (from UBI) was the same as that used by Usuda et al. (1991). Its labelling of rat retina was similar to that obtained with the MC5 antibody and similar to that reported by Usuda et al. (1991) for the rabbit retina; one difference is that, in our sections, the Müller cell endfeet (in the ganglion cell layer) appeared labelled (Fig. 7d; note that in this 0.5-μm section, the bipolar cell axons cannot be followed along their entire length). In contrast, two other antibodies (M6 and M9) did not label the bipolar cell axons or synapses well, and they labelled some additional cells (Fig. 7a–c). Different labelling of cell bodies in the inner nuclear layer can be seen by comparing Fig. 7a and b with Fig. 7d. In Fig. 7a and b, most of the labelled cell bodies are in the proximal part of the inner nuclear layer, whereas in Fig. 7d most are in the distal part. Moreover, as shown in Fig. 7a–c, horizontal cell bodies (e.g., arrowhead in Fig. 7c) and axons (in the outer plexiform layer) and ganglion cell bodies are labelled. These differences in labelling by what appear to be PKCα-specific monoclonal antibodies suggest that the precise distribution of PKCα in the inner retina should be explored further. Inner retinal labelling by most of the other antibodies used was not examined in detail; this region was usually overexposed in micrographs to see if any light labelling of the outer segments could be detected. For our pur-

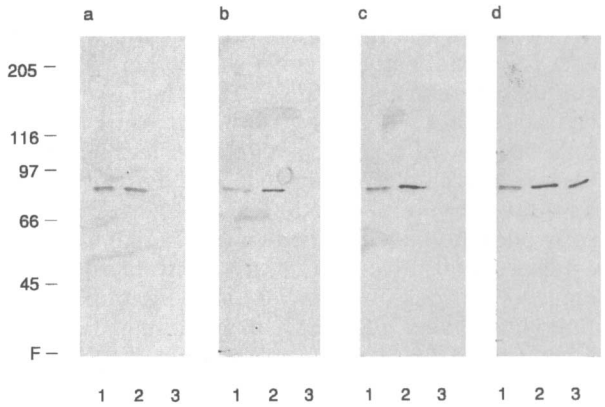

FIG. 6. Western blot analysis of the PKCα antibodies used in Fig. 7 and a PKC pseudosubstrate antibody. In each panel, lane 1 contained 100 μg of protein from rat retinal lysate, lane 2 contained 10 ng of purified brain PKCα, and lane 3 contained 10 ng of purified brain PKCβ. **a:** PKCα monoclonal antibody (M6) against an epitope in the catalytic domain of the isozyme. **b:** PKCα monoclonal antibody (M9) against an epitope in the regulatory domain of the isozyme. **c:** PKCα monoclonal antibody (from UBI) against an epitope in the regulatory domain of the isozyme. **d:** Polyclonal antibody against the peptide corresponding to the pseudosubstrate domain of rat PKCα and β.

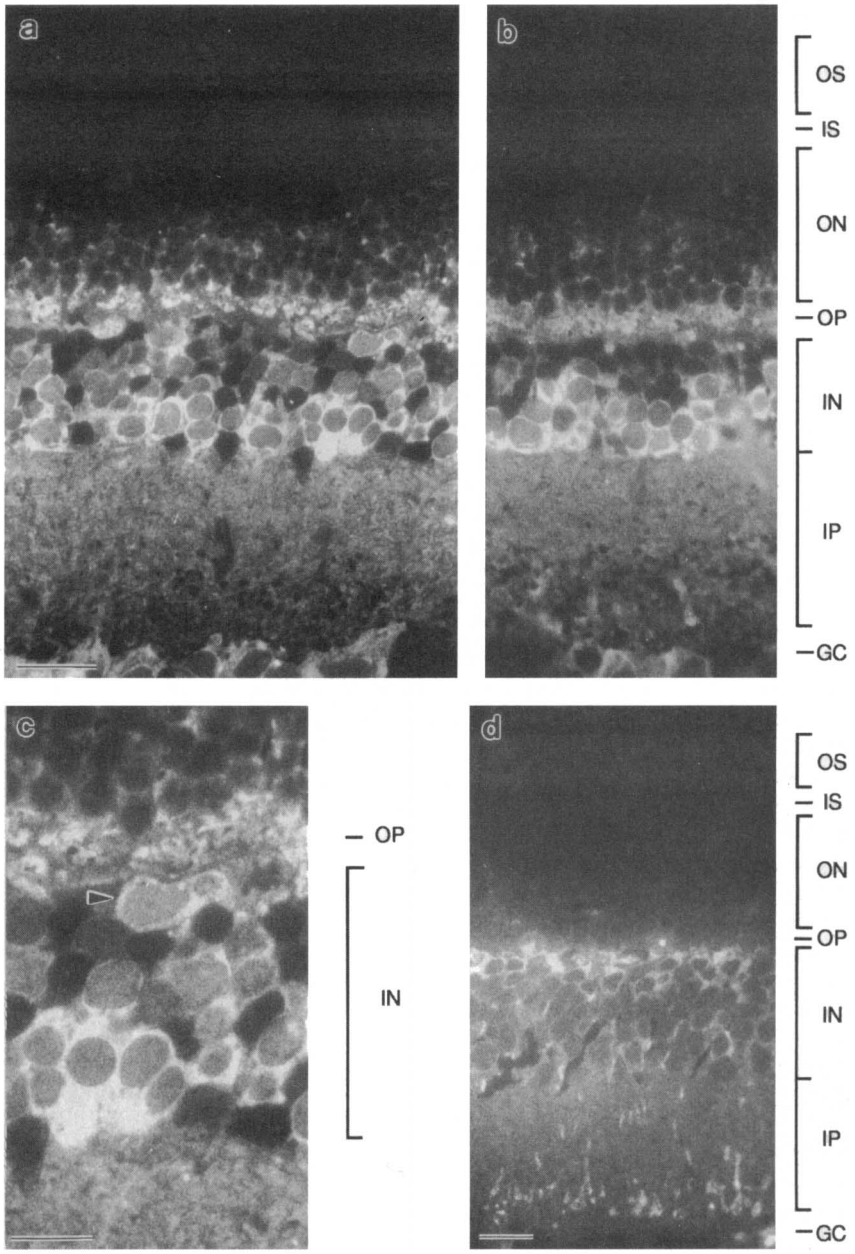

FIG. 7. Immunofluorescence of rat retinal cryosections (0.5 μm thick) labelled with antibodies against PKCα. **a:** PKCα monoclonal antibody (M6) against an epitope in the catalytic domain of the isozyme; light-adapted retina. **b:** PKCα monoclonal antibody (M9) against an epitope in the regulatory domain of the isozyme; dark-adapted retina. **c:** Higher magnification of part of a. **d:** PKCα monoclonal antibody (from UBI) against an epitope in the regulatory domain of the isozyme; dark-adapted retina. OS, photoreceptor outer segment layer; IS, photoreceptor inner segment layer; ON, outer nuclear layer; OP, outer plexiform layer; IN, inner nuclear layer; IP, inner plexiform layer; GC, ganglion cell layer. Scale bar in a is 20 μm, in c 10 μm, and in d 20 μm; a and b are the same magnification. In a and b, cell bodies in the inner nuclear layer and ganglion cell layer are labelled. Axons in the outer plexiform layer are labelled. In c, the arrowhead indicates a large labelled cell body at the distal border of the inner nuclear layer, most likely a horizontal cell. Many of the other labelled cell bodies belong to bipolar cells. In d, only cell bodies in the distal inner nuclear layer (mostly belonging to bipolar cells) are labelled. Bipolar cell axons and synaptic terminals are also labelled. Only parts of labelled axons are visible; in contrast to thick sections, this semithin section does not include the entire cell, so the labelled axons are only partially visible, i.e., where they are in the plane of the section. The ganglion cell bodies are not labelled by this antibody, but the Müller cell endfeet in the ganglion cell layer do contain some label. In all of a–d, the unlabelled areas that appear to fill the spaces between cell bodies in the inner nuclear layer are most likely Müller cell bodies.

poses, it was sufficient to conclude that none of the antibodies labelled the photoreceptor outer segments.

DISCUSSION

Evidence for PKC in photoreceptor outer segments

There is considerable biochemical evidence that PKC is present in rod outer segments. First, substantial PKC activity has been detected in purified bovine and rat photoreceptor outer segments (Kelleher and Johnson, 1985, 1986; Wolbring and Cook, 1991; present study), with activity measurements consistent with about one PKC molecule per 10^3 rhodopsin molecules (Kelleher and Johnson, 1985, 1986). Second, treatment of intact retinas with phorbol esters alters the phosphorylation of rhodopsin, indicating that PKC and this rod outer segment-specific protein are present in the same compartment (Newton and Williams, 1991). Third, phorbol esters, which are specific PKC ligands, bind outer segment membranes from purified outer segment preparations (this study) and in retinal sections (Zarbin et al., 1989; Janssen-Bienhold et al., 1995). Binding of [^{3}H]PDBu to outer segments indicates about one PKC molecule per 2,000 rhodopsin molecules, a ratio that is similar to that for rhodopsin kinase and rhodopsin (see Hamm and Bownds, 1986) and is consistent with PKC activity measurements.

Identity of PKC in photoreceptor outer segments

The present immunocytochemical results, like most previously published results, are remarkable for the lack of labelling of photoreceptor outer segments by PKC antibodies. We are aware of only one study that shows immunolabelling of all outer segments. In this study, Wood et al. (1988) used a polyclonal antibody against brain PKC to label rat retinal sections. Subsequently, a minority of outer segments were shown to be labelled by a commercial PKCα antibody (monoclonal MC5, from Amersham) in rabbit (Osborne et al., 1992) and rat (Ohki et al., 1994) retinas. However, in publications by others, labelled outer segments are not evident in micrographs of rabbit (Greferath et al., 1990; Koistinaho and Sagar, 1994) or rat (Zhang and Yeh, 1991; Osborne et al., 1992) retinas that have been labelled with this same commercial antibody. So, although the published literature contains some contradictions, the majority of the evidence indicates that the outer segments are not labelled by antibodies against the known PKC isozymes (Greferath et al., 1990; Osborne et al., 1991, 1992, 1994; Usuda et al., 1991; Zhang and Yeh, 1991; Kolb et al., 1993; Koistinaho and Sagar, 1994). This conclusion is corroborated by the present study, in which we have used a greater variety of antibodies than used previously. It follows that either none of the known PKCs is present in photoreceptor outer segments, or their epitopes are masked in tissue sections. Given the unusual organization of photoreceptor outer segments—essentially a stack of tightly packed membranes—it seems reasonable that a protein, especially one that binds membranes, might be more difficult to immunodetect in outer segments than in a more typical cell environment. However, countering this argument, is the successful immunodetection of other membrane-associating proteins in outer segments, such as calpain, by using the same procedure (Azarian et al., 1993). Furthermore, our findings using antibodies against many different domains of the one known isozyme that was detected in rod outer segment preparations, PKCα (Table 1), suggest that PKCα is not present in photoreceptor outer segments. Thus, the presence of PKCα in isolated rod outer segment preparations appears to result from contamination from disrupted inner retinal cells. Such contamination plagues all photoreceptor outer segment preparations (Williams et al., 1994) and is potentially greatest for soluble proteins (e.g., Feraudi, 1983), which may diffuse in and out of leaky outer segments (note that even sealed outer segments were leaky at some stage, at least during breakage from the inner segment).

The present biochemical analyses show that the PKC activity in rod outer segments is regulated by Ca^{2+} and lipid, so that this isozyme(s) belongs to the subfamily of conventional PKCs. The affinity of this isozyme for Ca^{2+} is higher than that of PKCβII and about the same as that of PKCα. Its affinity for phosphatidylserine is slightly higher than that of any of the known conventional PKCs. Combining western blot analyses with activity measurements revealed that, in rod outer segment preparations, PKCα could account for no more than half the Ca^{2+}-dependent PKC activity. Specifically, when samples containing equal amounts of activity of rod outer segment PKC and rat brain PKC were probed with antibodies against PKCα or the conserved pseudosubstrate, the rod outer segment PKC was consistently labelled only 40% as intensely as brain PKC.

Together, then, our biochemical results and our negative immunocytochemical results indicate that rod outer segments contain a novel Ca^{2+}- and lipid-dependent PKC. It is noteworthy that Nishizuka and coworkers have detected an immunologically distinct PKC isozyme in retinas (Fujisawa et al., 1992). They found that retinal PKC resolved into four peaks of Ca^{2+}- and lipid-stimulated activity on hydroxylapatite chromatography. Three correspond to known isozymes: PKCα (major peak), PKCβ (minor peak), and PKCγ (barely detectable peak). A fourth peak of activity contained protein that was not labelled with antibodies against the known isozymes, leading to the conclusion that it contained a "structurally unknown member of the PKC family" (Fujisawa et al., 1992). Whether this novel retinal isozyme corresponds to that found in rod outer segments remains to be established. The existence of a photoreceptor-specific PKC would not be unique: Zuker and co-workers have identified a photoreceptor-specific PKC in the *Drosophila* visual system (Schaeffer et al., 1989).

Function of PKC in photoreceptor outer segments

As noted in the introductory section, PKC has been suggested to play a role in the regulation of rhodopsin (Kelleher and Johnson, 1986; Newton and Williams, 1991, 1993; Greene et al., 1995), transducin (Sagi-Eisenberg et al., 1989), cyclic GMP-phosphodiesterase (Udovichenko et al., 1994), the cyclic GMP-gated channel (Liu et al., 1994), arrestin (Weyand and Kühn, 1990), and guanylate cyclase activity (Wolbring and Schnetkamp, 1995). Some of these specific photoreceptor functions might require a PKC isozyme(s) that is unique to the photoreceptor outer segment.

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