Differential Localization of Protein Kinase C Isozymes in Retinal Neurons

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Abstract. We report the immunohistochemical localization of protein kinase C isozymes (types I, II, and III) in the rabbit retina using the monospecific monoclonal antibodies MC-1a, MC-2a, and MC-3a. Using immunoblot analysis of partially purified protein kinase C preparations of rabbit retina, types II and III isozymes alone were detected. The activity of type III was the stronger. By light microscopic immunohistochemical analysis, retinal neurons were negative for type I and positive for type II and type III isozymes. Type II was more diffusely distributed through the retinal layers, but was distinctive in ganglion cells, bipolar cells, and outer segments. The immunoreactivity was stronger for type III isozyme, and it was observed in mop (rod) bipolar cells and amacrine cells. By using immunoelectron microscopy, the cytoplasm of the cell body, the axon, and dendrites of the mop bipolar cells were strongly immunoreactive for type III. The so-called rod bipolar cells were for the first time seen to form synapses with rod photoreceptor cells. These differential localizations of respective isozymes in retinal neurons suggest that each isozyme has a different site of function in each neuron.

PROTEIN kinase C is a diacylglycerol-phospholipid-, and calcium ion–dependent protein kinase, and is generally accepted as playing important roles in cell immunity (40), mitosis (12), cytoskeletal movement (34, 37), and carcinogenesis (25) through transmembrane signaling (26). This important enzyme is known to be located in various tissues including brain tissues (26, 41). Its functions in nerve tissues are supposed to be related to neural transmission through change in membrane ion flux (3, 24) or the release of transmitters (32).

We first purified protein kinase C from rabbit retina and showed the activation of protein kinase C by retinoic acid (27). The presence of protein kinase C activity in the outer segment of bovine retina (18) and its phosphorylation of rhodopsin (19) also indicated its importance in the retina. Immunohistochemical study using polyclonal antibody showed a broader distribution of protein kinase C in the retina other than the outer segments (41). Pharmacological studies also suggested a possible role for protein kinase C in the retina (5). In spite of these biochemical, pharmacological, and morphological studies, however, almost nothing concrete is known about the function of protein kinase C in other neurons in the retina.

Brain protein kinase C was further resolved into three fractions; type I, type II, and type III, upon chromatography of a hydroxyapatite column (15, 17). Each isozyme has different characteristics in its mode of activation (35) and the susceptibility to hydrolysis (14). Though protein kinase C is composed of a family of isozymes with different characteristics, most studies on the substance have been done on total protein kinase C. This may help to explain why the extensive research that has been carried out on protein kinase C has done little to really clarify its nature. To facilitate research, we developed monospecific monoclonal antibodies: MC-1a, -2a, and -3a for type I, type II, and type III isozymes (9), and specific cDNA probes for each isozyme (28). Using these antibodies and cDNA probes, we were able to demonstrate the tissue-specific expression of isozymes in the cerebellum, the spleen, leukemic cells, pancreatic endocrine cells, and glioma cells (9, 16, 28, 31, 36). In the present study, immunohistochemical analysis using monoclonal antibodies of rabbit retina was done to throw some light on protein kinase C in the retina.

Materials and Methods

Animals and Tissue Processing for Immunohistochemistry

Male rabbits were anesthetized with pentobarbiturate. The retina and the cerebellum were fixed by 4% paraformaldehyde/0.1 M sodium phosphate, pH 7.4 by perfusion through left heart ventricle for 15 min and then by immersion for 3 h. After washing in 0.1 M lysine/0.1 M sodium phosphate, pH 7.4/0.15 M sodium chloride for 1 h, they were immersed in graded sucrose solutions: 10, 15, and 20% for 10 h each. Frozen in a mixture of dry ice and n-hexane, 6-μm sections were cut on a cryostat microtome and collected on gelatin-coated glass slides.

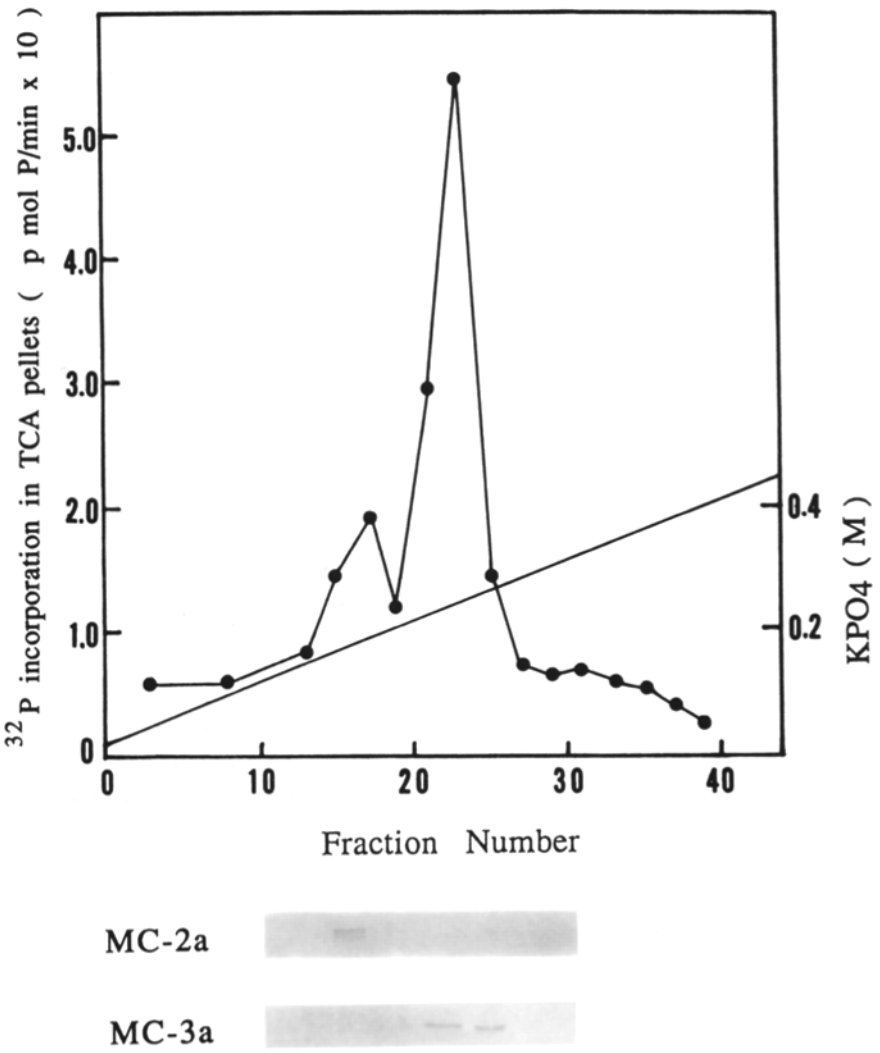


Figure 1. Immunoblot analysis of partially purified protein kinase C with three antibodies for each isozyme, type I, type II, and type III. Upper half of the figure shows the elution profile of protein kinase C activity on hydroxyapatite column chromatography. Two peaks of protein kinase C activity were detected. Lower half of the figure shows the result of the immunoblot analysis using monoclonal antibodies for protein kinase C isozymes, type II (MC-2a) and type III (MC-3a). The immunoreactivity for MC-2a was detected in the first peak and that for MC-3a in the second peak.

Antibodies

Protein kinase C was purified from rabbit cerebellum as reported previously (9). It was injected into 4-wk-old BALB/c mice. After a booster injection of the antigen, spleen cells were harvested and fused with SP2/0 myeloma cells. Hybrid clones were tested and selected by standard procedures (21). Positive clones were subcloned by the soft agar method and they were injected into pristane-primed BALB/c mice. Three types of monoclonal antibodies were purified from ascitic fluid by affinity chromatography on a protein A–coupled Sepharose column. They were designated MC-la, MC-2a, and MC-3a, recognizing protein kinase C isozymes type I, type II, and type III, respectively.

Assay for Protein Kinase C Activity and Immunoblot Analysis

Protein kinase C activity was measured by quantitation of the rate of [γ-^{32}P]phosphate incorporation into protein substrate, in the reaction mixture containing 25 mM Tris-HCl, pH 7.5, 10 mM magnesium chloride, 0.1 mM calcium chloride or 2 mM EGTA, 20 μg phosphatidyl serine, 40 μg histone III-s, 10 μM [γ-^{32}P]ATP (8×10^5 cpm), and 20 μl of protein kinase C fraction in a final volume of 200 μl (7). Western blot analysis was performed by SDS-PAGE according to the method of Laemmli (23) on 10% polyacrylamide gels followed by electrophoretical transfer to a nitrocellulose membrane (39). Immunoreactive proteins were stained as previously described (20).

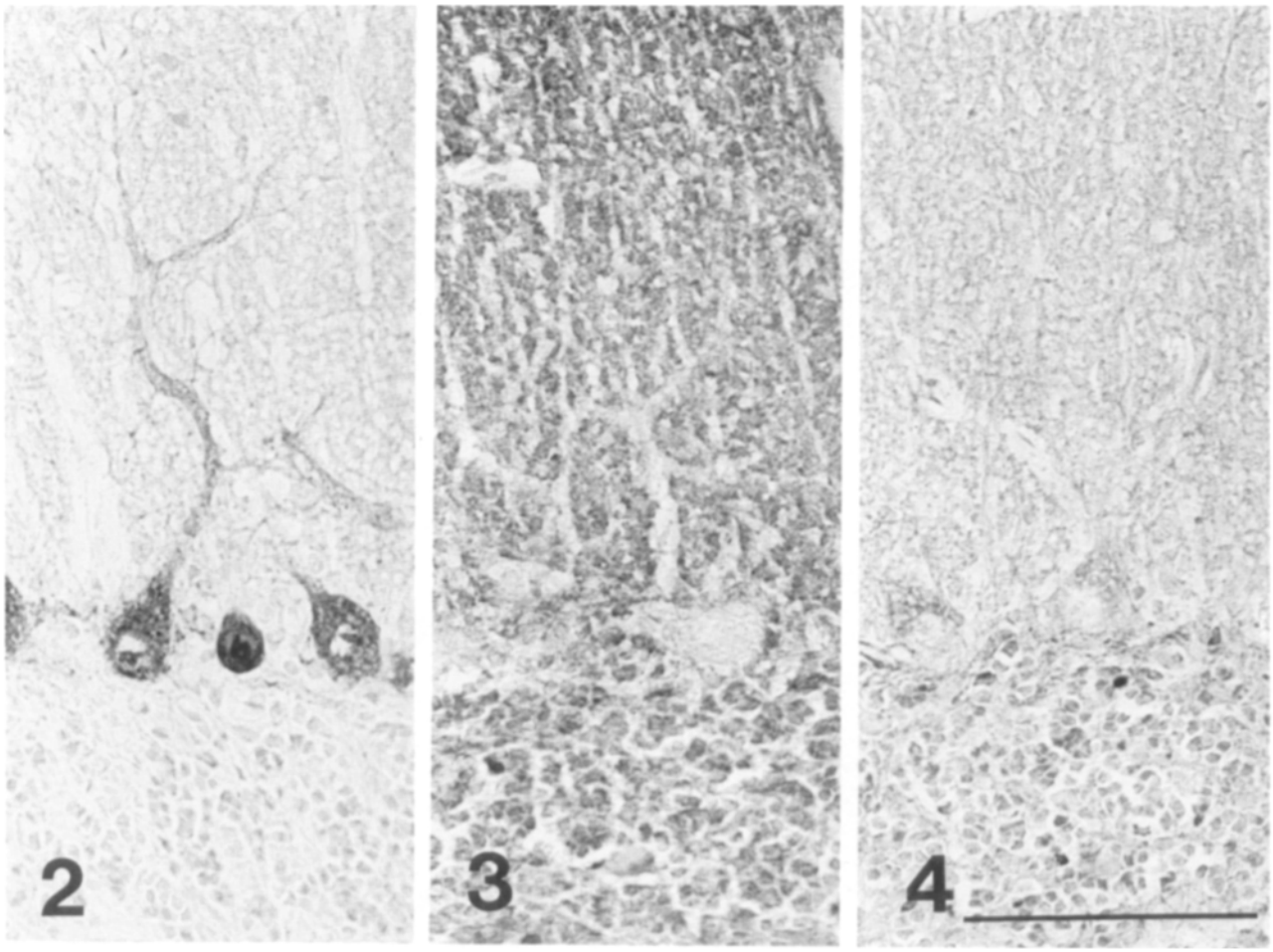
Purification of Protein Kinase C

Protein kinase C was purified from frozen rabbit retina (2 g) by a modification of Hidaka's procedure (7). Briefly, after DEAE-cellulose (0–0.4 M NaCl gradient) and butyl-Toyopearl 650M (1.0–0.1 M NaCl gradient) column chromatographies, active protein kinase C fractions were collected and dialyzed against a solution containing 20 mM potassium phosphate, pH 7.5/1 mM dithiothreitol/10% (vol/vol) glycerol/0.001% leupeptin before being applied to a hydroxyapatite column that was connected to a FPLC system. After the column was eluted with the same solution, bound protein kinase C was eluted with a linear gradient of potassium phosphate (20–200 mM).

Immunostaining

Peroxidase-antiperoxidase staining (PAP method) was performed according to the method of Sternberger (38). The sections were stained in the sequence of 2% normal rabbit serum for 30 min; primary antibodies (10 μg/ml of protein concentration of IgG fraction or 100 times diluted mouse ascitic fluid) for 2 h; rabbit anti-mouse IgG (DAKO, Copenhagen, Denmark) diluted 1:50, for 1 h; mouse peroxidase-antiperoxidase complex (DAKO) diluted 1:250, for 1 h. All three antibody solutions were made with 50 mM Tris-HCl, pH 7.5/150 mM NaCl containing 1% normal rabbit serum. All the sections were then incubated in the medium for peroxidase which contained 0.05% 3,3'-diaminobendizine tetrachloride and 0.01% H_2O_2 in Tris-HCl, pH 7.2 for 15 min, and counterstained with 1% methyl green solution. They were examined and photomicrographed with an Olympus AHB-LB microscope.

For electron microscopy, tissue sections that were immunostained for light microscopy with the antibody for protein kinase C (isozyme type III) were postfixed with osmium tetroxide buffered with 0.1 M sodium phosphate, pH 7.4 and then dehydrated with graded ethanol and acetone. After embedding in Epok 812 (Oken, Tokyo, Japan), glass slides were detached



Figures 2, 3, and 4. Immunohistochemical localization of protein kinase C isozyme; type I (*Fig. 2*), type II (*Fig. 3*), and type III (*Fig. 4*), in cerebellar cortex shown as a control for the staining. Bar, 100 μm.

from tissue sections. Ultra-thin sections of 0.1 μm were made parallel to them with an ultramicrotome (Porter Blum MT2B; Dupont-Sorval Instruments, Newtown, CT). They were stained with uranyl acetate and lead citrate, and observed in a Hitachi H-700 electron microscope at an accelerating voltage of 150 kV.

Results

Enzyme Activity and Immunoblotting

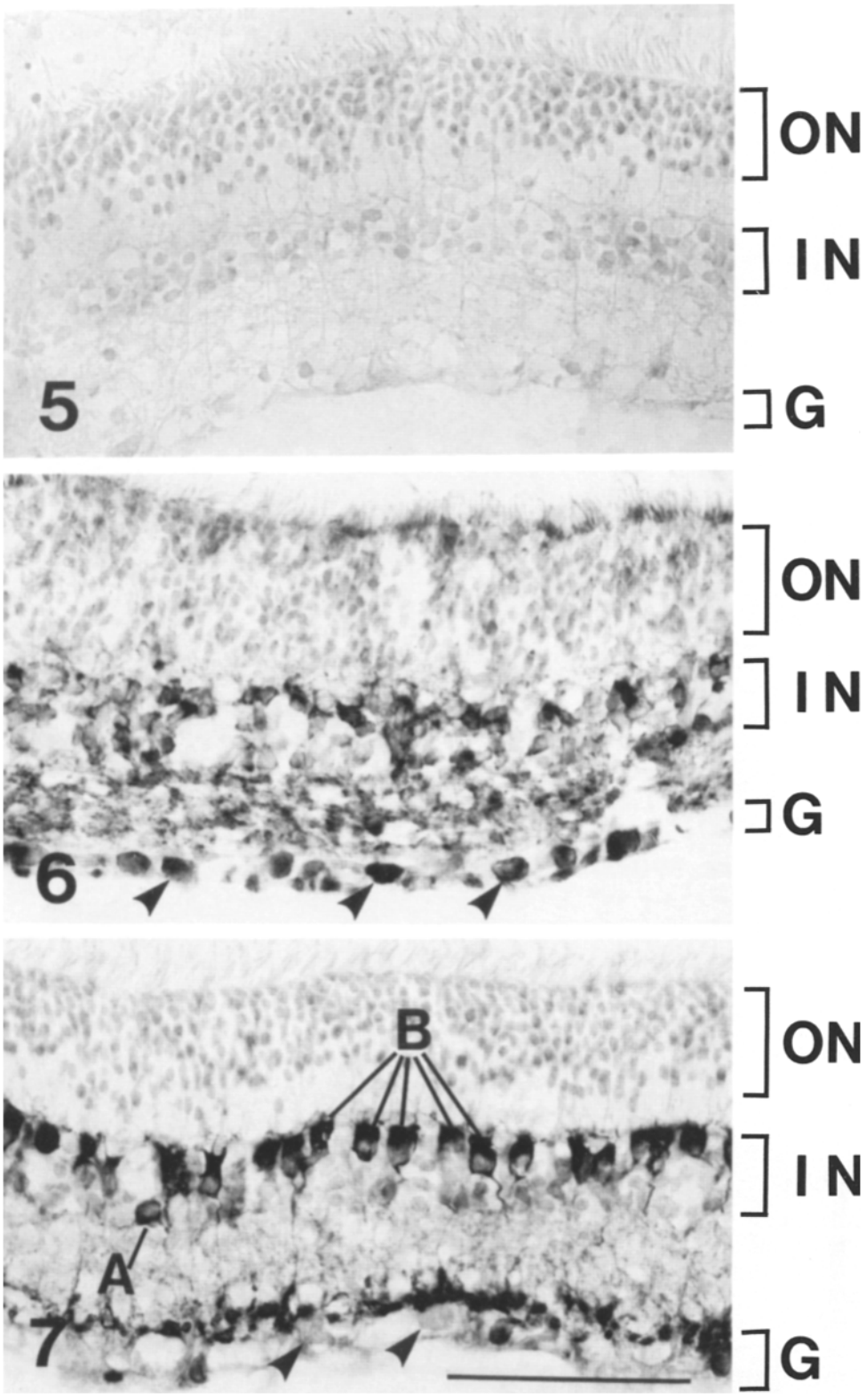
Two peaks of kinase activity were detected in protein kinase C fractions of the homogenate of rabbit retina (Fig. 1). Immunoblot analysis indicated that an 80-kD protein, eluted from hydroxyapatite column as the first peak, reacted to the MC-2a antibody, and the second peak reacted to the MC-3a antibody. The same experiment was done with rabbit cerebellum as a control tissue. In this case, however, three peaks of protein kinase C activity were detected, which were reactive with MC-la, MC-2a, and MC-3a antibodies, respectively (data not shown).

Immunohistochemistry

Fig. 2, 3, and 4 summarizing the localization of protein kinase C isozymes in cerebellar cortex are presented as a control to show the specificity of the immunostaining procedure. Type I isozyme was localized in the cytoplasm of Purkinje cell and its processes (Fig. 2). The granular layer excluding Purkinje cells was stained with the antibody for type II (Fig. 3). Weak diffuse staining was observed for type III (Fig. 4).

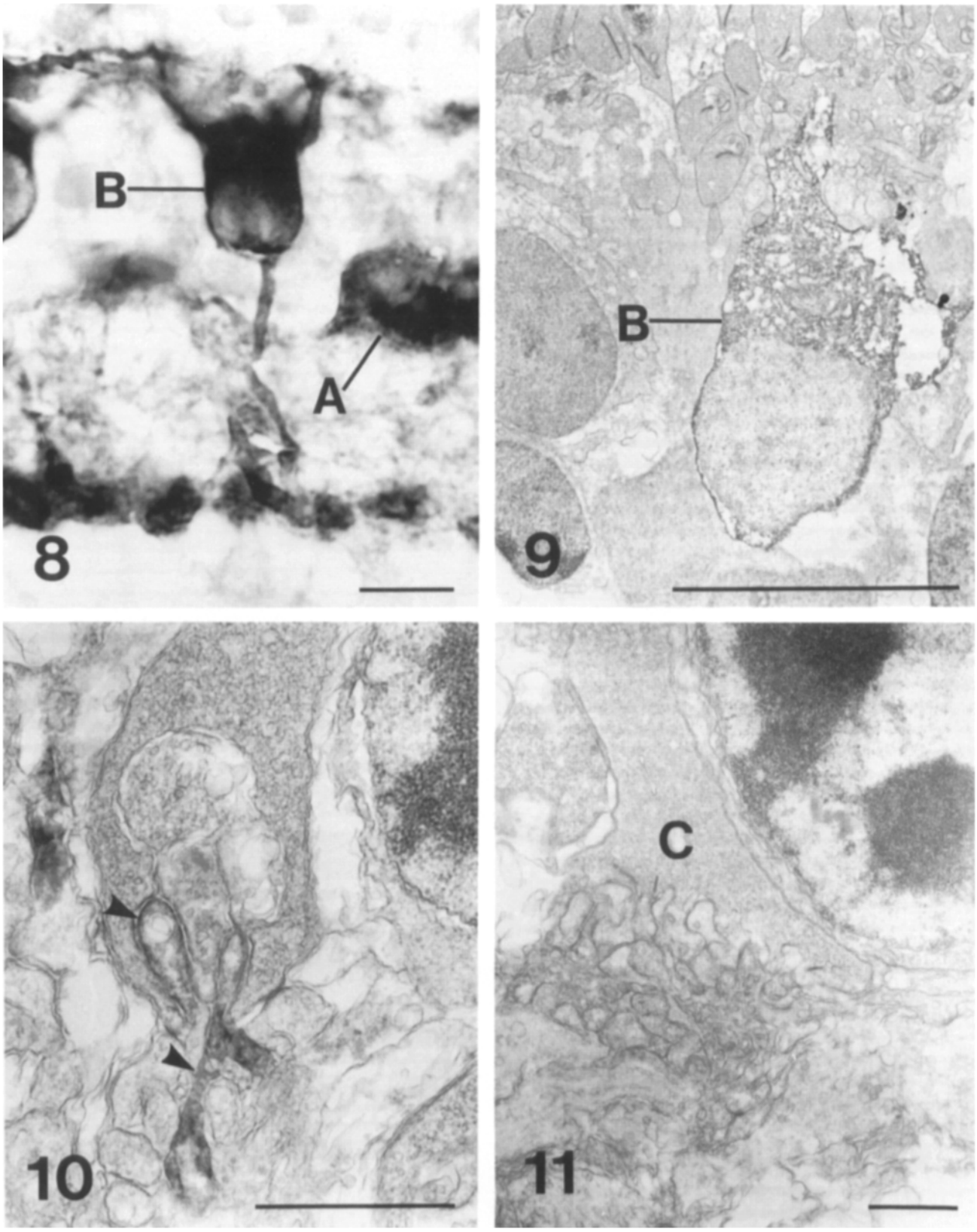
In Figs. 5, 6, and 7, the immunoreactivity of the retina for protein kinase C isozymes is shown. The retina was negative for type I isozyme (Fig. 5). However, characteristic staining patterns were observed for type II (Fig. 6) and type III (Fig. 7). Of the two, the retina was more strongly reactive for type III isozyme. The staining for type II was more diffuse than that for type III. Type II isozyme was localized in the outer segment, bipolar cells in the inner nuclear layer, in the inner plexiform layer and in the ganglion cells in the ganglion cell layer (Fig. 6). The reaction product for type III isozyme was more densely visualized than that for type II, and its distribution was more restricted to certain kinds of neuron (Fig. 7). It was localized in bipolar cells whose cell bodies were located in the outer half of the inner nuclear layer and amacrine cells through the inner nuclear layer to the inner plexiform layer (Fig. 7). These immunoreactive bipolar cells were arranged at the same intervals.

Fig. 8, a high-power magnification light-microscopic photograph, shows the characteristic shape of the bipolar cell immunoreactive for type III isozyme. It has an unbranched axon ending in a knot-like termination in the inner plexiform layer, and has abundantly branching dendrites. On the basis



Figures 5, 6, and 7. Immunohistochemical localization of protein kinase C isozyme; type I (*Fig. 5*), type II (*Fig. 6*), and type III (*Fig. 7*) in the retina. (*Arrowheads*) Ganglion cells. Ganglion cell layer (*G*), inner nuclear layer (*IN*), outer nuclear layer (*ON*). Note that ganglion cells are positive for type II isozyme (*Fig. 6*) but negative for type III isozyme of protein kinase C (*Fig. 7*), and that different types of bipolar cells were stained for type II and type II isozymes respectively (*Figs. 6* and *7*). Note also the strong immunoreactivity of small numbers of bipolar cells (*B*) in the inner nuclear layer for type III isozyme of protein kinase C (*Fig. 7*). Bar, 100 μm.

of its morphology, it is called a "mop bipolar cell" (10). The immunoreactivity could be detected through the axon, the cell body, and dendrites, except for the nucleus. By electron microscopy, the cytoplasm of the cell body was diffusely immunoreactive for protein kinase C type III, and the nucleus was not immunoreactive (Fig. 9). In the outer plexiform layer, dendritic processes of bipolar cells that form synapses with rod sphericles, the oval terminals of the internal axon-like processes of rod photoreceptor cells, were immunoreactive (Fig. 10). However, neural processes forming synapses



Figures 8, 9, 10, and 11. (*Fig. 8*) High-power magnification photomicrograph of the inner half of the retina stained for type III isozyme of protein kinase C. Amacrine cells (*A*) and bipolar cells (*B*). Bar, 10 μm. (*Fig. 9*) Electron microscopy photomicrograph of bipolar cell (*B*) immunoreactive for type III isozyme of protein kinase C. Bar, 10 μm. (*Fig. 10*) Electron microscopic photomicrograph of a synapse of the rod sphericles stained with protein kinase C isozyme type III. Note that the dendritic process of the bipolar cell (*arrowheads*) is immunoreactive for it. Bar, 1 μm. (*Fig. 11*) Electron microscopic photomicrograph of a synapse of the cone pedicle (*C*) stained with protein kinase C isozyme type III. Note that the dendritic processes are negative for it. Bar, 1 μm.

with cone pedicles, the cone-shaped terminals of the internal axon-like processes of cone photoreceptor cells, showed no immunoreactivity (Fig. 11).

Discussion

In the present study, monoclonal antibodies MC-1a, -2a, and -3a that specifically bind to the three isozymes of protein kinase C, type I, type II, and type III (9), were used for immunochemical and immunohistochemical analysis of rabbit retina. Brain protein kinase C was separated into three distinct types: I, II, and III on a hydroxyapatite column (15, 17). Independent of protein chemistry studies, molecular cloning analyses showed that protein kinase C is a family of subspecies having α, β (β1 and β2), and γ sequences (2, 11, 22, 29). Three subfractions, types I, II, and III, have been shown to correspond to the products of γ-, β-, and α-type genes, respectively, as based on identification of immunohistochemical and in situ hybridization histochemical localization and the expression in COS cells transfected with three types of cDNA (1, 9, 13). As the subspecies β1 and β2 have the same regulatory domain (NH_2-terminal side) and the monoclonal antibody MC-2a reacts with the NH_2-terminal side of the protein kinase C molecule, MC-2a reacts with both subspecies β1 and β2 (6). Recently, additional cDNA clones δ, ϵ, and ζ have been isolated and termed "nPKC" (29, 30). Although the existence of a protein molecule encoded by the ϵ gene was suggested (30), protein molecules corresponding to δ or ζ have not been identified, except in expression experiments.

Immunoblot analysis of the protein kinase C preparation from rabbit retina showed type III isozyme to be the main one, while type I could not be detected. The immunoreactivity shown by immunohistochemistry was the most intense with type III antibody for limited kinds of bipolar cell. The immunoreactivity of retinal neurons varied among cell types for type II antibody and was generally less intense than for type III antibody. Type I isozyme could not be visualized by immunostaining. These data obtained by immunoblot analysis and immunohistochemistry showed good agreement in the modality of the distribution of isozymes.

Light microscopic localization of protein kinase C showed a characteristic distribution in the retina. The retinal neurons showed rather diffuse immunoreactivity for type II through photoreceptor cells, bipolar cells, and ganglion cells. Amacrine cells and mop bipolar cells, judged by their characteristic shapes (10) in the inner nuclear layer, showed strong immunoreactivity for type III. However, photoreceptor cells and ganglion cells were not immunoreactive for this type. The characteristic differential distribution of type II and type III isozymes suggests that type II and type III isozymes play some role in the transmission of signals in different kind of neuron.

Further, the specific localization of type III isozyme in amacrine cells, whose subpopulation could not be well characterized here, might indicate a specialized function of type III in these cells. In ganglion cells, type II was almost the sole isozyme present. These data about the location of isozymes in each neuron of the inner nuclear layer and ganglion cells suggest specific functions for them. Nothing, however, is known about what kind of function each specific protein kinase C isozyme plays in these neurons. The role of protein kinase C in rod photoreceptor cells has been examined as described in the introduction. As type II isozyme was the only isozyme in the outer segments, probably cones and rods, this isozyme might be the only one performing protein kinase C function in the photoreceptor cells. Recently, though, genes coding putative new protein kinase C were found in *Drosophila* photoreceptor cells (33). In vertebrate photoreceptor cells, the localization of new putative protein kinase C isozymes has not been checked yet.

By electron microscopic immunohistochemical analysis, the localization of type III isozyme could be visualized. The reaction products for protein kinase C were localized in the cytoplasm of the bipolar cell with a large cell body and abundantly branching dendritic processes, namely mop or rod bipolar cells (10). A high-power magnification photograph of the dendritic process (Fig. 10) showed the synapse between rod photoreceptor cells and mop bipolar cells. A popular textbook (4) claims that the bipolar neurons with their external process form numerous dendritic branches that, in the outer plexiform layer, make synaptic contact with several rod sphericles for each rod bipolar neuron in question. Our picture showing the protein kinase C isozyme type III–reactive dendrites with rod sphericles is in doubtless the first to reveal that mop bipolar cells are truly rod bipolar cells.

To throw light on the function of protein kinase C in the retina, we attempted to compare the localization of protein kinase C isozymes between animals in dark-adapted and light-adapted states. No difference in localization and intensity of staining between dark-adapted and light-adapted animals could be obtained (data not shown). A phenomenon like translocation of protein kinase C, which occurs when cultured cells are treated with phorbol esters (16), could not be detected. Nothing concrete is known about the functional role of protein kinase C or its isozymes in the retinal neurons except for the photoreceptor cells. The new findings presented in this paper about the localization of protein kinase C should lead to more detailed description of its function in the retina.

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