Immunocytochemical Localization of Phosphatidylinositol-4,5-Bisphosphate in Dark- and Light-Adapted Rat Retinas

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ABSTRACT. Light-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate (TPI) to 1,2-diacylglycerol and D-myo-inositol 1,4,5-triphosphate (IP3) has been reported in the visual photoreceptor cells. We have investigated the localization of the TPI antigenic sites in dark- and light-adapted rat retinas using rabbit anti TPI antibodies (Ab). Sprague-Dawley rats were dark-, or light-adapted, or were exposed to a light flash. The eyes were fixed immediately and the tissue sections stained with the rabbit anti TPI Ab. The peroxidase-antiperoxidase method was used to find the localization of the TPI antigenic site. Image analysis of the sections was performed to obtain optical density profiles of the stain. Dark-adapted retinas showed intense staining of the rod outer segment (OS) layer but much less staining of the rod inner segment layer. Compared with the OS of dark-adapted retinas, those of the flash-bleached retinas were stained much less. The OS of fully bleached retinas showed little or no staining. The anti TPI Ab-protein A-gold conjugate intensely stained disks from dark-adapted retina but those from bleached retina much less. Our results suggest that rapid hydrolysis of TPI in rat rod outer segments occurs in vivo in response to light.

Increased phosphatidylinositol turnover in response to extracellular stimuli seems to be a general mechanism of signal transmission in various receptor systems (3, 16). The hydrolysis of phosphatidylinositol-4,5-bisphosphate (TPI) gives rise to two intracellular effectors; 1,2-diacylglycerol which activates protein kinase C (17) and inositol 1,4,5-triphosphate (IP3) which causes calcium flux across the cell membrane (3).

Light stimulates the rapid hydrolysis of TPI in vertebrate (8, 11) and invertebrate (5, 23) photoreceptors. TPI turnover also seems to function in Drosophila photoreceptors (26). Recent evidence indicates that IP3 mimics the effect of light in modulating the membrane potential of the photoreceptors. Intracellular injections of IP3 in the dark induce the depolarization of Limulus ventral photoreceptors reversibly.

Abbreviations used: TPI, phosphatidylinositol-4,5-bisphosphate; DPI, diphosphoinositide; PA, phosphatidic acid; IP3, D-myo-inositol 1,4,5-triphosphate; OS, outer segment; Ab, antibodies.

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and without detectable latency (5, 7) and the hyperpolarization of salamander rod photoreceptors (24). Calcium has an important role in the photoreceptors (9, 10, 25), and recent evidence suggests that IP3 induces an increase in the intracellular calcium in Limulus photoreceptors (4). Therefore the IP3 generated by the hydrolysis of TPI, may function in controlling intracellular calcium concentration in the photoreceptors.

We have determined the localization of TPI (antigenic sites) in the rat retina, using TPI-specific polyclonal antibodies. We also investigated whether the concentration of TPI in the photoreceptors changes between dark and light adaptations.

**MATERIALS AND METHODS**

**Materials.** TPI-hapten suspensions were prepared according to Kataoka and Nojima (14). Rabbits (New Zealand white) were immunized by an intravenous injection of TPI-hapten suspension every other day for three weeks. Immunoglobulin G fractions containing antibodies to TPI were purified by affinity absorption to Protein A. The relative immuno-reactivity of the antibodies for different kinds of phospholipid, as determined by a radioimmunoassay (13), was TPI (100%), diphosphoinositide (DPI) (6%), phosphatidylinositol (4%), phosphatidic acid (PA) (9%), and phosphatidylethanolamine (2%). Because nonspecific binding was no greater than 10% of the TPI binding under our assay conditions, the antibodies were considered to be highly specific for TPI. The anti TPI antibodies were successfully employed for detection of TPI in hair cells (13, 22). Rabbit immunoglobulin G, goat antirabbit immunoglobulin G, rabbit peroxidase antiperoxidase and 3,3'-diaminobenzidine tetrahydrochloride were purchased from Sigma Chemical Co., (St. Louis, MO, USA). All the other reagents used were from Fisher Scientific Co., (Pittsburgh, PA, USA).

**Tissue Preparation.** Sprague-Dawley rats were maintained under normal lighting conditions or dark-adapted in light-tight cages in a dark room for 36 h. Some of the dark-adapted rats were exposed to a 1-msec. flash of light (2,000 beam candle power) from a camera strobe unit (model 273, Vivitar, NY, USA) at 1 m distance. The animals were killed by cervical dislocation, and their globes enucleated under light or dim red light (>650 nm) conditions were fixed in 4% paraformaldehyde in cacodylate buffer, pH 7.2. The time between death and tissue fixation was less than a minute. The globes then were dehydrated by treating them with several concentrations of ethanol, after which they were cleared in cedarwood oil then embedded in paraffin. Sections 4 µm wide were cut on a Reichert-Jung 2,030 Biocut microtome (Reichert Scientific Instruments, Buffalo, NY, USA) then mounted on cleaned microscope slides.

**Localization by the peroxidase-antiperoxidase method.** The procedure of Sternberger et al. (21) was followed. Briefly, tissue sections were cleaned of paraffin then rehydrated and placed in phosphate-buffered saline for 10 min. These tissue slides then were incubated for 5 min with 5–6 drops of 3% hydrogen peroxide to quench endogenous peroxidase activity, after which they were rinsed with phosphate-buffered saline and incubated at 4°C for 12 h in a humidified chamber with rabbit anti TPI antibody, or rabbit immunoglobulin G, or TPI-preabsorbed anti TPI (1 mg/ml). The sections then were washed and incubated at room temperature for 90 min with goat antirabbit immunoglobulin G followed by incubation at room temperature for 90 min with rabbit peroxidase-antiperoxidase. After being washed, the slides were incubated with 3,3-diaminobenzidine tetrahydrochloride as the chromogen. All the sections were processed under identical conditions and treated with the same concentration of antibody. Stained sections were examined then photographed with a Nikon
LABOPHOT microscope equipped with a UFX camera (Nikon Incorp., Garden City, NY, USA).

Image Analysis. Images of the stained sections were digitized and measured using a Spatial Data systems EyeCom II image analysis system interfaced to a Digital Equipment Corp. MINC 11/23 minicomputer. Data collection program “BANDS” was used to derive the optical density profiles starting from the outer segments across the cellular layers of the retina and ending just beyond the ganglion cell layer into the vitreous. This technique has been described by King et al. (15) who generously loaned us their facilities. Each section was sampled with five equally spaced points along the retinal cell layers; the connecting points formed a line perpendicular to the inner limiting membrane (see Fig. 2). The computer used these lines as tracks and sampled the digital grey level value (black is equal to 0 and the whitest grey level to 255) at each pixel (screen picture element) along the transverse. The average grey level of a 50-pixel-wide sample line centered on the perpendicular to the transverse determined the raw screen intensity for each point along the profile line. The optical density profiles were averaged and automatically plotted on the screen by the computer.

Ultrastructural localization of TPI antigenic sites. Ultrastructural localization of the TPI antigenic sites was performed as described by Tachibana et al. (22). Briefly, light- or dark-adapted eyes were fixed in 4% paraformaldehyde-0.1% glutaraldehyde in cacodylate buffer, pH 7.2. After the anterior half had been removed, the posterior cup was washed in 10 mM phosphate buffer saline (PBS), dehydrated in half-sections and embedded in epon-araldite. Ultrathin sections (70 nm) on nickel grids were treated with 1% ovalbumin in PBS for 30 min to block nonspecific binding, then floated face down on a drop of TPI antibody (1:500 dilution) and incubated for 48 h at 4°C. After washing them with PBS, these sections were treated for 30 min with a 1:5 dilution of protein A-gold conjugate 15.3–16.4 μm in diameter [prepared by Dr. T.J. Raub according to DeMey (6)] then washed with PBS, stained with uranyl acetate and lead citrate and examined in a Zeiss 10C electron microscope (Carl Zeiss Inc., Thornwood, NY, USA).

RESULTS

Immunocytochemical localization of TPI antigenic sites in rat retinas. The peroxidase-antiperoxidase method was used to find the location of phosphatidylinositol-4,5-bisphosphate (TPI) in dark-adapted (Fig. 1a), partially bleached (Fig. 1b), and light-adapted (Fig. 1c) retinas. Fig. 1d shows a cross section of the control retina (dark-adapted, not treated with TPI antibodies). The retinal rod OS in this dark-adapted retina showed intense staining for TPI. This staining diminished markedly in retinas exposed to a brief flash of light and was barely detectable in fully light-adapted retinas. The inner segments did not stain appreciably for TPI in all retina samples. The outer nuclear layer was stained for TPI throughout the cell layer in the dark-adapted retinas; whereas, in partially bleached ones only a specific population of cells was stained. In light-adapted retinas the outer nuclear layer did not stain for TPI. The inner nuclear layers of all the retina samples did not appear to be stained intensely. The staining profiles of the ganglion layers were essentially identical in all the retina samples. In other words, light had little effect on the staining of both the inner nuclear and ganglion layers. Some background staining was present in control retinas from dark-adapted animals (Fig. 1d) as well as in control retinas from fully light-adapted, or flash-bleached animals (data not shown). Close examinations of the retinal sections indicate that cone cells are not stained as intensely as are rod cells.
Lack of cone staining also was noted in partially bleached bovine retina (data not shown). Further experiments are required to confirm this observation. The digitized image of a dark-adapted rat retina generated by computer analysis is shown in Fig. 2. A representative optical density pattern (inset of Fig. 2) for grey level values was obtained from different fields. The optical density profiles obtained by scanning the dark-adapted, flash-bleached, fully light-adapted, and control retinas shown in Fig. 1 are presented in Fig. 3. The digital gray level values for OS, inner segment and ON layers were OS 144.5 ± 8.9 (dark), 226.2 ± 7.4 (flash bleach), 243.2 ± 3.6 (full bleach); inner segment 190.5 ± 11.9 (dark), 237.1 ± 6.1 (flash bleach), 248.5 ± 1.6 (full bleach); ON 154.0 ± 7.6 (dark), 199.9 ± 10.4 (flash bleach), 230.5 ± 7.7 (full bleach). The numbers are given the means ± standard deviations (n = 5) obtained from scanning five adjacent areas of each tissue sample. The gray level values are inversely related to the intensity of staining. It is evident from the semiquantitative analysis...
that the magnitude of staining in the retinal OS region is reduced markedly after a 1-ms flash bleach and becomes essentially zero in fully bleached retinas.

**TPI antigenic sites in the rod disk membrane.** Staining of dark- and light-adapted rod outer segments by TPI antibodies conjugated with gold-labeled Protein-A is shown in Fig. 4. A dense population of gold particles is associated with the membranes of dark-adapted disks (arrows in Fig. 4 A). The detailed structure of the outer segment is not recognizable in Fig. 4 A because the camera was focused on the gold particles to increase the staining contrast. Some gold particles that appear to be in the subretinal space are actually associated with the barely discernible rod membranes. In contrast, light-adapted rod membranes bind very few gold particles (Fig. 4 B). It should be noted that all the particles are associated with the membranes and are absent in inter- and intra-discal spaces (arrows). These results are strong evidence that TPI associated with the rod disk membranes is hydrolyzed rapidly in response to light.

**DISCUSSION**

Studies of isolated frog (8) and rat (18) retinas show that the incorporation of \[^{3}H\] inositol into phosphatidylinositol is stimulated markedly by light. Light also increases the incorporation of \[^{14}C\] glycerol into phosphatidylinositol in isolated rat retinas (19). Furthermore, the phosphorylation of phosphatidylinositol to TPI
in excised rat retinas is enhanced by light (19). These results are consistent with the hypothesis that the metabolism of phosphatidylinositol, including polyphosphoinositides in vertebrate photoreceptors is stimulated by light. Recent studies report the light-induced breakdown of TPI in frog photoreceptors (8, 11), the light mediated decrease of TPI determined chemically after extraction of lipid is not very large [L/D = 0.63 (8) to 0.82 (11)]. This may be attributed to the hydrolysis of TPI during preparation of rod outer segments in the dark (8). Contamination of the outer segment preparations by other cellular organelles also affects the reliability of the procedure. To demonstrate light-induced changes in TPI in vertebrate photoreceptor in vivo, we exposed the whole animal to an illumination and analyzed the TPI content in the retina using purified antibodies specific for TPI.

Compared with the procedure that involves the radiolabeling of excised tissue, lipid extraction and the assay of TPI, the immunocytochemical method is more direct.

Fig. 3. Optical density plots for the TPI-stained bandwidth. (a) Dark-adapted section, (b) flash-bleached section, (c) light-adapted section and (d) control tissue section.

Fig. 4. Ultrastructural localization of TPI antigenic sites in rat rod outer segments. (A) Dark-adapted section, (B) Light-adapted section. Original magnification: ×25,000. Bar: 1.0 μm.
and analyzes TPI under more physiological conditions. One drawback of this latter method is that no TPI that is inaccessible to the antibodies can be detected; and therefore, the amount of TPI estimated from the extent of staining is only semi-quantitative. The specificity of antibodies is always a problem in immunocytochemical localization studies. Because the antibodies used have low reactivity with phospholipids such as phosphatidylcholine, it may be argued that the light-induced decrease in staining found represented a loss of phospholipids other than TPI.

In rat photoreceptor membranes phosphatidylcholine is the major phospholipid (about 50 mole % of the total phospholipid), whereas phosphatidylinositol accounts for about 1.6 mole % (1). If phosphatidylcholine was responsible for the staining pattern observed in this work, the phospholipid would have had to disappear almost completely from the light-illuminated photoreceptor membrane which would have caused degeneration of the rod membranes, but no disintegration of the photoreceptor membranes was detected upon bleaching under the experimental conditions.

Another possibility is that the antibodies react with DPI and PA. In frog photoreceptors, the DPI/TPI ratio is about 2 and the PA/TPI ratio about 1 (11). If similar ratios are assumed for these compounds in rat photoreceptors, about 12% of the antibody binding would be due to DPI and about 9% to PA. This leaves about 80% of antibody binding to react with TPI. For these reasons, we conclude that the antibodies used primarily bound to the TPI antigenic sites in the membrane. Labeling of the dark-adapted rod outer segments with gold-labeled antibodies showed the TPI antigenic sites to localize on the disk membrane. In contrast, staining of light-adapted disk membranes was very slight. These results suggest strongly that TPI in rat rod disk membranes undergoes a rapid and precipitous decrease in light.

TPI hydrolysis in isolated frog photoreceptors becomes detectable after exposure to a 5-sec. illumination (8, 11). In our experiment, exposure of the whole animal to a 1 msec. flash illumination followed by the fixing of the tissue within a minute was sufficient to cause a marked decrease in the TPI content in the photoreceptor layer. Therefore, light-induced hydrolysis of TPI in vivo may be fast enough to have an important function in the visual process. The initial event in light-induced TPI hydrolysis must be photon absorption by the visual pigments. Hydrolysis of TPI to IP3 is catalyzed by a TPI-specific phospholipase C (20); nothing, however, is known of how photoactivated rhodopsin activates phospholipase C.

The outer nuclear layer is also stained intensely by anti-TPI antibodies and this staining is reduced markedly by light. The stained cells are probably horizontal ones although the synaptic ends of visual cells also may be stained. The phosphoinositide metabolism in toad horizontal cells reportedly is activated by light (2). If the stained cells are horizontal ones, intercellular communication between the photoreceptor cells and the horizontal cells must evoke a metabolic response in the secondary neurons. Neurotransmitter release from the synaptic end of vertebrate photoreceptors occurs in the dark and is inhibited in light. Divalent ions that inhibit neurotransmitter release from the photoreceptor cells (but not those cations that potentiate neurotransmitter release) stimulate phosphatidylinositol metabolism in horizontal cells (2).

If light-induced hydrolysis of TPI does have a function in vertebrate photoreceptors, the original amount of TPI must be restored in the dark. Further studies are in progress to determine how quickly TPI is resynthesized during dark-adaptation of the animal.
Acknowledgments. This research was supported in part by an unrestricted departmental grant from Research to Prevent Blindness, Inc., New York City and by U.S. Public Health NIH grant EY03807. We thank Mr. Sue Semple-Rowland for the gift of inbred Sprague-Dawley rats, Ms. Pat Lewis for sectioning the retinas, Dr. Bruce Hunter and Mr. W. Creegan for allowing us to use their computer facilities, and Ms. Mable Wilson and Ms. Roxanne M. Bowman for typing the manuscript.

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(Received for Publication, January 18, 1986)