Histochemical evidence of cyclic nucleotide phosphodiesterase in photoreceptor outer segments

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A histochemical demonstration of cyclic 3',5'-nucleotide phosphodiesterase in retinal photoreceptor outer segments is reported for the first time. The enzyme appears to be located on outer segment lamellae. It is not found in adjacent inner segment or nuclear regions. This histochemical localization is consistent with previously reported biochemical studies showing high phosphodiesterase activity in preparations of isolated rod outer segments.

Key words: histochemistry, phosphodiesterase, cyclic nucleotide, cyclic AMP, retina photoreceptor outer segments.

In recent years, there has been heightened interest in cyclic nucleotide research, specifically in the role cyclic nucleotides might play in photoreceptor function. Biochemical evidence of synthetic and degradative enzymes for cyclic nucleotides has been found in preparations of isolated photoreceptor outer segments.

Further, a deficiency of cyclic nucleotide phosphodiesterase (PDE) has been proposed as an early defect in hereditary retinal degeneration in mice. Early attempts at a histochemical localization of PDE in tissues suffered from procedural limitations. Recently, however, an improved technique has been successfully employed for the electron microscopic demonstration of the enzyme in cerebral cortex. A modification of this technique has been used in the following study to achieve an anatomic localization of PDE in normal retinal tissue.

Methods

Adult mice of the C37BL/6J inbred strain, heterozygous at the genetic locus for retinal degeneration (and, therefore, phenotypically normal), were used in the study in anticipation of the desirability of subsequently examining their homozygous littermates (who would be affected with the recessively inherited retinal degeneration). The animals were killed with an intra-
peritoneal injection of pentobarbital and were immediately decapitated. Eyes were removed in room illumination and were immersed in 2 per cent glutaraldehyde in 0.05 M cacodylate-nitrate buffer (pH 7.4) containing 0.25 M dextrose. While in this fixative, the eyes were opened under an operating microscope. The retina was separated gently from pigmented epithelium and was cut into pieces approximately 1 mm. square. Total fixation time was no longer than 20 minutes.

The pieces of retina were first incubated for 30 minutes at room temperature in a solution containing 60 mM Tris maleate buffer {pH 7.4), 2 mM MgCl₂, and 0.25 M sucrose (TMS buffer), to which was added 5'-nucleotidase in the form of snake venom of Crotalus atrox: 3 mg. dry venom per milliliter. The tissue was then incubated for 30 minutes at 37° C. with slow agitation in TMS buffer containing 2 mM lead nitrate, 5'-nucleotidase (3 mg per milliliter), and cyclic nucleotide substrate: either 3 mM adenosine 3',5'-monophosphate (cyclic AMP) or 3 mM guanosine 3',5'-monophosphate (cyclic GMP). Control experiments were performed without added 5'-nucleotidase, or with 5'-nucleotidase in only the first incubation and 5'-adenosine monophosphate as substrate in the second.

After incubation, the retinal tissue was briefly washed in distilled water, dehydrated in graded alcohols, treated with propylene oxide, and embedded in Epon 812. Thin sections were cut, stained with uranyl acetate, and examined with the electron microscope.

Using similar techniques, the retina of a cow obtained at a local abattoir and the retina of a rhesus monkey were examined for enzymatic activity.

**Results**

Cyclic 3',5'-nucleotide phosphodiesterase hydrolyzes cyclic AMP to 5'-adenosine monophosphate (5'-AMP). The basis of the histochemical procedure outlined above is the further conversion of 5'-AMP to adenosine and inorganic phosphate with an excess of 5'-nucleotidase and the precipitation of the newly formed inorganic phosphate with lead ions, thus forming an electron-opaque...
Fig. 2. Higher magnification of rod outer segment, showing reaction product from hydrolysis of cyclic AMP. Precipitate follows the contour of lamellae closely (arrows) and does not appear between lamellae. A lighter precipitate appears on plasma membrane. Magnification, x80,000.

product at or near the site of phosphodiesterase activity.

When pieces of mouse retina were incubated in medium containing cyclic AMP as substrate, a reaction product formed on the lamellae of photoreceptor outer segments (Fig. 1). At higher magnifications it was evident that the lead phosphate precipitate followed the contour of individual lamellae closely and did not occur in the spaces between lamellae (Fig. 2). A light precipitate was occasionally present on the outer segment plasma membrane. No reaction product was found in photoreceptor inner segments or nuclei (Fig. 3). When the same retina was incubated with cyclic GMP as substrate, the precipitate was much less dense and somewhat inconstant from one area to another. When the cyclic nucleotide substrate was omitted altogether, no precipitate formed (Fig. 4). Incubation with cyclic AMP plus papaverine hydrochloride resulted in a marked reduction of the amount of reaction product, consistent with biochemical data indicating that papaverine effectively blocks PDE.
Fig. 3. Inner segments (IS) and cilium (CIL) show no reaction product, whereas outer segments have precipitate, even at their base. Double arrows point to lighter staining ribosomal particles in inner segment. Magnification, ×35,000.

Fig. 4. Incubations carried out without substrate show no reaction product on inner segment (IS) or outer segment (OS) of photoreceptor cells. Magnification, ×32,000.
activity. Incubations of cattle and monkey retina gave similar results (Figs. 5 and 6).

The preservation of tissue architecture in the outer plexiform layer and in inner retinal layers was less good than in the photoreceptor layer. No definite localization of reaction product could be recognized in synaptic structures or elsewhere in the inner retina. The appearance of precipitate along the inner retinal surface and between some cell processes of the nerve fiber layer in incubations with heavy outer segment precipitate was of uncertain significance (Fig. 7). Since the inner retinal surface was in direct contact with incubation medium during the histochemical reaction, it is possible that this precipitate diffused in from the surface between cells. No reaction product was seen intracellularly in this area.

Incubations performed without added exogenous 5'-nucleotidase did result in the formation of some precipitate on outer segments, indicating the presence of a certain amount of endogenous retinal 5'-nucleotidase, as previously reported by Lessell and Kuwabara. On the other hand, when 5'-AMP was used as substrate, even after initial incubation with exogenous 5'-nucleotidase, the expected diffuse distribution of reaction product was present only in photoreceptor and outer plexiform layers, suggesting that either substrate or 5'-nucleotidase was not available to inner retinal layers.

Discussion
The histochemical evidence for cyclic nucleotide PDE activity in outer segments of photoreceptor cells is consistent with biochemical studies of the localization of this enzyme in the retina. Although pieces of whole retina were used in the histochemical procedure, there is little to suggest that reaction product diffused into outer segments from some other primary site of enzyme activity. Close examination of the pattern of precipitation clearly suggests that the reaction product was primarily formed on outer segment lamellae. In those incubations in which particularly heavy precipitate was formed, some possible diffusion of reaction product was observed on the inner surface of the retina, but this was extracellular in location and limited to the area immediately adjacent to the inner limiting membrane.

Moses and Rosenthal have pointed out some of the pitfalls of using lead ions in histochemical procedures involving nucleoside phosphatases, specifically the non-enzymatic hydrolysis of adenosine triphos-
Fig. 6. Light precipitate from hydrolysis of cyclic AMP on outer segments of rhesus monkey. At left, the outer segment lamellae are cut in cross-section, whereas on the right, a nearly flat section shows even distribution of reaction product. Magnification, x57,000.

Fig. 7. Precipitate between cells and axons (double arrows) of the nerve fiber layer and along the inner limiting membrane (ILM) of the mouse retina was interpreted as a diffusion artifact since no intracellular localization was evident. Magnification, x12,000.
phate by lead in millimolar concentrations. The same authors, however, found no appreciable dephosphorylation of either 5'-AMP or cyclic AMP in the presence of 4.0 mM lead nitrate. This would seem to rule out nonenzymatic hydrolysis as a problem in our incubations. Our own control incubations without substrate and with substrate plus papaverine hydrochloride as PDE inhibitor support the specificity of the histochemical technique.

The lack of reaction product with cyclic GMP as substrate is puzzling in view of the findings of Pannbacker, Fleischman, and Reed and of Chader and co-workers that cyclic GMP as well as cyclic AMP is hydrolyzed by rod outer segment preparations. It is still not certain whether the same or different enzymes hydrolyze these closely related substrates. There are some differences in the kinetics of PDE activity for cyclic AMP and cyclic GMP when the latter are used in millimolar concentrations. Differential susceptibility of separate enzymes to glutaraldehyde fixation may offer an explanation for the less impressive results obtained with cyclic GMP as substrate.

A preliminary incubation with added exogenous 5'-nucleotidase was employed primarily to provide an excess of this enzyme in the tissue being studied. At the same time the initial incubation served to drain off any endogenous substrate, either cyclic AMP or 5'-AMP, left in the tissue after fixation. That there was some limitation in the diffusion of 5'-nucleotidase, or of added substrate, through the retinal tissue was indicated by the lack of diffusely distributed reaction product when 5'-AMP was used as substrate. Since a diffuse precipitate was seen only in the photoreceptor and outer plexiform layers, no statement can be made about the presence or absence of PDE activity in inner retinal layers on the basis of these studies. Biochemical evidence has consistently shown higher specific activity of PDE in outer segment preparations than in whole retina or inner retinal layer homogenates.

The brief fixation of tissues in glutaraldehyde resulted in some compromise of morphological integrity, but longer periods of fixation reduced enzyme activity to the point where little or no reaction product could be seen. Osmium tetroxide was eliminated as a postfixative after incubation because the customary agitation of this aqueous solution resulted in severe washout of the reaction product. Attempts to substitute formaldehyde for glutaraldehyde as a primary fixative resulted in poorer preservation of morphologic detail and less well-localized precipitation of reaction product.

Studies using this same histochemical technique for localization of PDE activity are being carried out on the developing retina of normal mice and mice with hereditary retinal degeneration and will be reported separately.

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REFERENCES