Alteration of Retinal Choline Metabolism in an Experimental Model for Photoreceptor Cell Degeneration

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The choline analog hemicholinium-3 causes selective degeneration of the cones and rods in the rabbit retina. These experiments examine some biochemical effects of hemicholinium-3 on metabolic pathways involved in choline metabolism. No effect of the drug on several important photoreceptor biosynthetic functions was found: leucine incorporation into TCA-precipitable material, glycerol incorporation into phospholipids, and phosphatidylcholine formation by either base exchange or choline phosphotransferase activity. However, hemicholinium-3 does selectively affect free choline use: high-affinity uptake, phosphorylation and subsequent incorporation into lipid. In each instance, hemicholinium-3 causes a significant inhibition of control activity at a concentration of 30 |M, a dosage that causes complete photoreceptor outer segment degeneration in mammalian retinas. Thus, it seems that the enormous synthetic requirements for phosphatidylcholine leave the photoreceptor cell in fragile metabolic balance, and transient alteration of this balance may destroy the outer segment.


Membrane renewal in the photoreceptor outer segment involves replacement of both protein and phospholipid. The necessary amount is enormous: mammalian rods commonly produce about 400 |m^2 of new membrane daily. Undoubtedly, photoreceptor cells are metabolically specialized to sustain this extraordinary synthetic rate. One specialization is the high-affinity choline uptake. Mammalian photoreceptors do not synthesize acetylcholine, but instead need this system to produce phospholipid. Phosphatidylcholine comprises about 45% of the total outer segment phospholipid, and probably efficient continual choline accumulation is concomitant to ensure phospholipid synthesis. This suggests that altering choline metabolism could have consequences for normal outer segment renewal.

The choline analog hemicholinium-3 has long been known to deprive cells of choline. When mammalian retinas are acutely exposed to about 20 |M hemicholinium-3 by intravitreal injection, a specific outer segment degeneration results. Within one week, all outer segments are gone, but the remaining retina, including the receptor nuclei, appears morphologically and physiologically normal.

Choline is used exclusively in mammalian photoreceptor cells for phospholipid synthesis (mainly phosphatidylcholine). As shown in Figure 1, photoreceptor cells synthesize phosphatidylcholine from choline via either base exchange (Reaction I) or the Kennedy pathway (Reaction II). The experiments in this paper examine the effects of hemicholinium-3 on metabolic steps involved in each of these pathways.

Materials and Methods

Incubations with Isolated Retinas

Rabbit eyes were enucleated under anesthesia, and the retina was removed from the eyecup under oxygenated mammalian Ringer. This medium contained (mM): NaCl, 150; KCl, 4; CaCl_2, 2; MgCl_2, 2; Na_2HPO_4, 0.2; NaHCO_3, 5; Hepes (pH 7.4), 20; glucose, 5.6. The isolated retina was cut with a razor blade into about ten pieces. Incubation volume was 2 ml including the appropriate concentration of radiisotope. For choline uptake experiments, in addition to ^3H-choline, 1-10 |M ^14C-mannitol (SA = 45 mCi/mmol) was used as an extracellular maker. The radioactivity of the media never decreased by more than 5% during experiments. These procedures
occurred under normal room lights and at room temperature (22–23°C).

After incubation, retinas were placed into cold 10% TCA for at least 1/2 hr, and an aliquot of the TCA-soluble material was taken for scintillation counting. In studies involving leucine and choline, the TCA-precipitable material was washed twice with distilled water, solubilized with 1 N NaOH, and aliquots of the solubilized material were used for scintillation counting and protein determination.

In 2-3H-glycerol incorporation studies, the phospholipid was extracted from the TCA-precipitate by adding 5 volumes of chloroform:methanol (2:1, v/v). The organic layer was washed three times with a mixture of chloroform:methanol:H2O (3:47:50, by vol), and the chloroform layer was evaporated to dryness and resuspended in 200 μl chloroform:methanol (2:1). Duplicate phosphorus determinations were made on aliquots of the extract. Twenty-five microliter aliquots were spotted on silica gel HR plates, and the phospholipids were separated by two-dimension, thin-layer chromatography. Since the relative phospholipid proportions remain constant, we present the glycerol incorporation data as the radioactivity present in each phospholipid divided by the total phosphorus applied to the thin-layer chromatography plate.

Cell-free Biochemical Studies

These studies were performed on both rabbit and bovine retinas. Bovine eyes were obtained early in the morning from local slaughterhouses and the retinas were harvested immediately. Rabbit eyes were enucleated, and retinas were isolated under 0.32 M sucrose. The preparative techniques for both tissues were the same. After gentle homogenization in a Teflon and glass homogenizer, the tissue was spun first at 500 × g for 10 min, followed by 15,000 × g for 15 min. Both pellets were discarded, and the supernatant was spun at 100,000 × g for 1 hr. This high speed supernatant was frozen and used within 6 weeks for measuring choline kinase activity. The pelleted microsomes were diluted in 0.32 M sucrose, sus-

Fig. 1. The two pathways of phosphatidylcholine synthesis from choline. In Reaction I, phosphatidylcholine is formed directly from endogenous phospholipids by the base exchange reaction. Shown below is the Kennedy Pathway (Reaction II), which is the major route for de novo synthesis of retinal phosphatidylcholine. Glycerol is incorporated into the phospholipid backbone by the pathway shown at the bottom.

Leucine Incorporation
TCA Ppt

Fig. 2. Incorporation of 3H-Leucine into TCA-precipitable material in rabbit retinas incubated for 1 hr in the absence or presence of hemicholinium-3 (HC-3). Leucine concentration is 3 μM (SA = 30 Ci/mmol). Each point is the mean of at least eight experiments.
Table 1. DPM[μG total lipid phosphorus]

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>2-3H-Glycerol</th>
<th>2-3H-Glycerol + 30μM HC-3</th>
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<tr>
<td>Phosphatidylcholine</td>
<td>10,580 ± 1900</td>
<td>10,334 ± 2210</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>19,888 ± 3760</td>
<td>19,738 ± 3564</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>1,284 ± 353</td>
<td>1,406 ± 540</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>7,309 ± 1750</td>
<td>7,841 ± 2011</td>
</tr>
<tr>
<td>Phosphatidyserine</td>
<td>1,813 ± 127</td>
<td>1,410 ± 460</td>
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The amount of 3H-glycerol incorporated into phospholipid for rabbit retinas incubated for 3 hours in the presence or absence of 30 nM HC-3. Glycerol concentration was 100 nM (SA = 500 mCi/mmol). Each value is the mean of at least 15 determinations.

Choline kinase activity was determined by taking a 200 μl aliquot of the high speed supernatant and adding it to an equal volume of reaction mix. The final concentrations in the assay mix were (mM): Tris (pH 8.0), 50; Na·ATP, 3.3; MgCl₂, 4. Appropriate concentrations of 3H-choline and hemicholinium-3 were added. After 1 hr incubation at 37 C, the reaction was terminated by addition of 50 μl 1 M TCA. This mixture was spun at 5,000 × g for 5 min at 4 C. The pellet was solubilized and used for protein determination. After spotting a 10 μl aliquot of the supernatant on Whatman 3MM chromatography paper, the phosphorylcholine and choline were separated by a published procedure. Authentic 3H-choline and 14C-phosphorylcholine were run concurrently as standards. After development, the paper was exposed to iodine vapor, and the lanes containing choline and phosphorylcholine were marked, cut, and placed into scintillation vials. The label was eluted overnight with 1 ml H₂O; 10 ml of scintillation fluid was added and the samples counted. Recovery of standards was greater than 90%. No attempt was made to separate phosphorylcholine from CDP-choline.

Results

Effect of Hemicholinium-3 on Retinal Protein Synthesis

Since outer segments require renewal of both protein and phospholipid, we tested the effect of hemicholinium-3 on protein synthesis. Retinas were incubated in 3H-leucine in the presence or absence of 3.0 μM HC-3. Incubation time was 1 hr. The apparent Km is 258 μM for choline; apparent Vₘₐₓ is 1.1 nmoles/hr/mg protein. Each point is the mean of three experiments. Hemicholinium-3 also had no effect on base exchange in bovine retina microsomes. In bovine the apparent Km is 619 μM for choline and Vₘₐₓ is 17.4 nmoles/hr/mg protein.
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1500-
1000-
500-

CDP-Choline
Phosphatidylcholine

Fig. 4. Rabbit retina microsomal phosphatidylcholine production from 125 μM 14C-CDP-choline (SA = 53 mCi/mmol) in controls and in presence of 30 or 300 μM hemicholinium-3. Each point is the mean of five experiments. For bovine microsomes, 1221 ± 8 nmoles/hr/mg protein of phosphatidylcholine was formed under control conditions and in the presence of 30 μM hemicholinium-3 1196 ± 65 nmoles/hr/mg protein were produced.

Various concentrations of hemicholinium-3. At concentrations of up to 300 μM hemicholinium-3 (Fig. 2), there was no inhibition of label incorporation into TCA-precipitable material.

Effect of Hemicholinium-3 on Glycerol Incorporation into Phospholipids

Incubation of isolated retinas with 3H-glycerol resulted in substantial incorporation of radioactivity into phospholipids. Each particular phospholipid isolated from retinas incubated in the presence of 30 μM hemicholinium-3 had the same relative specific radioactivity as that found in controls (Table 1). This result was confirmed even if the retinas incubated in hemicholinium-3 were obtained from eyes two days after pre-exposure to the drug by intraocular injection.

Effect of Hemicholinium-3 on Choline Metabolism

The lack of effect of hemicholinium-3 on leucine and glycerol incorporation suggested that hemicholinium-3 selectively affects choline metabolism. Choline is incorporated directly into phosphatidylcholine by a calcium dependent base exchange reaction. When retinal microsomes were incubated with 30 μM hemicholinium-3, there was no effect on phosphatidylcholine formation from labeled choline over a wide range of substrate concentrations (Fig. 3).

Microsomes also produce phosphatidylcholine from CDP-choline and endogenous diglycerides by the choline phosphotransferase reaction. This reaction, like base exchange, was unaffected by hemicholinium-3 (Fig. 4).

Two important steps in the biosynthesis of CDP-choline are the uptake of choline and its phosphorylation. We examined the effect of hemicholinium-3 on uptake by incubating retinas for either 20 min or 2 hrs in the presence of 5 μM choline, the physiologic level.12 After incubation, the radioactivity in the TCA-soluble and TCA-precipitable fractions was...
counted. The soluble fraction contained label in choline, phosphorylcholine, and acetylcholine; no attempt was made to separate these. The insoluble portion contained the phospholipids; greater than 95% of the label was associated with phosphatidylcholine. In the 20-min incubations, there was negligible incorporation of choline into TCA-precipitable counts, presumably because this was insufficient time for choline to be converted into phosphatidylcholine by the Kennedy pathway. However, there was significant radioactivity in the soluble fraction, and hemicholinium-3 inhibited its accumulation. This effect, observed in 20-min incubations, was fundamentally the same at 2 hrs. The latter data are presented in Fig. 5 since in the longer incubations, the ultimate effect of hemicholinium-3 on choline incorporation into lipid could also be demonstrated. In the TCA-soluble portion, hemicholinium-3 inhibited retinal choline accumulation by about 40%. This result was similar to the observed inhibition of phosphorylcholine production under similar conditions. The decreased uptake eventually reduced lipid formation as observed in the lowered amount of label in TCA-precipitable material.

The effects of hemicholinium-3 on retinal choline accumulation probably occur extracellularly and resemble its classic mode of action at the cholinergic synapse. However, studies with labeled hemicholinium-3 have also shown a net intracellular accumulation in cells possessing high-affinity choline uptake. Choline kinase is soluble and it has been shown in brain preparations that hemicholinium-3 inhibits its activity. Retinal high speed supernatants possess high choline kinase activity and this is also inhibited by hemicholinium-3 (Fig. 6). At lower drug concentrations, this inhibition appeared competitive, but at higher levels mixed inhibition occurred (Table 2). Thus, the decreased incorporation of labeled choline into phosphatidylcholine may result from a decrease in both uptake and phosphorylation.

Table 2. Apparent kinetic parameters obtained for bovine retina choline kinase activity

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<th>Control</th>
<th>30 μM HC-3</th>
<th>30 μM HC-3</th>
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<tbody>
<tr>
<td>K_m (μM)</td>
<td>275</td>
<td>346</td>
<td>580</td>
</tr>
<tr>
<td>V_max (nmol/hr/mg protein)</td>
<td>191</td>
<td>196</td>
<td>144</td>
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Discussion

These experiments examine the effects of hemicholinium-3 on portions of the biosynthetic pathway...
for phosphatidylcholine in the rabbit and bovine retina. We have also verified that mammalian retinal microsomes synthesize phosphatidylcholine by both base exchange and choline phosphotransferase activity. Additionally choline kinase activity has been confirmed in the high speed supernatant of both retinal homogenates.

In interpreting these experiments we make one implicit assumption: biochemical reactions that occur almost exclusively in photoreceptor cells can be studied in whole retina. Certainly retinal choline metabolism is dominated by the photoreceptor cells and presumably most glycerol incorporation into phospholipid also occurs there.

The incubation studies involving labeled choline demonstrate dramatic inhibitory effects of hemicholinium-3. Thirty micromolar hemicholinium-3 causes nearly half inhibition of both choline uptake and its incorporation into lipid. This concentration is about the threshold level required for total photoreceptor degeneration in mammals.

The paradoxical finding that phosphatidylcholine synthesis from choline is severely reduced by 30 μM hemicholinium-3 but proceeds normally from glycerol is most simply explained by assuming that photoreceptor cells maintain a large endogenous phosphorylcholine pool. Net phosphatidylcholine synthesis from glycerol can be normal if the phosphorylcholine concentration is sufficient to saturate the enzyme that forms CDP-choline (Fig. 1), since this is the rate limiting step in the Kennedy pathway. However, when choline is the precursor, its uptake and phosphorylation are inhibited by hemicholinium-3, and thus, the formation of labeled phosphorylcholine is much less than in control retinas.

These effects of hemicholinium-3 on choline metabolism probably result in a reduced phospholipid renewal rate in photoreceptor cells. However, it is unreasonable to assume that the degeneration is caused solely by the lowered rate. Outer segment renewal rates are experimentally altered by both lighting conditions and temperature (in poikilothersms) and certainly neither of these conditions need result in degeneration. Renewal rates are thus regulated and mutable but receptors do tolerate these changes in synthesis and degradation.

Our best present clue to the action of hemicholinium-3 is the anatomical observation of small vesicles in the base of the rod outer segments. These vesicles, which are seen almost immediately after the receptors are exposed to hemicholinium-3, are morphologic structures never present in control retinas. Perhaps the photoreceptor produces membrane deficient in phosphatidylcholine relative to the other phospholipids or else hemicholinium-3 become interpolated in the membrane causing vesiculation. Studies are currently underway to distinguish between these possibilities.

Key words: photoreceptor degeneration, choline, high-affinity uptake, hemicholinium-3, phospholipid renewal, phosphatidylcholine

References

5. Pu GA and Masland RH: Biochemical alteration of photoreceptor cell phospholipid renewal. Submitted for publication.