Rhodopsin Phosphorylation in Developing Normal and Degenerative Mouse Retinas

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The developmental pattern of rhodopsin phosphorylation in degenerative (rdle homozygote) and normal (rd/+ heterozygote) mouse retina has been investigated. The results indicate that rhodopsin levels are comparable in the 2 retinas up to about 10 days of age but that rhodopsin phosphorylation is not. The phosphorylation of rhodopsin is substantially reduced in the degenerative retina during development. This abnormality may be an expression of the rd lesion. The rhodopsin kinase/phosphatase system, the G protein, and the visual pigment are all involved in the modulation of cGMP-phosphodiesterase activity in normal retinas. A defect in any of these components could account for the reduced level of cGMP accumulation and subsequent photoreceptor degeneration. Invest Ophthalmol Vis Sci 27:264-268, 1986

Mice which have the homozygous rd gene suffer photoreceptor degeneration. A comprehensive study reported many years ago suggested that the retina of the C3H mouse, a strain which has the rd gene, forms differentiating photoreceptors which appear normal in size and shape (at the light microscope level) as well as in the quantity of rhodopsin until the age of 11 days.1 Thereafter, the process of degeneration occurs rapidly and by day 20, most rod visual cells have died.2 The earliest morphological changes can be observed with the electron microscope at about the eighth postnatal day.3 Affected rd photoreceptors begin to accumulate cGMP after postnatal day 6.4 This accumulation of cGMP is due to a deficiency of rod outer segment (ROS) cGMP phosphodiesterase (PDE) activity.5 Previous experiments indicated that the enzyme is present in the rod cells; however, it does not respond to activation as does the cGMP-PDE of control photoreceptors.6

In normal ROS, photolyzed rhodopsin catalyzes the formation of a complex between a regulatory protein with GTPase activity (G protein) and GTP,7 and this complex activates cGMP-PDE. At the same time an- other process occurs but at a slower rate: photolyzed rhodopsin is phosphorylated by rhodopsin kinase. Since phosphorylation renders rhodopsin less able to activate PDE,8 it is necessary to establish whether or not the rhodopsin phosphorylation/dephosphorylation system is intact and functional in rd mouse photoreceptors. To this end we investigated the extent of rhodopsin phosphorylation in the retinas of C3H (homozygous rd), C57BL (normal), rdle (homozygous rd gene linked to the le gene for light ears), and the rd/+ (heterozygous littermate to rdle) mice. We also compared the levels of rhodopsin in these retinas since the presence or absence of rhodopsin is directly related to the ability to activate PDE. These studies were conducted using mice in the age range 7-15 days because this is a critical time for normal differentiation and for the degeneration of ROS.

Our results indicate that there may be a lesion in the rhodopsin phosphorylation/dephosphorylation system in the rd mouse retina. This lesion could explain the lack of activation of the ROS PDE, or it may indicate that the actual genetic defect is more complex than what is currently thought.

Materials and Methods. Animals studied were either from Simonsen Laboratories (Gilroy, CA) or from our colonies. The rdle homozygotes and heterozygotes compared were littermates. We have adhered to the ARVO Resolution on the Use of Animals in Research.

Rhodopsin was purified from dark adapted rat ROS or from whole retinas by detergent solubilization and affinity chromatography on Concanavalin A Sepharose as described in the literature.9 The ROS were prepared using a previously published technique.10 Antiserum to rat opsin was produced by injecting

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purified, light exposed rhodopsin into a female New Zealand white rabbit. Approximately 50 μg of pure rhodopsin were emulsified in 2 vol of Complete Freund's adjuvant and injected near the popliteal lymph node. The rabbit was given 2 boosts of rhodopsin in incomplete adjuvant after 3-wk intervals. Blood was collected by ear vein puncture, allowed to coagulate overnight at 4°C and centrifuged to collect the clear supernatant.

To determine the amount of rhodopsin present, aliquots of retinal homogenates were dissolved in SDS, run on gradient gels and the separated proteins were transblotted to nitrocellulose paper. Unbound sites on the paper were blocked with BSA, and the nitrocellulose sheet was then incubated in rhodopsin antiserum (diluted with buffer). After washing the sheet, bound antibody was detected using a second antibody (goat antirabbit) coupled to horseradish peroxidase. The technique and materials were essentially those published and produced by BioRad Laboratories (Richmond, CA).

To determine rhodopsin phosphorylation, retinas were dissected and homogenized in Hepes buffer (20 mM), pH 7.2, containing MgCl₂ (2-5 mM) and dithiothreitol (0.5-1 mM), to a final protein concentration of about 3 mg/ml. The reactions were initiated by adding (γ³²P)ATP and terminated by adding SDS. Proteins were resolved on SDS PAGE gradient gels and the gels were dried and autoradiographed as previously described.¹⁰

**Results.** We initially compared rhodopsin phosphorylation in 12-day-old C3H and C57BL retinas because Caravaggio and Bonting had shown that the rhodopsin concentration was highest at this age in the C3H retina.¹ Although rhodopsin was phosphorylated in the normal tissue, we were unable to detect incorporation of ³²P into rhodopsin in the C3H retina (results not shown). However, at 12 days the degenerative process is occurring in the C3H retina and, as a result, its rhodopsin content is less than the normal. Thus we considered it necessary to compare phosphorylation in retinas of younger animals with similar rhodopsin content.

Use of the rdle instead of the C3H mouse has certain advantages in this type of comparison. Homozygous rdle and heterozygous rd/+ animals from the same litter have identical ages and are genetically the same except for heterozygosity at the rd and le genes (the le gene is not thought to affect the growth pattern of the animal).² For our experiments, we assumed that the developmental patterns of rhodopsin concentration which Caravaggio and Bonting found in C3H and DBA mice³ were similar in the rdle homozygote and rd/+ heterozygote littermates; however, this remained to be shown.

The results of the phosphorylation reaction carried out with retinas from littermates are shown in Figure 1. Lane 1 has the phosphorylated retinal proteins from rd/+ and rd/− retinas of 11 days of age. Lane 2, rdle, and lane 3, a mixture of 40% rd/+ retina and 60% rdle retinal homogenates. Rhodopsin phosphorylation is indicated by the letter R.
Fig. 2. Antibody binding to opsin in retinas of different ages. Retinal proteins were separated by SDS gel electrophoresis, transblotted to nitrocellulose membranes, and processed for antibody binding. Lanes 1 and 10 contain opsin. Lanes 2 through 9 contain 7 days normal (C57BL), 15 days rdle, 11 days rdle, 10 days rd/+, 10 days rdle, 9 days rdle, 8 days rd/+, and 8 days rdle proteins, respectively. The rd/+ and rdle retinas were from littermates.

were equal in rhodopsin content, while at day 11, the DBA retina contained about twice as much rhodopsin as that of the rd mouse. We determined that our inability to see rhodopsin phosphorylation in the rdle retina is unlikely to be due to detection problems. Lane 3 shows that rhodopsin incorporated $^{32}$P in a reaction mixture of 40% rd/+ and 60% rdle homogenates.

Since our results, which indicate a lack of rhodopsin phosphorylation in the rdle retina, depend upon the presence of comparable amounts of rhodopsin for significance, our next task was to actually demonstrate that this was the case in the two types of retinas at early ages. This was accomplished as described in Materials and Methods. Figure 2 shows that the rhodopsin content of rdle homozygous retinas increased and was comparable to rd/+ heterozygous retinas up to about day 10, and it reached a plateau by day 11. Thereafter, rhodopsin content decreased sharply in rdle retinas, and by 15 days of age minimal amounts were left. These results indicate that comparisons of 10-day-old retinas with regard to rhodopsin phosphorylation are valid since these tissues have similar rhodopsin concentration.

We dissected retinas from mice at different developmental ages and attempted to phosphorylate the rhodopsin. Our results, shown in Figures 3 and 4, demonstrate that this phosphorylation did occur in normal but not in degenerative retinas at a time when the rhodopsin levels were comparable. The content of rhodopsin in 7–8-day retinal samples is very low, and its phosphorylation was difficult to detect. These figures also demonstrate that rhodopsin phosphorylation in the rd/+ was comparable to that of normal C57BL retina.

Discussion. Elucidation of the biochemical mechanisms which cause the degeneration of the photore-
ceptor cells in the inherited disease of rd mice has been the interest of our laboratory for several years. Our present results extend and relate to previous findings which implicated a deficiency in cGMP-PDE activity for the accumulation of high levels of cGMP in rd photoreceptors.5

As evidenced by antibody binding on electrophoresed and blotted proteins, the rdle and rd/+ retinas are comparable in rhodopsin content through day 10, in agreement with the spectrophotometric findings of Caravaggio and Bonting1 in their work with the C3H (rd) and DBA (control) mice. However, our data on rhodopsin phosphorylation show that the rdle and rd/+ retinas are not comparable in the ability to incorporate 32P into rhodopsin in vitro: we observe substantially reduced labeling of rhodopsin after incubation of rdle retinal homogenates with (γ32P)ATP. This could indicate an abnormality in rhodopsin kinase, rhodopsin phosphatase, or rhodopsin itself (ability to be phosphorylated or dephosphorylated, packing in the membrane, etc) of the rd retina. While we cannot make a definitive statement about rhodopsin phosphorylation in 7- or 8-day-old normal and rdle mouse retinas, we feel the differences we detect in the tissues of 9–10-day-old rdle and rd/+ mice may extend to these earlier ages, but the low rhodopsin content of the younger retina precludes our detection of rhodopsin phosphorylation even in the heterozygote.

Detection of rhodopsin phosphorylation in our assay requires dephosphorylated rhodopsin which is then phosphorylated with (γ32P)ATP; thus, it is possible that the rdle rhodopsin is stably phosphorylated in vivo. This could result in a situation consistent with previously reported data.5 Rhodopsin which is phosphorylated in vivo, but not dephosphorylated, would not support PDE activation8 and could not be detected using our techniques.

During our attempts to establish the basis for the differences in rhodopsin phosphorylation between the two types of retinas, we carried out several mixing experiments and also looked for conditions which would allow rdle rhodopsin phosphorylation to occur. We separated soluble and membrane fractions from control and rdle dark-adapted retinas and then remixed the fractions. Each set of membranes (rdle and rd/+ ) was mixed with an appropriate amount of the original supernatant or supernatant from the other retinal type. The amount of 32P incorporation into rhodopsin depended upon the origin of the pellet (membranes and adhering materials), regardless of whether the supernatant was homologous or heterologous. Furthermore, addition of supernatants extracted from rat ROS or the addition of GTP to rdle retinas did not enhance the phosphorylation of rdle rhodopsin.

Thus, visual cell degeneration in the rd mouse may arise from an abnormality occurring prior to the action of cGMP-PDE. However, since we are working with whole retina or ROS homogenates and not with the purified enzymes or substrates, it is not possible at this time to come to a conclusion about which of the components of the system is defective. Our results are supported by the presence of the same type of phenomenon (lack of phosphorylation of rhodopsin) in the degenerative retina of the Irish Setter dog (Dr. S. Schmidt, personal communication). Further studies are in progress which may allow us to better define the locus of the rd mouse disease.

Key words: rhodopsin, phosphorylation, development, retinal degeneration

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Fig. 4. An autoradiogram of phosphorylated proteins from light adapted retinas as in figure 3. The A lanes are from 9–10-day-old and the B lanes are from 11-day-old animals.


Diurnal Rhythm in the Human Rod ERG

Relationship to Cyclic Lighting

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Five normal subjects were monitored with periodic full-field ERG testing during entrainment to a 14L:10D lighting cycle. On the second day of entrainment, mean ERG threshold was significantly higher 1.5 hr after light onset than at other times of day and was comparable to a previously reported threshold elevation following 7 days of entrainment. This diurnal rhythm was not detectable in eyes entrained for 3 days that were then maintained in darkness for 1 day. The findings support the idea that 2 days of entrainment are required to maximize the diurnal rhythm in the human rod ERG and that an adapting light may be required to trigger this rhythm. Invest Ophthalmol Vis Sci 27:268–270, 1986

Six normal adult subjects who were monocularly entrained for 7 days to a 14-hr light:10-hr dark (14L:10D) lighting cycle showed a diurnal rhythm in their dark-adapted, full-field rod ERG. An analysis of variance showed that time of day had a significant effect on the rod ERG in the entrained eye, with b-wave threshold 0.13 log-unit higher 1.5 hr after light onset than at other times of day. No effect of time of day was seen in the unentrained eye, suggesting that this phenomenon was regulated within the eye. The amplitude of the a-wave was significantly smaller 1.5 hr after light onset in the entrained eyes, indicating that the diurnal rhythm was present in the rod photoreceptors and not due to possible diurnal fluctuations in synaptic transmission or post-receptor function. The increase in b-wave threshold in this electroretinographic study in humans was compatible with anatomical studies in the rat, frog, cat, and monkey that have shown a daily burst of rod outer segment (ROS) disc shedding in light-entrained animals 1.5–2 hr after light onset. The minimum number of days of entrainment required to detect this diurnal rhythm in the human ERG was not evaluated.

Previous studies in the rat suggested that the daily burst in ROS disc shedding followed a circadian rhythm since it persisted in continuous darkness for at least 2 wk without a light trigger. In other species (eg, Rana pipiens) the diurnal disc shedding rhythm has appeared directly related to light onset and had a weak or absent circadian component.

The present study was done in part to determine the minimum number of days of cyclic (14L:10D) illumination necessary to produce a diurnal rhythm in rod b-wave threshold comparable to that measured previously following 7 days of entrainment. A second goal was to determine whether the diurnal rhythm in the rod ERG of entrained human subjects persists in continuous darkness and thus fulfills one criterion for a circadian rhythm.

Materials and Methods. Five adult subjects (ages 18 to 35) with normal ophthalmic examinations and normal rod and cone full-field ERGs were tested following at least 1 hr of dark-adaptation at 7:45 AM, 9:30 AM, and 4:00 PM and/or 9:00 PM for 5 successive days with different light–dark cycle protocols. Informed consent was obtained from all subjects after the details of the following procedures had been explained fully. On day 0 (control day) subjects were retested immediately following the 7:45 AM test session, with no additional light-exposure prior to the 9:30 AM test session. With the exception of 1 hr of dark-adaptation prior to the 4:00 PM and 9:00 PM test sessions, subjects were exposed to ambient illumination for the remainder of the day. Subjects were then monocularly patched from 10:00 PM until 8:00 AM in order to begin to establish a 10-hr dark:14-hr light cycle. The same eye was patched from 10:00 PM to 8:00 AM for 3 additional nights. On days 1–3 (entrainment days) the entrained eye was exposed for 10 min to a steady 34 cd/m² background at 8:00 AM and then to ambient illumination for the remainder of the day. Testing was