Phosphodiesterase-Probes Show Distinct Defects in \textit{rd} Mice and Irish Setter Dog Disorders

Rehwa H. Lee,*‡ Bernice S. Lieberman,† Richard L. Hurwitz,‡ and Richard N. Lolley†*

The phosphodiesterase from the visual cells of \textit{rd} mice and affected Irish setter dogs has been analyzed, using biochemical, biophysical, and immunological techniques. The authors' findings demonstrate that the mechanisms that cause a deficiency in phosphodiesterase activity in \textit{rd} mice and Irish setter dogs are distinctly different. Apparently, the phosphodiesterase complex is normal in affected Irish setter dogs but is abnormal in \textit{rd} mice. The criteria used for determining the normalcy of the phosphodiesterase complex were sedimentation characteristics, immuno-cross-reactivity, and histone-activation, which is shown to be a unique characteristic of the visual cell enzyme. According to these criteria, the phosphodiesterase complex in the visual cells of \textit{rd} mice is either absent or abnormal from the onset of visual cell differentiation until degeneration, because (1) it exhibits no cross-reactivity with antibody to phosphodiesterase; (2) it is not activated by histone; and (3), if present, it exhibits abnormal sedimentation characteristics and perhaps subunit structure. On the other hand, phosphodiesterase from the visual cells of affected Irish setter dogs is normal by the same criteria, because (1) it cross-reacts with antibody against phosphodiesterase; (2) it is activated by histone; and (3) it exhibits normal sedimentation and electrophoretic patterns. It is proposed that depressed levels of phosphodiesterase activity in affected setter photoreceptors are due, perhaps, to a defect in the light-initiated cascade which activates the enzyme normally, in situ. Invest Ophthalmol Vis Sci 26:1569–1579, 1985

Guanosine cyclic 3',5'-monophosphate (cyclic GMP) is a natural constituent of rod photoreceptor cells which appears to be associated with the visual process. Its metabolism is altered by light, with photon capture initiating a series of biochemical reactions that culminate in the activated hydrolysis of cyclic GMP by the enzyme, phosphodiesterase (PDE).\textsuperscript{1–6} The exact role that cyclic GMP or cyclic GMP hydrolysis plays in visual cell function has yet to be resolved, but evidence suggests that cyclic GMP participates in visual transduction or in the mechanisms of visual adaptation. Whatever the ultimate resolution of this issue, cyclic GMP appears to play a role that is essential for the maintenance of visual cell viability.

In the inherited disorders of \textit{rd} (retinal degeneration) mice and affected Irish setter dogs, an abnormality in cyclic GMP hydrolysis is observed before the affected visual cells show signs of morphological pathology.\textsuperscript{7,8} In both cases, it has been demonstrated, using retinal homogenates or microdissected layers of the retina, that PDE activity is depressed and that cyclic GMP accumulates in the affected photoreceptors.\textsuperscript{9–11} Kinetic analyses of PDE activities were useful in identifying the photoreceptor-specific PDE, but they have been of limited value in assessing the nature of the biochemical defect in the animal disorders.

With the identification of the cascade of reactions that links the absorption of light by rhodopsin to the activation of PDE,\textsuperscript{12,13} it became evident that a deficiency in PDE activity could arise from several sources other than a defect in the PDE enzyme complex itself. In order to unravel the basic defect in the inherited animal disorders, we chose first to analyze the activity and activatability of the PDE enzyme complex. It is known that the PDE enzyme complex is composed of three polypeptides which adhere to rod outer segment (ROS) membranes in the presence of magnesium.\textsuperscript{14} When solubilized, PDE activity is low, due probably to inhibitory constraints by the smallest subunit of the PDE complex.\textsuperscript{15} However, the solubilized enzyme can be activated by incubation with polycations or polyanions or by partial proteolysis.\textsuperscript{16} In this paper, we demonstrate that histone-activation of PDE is unique to the enzyme of photoreceptor cells, and we use this feature in the analysis of the enzymes from affected

\textsuperscript{*}From the Department of Anatomy, University of California School of Medicine, Los Angeles, California, and the Developmental Neurology Laboratories, Veterans Administration Medical Center, Sepulveda, California, and the Department of Pharmacology, University of Washington School of Medicine, Seattle, Washington.

\textsuperscript{†}Supported by USPHS Grant EY-00395 (RNL) and by the Medical Research Service of the Veterans Administration.

\textsuperscript{‡}Currently at the Departments of Pediatrics and Cell Biology, One Baylor Plaza, Baylor College of Medicine, Houston, Texas.

\textsuperscript{§}Submitted for publication: December 26, 1984.

\textsuperscript{||}Reprint requests: Rehwa H. Lee, PhD, Developmental Neurology Laboratory (151B), V.A. Medical Center, 16111 Plummer Street, Sepulveda, CA 91343.
visual cells. Histone-activation studies, with monoclonal antibodies against PDE, and biophysical measurements of molecular weight provide the basis for analyzing the PDE complex in retinas of developing animals with inherited blindness. Our findings demonstrate that the rd mouse and affected Irish setter dog disorders differ in the mechanisms which cause a deficiency in PDE activity.

Materials and Methods

Phosphodiesterase Assay

The activity of ROS-PDE was assayed using the two-step procedure of Thompson and Appleman. The reaction mixture, in a final volume of 500 µl, contained 50 mM Tris/HCl, pH 7.5, 2 mM MgCl₂, 2 mM β-mercaptoethanol, 0.05% bovine serum albumin and 1 mM ³H-cGMP (specific activity about 800 cpm/nmole; Amersham Corp.; Arlington Heights, IL) with or without 1 mg/ml (unless indicated otherwise) histone (type II, Sigma Chemical Co.; St. Louis, MO). The tubes were pre-equilibrated at 30°C (unless indicated otherwise), and the reaction was started by adding appropriate amounts of tissue homogenate or extract. At four time-intervals, 100-ul aliquots were removed and heated to 100°C for one min to stop the reaction. The samples were then incubated at room temperature with six units of alkaline phosphatase (Worthington Diagnostics; Freehold, NJ) for 20 min, before 1 ml of resin (Bio-Rad [Richmond, CA] AG1X2, 50-100 mesh, 1:3 slurry in water) was added. The suspension was vortexed and let stand for 30 min before spinning in a clinical centrifuge. Two hundred and fifty microliters of the resulting supernatant were counted in 5-ml scintillation cocktail (Amersham, PCS:xylene, 2:1). After correction for recovery of ³H-guanosine from the resin, the value at each time interval was plotted, and phosphodiesterase activity was determined from the linear slope of the line passing through the individual points. The protein concentration was determined according to Ross and Schatz. Phosphodiesterase activity is expressed as nanomole of cyclic GMP hydrolyzed per minute per milligram of protein.

Preparation of Tissue Extracts for the Survey of Phosphodiesterase Activity

Tissue from rats, toads, and mice was obtained from light-adapted animals which were maintained in our vivarium and killed by decapitation immediately prior to the experiments. The bovine tissue was obtained fresh from a local slaughterhouse. Homogenization of the tissues was carried out at 4°C in a glass homogenizer containing appropriate volumes of buffer (10 mM Tris/HCl, pH 7.5, and 2 mM β-mercaptoethanol). The following tissue to buffer ratios (wet weight:volume) were routinely used for rat, bovine and toad homogenates: 1:10 for liver, cerebellum, heart, and cerebral cortex; and 1:5 for spleen, testes, kidney and lung. For the bovine retina and bovine rod outer segments, one retina or one ROS pellet (from 6 bovine retinas) was homogenized in 4 ml of buffer. The homogenates were centrifuged at 10,000 x g for 20 min, and the resulting supernatant was assayed for PDE activity. For rat, toad, and mouse retinas, retinal homogenates were assayed for PDE activities. Homogenization was carried out in the ratio of two retinas per 500 ul buffer, with the exception of the C3H retinas which were homogenized in the ratio of four per 500 ul.

Assay of PDE Activity in the Developing Retinas of rd Mice and Affected Irish Setter Dogs

The C57BL/6, C3H/HeJ rd (++/++) and C57BL rdle (++/—) mice of various postnatal ages were obtained from breeding colonies in our vivarium. The mice were killed by decapitation, and the retinas dissected under room light immediately before homogenization. The retinas of affected Irish setter dogs were obtained from Dr. Greg Acland, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA. The eyes were removed under anesthesia and the retinas dissected immediately before freezing in liquid nitrogen and storage at −70°C. The retinas of visually-normal dogs were obtained from the animal facility at the VA Medical Center, Sepulveda, CA. The dogs were killed by anesthesia, and the retinas were dissected within one hr postmortem. The retinas were homogenized in ice-cold medium containing 10 mM Tris/HCl, pH 7.5 and 2 mM β-mercaptoethanol in the following retina to buffer ratio: two C57 retinas or four C3H retinas per 500 µl, or one dog retina per 1.5 ml buffer. The basal and histone-activated PDE activity was measured in duplicate, as described above. The animals in this study have all been treated according to the ARVO Resolution on the Use of Animals in Research.

Immunoadsorption and SDS-Gel Electrophoresis of ROS-PDE from Mice and Dogs

The immunoadsorption and sodium dodecyl sulfate (SDS)-gel electrophoresis of the immunoadsorbed ROS-PDE was performed by Richard L. Hurwitz in the laboratory of Dr. J. Beavo, Department of Pharmacology, University of Washington, Seattle, WA. The ROS-1 monoclonal antibody was generated and coupled to Sepharose as described by Hurwitz et al. For extraction of ROS-PDE, retinas were homogenized at 4°C in 1 ml of 10 mM Tris/HCl, pH 7.8,
containing 0.5% Triton X-100 and 200 uM of phenylmethylsulfonyl fluoride (PMSF). Various retinal samples were prepared and processed concurrently: a visually-normal dog, a 34-day-old affected Irish setter, a bovine, ten retinas from 21-day-old C57BL mice, and ten retinas from 10-day-old C3H mice. All retinas were light-adapted and freshly dissected, except those of the cattle and Irish setter dog which were stored frozen at -70°C prior to homogenization. The homogenates were centrifuged at 10,000 X g for 15 min, and the resultant retinal supernatants were maintained at 4°C and sent to Richard L. Hurwitz or the cross-reactivity experiments. Aliquots of the Triton X-100 supernatants, containing extracted ROS-PDE, were incubated with ROS-1-Sepharose for 18 hr at 4°C and then centrifuged. The immunoadsorbed supernatants as well as untreated aliquots of the original Triton X-100 supernatants were assayed for PDE activity in the presence of histone. 21 The pelleted resins were washed; the protein was eluted with 8 M urea, boiled under reducing conditions, and applied to a 15% total acrylamide, 0.08% bis-acrylamide SDS gel, under conditions described by Laemmli 22 and Baehr et al. 14 Polypeptides which were immunoadsorbed by ROS-1 were detected by silver staining (Bio-Rad).

Sedimentation of ROS-PDE on Sucrose Density Gradient

The ROS-PDE was extracted with a hypotonic buffer from the retinas of normal and diseased mice and dogs before centrifugation on the sucrose density gradients. Retinas were homogenized in 10 mM Tris/HCl, pH 7.5, 2 mM β-mercaptoethanol, with the following tissue to buffer ratios: one 12-day-old C57BL retina, or two 10-day-old C3H retinas in 250 ul buffer; and one visually-normal dog retina, or one 35-day-old affected Irish setter dog retina in 1.5 ml buffer. The homogenates were centrifuged at 15,000 X g for 20 min. Two hundred microliters of each hypotonic extract were layered on 5-20% linear sucrose density gradients and centrifuged as described previously. 23 Following centrifugation, the gradients were fractionated into 500-μl fractions, and each fraction was assayed for PDE activity in the presence or absence of histone.

Protein components in each ROS-PDE fraction were further analyzed by SDS-gel electrophoresis. Each fraction containing PDE activity from three identical gradients was combined, dialyzed overnight in water containing 0.1 mM PMSF, concentrated to about 500 microliters using Aquacide I (Behring Diagnostics; La Jolla, CA), and lyophilized to dryness. Each resulting residue was taken up in 100 ul of 2% SDS and 2% β-mercaptoethanol and applied to a 10% SDS gel under the conditions of Laemmli. 22 Proteins in each fraction were detected by silver staining (Bio-Rad).

Results

Histone-Activation of ROS-PDE

Polycations or polyanions activate the PDE of frog ROS. 16 Using crude or purified PDE from mammalian ROS and retinas, we found similar activation. Histone (Sigma type IIS and type VIIIS) consistently exhibits the highest extent of activation in comparison to other polycations (eg, other types of histone, protamine, polylysine, or polyarginine). Our laboratory has routinely used the type IIS histone for the activation of ROS-PDE. Figure 1 depicts the time-course of cyclic GMP hydrolysis catalyzed by a hypotonic extract from bovine ROS. Hydrolysis proceeds linearly in the absence of histone for at least 30 min. In the presence of histone, the reaction proceeds linearly at an accelerated rate, after an initial lag. Depending on the activity of
Fig. 2. Effect of assay temperature on the activity of bovine ROS-PDE. a. The activity of bovine ROS-PDE in the presence (O---O) or absence (●---●) of histone was assayed at 0, 10, 22, 30, 37 and 40°C, as described in Methods. b. Arrhenius plot of the histone activation of PDE. c. Time-course of cGMP hydrolysis at various assay temperature. The hydrolysis of cGMP by bovine ROS-PDE was carried out in the presence of 1 mg/ml of histone at the temperature indicated. The reaction was started with the addition of the enzyme. The data are representative of three independent experiments.

PDE, the hydrolysis usually stops within three to five min apparently because of substrate depletion, since further hydrolysis occurred upon addition of fresh 3H-cGMP. Accumulation of the product, 5'-GMP, is not inhibitory (results not shown). In order to assure accurate estimates of the rate at which cGMP is hydrolyzed in the presence of histone, it is essential to estimate the rate from the linear portion of the slope.

The rate at which activated PDE hydrolyzes cyclic GMP is dependent on the concentration of added histone. At 30°C, the extent of histone-activation increases from threefold of the basal activity in the presence of 0.1 mg/ml histone to a maximum of 20-fold in the presence of 1 mg/ml histone. Higher histone concentrations of 1 to 5 mg/ml yield little increase in enzyme activation. Routinely, the assay was carried out in the presence or absence of 1 mg/ml of histone.

The rate at which activated PDE hydrolyzes cyclic GMP is dependent also on the assay temperature (Fig. 2A). In the absence of histone, the rate of hydrolysis increases twofold when the temperature increases from 0°C to 30°C, and it decreases slightly between 37°C and 40°C probably because of heat denaturation of the PDE enzyme. In the presence of 1 mg/ml histone, activation of the PDE activity increases from onefold of the basal activity at 0°C to about 20-fold of the basal activity at 37°C and 40°C, indicating that activation of PDE by histone is an endothermic reaction. Similar effects of the assay temperature were also observed on the histone-activated PDE activities of mouse and rat retinas. An Arrhenius plot (Fig. 2B) of the same data shows that the heat of PDE activation is about 11 Kcal/mole. In order to compare histone-activation from different studies, it is imperative to keep the assay temperature constant. Unless stated otherwise, PDE was routinely assayed at 30°C.

In following the time-course of histone-activated cyclic GMP hydrolysis, we have observed a lag in the reaction rate immediately after the start of the assay before PDE hydrolysis is at its fully-activated rate (Fig. 2C); the lag in cyclic GMP hydrolysis by bovine ROS-PDE was observed at 30°C or lower assay temperatures.
The time-lag in histone activation at 30°C was confirmed with measurements of PDE activity at 20 and 40 sec after start of the reaction (results not shown). At temperatures of 37°C or higher, cyclic GMP was hydrolyzed so rapidly that the lag was not detected by our experimental procedures. The lag was not removed by preincubation of the enzyme and/or reaction mixture at 30°C. A temperature-dependent time-lag in histone-activation was observed also using retinal homogenates of mice.

Histone apparently activates PDE by direct interaction with the enzyme complex. The cascade of enzymic interactions that are required in the activation of PDE by light are unnecessary during activation by histone. At 1 mg/ml, histone activates purified bovine ROS-PDE by 20- to 70-fold (results not shown); this enzyme preparation was homogeneous as judged by the presence on silver-stained SDS gels of a single doublet at 88, 84K. Histone-activation was observed also with retinal homogenates or ROS which contained all components of the cascade, in light or darkness, and in the presence or absence of Gpp(NH)p (Table 1). Therefore, the ability of histone to activate the visual cell PDE can be assessed regardless of the purity/crudeness or light/dark adaptation of the samples. Furthermore, the histone-activated PDE activity is significantly higher than that obtained by light activation, in vitro.

Table 1. Effect of histone on the activity of phosphodiesterase in dark- or light-adapted retinas*

<table>
<thead>
<tr>
<th>Animal/tissue</th>
<th>− Histone</th>
<th>+ Histone</th>
<th>+ Histone/− Histone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat: retina</td>
<td>10.30 ± 1.62 (2)</td>
<td>294.40 ± 86.5 (3)</td>
<td>28.58</td>
</tr>
<tr>
<td>Cerebral</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>100.03 ± 31.26 (4)</td>
<td>70.28 ± 23.90 (4)</td>
<td>0.69</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>11.08 (1)</td>
<td>5.26 (1)</td>
<td>0.47</td>
</tr>
<tr>
<td>Heart</td>
<td>3.02 ± 1.20 (4)</td>
<td>1.27 ± 0.87 (4)</td>
<td>0.42</td>
</tr>
<tr>
<td>Liver</td>
<td>4.62 ± 0.35 (4)</td>
<td>1.83 ± 0.24 (4)</td>
<td>0.40</td>
</tr>
<tr>
<td>Lung</td>
<td>3.20 ± 0.57 (4)</td>
<td>0.96 ± 0.34 (4)</td>
<td>0.30</td>
</tr>
<tr>
<td>Spleen</td>
<td>3.39 (1)</td>
<td>0.77 (1)</td>
<td>0.23</td>
</tr>
<tr>
<td>Testes</td>
<td>0.30 (1)</td>
<td>0.21 (1)</td>
<td>0.70</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.41 (1)</td>
<td>0.56 (1)</td>
<td>0.40</td>
</tr>
<tr>
<td>Cattle: retina</td>
<td>2.20 ± 0.63 (5)</td>
<td>51.81 ± 16.34 (5)</td>
<td>23.50</td>
</tr>
<tr>
<td>ROS</td>
<td>68.30 ± 6.01 (4)</td>
<td>4781.1 ± 51.2 (4)</td>
<td>70.00</td>
</tr>
<tr>
<td>Brain</td>
<td>45.50 ± 2.05 (4)</td>
<td>37.44 ± 2.01 (4)</td>
<td>0.82</td>
</tr>
<tr>
<td>Heart</td>
<td>12.40 (1)</td>
<td>7.21 (1)</td>
<td>0.38</td>
</tr>
<tr>
<td>Toad: retina</td>
<td>103.79 ± 22.08 (4)</td>
<td>750.62 ± 65.45 (4)</td>
<td>7.20</td>
</tr>
<tr>
<td>Heart</td>
<td>14.26 ± 1.83 (3)</td>
<td>2.39 ± 0.09 (3)</td>
<td>0.17</td>
</tr>
<tr>
<td>Liver</td>
<td>1.27 ± 0.22 (3)</td>
<td>0.29 ± 0.23 (3)</td>
<td>0.23</td>
</tr>
<tr>
<td>Mouse: C57, Adult retina</td>
<td>17.28 ± 1.82 (4)</td>
<td>395.40 ± 38.93 (4)</td>
<td>17.8</td>
</tr>
<tr>
<td>C3H, Adult retina</td>
<td>8.01 ± 1.64 (3)</td>
<td>7.40 ± 1.23 (3)</td>
<td>0.92</td>
</tr>
</tbody>
</table>

* Hypotonic extracts from various tissues were prepared and assayed for phosphodiesterase activity, as described in Methods.
† Mean ± SEM. The numbers in parentheses indicate the number of independent measurements.
‡ Retinal homogenates were assayed.

Specificity of Histone Activation

The effect of histone on the activity of PDE obtained from various tissues from several vertebrate species is summarized in Table 2. The levels of PDE activity varied greatly in the different tissues as well as in the same tissue from different animals. Nevertheless, it is clear that PDE activity in all non-retinal tissues is either unaffected or inhibited by histone. The PDE activity in each retinal extract (cattle, rat, mouse and toad) was activated between eight- and 24-fold by histone.

Histone-activation of PDE in the retina is restricted
Table 3. Effect of histone on the activity of phosphodiesterase in the retinas of visually normal dog and affected Irish setters*  

<table>
<thead>
<tr>
<th></th>
<th>- Histone</th>
<th>+ Histone</th>
<th>+ Histone/ - Histone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Dog</td>
<td>7.0</td>
<td>144.4</td>
<td>20.6</td>
</tr>
<tr>
<td>Affected Irish Setters:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18-day-old</td>
<td>14.5</td>
<td>28.3</td>
<td>2.0</td>
</tr>
<tr>
<td>23-day-old</td>
<td>18.6</td>
<td>53.1</td>
<td>3.0</td>
</tr>
<tr>
<td>28-day-old</td>
<td>10.6</td>
<td>49.7</td>
<td>4.5</td>
</tr>
<tr>
<td>39-day-old</td>
<td>14.3</td>
<td>71.3</td>
<td>5.0</td>
</tr>
</tbody>
</table>

* The retinas of visually normal dogs and affected Irish setters were homogenized, as described in Methods, and assayed at 30°C in the presence or absence of 1 mg/ml histone. The data are representative of two independent experiments in which each value was derived from 5-6 determinations. Three other independent experiments using retinas from 34- to 35-day-old affected Irish setters also gave similar results (average histone-activation of 4.2-fold).

to the visual cells. For example, the mature retina of normal mice (C57BL) contains functional photoreceptors, and homogenates of the retina exhibit PDE activity that is activated 18-fold by histone. By comparison, mature C3H mice possess no photoreceptors, and retinal homogenates of these retinas possess PDE that is unaffected by histone. Our observations suggest that histone-activation of PDE is a feature that is unique to the PDE of the photoreceptor cells.

Evaluation of the ROS-PDE Enzyme Complex in Animals with Inherited Blindness

Affected Irish setters: The ability of histone to activate visual cell PDE was measured using retinal homogenates of a visually-normal dog and affected Irish setters (Table 3). In the normal dog, the enzyme was activated about 20-fold. About two- to five-fold activation was observed in the 18- to 39-day Irish setters; at these ages, the visual cells have differentiated, and they exhibit early signs of morphological pathology. Since activation by histone is unique to the photoreceptor PDE, this observation suggests the presence of a normal PDE complex in the visual cells of affected Irish setter dogs. The failure of the affected photoreceptors to hydrolyze cyclic GMP in vivo is due apparently to defect(s) other than at the catalytic site of the ROS-PDE.

Density gradient centrifugation experiments were used to assess the sedimentation coefficient of the PDE complex (Fig. 3). Following centrifugation, the PDE both of normal and affected retinas was found to have a sedimentation coefficient of approximately 9.9 S.

Fig. 3. Sedimentation on sucrose density gradient of ROS-PDE from normal dog and affected Irish setters. The ROS-PDE from the retinas of a normal dog (left) and affected Irish setter (right) was extracted and centrifuged in parallel on sucrose density gradients, as described in Methods. Following centrifugation, the gradients were fractionated and assayed for basal (○) and histone-activated (●) PDE activity. Fraction 1 represents the bottom fraction of the 5 to 20% gradient.
These observations indicate that the affected ROS-PDE enzyme complex is similar in molecular weight and conformation to that of normal PDE. As shown in a later section, the PDE enzyme from affected dogs also contains apparently normal subunits.

Several high-affinity monoclonal antibodies have been generated against the PDE enzyme complex of bovine ROS. One antibody, designated ROS-1, cross-reacts selectively with the ROS enzyme and inhibits its catalytic activity. Furthermore, ROS-1 cross-reacts with ROS-PDE from various retinas, including those of human, monkey, rat, mouse and goldfish. To confirm that histone-activation is specific for ROS-PDE, we have investigated the ability of ROS-1 to adsorb and precipitate the histone-activated PDE. Detergent-solubilized extracts from the retinas of normal and diseased dogs were each incubated with either Sepharose-bound ROS-1 or ethanolamine-Sepharose. After centrifugation of the resin, histone-activated PDE activities remaining in the immunoadsorbed supernatants were compared to those in the ethanolamine-Sepharose-incubated supernatants (taken respectively as 100% of the PDE activity present in the normal or diseased retinas). It was found that more than 90% of the histone-activated PDE activity present in the retinas of the visually-normal dog and the affected Irish setter were adsorbed to the ROS-1-Sepharose. A comparable level of enzyme activity from the bovine extract was also adsorbed, but the data are not shown.

The ROS-1-bound polypeptides were later solubilized, run on SDS-polyacrylamide gels, and detected by silver staining. Consistent with previous reports, subunits from the ROS-PDE of visually-normal dogs, affected Irish setters, and bovine (Fig. 4, Lanes b, c, and d, respectively) exhibit molecular weights ranging between 94,000 and 84,000. This observation shows conclusively the presence of apparently normal PDE in the visual cells of affected setters.

![Fig. 4. SDS-gel electrophoresis of immunoadsorbed ROS-PDE from the retinas of normal and rd animals. Triton X-100-solubilized ROS-PDE from a visually normal adult dog (Lane b), a 34-day-old affected Irish setter (Lane c), a bovine (Lane d), 21-day-old C57BL mice (Lane e), and 10-day-old C3H mice (Lane f) were immunoadsorbed by ROS-1-Sepharose, electrophoresed on SDS-polyacrylamide gels, and detected by silver staining, as described in Methods. Lanes a and g contain molecular-weight markers purchased from Bio-Rad.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933120/)

Both histone-activation (Table 4) and immunad- sorption data (Fig. 4) suggest that the amount of PDE complex is less than normal in affected setter retinas. A reduced level of PDE protein could be a secondary feature of the degenerative disease resulting from the reduced size of the inner and outer segments of affected visual cells as well as from a reduced rate of PDE syn-

### Table 4. Basal and histone-activated phosphodiesterase activity in retinas of developing mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Age</th>
<th>Phosphodiesterase Activity* (nmol cGMP hydrolyzed/min/mg)</th>
<th>— Histone</th>
<th>+ Histone</th>
<th>+ Histone/— Histone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal†</td>
<td>1 d</td>
<td>5.65 (2)</td>
<td>N.D.</td>
<td>71.44 ± 14.70 (4)</td>
<td>15.8</td>
</tr>
<tr>
<td></td>
<td>7–8 d</td>
<td>8.15 ± 2.43 (4)‡</td>
<td>395.4 ± 38.93 (4)</td>
<td>17.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11–12 d</td>
<td>22.26 ± 4.04 (4)‡</td>
<td>272.18 ± 14.9 (4)</td>
<td>15.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>23 d</td>
<td>17.28 ± 1.82 (4)‡</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>rd† (++/++)</td>
<td>1 d</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>7–8 d</td>
<td>5.61 ± 1.51 (4)‡</td>
<td>6.41 ± 3.03 (4)</td>
<td>1.14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11–12 d</td>
<td>6.65 ± 1.05 (5)‡</td>
<td>7.64 ± 1.11 (5)</td>
<td>1.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>23 d</td>
<td>8.01 ± 1.64 (3)‡</td>
<td>7.40 ± 1.23 (3)</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td>rdle† (++/—)</td>
<td>12–14 d</td>
<td>15.00 ± 1.15 (7)</td>
<td>300.10 ± 73.2 (7)</td>
<td>20.0</td>
<td></td>
</tr>
</tbody>
</table>

* The retinas of developing mice were homogenized and assayed, as described in Methods.
† Normal, C57BL/6; rd (++/++), C3H/HeJ; rdle (++/—). C57BL rdle
‡ Mean ± SEM. The numbers in parentheses indicate the number of independent measurements.
The sedimentation coefficient of the ROS-PDE enzyme complex from the retina of a C57BL mouse was evaluated by density gradient centrifugation (Fig. 5). The histone-activatable PDE sediments on a sucrose gradient to a location comparable to that of the dog (Fig. 3) and bovine (results not shown) enzymes. Electrophoretic analysis of each gradient fraction shows that the two large subunits of ROS-PDE migrate as 88,000 and 84,000 molecular weight bands; their protein-staining intensity correlates with the PDE activity from the same fractions. A parallel experiment also was performed using a hypotonic extract from the retinas of a 10-day-old C3H mouse; care was taken to match the amount of protein in the rd and control samples. No basal or histone-activatable PDE activity was observed in the entire gradient. Electrophoretic analysis and silver staining also failed to show either component of the 88K and 84K doublet in the fractions where normal PDE is located. These observations indicate that either the ROS-PDE is missing in the rd visual cells or, if present, the affected ROS-PDE enzyme complex is abnormal in its conformational structure as well as its catalytic site.

A cross-reactivity study, using ROS-1 monoclonal antibody, was done also to determine the normalcy of the PDE enzyme complex from rd visual cells. Detergent-solubilized extracts from the retinas of 21-day C57 mice and 10-day C3H mice were immunoadsorbed with ROS-1-Sepharose (see Methods). In the C57 mice, more than 90% of the histone-activated PDE activity (compared to the ethanolamine-Sepharose-incubated control sample) was adsorbed by the antibody. On the other hand, in C3H mice, less than 5% of the retinal PDE activity was precipitated by ROS-USepharose, and this activity was not activated by histone. Gel electrophoresis and silver staining of the adsorbed protein of the C57 sample show clearly the 88K and 84K doublet (Fig. 4, lane e), whereas no polypeptide band was detected in the C3H sample (Fig. 4, lane f). These observations further strengthen the conclusion that the PDE complex in rd visual cells is abnormal or missing.

The expression of the rd gene could result in the synthesis of an abnormal PDE complex in rd visual cells. To test this possibility, we have measured PDE activity in the retinas of 12- to 14-day-old first generation hybrids (C57BL rdle ++/−), obtained from breedings of C57BL/6 and C57BL rdle (+/+ ++) mice; these mice are heterozygous for the rd gene and are visually normal. In the heterozygous retinas, PDE activity was activated about 20-fold by histone. Further-
more, the basal and histone-activated activities are comparable to those of the control mice (C57BL/6) of the same postnatal ages (Table 4).

Discussion

A deficiency in PDE activity and an accumulation of cyclic GMP characterize the inherited visual disorders of rd mice and affected Irish setter dogs. Both disorders arise in early postnatal life, and they result in visual cell degeneration, with rod degeneration preceding cone death. Biochemical and morphological studies of the developing retinas show that, in the rd mouse disorder, cyclic GMP levels rise two- to threefold above normal in the second and third postnatal week and that rod photoreceptor cell degeneration is complete by the end of the third week. On the other hand, cyclic GMP levels rise eight- to tenfold above normal by one month of postnatal life in affected setters, and rod visual cell degeneration is complete by about one year. The ability of visual cells of affected setters to survive for weeks in the presence of high levels of cyclic GMP might reflect species variation, or it could indicate that the inherited disorders of rd mice and affected setters have distinct biochemical lesions, which disrupt different aspects of visual cell metabolism or function.

In order to understand the mechanism which underlies the deficiency of PDE activity in these animal disorders, we have sought biochemical, biophysical and immunological characteristics which can be used to evaluate the function and structure of the affected PDE enzyme complex. Phosphodiesterase from the rod outer segments of cattle and frog has been purified to homogeneity. Our investigation of the PDE from mice and rat retinas shows that the ROS-PDE enzyme complex of various animal species has similar molecular weight and subunit structure. The two large subunits of ROS-PDE can be readily identified on SDS-polyacrylamide gels (as doublets with molecular weights ranging from 94K to 84K). Miki et al. have shown that solubilized PDE from frog rod outer segments exhibits low catalytic activity and can be activated by incubation with polycations or polyanions. These criteria along with the immuno-cross-reactivity of the enzyme with monoclonal antibody ROS-1 serve as a fingerprint of the ROS-PDE and allow the identification of normal from abnormal PDE.

The use of histone-activation in identifying the visual cell PDE is only effective if it can be shown that this feature is unique to the visual cell PDE. In this paper we have given evidence that verifies this uniqueness. The mechanism by which histone activates ROS-PDE is probably related to the mechanism by which this enzyme complex is activated by light. In the absence of histone, the PDE enzyme complex exhibits low catalytic activity, apparently as a result of the inhibition by the 13K subunit. When this constraint is removed, in vitro, the PDE complex becomes activated. This can be achieved by partial proteolysis with trypsin destroying the small subunit or by dilution of the enzyme complex, which facilitates dissociation of the 13K subunit. The detailed mechanism by which the membrane-bound PDE is activated by the transducin-GTP complex (T • GTP), in situ, remains to be elucidated. Fung and Nash observed that the T • GTP complex activates PDE most effectively in the presence of reconstituted membranes consisting of positive, charged phospholipids such as phosphatidylcholine. It is conceivable that histone, through its positive electrostatic charges, mimicks the light activation of PDE and induces conformational changes in the 13K subunit of PDE, revealing the catalytic site and allowing activation of the enzyme. In the experiments depicted in Figure 2c, we have observed an initial lag in histone-activation that can be reduced or eliminated by increasing the assay temperature. This observation suggests that the PDE enzyme complex undergoes slow conformational changes during histone-activation.

Histone or trypsin treatment activates PDE to a comparable extent, and both achieve levels of activity that are considerably higher than that initiated by light and a reconstituted cascade. Histone-activation of ten- to 20-fold is common in crude retinal homogenates, and it can be used to give an approximation of maximal PDE activity. The extent of histone-activation can be modified by several factors; for example, it increases with increasing assay temperature and with the amount of added histone. The extent of activation can be diminished if the basal PDE activity is elevated by dilution of the sample or by the presence of other PDE isoenzymes. For our purpose, histone-activation has been used as a diagnostic tool rather than a means to quantitate the enzyme content; the presence of histone-activation indicates a functional PDE complex and the absence indicates an abnormal PDE.

Applying the above criteria to developing normal mice and to those with inherited blindness, the PDE complex of rd mice appears either missing or abnormal from the earliest postnatal life. No histone-activatable PDE activity was observed in the affected retinas at any age studied; no significant PDE activity or any polypeptide of 94–84K molecular weight was immunoabsorbed by the ROS-1 monoclonal antibody. Moreover, the two familiar subunits of a normal enzyme complex did not appear at the location in the sucrose density gradient where the normal enzyme sediments. Hurwitz et al. showed that the ROS-1 antibody recognizes only the inhibited conformation of
The present finding could indicate that some locus of the Irish setter do produce a PDE with features typical of the switch hypothesis because affected photoreceptors show a typical visual cell enzyme that is calmodulin-insensitive. The current findings are inconsistent with the hypothesis that affected photoreceptors fail, during cell differentiation, to switch to a calmodulin-dependent state. They suggest further that affected photoreceptors fail, during cell differentiation or growth, to switch to a calmodulin-dependent state. The present finding could indicate that some locus of the PDE complex. We cannot rule out the possibility that the PDE might exist in an already activated state and is therefore unreactive with the antibody; however, the low levels of PDE activity that are observed in the rd retina make this possibility unlikely. From this investigation we conclude (1) that rd visual cells possess either a radically abnormal PDE both catalytically and conformationally, or they fail to produce the PDE enzyme complex altogether; and (2) that the PDE activity which is observed in the rd retina is associated with the neurons of the inner retina, since it is not activated by histone, and its activity persists after the visual cells have degenerated.

The absence of PDE or the production of an abnormal PDE by rd visual cells encourages the speculation that the genes for PDE and rd could be identical. Experiments were carried out which attempted to resolve whether the structural gene for PDE might be abnormal in rd retinas. Mice that were heterozygous for the rd gene (C57BL rdle +/−) were investigated for the presence of PDE activity and for the ability of histone to activate the PDE observed. The rationale was that one half of the rd gene dosage would be present in the heterozygous animal resulting in lower than normal levels of PDE activity and about one half of the normal level of histone-activated PDE activity. Instead, we observed PDE activity and histone-activation levels that were comparable to normal retinas of the same age (12–14 days, Table 4; adult data not shown). These observations suggest that the structural gene for visual cell PDE is not identical to the rd gene.

Using the same approach to evaluate the visual cell PDE of developing Irish setter dogs, we found that (1) the PDE complex in the affected retinas showed normal composition and sedimentation characteristics; (2) it displayed normal antibody cross-reactivity; and (3) it showed substantial histone-activation. These observations indicate that affected visual cells of the immature Irish setter retina contain a photoreceptor-specific PDE which has a variety of normal characteristics. This conclusion is different from that reported by Liu et al.26; they proposed that the visual cells of affected setters produce an abnormal PDE. They suggest further that affected photoreceptors fail, during cell differentiation or growth, to switch to a calmodulin-dependent to a typical visual cell enzyme that is calmodulin-insensitive. The current findings are inconsistent with the switch hypothesis because affected photoreceptors of the Irish setter do produce a PDE with features typical of the visual cell enzyme of normal retinas.

It remains to be elucidated why the affected setter visual cells are unable to hydrolyze cyclic GMP at a rate which prevents cyclic GMP accumulation, in situ. The present finding could indicate that some locus within the PDE activation cascade is defective other than PDE. It is also possible that our experimental paradigm missed an abnormality in the PDE complex which prevents activation by the light-triggered cascade. The answer will come with additional experimentation. But, even now, a point of distinction can be drawn between the rd disorder of mice and the inherited degeneration of Irish setter dogs. While both disorders share an apparent abnormality in cyclic GMP hydrolysis, they differ in the mechanisms which underlie the deficiency in visual cell PDE activity.

Key words: retinal degeneration, rod visual cell, phosphodiesterase, histone-activation, immuno-cross-reactivity

Acknowledgments

We thank Dr. Joseph Beavo for helpful discussions, Dr. Greg Acland for supplying retinas from affected Irish setter dogs, Ms. Elizabeth Racz for dissecting the retinas, and Ms. Louise V. Eaton for assistance in manuscript preparation.

References

12. Wheeler GL and Bitensky MW: A light-activated GTPase in


