

Light Responses in Rods of Vitamin A–Deprived Xenopus

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PURPOSE. Accumulation of free opsin by mutations in rhodopsin or insufficiencies in the visual cycle can lead to retinal degeneration. Free opsin activates phototransduction; however, the link between constitutive activation and retinal degeneration is unclear. In this study, the photoresponses of Xenopus rods rendered constitutively active by vitamin A deprivation were examined. Unlike their mammalian counterparts, Xenopus rods do not degenerate. Contrasting phototransduction in vitamin A–deprived Xenopus rods with phototransduction in constitutively active mammalian rods may provide new understanding of the mechanisms that lead to retinal degeneration.

METHODS. The photocurrents of Xenopus tadpole rods were measured with suction electrode recordings, and guanylate cyclase activity was measured with the IBMX (3-isobutyl-1-methylxanthine) jump technique. The amount of rhodopsin in rods was determined by microspectrophotometry.

RESULTS. The vitamin A–deprived rod outer segments were 60% to 70% the length and diameter of the rods in age-matched animals. Approximately 90% of its opsin content was in the free or unbound form. Analogous to bleaching adaptation, the photoresponses were desensitized (10- to 20-fold) and faster. Unlike bleaching adaptation, the vitamin A-deprived rods maintained near normal saturating (dark) current densities by developing abnormally high rates of cGMP synthesis. Their rate of cGMP synthesis in the dark (15 seconds−1) was twofold greater than the maximum levels attainable by control rods (~7 seconds−1).

CONCLUSIONS. Preserving circulating current density and response range appears to be an important goal for rod homeostasis. However, the compensatory changes associated with vitamin A deprivation in Xenopus rods come at the high metabolic cost of a 15-fold increase in basal ATP consumption.

Vitamin A is the precursor to 11-cis retinal, the chromophore that binds to opsin to produce rhodopsin, the light-absorbing pigment. Without vitamin A, the amount of free opsin increases, leading to a loss in quantum catch and sensitivity that resembles the desensitization caused by bright, bleaching lights. Long-term vitamin A deprivation causes retinal degeneration in mammals but not in Xenopus. Mutations that produce constitutive activation of phototransduction cause retinal degeneration in mammals. The point mutation G90D in rhodopsin is associated with congenital stationary night blindness. Mutations in RPE65, the gene that codes for retinoid isomerase in the pigment epithelium, cause Leber congenital amaurosis. Constitutive activation of phototransduction by bleaching adaptation or caused by mutations in rhodopsin leads to desensitization, speeding up of the light response, faster cGMP turnover, and a reduction of the dark circulating currents. The lack of chromophore in RPE65−/− mice produces similar effects. However, the precise link between constitutive activation of the phototransduction pathway and rod degeneration remains unclear. The equivalent light hypothesis posits that a persistent activation of the transduction pathway triggers photoreceptor degeneration. This idea rests on the notion that a persistent decrease in intracellular calcium triggers cell death.

Understanding the reasons that constitutively active Xenopus rods do not degenerate may help us understand the mechanisms of retinal degeneration. We report that vitamin A–deprived rods in Xenopus are chronically desensitized, exhibiting constitutive activation of their phototransduction cascade and response features characteristic of light and bleaching adaptation. However, vitamin A–deprived rods preserve normal circulating current density levels by developing abnormally high levels of basal guanylate cyclase (GC) activity that counter the enhanced rates of cGMP hydrolysis.

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Materials and Methods

Husbandry

Xenopus laevis tadpoles were generated by in vitro fertilization and reared as described previously. All procedures were approved by the SUNY Upstate Medical University Institutional Animal Care and Use Committee and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (1996, National Academy of Sciences, Washington, DC) and in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Control tadpoles were raised on a standard diet consisting of nettle (Wunderlich-Dietz, Hasbrouck Heights, NJ) and tadpole powder (Xenopus Express, Plant City, FL). To generate vitamin A–deprived animals, tadpoles were raised on a custom diet lacking vitamin A (MP Biomedicals Inc., Aurora, OH). The developmental stages of the animals were determined according to standardized morphologic characteristics. However, as shown in Figure 1A, metamorphosis of the vitamin A-deprived animals was absent or delayed because of the absence of retinoic acid, a derivative of vitamin A. Vitamin A–deprived animals
Electrophysiological Recordings

We prepared the cells and performed suction electrode recordings as described previously.20 Recordings were obtained exclusively from rods in small clusters with intact inner segments. Changes in circulating current elicited by 20-ns (520-nm wavelength), nonpolarized light flashes were recorded with an amplifier (Axopatch 1D; Axon Instruments, Union City, CA) and analyzed with commercial software (Clampfit; Axon Instruments, and SigmaPlot and SigmaStat; SPSS, Inc., Chicago, IL). Error bars in graphs indicate the SEM. The sample size (n) is indicated in the text.

Ensemble variance analysis22 was used to estimate the collecting area of rods as described previously.20 In short, the square of the average of 16 or more responses to dim flashes was compared with the excess ensemble variance elicited by the same flashes. The number of photoisomerizations per flash was estimated from the scaling factor needed to make the onset of the waveforms overlap. Hence, the collecting area determined by this approach is defined as the ratio of the number of photoisomerizations to the number of photons reaching the rod during a flash (photons per square micrometer).

We measured relative cyclase activity (α) by using the IBMX (3-isobutyl-1-methylxanthine) jump recording technique.20 We improved the method by recording from rods with intact inner segments. We have reported that the inner segments of Xenopus rods usually do not preserve well after cell isolation procedures, resulting in faster than normal responses.20 To ensure that we recorded from rods with intact inner segments, we selected tissue pieces containing a pair of rods. One rod was drawn fully into the electrode, and continued suction pulled the inner segment of the second rod into the pipette until its ciliary stalk was in line with the edge of the pipette’s tip. The advantages of this painstaking maneuver were reflected in the extended duration of the responses of control stage 48 to 51 tadpole and juvenile frog rods to saturating flashes. During light responses, the changes in cyclase activity levels of rods recorded in this manner increased by a factor of two compared with those reported in our prior study,20 although basal cyclase activity levels were lower.

Pigment Reconstitution

Retinas were incubated for 40 minutes in 50 μM 11-cis retinol solution in Xenopus Ringer’s. The retinal solution was prepared as a 0.1% dilution of a stock solution of 11-cis retinol in ethanol.25

Estimation of Collecting Area by Using Physical Properties of Rod Outer Segments

Collecting areas were estimated by using the expression22,

\[
CoA = \frac{\pi dl^2}{4} Q_{iso} f 2.3 \alpha
\]

where CoA is the collecting area, d and l are the diameter and length of the ROS, respectively, Q_{iso} is the quantum efficiency of isomerization (0.7), f is the polarization factor (takes a value of unity for linearly polarized light traveling transversely across the outer segment, or a value of 0.5 for nonpolarized light), and α is the specific optical density measured by microspectrophotometry and defined as the optical density per unit distance for plane polarized light traveling transversely across the outer segment.

Microspectrophotometry and Template Fits

Control and deficient tadpoles were transported to Cornell University in the dark-adapted state and examined immediately on arrival. All procedures were performed under dim far-red light or under infrared illumination, with the use of image converters. After the tadpoles were euthanatized in MS-222, the eyes were removed and hemisected, and the retina was floated out of the eyecup under PBS (pH 7.4) with 6% sucrose used to increase the osmolarity. Pieces of retina were chopped on a coverslip and then sandwiched with a second coverslip edged
with silicone grease. The computer-controlled, single-beam microspectrophotometer (MSP) used in this study has been described.2,4 A 2 × 3-μm measuring beam was produced by demagnification with a condenser (Ultrafluor; Carl Zeiss Meditec, Inc. Dublin, CA) set to a numerical aperture of 0.4. A 100× condenser (0.85 NA) collected the transmitted light and focused it onto the photomultiplier photocathode. Isolated rod outer segments were identified and selected for examination based on appearance. That is, the edges had to be straight and sharp as the image was moved through focus, and there could be no obvious vesiculation or disc disruption. Baseline and sample spectra were obtained at 100 nm/s from 750 to 350 nm, and back from 350 to 750 nm, with a wavelength accuracy of ± 1 nm.2,4 Calipers were used to obtain the diameter of each measured rod from the IR video image. Absorbance spectra were analyzed, and \( k_{\text{max}} \) determined, according to the methods of Mansfield25 and MacNichol.26 The porphyropsin data of Bridges27 were used for template fitting to the data. The best-fit template curve was scaled to the original absorbance data and fit by linear regression was performed on the 51 data points from 699 to 750 nm with the central data point of the regression assumed to be 0 absorbance. This value was subtracted from the absorbance of the template curve to obtain the outer segment absorbance. We calculated the specific optical density by dividing this absorbance value by the measured diameter of the outer segment.

**Immunohistochemistry**

Light-adapted animals were euthanatized by immersion in 0.2% tricane solution and their eyes removed, fixed, embedded, and sectioned with a cryostat at 12-μm thickness. To identify the rods and cones, the cones were immunolabeled with rabbit polyclonal antibody against calbindin at a 1:500 dilution, with goat anti-rabbit Alexa 594 used as a secondary antibody. The rods were subsequently immunolabeled with mouse anti-rhodopsin at 1:5000,28 with goat anti-mouse Cy2 used as a second-antibody. The rods were subsequently immunolabeled with rabbit polyclonal antibody against calbindin at a 1:500 dilution, with goat anti-rabbit Alexa 594 used as a secondary antibody. Samples were counterstained with DAPI (Molecular Probes, Eugene, OR) to label nuclei according to the manufacturer’s instructions.

**Analytical Model**

The phototransduction pathway can be modeled as a cascade of three first-order integrating stages.29 In the frequency domain, the energy-density spectrum, normalized relative to the saturating currents, is:20

\[
|R(f)|^2 = A^2 \left( \frac{1}{(2 \pi f)^2 + k_1^2} \right) \left( \frac{1}{(2 \pi f)^2 + k_2^2} \right) \left( \frac{1}{(2 \pi f)^2 + \beta_o^2} \right)
\]

where \( A \) is amplification, \( k_1 \) and \( k_2 \) are the rate constants of inactivation of rhodopsin and phosphodiesterase (PDE) respectively, and \( \beta_o \) is the phosphodiesterase activity rate in the dark. Note that the single-photon response is a transient signal with finite energy. Thus, equation 2 refers to the energy and not to the power density spectrum, as we stated in a previous report.29 The unit of the energy-density spectrum is joules per hertz (J/Hz) or picoamps squared per second divided by hertz (pA²/s/Hz; for a 1-Ω resistor). For normalized signals the picoamps squared (pA²) term cancels out and the unit becomes seconds per hertz (s/Hz).

When the calcium-dependent feedback loop operating by way of G C is considered,20 the normalized expression of the energy spectrum is:

\[
|R(f)|^2 = A^2 \left( \frac{(2 \pi f)^2 + \beta_o^2}{(2 \pi f)^2 + k_1^2} \right) \left( \frac{(2 \pi f)^2 + \beta_o^2}{(2 \pi f)^2 + k_2^2} \right) \left( \frac{(2 \pi f)^2 + \beta_o^2}{(2 \pi f)^2 + (\beta_o + \beta_e)^2} \right)
\]

where \( H_1 \) and \( \beta_e \) are the gain and rate constants of the feedback stage, respectively. In the presence of constitutive activity, \( \beta_e \) rises to levels such that in the frequency range of interest \( 2\pi f \ll \beta_e \), and if the relations \( \beta_o \gg \beta_e \) and \( \beta_o \beta_e \gg H_1 \) hold, then equation 3 simplifies to:

\[
|R(f)|^2 = A^2 \left( \frac{1}{(2 \pi f)^2 + k_1^2} \right) \left( \frac{1}{(2 \pi f)^2 + \beta_o^2} \right)
\]

As the value of \( \beta_o \) increases (beyond the upper frequencies of the measurable power spectrum), integration by the third filter in equation 2 becomes negligible and the energy spectrum is described by a second-order system (equation 4). The magnitude of the spectrum is scaled by the factor \( \beta_o^{-2} \) and the high-frequency asymptote is rather shallow, decreasing at a rate of 4 log units/decade (compared with slope values of 6 log units/decade characteristic of third order systems). We used equation 4 to fit the data of the vitamin A–deprived rods.

**Results**

**Outer Segment Size of Vitamin A–Deprived Rods**

Rod-like rods of the vitamin A–deprived *Xenopus* developed normally during the early stages, probably due to a maternal supply of retinoids.3 However, their outer segments were only 60% to 70% the length and diameter of the rods in age-matched control animals (Fig. 1). Despite having shorter outer segments, the inner retina appeared to be of normal thickness. Terminal uridine nick-end labeling (TUNEL) was negative in these retinas (data not shown), and the number of nuclei in the outer layer appeared normal. These results indicate that the rate of rod outer segment growth was slower than normal although the retinas did not show obvious signs of degeneration.

**Pigment Density Levels of Vitamin A–Deprived Rods**

We used microspectrophotometry to determine the absorbance of single rods (Fig. 1C). The maximum absorbance of the control rods was \( 87 \pm 13 \times 10^{-3} \) at \( k_{\text{max}} \) of 519 ± 0.3 nm (\( n = 14 \)). The absorbance of the rods in the vitamin A–deprived tadpoles was approximately 10-fold lower than that of the control, \( 8 \pm 2 \times 10^{-3} \), at virtually the same \( k_{\text{max}} \) of 520 ± 0.6 nm (\( n = 9 \)). Correspondingly, the specific optical density in the control rods was \( 10 \pm 0.5 \times 10^{-3} \) μm⁻¹ (similar to values reported previously by Liebman30 versus 0.9 ± 0.09 \times 10^{-3} \) μm⁻¹ in the vitamin A-deficient rods. We were unable to measure the specific optical density of the rods after incubation in 11-cis retinal, perhaps as a consequence of masking by the large amounts of chromophore that accumulate in the rod membranes.

The smaller physical dimensions of the rod outer segments combined with their lower absorbance diminished the capacity of the vitamin A–deprived rods to absorb incident light. We estimated the collecting area of the vitamin A–deprived and control rods by using equation 1 (the Methods section), and the physical dimensions listed in Table 1. For nonpolarized incident light normal to the axis of the outer segment (\( f = 0.5 \) in equation 1), the collecting area of the vitamin A–deprived rods was \( 0.38 \pm 0.02 \) μm² (\( n = 12 \)). This area is approximately 14-fold smaller than that estimated for control stage 48 to 51 tadpoles (5.3 ± 0.14 μm²; \( n = 14 \)) and 40-fold smaller than the collecting areas of rods in postmetamorphic juvenile frogs (15.3 ± 0.42 μm²; \( n = 15 \)). The collecting areas of the control rods estimated with equation 1 were in close agreement with those estimated independently by using ensemble variance analysis20 (Table 2).
**Table 1. Physical and Electrical Parameters of Rod Outer Segments**

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Vitamin A–Deprived</th>
<th>Reconstituted Pigment</th>
<th>Tadpole Control</th>
<th>Juvenile Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mo)</td>
<td>4–6</td>
<td>4–6</td>
<td>1–2</td>
<td>6–9</td>
</tr>
<tr>
<td>Length (µm)</td>
<td>35.0 ± 1.5</td>
<td>32.0 ± 1.3</td>
<td>23.7 ± 0.6</td>
<td>68.4 ± 2.2</td>
</tr>
<tr>
<td>Diameter (µm)</td>
<td>4.5</td>
<td>4.5</td>
<td>6.5</td>
<td>6.5</td>
</tr>
<tr>
<td>Envelope surface (µm²)</td>
<td>494 ± 21</td>
<td>459 ± 19</td>
<td>480 ± 13</td>
<td>1400 ± 45</td>
</tr>
<tr>
<td>Saturating current (pA)</td>
<td>6.6 ± 0.5</td>
<td>11.7 ± 0.8</td>
<td>7.3 ± 0.5</td>
<td>25.4 ± 2.4</td>
</tr>
<tr>
<td>Current density (10⁻⁷ pA/µm²)</td>
<td>13.5 ± 1.0</td>
<td>26 ± 2</td>
<td>15 ± 1</td>
<td>18 ± 2</td>
</tr>
</tbody>
</table>

Parameters in vitamin A–deprived and reconstituted rods were compared to the parameters of rods in control stage 48–51 tadpoles and juvenile frogs. Rod outer segment length was measured from the video monitor with a calibrated scale. The diameter is the aperture of the suction pipettes used for the recordings. These measures were used to estimate envelope surface area. Control tadpole and juvenile frog data are from Solessio et al. Data are the mean values ± SEM; vitamin A–deprived, n = 12; reconstituted, n = 8; tadpole, n = 14; juvenile, n = 12.

**Constitutive Activation of the Phototransduction Pathway by Free Opsin**

Figures 2A and 2B show typical photoresponses of the vitamin A–deprived rods before and after regeneration of the photopigment with 11-cis retinal. Consistent with bleaching adaptation, the responses of the vitamin A–deprived rods were considerably less sensitive and had faster recovery times than those of the rods treated with 11-cis retinal. After pigment regeneration, the intensity–response curves shifted 100-fold to the left along the intensity axis (Fig. 2C), consistent with regeneration of functional rhodopsin by 11-cis retinal. The enhanced sensitivity of the treated rods matched that of the control rods (Table 2).

We examined the dim-light responses of the vitamin A–deprived rods and found no variability in the responses from flash to flash (Fig. 3A) consistent with constitutive activation and desensitization of the transduction pathway by free opsin. The low variability indicates that the amplitude of the response to a single photoisomerization is very small and that multiple photoisomerizations per flash are necessary to elicit a measurable response (described later). After pigment regeneration the rods exhibited a large degree of flash-to-flash variability in their dim-light responses (Fig. 3B) similar to those observed in normal rods and consistent with a full complement of rhodopsin. In this case a single photoisomerization was sufficient to elicit a measurable response of approximately 0.6 pA in amplitude. The variability in the response to repeated trials reflects the random nature of photon absorption and rhodopsin activation. In 2 of 11 retinas incubated with 11-cis retinal, the rods showed little sign of recovery. The intensity–response curves shifted 10-fold to a lesser degree, and their dim responses did not exhibit variability (not shown), suggesting that only a fraction of their opsin content was regenerated by the exogenous application of retinal. These rods (n = 9) were excluded from the analysis.

**Table 2. Response Properties of Rods**

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Vitamin A–Deprived</th>
<th>Reconstituted Pigment</th>
<th>Tadpole Control</th>
<th>Juvenile Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collecting area, µm² (by equation 1)</td>
<td>0.38 ± 0.02</td>
<td>3.5 ± 0.2</td>
<td>5.3 ± 0.2</td>
<td>15.3 ± 0.5</td>
</tr>
<tr>
<td>Collecting area, µm² (from ensemble variance analysis)</td>
<td>0.035 ± 0.005</td>
<td>0.68 ± 0.07</td>
<td>0.6 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Sensitivity (pA/R*)</td>
<td>0.5 ± 0.02</td>
<td>2.0 ± 0.5</td>
<td>2.0 ± 0.12</td>
<td>1.3 ± 0.06</td>
</tr>
<tr>
<td>Time to peak (s)</td>
<td>1.0</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Dominant time constant (s)</td>
<td>14.8 ± 0.75</td>
<td>0.5 ± 0.1</td>
<td>1.1 ± 0.14</td>
<td>0.6 ± 0.05</td>
</tr>
<tr>
<td>Envelope surface (µm²)</td>
<td>24.0 ± 2.3</td>
<td>11.0 ± 1.5</td>
<td>7.3 ± 0.5</td>
<td>9.0 ± 0.6</td>
</tr>
</tbody>
</table>

Control tadpole and juvenile frog data are from Solessio et al. Data are the mean ± SEM; vitamin A–deprived, n = 12; reconstituted, n = 8; tadpole, n = 14; juvenile, n = 12.

Based on the variability of the dim responses, we estimated the collecting areas of the regenerated vitamin A–deprived rods by applying ensemble variance analysis (see the Methods section). Average collecting areas measured with this approach were 5.0 ± 1.3 µm² (n = 10), which represents a 10-fold increase over the collecting areas of the untreated vitamin A–deprived rods (estimated with equation 1) and is similar to the collecting areas of control stage 48 to 51 tadpole rods. Thus, the density of opsin in rods of the vitamin A–deprived animals did not vary significantly from that in control rods, in agreement with Engbretson and Witkovsky, but only ~10% of the opsin bound the chromophore 11-cis retinal.

**Photoresponses to Dim Flashes**

We inferred the time course of the ensemble single-photon responses by scaling the rod responses to dim flashes by the average number of photoisomerizations elicited by the flash (Fig. 3C). Times to peak and recovery to baseline were shorter in the vitamin A–deprived rods than in the control rods (Table 2). After regeneration with 11-cis retinal, the responses of the vitamin A–deprived rods recovered in amplitude and were almost indistinguishable from those of the control tadpole rods. The estimated amplitudes of the average single-photon responses were 0.035 ± 0.005 pA/R* (n = 8) and 0.68 ± 0.07 pA/R* (n = 10) for the vitamin A–deprived and fully reconstituted rods.
ated rods, respectively. The desensitization and faster kinetics are consistent with constitutive activity in the vitamin A–deprived rods.

Saturating Currents of Vitamin A–Deprived Rods

We compared the saturating currents that we took to represent the circulating currents in the dark. Because the control and vitamin A–deficient rods had different physical dimensions (Table 1) we computed the saturating current densities (ratio of the saturating currents to their respective rod envelope surfaces). Figure 4 shows that the current densities of control stage 48, 56, and postmetamorphic *Xenopus* rods increased slightly as a function of envelope surface area (adapted from Ref. 20). The plot shows that the vitamin A–deprived rods and control (stage 48) rods had similar surface areas. Surprisingly, the vitamin A–deprived rods and control (stage 48) rods also shared similar saturating current densities. This is an unexpected finding because constitutive activity by free opsin reduces the circulating currents. After pigment regeneration with 11-cis retinal the saturating currents increased almost twofold (Table 1; Fig. 4) consistent with silencing of constitutive activity. Thus, we conclude that vitamin A–deprived rods are constitutively active, but their circulating current levels are normal.

Basal GC Activity Levels in Vitamin A–Deprived Rods

We measured the activity levels of GC by using the IBMX jump approach (Fig. 5A). Relative GC activity levels were estimated from the rate of change in the rising phase of the responses to short pulse applications of 0.5 mM IBMX (Fig. 5B). Basal relative GC levels in the vitamin A–deprived rods (1.1 second; Table 2) were 15- to 20-fold greater than the basal relative activity levels in tadpole (1.1 second) and juvenile frog rods (0.6 seconds; Table 2). To determine maximum GC activity, we stimulated the rods with saturating flashes eliciting ~6000 photoisomerizations and measured GC activity at fixed intervals after the flash (Fig. 5C). Differences in the build-up of GC activity in the control and vitamin A–de-
prived rods are probably an artifact arising from the failure of IBMX to block the high PDE activity induced by bright flashes.31 However, as described previously,20,31 GC activity levels grew progressively, reaching a maximum at the onset of response recovery, when the response saturation ends. Recovery from saturation in the control froglet and vitamin A–deprived rods occurred rapidly, within 5 to 6 seconds, whereas saturation in the control tadpole and regenerated rods was extended, with recovery occurring after 10 to 12 seconds.

In response to the saturating flashes, relative GC activity in the vitamin A–deprived rods increased from 14.8 to 24 seconds$^{-1}$. The maximum GC activity in the control tadpole and juvenile rods was 7.3 and 9.0 seconds$^{-1}$, which was considerably below the basal activity level in the vitamin A–deprived rods (Fig. 5D). Note that the maximum GC relative activity levels of the complete tadpole and frog rods used in these experiments were two times higher than the activity of the partial rods reported previously (see the Methods section). After full regeneration of the vitamin A–deprived rods with 11-cis retinal, the basal GC activity level decreased to 0.5 seconds$^{-1}$, matching the levels observed in the control rods. Maximum activity also returned to normal levels, in line with the silencing of constitutive activity (Figs. 5C, 5D). These results suggest that extraordinarily high basal GC activity levels sustain the normal circulating currents of vitamin A–deprived rods.

Amplification and Dominant Time Constant in Vitamin A–Deprived Rods

We measured the amplification of the vitamin A–deprived rods by fitting the rising phase of the normalized single-photon responses with the Pugh and Lamb model.32 Figure 6A shows the population responses of the vitamin A–deprived (black trace, right axis, $n = 7$), regenerated (gray trace, left axis, $n = 10$), and stage 48 control tadpole rods.20 Traces are the dim responses scaled by the estimated number of photoisomerizations elicited per flash ($R^*$).
activation does not alter the gain of the forward phototransduction cascade.

Figure 6B shows the time to 50% recovery plotted as a function of flash intensity for the vitamin A-deprived, regenerated, and control stage 48 tadpole rods. For identical flash intensities, the recovery times of the vitamin A-deprived rods were 10 to 15 seconds faster than those of the control tadpole and the regenerated vitamin A-deprived rods. Data were fit by the same piece-wise linear function, shifted along the temporal and intensity axes. For recovery times >10 seconds the curves have a limiting slope of 10 seconds/decade. The dominant time constant inferred from the shallow slopes of the curves is approximately 2.8 seconds and in close agreement with the time constants of the control stage 48 and juvenile rods (Table 2). The dominant time constant is a measure of the slowest step in the inactivation of the transduction cascade and is attributed to the rate of inactivation of transducin activity.

Effect of Calcium Feedback on the Dim Responses of Vitamin A-Deprived Rods

We incubated the rods in 1 \( \mu \text{M} \) BAPTA-AM (Sigma-Aldrich) to determine the role of calcium feedback loops in shaping the dim responses. BAPTA increases the buffering capacity of the cells, slowing down changes in intracellular calcium. Figure 6C shows that BAPTA had no effect on the light responses of the vitamin A-deprived rods. This result suggests that calcium feedback does not play a major role in shaping the dim-light responses of vitamin A-deprived rods.

Is increased basal PDE activity the basis for the faster responses, sensitivity loss, and lack of calcium feedback effects in the dim responses of vitamin A-deprived rods? To answer this question, we analyzed the responses to dim flashes in the frequency domain (Fig. 6D). Simple analysis of a linear model (equations 2 and 3) suggests that the effects of calcium feedback becomes less relevant as the value of \( \beta_o \) increases (equation 4, see the Methods section). We fit the energy-density spectra with a model system consisting of two first-order integrating reactions (equation 4). We assumed that amplification \( A \) and the dominant time constant \( T_e = K_\text{E}^{-1} \) does not change significantly in the control and vitamin A-deprived rods (Table 2). The same assumption extends to the time for rhodopsin inactivation \( T_R = K_\text{R}^{-1} \). We also assumed that, in steady state conditions, the PDE activity in the dark \( \beta_o \) is equal to the relative basal GC activity \( \alpha_v \).

Therefore, we assigned \( \beta_o \) the values 1.1 and 15 seconds \(^{-1} \) in the control and vitamin A-deprived rods, respectively (Table 2).

Remarkably, the model (equation 4) captures the essential features in the responses of the vitamin A-deprived rods. Raising the basal PDE activity to 15 seconds \(^{-1} \) in the model equation is sufficient to accurately predict the loss in amplitude, the increase in bandwidth, and the reduction in the slope of the high-frequency asymptote.

**Discussion**

**Preservation of Circulating Current Density in Vitamin A-Deprived Rods**

In line with the notion of constitutive activation by free opsin, desensitization of the phototransduction cascade in the vitamin A-deprived rods was accompanied by a speeding up of the response kinetics and an increase in the rate of basal GC activity. At apparent odds with this notion, the circulating current densities were not reduced by the constitutive activation of PDE. A simple interpretation of these results is that homeostatic mechanisms maintain the circulating currents at normal levels in chronically desensitized rods. Overexpression of the cGMP-gated channels in vitamin A-deprived rods may restore circulating current levels but will also lead to larger responses to single photons after pigment regeneration (Fig. 3), which is contrary to our findings. Another alternative is that the rods develop uncommonly high rates of cGMP synthesis (15-fold increase in basal rate) to balance the constitutive activation of the cGMP PDE (Fig. 5). GC is part of the calcium-sensitive feedback loop that regulates circulating currents and response kinetics. Diminished intracellular calcium concentrations by bleaching flashes or other forms of constitutive activation lead to an acceleration in GC rates. Lower calcium combined with an overexpression of GC or GCAPs enhances GC activity. However, the normal circulating current densities as well as the changes in GC activity elicited by saturating flashes (Fig. 5) do not support the idea of low, standing calcium levels in vitamin A-deprived *Xenopus* rods. Indeed, the maintenance of normal currents and intracellular calcium levels may be the key to rod survival.

Although saturating light flashes elicited changes in GC activity (Fig. 5), our experiments with BAPTA-AM suggest that responses to dim flashes are not shaped by calcium-induced changes in GC activity (Fig. 6). An explanation of this apparent discrepancy involves the impact that large values of \( \beta_o \), the constitutive PDE activity, have on sensitivity and recovery kinetics of the dim responses.

We can assume that the recovery phase of the dim responses is shaped both by calcium feedback effects and by the filtering effect of \( \beta_o \). As the value of \( \beta_o \) increases, the recovery is expected to speed up, leading to shorter responses that end before the (relatively slow) calcium feedback in *Xenopus* rods begins. This notion is supported by a linear model describing the energy density spectra of the dim response. Indeed, as \( \beta_o \) increased, the effects of calcium feedback diminished (Fig. 6). More experiments are needed to understand the exact relation between circulating currents, calcium levels, feedback, and the balance between basal GC and PDE activities.
The High Metabolic Cost of Constitutive Activity in Vitamin A–Deprived Rods

Vertebrate photoreceptors support two very demanding metabolic processes: phototransduction and maintenance of a circulating current. The turnover of cGMP during photoexcitation requires contributions of ATP from the inner segment, and maintaining the circulating currents accounts for 40% of total energy consumption. Fortunately for photoreceptors, the two processes do not overlap in time, allowing for a shunting of the energy resources to the process requiring them at a particular time. Our results suggest a very different shunting of the energy resources to the process requiring them if the two processes do not overlap in time, allowing for a maintaining normal circulating currents.

We can estimate the metabolic requirements of these rods by assuming that, in steady state conditions, the relative GC and PDE activity rates are equal, \( \alpha_{\text{dark}} = \beta_{\alpha} \), and that the turnover of cGMP is given by \( [\text{cGMP}]_{\text{free}} \cdot \beta_{\alpha} \). Given that the circulating currents were the same in the control and vitamin A–deprived rods, and that these circulating currents were largely determined by \( [\text{cGMP}]_{\text{free}} \), we assume that \( [\text{cGMP}]_{\text{free}} = 3 \mu M \) in both conditions. Based on our measurements of relative GC activity (Table 2), we estimate that the rate of ATP hydrolysis necessary to support the basal (dark) phototransduction cycle in control rods is \( \alpha'_{\text{dark}} \cdot [\text{cGMP}]_{\text{free}} = 0.18 \) mM/min where \( [\text{cGMP}]_{\text{free}} = 3 \mu M \) and \( \alpha' = 1 \text{ second}^{-1} \). Given that the stoichiometry of the cGMP cycle is 2 ATP per cGMP, then the rate of ATP consumption is \( 2\alpha' \cdot 0.36 \mu M/\mu M \) or \( 1.4 \times 10^9 \) ATP molecules assuming a rod outer segment cytosolic volume of 0.4 pL. In vitamin A–deprived rods, this basal rate increases by a factor of 15, to \( 5.4 \mu M/\mu M \) of ATP hydrolysis or \( 21.6 \times 10^9 \) ATP molecules/second.

These figures can be put in perspective by estimating the metabolic demands necessary to maintain circulating currents on the order of 10 pA in rods with ROS volumes of approximately 0.4 pL. The demands of ATP by the Na⁺-K-ATPase in the dark were estimated by assuming that (1) the inflow of Na⁺ = outflow of Na⁺ = \( I_{\text{dark}} \) (circulating current), and (2) the outflow of Na⁺ is mediated almost exclusively by the Na⁺-K-ATPase. Given that 1 ATP:3 Na⁺:2 K⁺ is the stoichiometry of the Na⁺-K-ATPase, then \( R \), the rate of ATP consumption by the ATPase is:
The intensity was 54 photons/rod. Amplification and circulating currents in vitamin A–deprived rods. (A) Population average time course of single-photon responses of vitamin A–deprived (n = 7) and regenerated (gray trace, n = 8) rods normalized by the respective saturating currents. The rising phase of the responses was fit with the Pugh and Lamb model. Amplification constant, 0.1 second^-1. The estimated rate of ATP hydrolysis by the ATPase (5.2 mM/min or 20 × 10^6 ATP/s) is comparable to that required to maintain the circulating currents, a small amount that is unlikely to stress the rods. Hence, it appears that mammalian rods that degenerate with constitutive activity are better prepared to manage the metabolic cost of constitutive activation than are Xenopus rods that do not degenerate under similar conditions. This notion suggests that the different capacities of these rods to recover their circulating currents probably arise from differences in the mechanisms governing the flux of cGMP. More studies are needed to decipher these mechanisms in mammalian and Xenopus rods.

\[ R = \frac{I_{\text{dark}}}{5FV_{\text{cyt}}} = 8.6 \cdot 10^{-10} \text{ moles/Lsec} = 5.2 \text{ mM/min} \]

where \( I_{\text{dark}} = 10 \text{ pA} \) is the circulating current in the dark, \( F \) is Faraday’s constant, and \( V_{\text{cyt}} = 0.4 \text{ pL} \) is the cytosolic volume of the rod outer segment. We conclude that the estimated rate of ATP hydrolysis by the ATPase (5.2 mM/min or 20 × 10^6 ATP/s) is comparable to that required to maintain constitutive GC activity (5.4 mM/min or 21 × 10^6 ATP/s). With the assumption that maintenance of the circulating currents accounts for 40% of total energy consumption, the simultaneous metabolic demands of both constitutive activity and circulating currents in vitamin A–deprived rods of Xenopus will combine to consume at least ~80% or more of the ATP produced by normal rods. How Xenopus rods can sustain the metabolic demands of both phototransduction and membrane potential while continuing to service other housekeeping needs is not at all clear. In mammalian rods, the allocation of energy resources allows large increments in cGMP synthesis without compromising the metabolic status of the cell. For example, in mouse rods the energy expenditure associated with the maintenance of the circulating currents is 57 × 10^6 ATP/s, whereas the ATP expenditure for cGMP synthesis (in the dark) is close to 0.1 × 10^6 ATP/s. Assuming a 15-fold increase in cGMP synthesis (in analogy to Xenopus rods), the energetic demands would rise to 1.5 × 10^6 ATP/sec. Such an increment represents ~< 2.5% of the energy required for maintaining the circulating currents, a small amount that is unlikely to stress the rods. Hence, it appears that mammalian rods that degenerate with constitutive activity are better prepared to manage the metabolic cost of constitutive activation than are Xenopus rods that do not degenerate under similar conditions. This notion suggests that the different capacities of these rods to recover their circulating currents probably arise from differences in the mechanisms governing the flux of cGMP. More studies are needed to decipher these mechanisms in mammalian and Xenopus rods.
References
