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Light Responses in Rods of Vitamin A–Deprived *Xenopus*

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Abstract

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.

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Vitamin A is the precursor to 11-*cis* retinal, the chromophore that binds to opsin to produce rhodopsin, the light-absorbing pigment.¹ 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.^{2,3} Long-term vitamin A deprivation causes retinal degeneration in mammals^{4,5} but not in *Xenopus*.^{2,6}

Mutations that produce constitutive activation of phototransduction cause retinal degeneration in mammals.⁷ The point mutation G90D in rhodopsin is associated with congenital stationary night blindness.⁸⁻¹⁰ Mutations in *RPE65*, the gene that codes for retinoid isomerase in the pigment epithelium,^{11,12} cause Leber congenital amaurosis.¹³ Constitutive activation of phototransduction by bleaching adaptation¹⁴ or caused by mutations in rhodopsin^{9,15,16} 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.^{17,18} 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.¹⁹ 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.

Materials and Methods

Husbandry

Xenopus laevis tadpoles were generated by in vitro fertilization and reared as described previously.²⁰ 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.²¹ 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.² Vitamin A–deprived animals grow to be oversized animals with features typical of stage 54 to 56 tadpoles. All experiments were conducted in vitamin A–deprived animals 4 to 6 months of age and 3 to 4 cm in body length. Control recordings were made from the rods of stage 48 to 51 tadpoles raised on a normal diet. These rods have similar outer segment surface area as the vitamin A–deprived rods used in the study.

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-ms (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 μ M 11-*cis* retinal solution in *Xenopus* Ringer's. The retinal solution was prepared as a 0.1% dilution of a stock solution of 11-*cis* retinal in ethanol.²³

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

Collecting areas were estimated by using the expression²²:

$$CoA = \frac{\pi d^2 l}{4} Q_{\text{isom}} f 2.3 \alpha \quad (1)$$

where CoA is the collecting area, d and l are the diameter and length of the ROS, respectively, Q_{isom} 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.²⁴ A $2 \times 3\text{-}\mu\text{m}$ 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 $100\times$ 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.²⁴ Calipers were used to obtain the diameter of each measured rod from the IR video image. Absorbance spectra were analyzed, and λ_{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 eye to fall in the middle of the record noise band. 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% tricaine solution and their eyes removed, fixed, embedded, and sectioned with a cryostat at $12\text{-}\mu\text{m}$ 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 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, is²⁰:

$$|R(f)|^2 = A^2 \left(\frac{1}{[(2\pi f)^2 + K_R^2][(2\pi f)^2 + K_E^2][(2\pi f)^2 + \beta_0^2]} \right) \quad (2)$$

where A is amplification, K_R and K_E are the rate constants of inactivation of rhodopsin and phosphodiesterase (PDE) respectively, and β_0 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.²⁰ The unit of the energy-density spectrum is joules per hertz (J/Hz) or picoamps squared per second divided by hertz ($\text{pA}^2 \text{ s/Hz}$; for a $1\text{-}\Omega$ resistor). For normalized signals the picoamps squared (pA^2) term cancels out and the unit becomes seconds per hertz (s/Hz).

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

$$|R(f)|^2 = A^2 \left(\frac{(2\pi f)^2 + \beta_i^2}{[(2\pi f)^2 + K_R^2][(2\pi f)^2 + K_E^2] \left\{ [H_i + \beta_o \beta_f - (2\pi f)^2]^2 + (\beta_o + \beta_i)^2 (2\pi f)^2 \right\}} \right) \quad (3)$$

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

$$|R(f)|^2 = A^2 \left(\frac{1/\beta_o^2}{[(2\pi f)^2 + K_R^2][(2\pi f)^2 + K_E^2]} \right) \quad (4)$$

As the value of β_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 β_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

Rods of the vitamin A-deprived *Xenopus* developed normally during the early stages, probably due to a maternal supply of retinoids.⁶ 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 a $\lambda_{\max}$ of 519 ± 0.3 nm ($n = 14$). The absorbance of 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 $\lambda_{\max}$ of 520 ± 0.6 nm ($n = 9$). Correspondingly, the specific optical density in the control rods was $10 \pm 0.5 \times 10^{-3} \mu\text{m}^{-1}$ (similar to values reported previously by Liebman³⁰) versus $0.9 \pm 0.09 \times 10^{-3} \mu\text{m}^{-1}$ 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 $0.38 \pm 0.02 \mu\text{m}^2$ ($n = 12$). This area is approximately 14-fold smaller than that estimated for control stage 48 to 51 tadpoles ($5.3 \pm 0.14 \mu\text{m}^2$; $n = 14$) and 40-fold smaller than the collecting areas of rods in postmetamorphic juvenile frogs ($15.3 \pm 0.42 \mu\text{m}^2$; $n = 15$). 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).

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 rods²⁰ 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 leftward 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.

Based on the variability of the dim responses, we estimated the collecting areas of the regenerated vitamin A–deprived rods by applying ensemble variance analysis²² (see the Methods section). Average collecting areas measured with this approach were $5.0 \pm 1.3 \mu\text{m}^2$ ($n = 10$), which represents a 13-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 Engbreton and Witkovsky,⁶ but only ~10% of the opsin bound the chromophore 11-*cis* retinal.

Figure 2D plots the intensity–response curve as a function of photoisomerizations per flash, where the number of photoisomerizations (R^*) is the product of the flash intensity by the collecting area (Fig. 2). The intensity–response curves of the vitamin A–deprived rods were shifted ~1 log to the right relative to the responses from the control and regenerated rods. Phototransduction in the vitamin A–deprived rods was 10 times less sensitive than that of the control tadpole rods, consistent with desensitization by constitutive activation.

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 $0.035 \pm 0.005 \text{ pA}/R^*$ ($n = 8$) and $0.68 \pm 0.07 \text{ pA}/R^*$ ($n = 10$) for the vitamin A–deprived and fully regenerated 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.¹⁴ 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 approach³¹ (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 ($\alpha' = 14.8 \text{ seconds}^{-1}$) were 15- to 20-fold greater than the basal relative activity levels in tadpole ($\alpha' = 1.1 \text{ second}^{-1}$) and juvenile frog rods ($\alpha' = 0.6 \text{ seconds}^{-1}$; 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–deprived 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 and regenerated rods (from Fig. 3C) normalized relative to their respective saturating currents. The early rising phase of their responses overlapped closely, suggesting that amplification did not change in the vitamin A–deprived and regenerated rods. The amplification estimated with the Pugh and Lamb model (0.1 second^{-2}) was similar to the amplification of the nondeprived rods (Table 2), suggesting that chronic constitutive 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 piecewise 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^{20,29} 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 cascade³³ and is attributed to the rate of inactivation of transducin activity.^{29,34}

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

We incubated the rods in 1 μ M BAPTA-AM (Sigma-Aldrich) to determine the role of calcium feedback loops in shaping the dim responses.^{20,35} 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 activity³⁶ 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 β_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 ($T_E = K_E^{-1}$) does not change significantly in the control and vitamin A–deprived rods (Table 2). The same assumption extends to the time for rhodopsin inactivation ($T_R = K_R^{-1}$). We also assumed that, in steady state conditions, the PDE activity in the dark (β_o) is equal to the relative basal GC activity (α_o').³¹ Therefore, we assigned β_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.^{14,37} 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 currents³⁸ and response kinetics.³⁹ Diminished intracellular calcium concentrations by

bleaching flashes⁴⁰ or other forms of constitutive activation^{18,41} lead to an acceleration in GC rates.¹⁴ Lower calcium combined with an overexpression of GC or GCAPs^{42,43} 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 β_o , the constitutive PDE activity, have on sensitivity and recovery kinetics of the dim responses.³⁶ We can assume that the recovery phase of the dim responses is shaped both by calcium feedback effects and by the filtering effect of β_o . As the value of β_o increases, the recovery is expected to speed up, leading to shorter responses³⁶ that end before the (relatively slow) calcium feedback in *Xenopus* rods begins.²⁰ This notion is supported by a linear model describing the energy density spectra of the dim response. Indeed, as β_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,⁴⁴ and maintaining the circulating currents accounts for 40% of total energy consumption.⁴⁵ 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.⁴⁶ Our results suggest a very different metabolic picture in vitamin A–deprived rods. In the dark, these rods supported a basal GC activity in the outer segment that was 15 times higher than normal while simultaneously 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, $\alpha'_{\text{dark}} = \beta_o$, and that the turnover of cGMP is given by $[\text{cGMP}]_{\text{free}} \cdot \beta_o$.³¹ 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 $[\text{cGMP}]_{\text{free}} = 3 \mu\text{M}$ ³² 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 $\alpha'_{\text{dark}} \cdot [\text{cGMP}]_{\text{free}} = 0.18 \text{ mM/min}$ where $[\text{cGMP}]_o = 3 \mu\text{M}$ and $\alpha' = 1 \text{ second}^{-1}$. Given that the stoichiometry of the cGMP cycle is 2 ATP – 1 cGMP, then the rate of ATP consumption is $2\alpha' = 0.36 \text{ mM/min}$ or $1.4 \times 10^6 \text{ ATP molecules}$ 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 mM/min of ATP hydrolysis or $21.6 \times 10^6 \text{ ATP molecules/sec}$.

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 $\text{Na}^+ \sim I_{\text{Dark}}$ (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:

$$R = \frac{I_{\text{dark}}}{3FV_{\text{cyt}}} = 8.6 \cdot 10^{-5} \frac{\text{moles}}{\text{Lsec}} = 5.2 \text{ mM/min}$$

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 $20 \times 10^6 \text{ ATP/s}$) is comparable to that required to maintain constitutive GC activity (5.4 mM/min or $21 \times 10^6 \text{ ATP/s}$). With the assumption that maintenance of the circulating currents accounts for 40% of total energy consumption,⁴⁵ 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 $57 \times 10^6 \text{ ATP/s}$, whereas the ATP expenditure for cGMP synthesis (in the dark) is close to $0.1 \times 10^6 \text{ ATP/s}$.⁴⁷ Assuming a 15-fold increase in cGMP synthesis (in analogy to *Xenopus* rods), the energetic demands would rise to $1.5 \times 10^6 \text{ ATP/sec}$. 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.

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Figure 1.

The vitamin A-deprived *Xenopus* rods. (A) Age-matched *Xenopus* (8 months) raised with normal diet (left) and with a vitamin A-deficient diet (right). (B) Retinal morphology in the vitamin A-deprived *Xenopus* (8 months). Cryostat sections of normal (left) and vitamin A-deprived (right) retinas. Rod outer segments were shorter and thinner in the vitamin A-deprived retinas. Green: rhodopsin (rods); red: calbindin (cones); blue: DAPI stain. PCL, photoreceptor cell layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar, 50 μ m. (C) Absorption spectra of a control and a vitamin A-deficient rod determined by microspectrophotometry. Data were fit with the template of a vitamin A2 rhodopsin² (solid lines). The animals were 6 months of age.

Figure 2.

Photoresponses of vitamin A-deprived rods. **(A)** Flash response-intensity family from a vitamin A-deprived rod. Flash intensities were 12.0, 54, 123, 514, 1742, 4446 photons/ μm^2 , respectively. Stimulus wavelength, 520 nm; flash duration, 20 ms. **(B)** Response-intensity family from a vitamin A-deprived rod after regeneration of the pigment with 11-*cis* retinal. Flash intensities were 0.32, 1.1, 4.8, 12.0, 54, and 123 photons/ μm^2 . **(C)** Average intensity-response relations for vitamin A-deprived rods ($n = 8$) and fully regenerated rods ($n = 10$) were compared with responses of nondeprived control tadpole rods (data from Ref. 20). Data were fit with Michaelis-Menten relations (*continuous line*): $R = R_{\text{max}} i / (i + i_0)$, where i_0 is 347 photons/ μm^2 for vitamin A-deprived rods, 2.9 photons/ μm^2 for reconstituted rods, and 2.7 for control (tadpole) rods. R_{max} is 7.3, 11.2 and 7.0 pA for vitamin A-deprived, regenerated, and control rods, respectively ($R^2 > 0.99$). **(D)** Intensity-response curves when the stimulus is expressed in terms of photoisomerizations per flash (R^*). The number of photoisomerizations was estimated by multiplying the flash intensity by the corresponding collecting area.

Collecting areas were estimated by applying equation 1 for the vitamin A-deprived rods and ensemble variance analysis for the control and regenerated rods (Table 2). Data were fit with Michaelis-Menten functions, where i_0 is 132 R^* for the vitamin A-deprived rods, 16.5 R^* for the reconstituted rods, and 10.5 R^* for the control (tadpole) rods. R_{max} is 7.3 and 7.0 pA for the vitamin A-deprived and control rods and 12.0 pA for the reconstituted rods ($R^2 > 0.99$).

Figure 3.

Responses to dim flashes. (A) *Top*: superimposed responses (16 gray traces) of a vitamin A-deprived rod to repeated applications of a dim flash (12 photons μm^{-2}). *Black trace*: the average; *bottom*: ensemble mean square (black trace) and negligible ensemble variance (gray trace) of the responses shown above. (B) *Top*: responses to dim flashes (0.3 photons μm^{-2}) after regeneration of the pigment with 11-cis retinal; *bottom*: ensemble mean square (and variance; gray trace) scaled so that the activation phases overlap closely. The scaling factor was used to estimate the number of photoisomerizations elicited per flash (R^*), as well as the average single-photon response amplitude and rod collecting areas. (C) Population average single-photon 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^*).

Figure 4.

Saturating current densities of vitamin A–deprived and control rods. Current densities estimated as the ratio of the saturating currents in the dark to the rod outer segment envelope surface (Table 1) plotted as a function of surface area. Current densities of control stage 48 ($n = 14$), stages 54 to 56 ($n = 9$), and juvenile ($n = 15$) rods increase linearly with surface area. Current densities of vitamin A–deprived ($n = 12$) rods match those of control rod outer segments with similar surface areas. Saturating current density of regenerated rods ($n = 8$) is significantly larger (ANOVA, $P < 0.001$). Control data adapted from Solessio et al.²⁰

Figure 5.

Relative GC activity levels in vitamin A-deprived rods. **(A)** Responses of the vitamin A-deprived rods to a brief (2 seconds) application of 0.5 mM IBMX before and after pigment regeneration with 11-*cis* retinal. The response of the vitamin A-deprived rod was faster and larger than the response of the rod with regenerated pigment, indicative of higher basal GC rates. Currents were normalized relative to their respective saturating currents (J/J_D). Recordings performed in the dark. **(B)** Derivatives of the square root of the responses in **(A)**. Relative GC was estimated from the maximum value of the derivative.²⁰ $J/J_D^{0.5}$ was fit with a sixth-order polynomial and the derivatives of the fitting functions estimated. Note the different amplitude and temporal scales in the axis of the graphs. **(C)** Relative GC activity of vitamin A-deprived ($n = 5$), regenerated ($n = 7$), tadpole control ($n = 8$), and juvenile control ($n = 5$) rods measured at fixed intervals during the response to saturating flashes eliciting 6000 R^* . **(D)** Basal and maximum relative GC levels measured in vitamin A-deprived, regenerated, and control rods. Basal GC activity in the vitamin A-deprived rods exceeded the maximum attainable levels in control rods. GC activity in the regenerated rods was normal.

Figure 6.

Amplification and dominant time constant 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.³² Amplification constant, 0.1 second^{-2} ; time delay, 30 ms. **(B)** Time to 50% recovery in response to saturating flashes. Recovery times of the vitamin A-deprived rods ($n = 8$) were faster than those of the regenerated ($n = 9$) and control tadpole rods. **(C)** Population average dim responses from vitamin A-deprived rods with (gray trace, $n = 7$) and without ($n = 8$) BAPTA-AM. The flash intensity was $54 \text{ photons}/\mu\text{m}^2$. **(D)** Energy-density spectra of the responses elicited by dim flashes in the vitamin A-deprived and control tadpole rods. Spectra of the normalized responses were estimated by using the Fast Fourier transform and averaged ($n = 7$). Data were fit with a simple model (equation 4). Both the vitamin A-deprived and control tadpole models shared the following parameter values: amplification $A = 0.10 \text{ seconds}^{-2}$; rate constant for inactivation of rhodopsin $K_R = 1/0.8 \text{ seconds}^{-1}$; and rate constant of inactivation of the PDE $K_E = 1/2.8 \text{ seconds}^{-1}$ (the value of the dominant time constant). The remaining parameter in the model is the PDE activity in the dark ($\beta_0 = \alpha'$), to which we assigned the measured values $\beta_0 = 15 \text{ seconds}^{-1}$ in vitamin A-deprived rods and $\beta_0 = 1.1 \text{ second}^{-1}$ in control tadpole rods.

Table 1**Physical and Electrical Parameters of Rod Outer Segments**

Quantity	Vitamin A–Deprived	Reconstituted Pigment	Tadpole Control	Juvenile Control
Age (mo)	4–6	4–6	1–2	6–9
Length (μm)	35.0 ± 1.5	32.0 ± 1.3	23.7 ± 0.6	68.4 ± 2.2
Diameter (μm)	4.5	4.5	6.5	6.5
Envelope surface (μm^2)	494 ± 21	459 ± 19	480 ± 13	1400 ± 45
Saturating current (pA)	6.6 ± 0.5	11.7 ± 0.8	7.3 ± 0.5	25.4 ± 2.4
Current density ($10^{-3} \text{ pA}/\mu\text{m}^2$)	13.5 ± 1	26 ± 2	15 ± 1	18 ± 2

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.²⁰ Data are the mean values $\pm$ SEM; vitamin A–deprived, $n = 12$; reconstituted, $n = 8$; tadpole, $n = 14$; juvenile, $n = 12$.

Table 2

Response Properties of Rods

Quantity	Vitamin A–Deprived	Reconstituted Pigment	Tadpole Control	Juvenile Control
Collecting area, μm^2 (by equation 1)	0.38 ± 0.02	3.5 ± 0.2	5.3 ± 0.2	15.3 ± 0.5
Collecting area, μm^2 (from ensemble variance analysis)		5.0 ± 1.3	3.8 ± 0.3	10.6 ± 0.9
Sensitivity (pA/R [*])	0.035 ± 0.005	0.68 ± 0.07	0.6 ± 0.1	0.6 ± 0.1
Time to peak (s)	0.5 ± 0.02	2.0 ± 0.3	2.0 ± 0.12	1.3 ± 0.06
Amplification (s ⁻²)	0.1	0.1	0.12	0.075
Dominant time constant (s)	3.0	2.8	2.5	2.5
$\alpha_o' = \beta_o(s^{-1})$	$14.8 \pm 0.75^*$	$0.5 \pm 0.1^*$	1.1 ± 0.14	0.6 ± 0.05
$\alpha_{\text{max}}' (s^{-1})$	$24.0 \pm 2.3^*$	$11.0 \pm 1.5^*$	7.3 ± 0.5	9.0 ± 0.6

Control tadpole and juvenile frog data are from Solessio et al.²⁰ Data are the mean $\pm$ SEM; vitamin A–deprived, $n = 12$; reconstituted, $n = 8$; tadpole, $n = 14$; juvenile, $n = 12$.

* $n = 5$.