Rod Sensitivity During *Xenopus* Development

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ABSTRACT We have measured the sensitivity of rod photoreceptors from overnight-dark–adapted *Xenopus laevis* through developmental stages 46–66 into adulthood by using suction-pipette recording. The dark current increased gradually from ~5 pA at stage 46 to ~20 pA at stage 57, compared with an adult (metamorphosed) current of ~35 pA. This increase in dark current largely paralleled the progressive increase in length and diameter of the rod outer segment (ROS). Throughout stages 46–66, the dark current increased approximately linearly with ROS surface area. At stage 53, there was a steep (~10-fold) increase in the rod flash sensitivity, accompanied by a steep increase in the time-to-peak of the half-saturated flash response. This covariance of sensitivity and time-to-peak suggested a change in the state of adaptation of rods at stage 53 and thereafter. When the isolated retina was preincubated with 11-cis-retinal, the flash sensitivity and the response time-to-peak of rods before stage 53 became similar to those at or after stage 53, suggesting that the presence of free opsin (i.e., visual pigment without chromophore) in rods before stage 53 was responsible for the adapted state (low sensitivity and short time-to-peak). By comparing the response sensitivity before stage 53 to the sensitivity at/after stage 53 measured from rods that had been subjected to various known bleaches, we estimated that 22–28% of rod opsin in stage 50–52 tadpoles (i.e., before stage 53) was devoid of chromophore despite overnight dark-adaptation. When continuously dark adapted for 7 d or longer, however, even tadpoles before stage 53 yielded rods with similar flash sensitivity and response time-to-peak as those of later-stage animals. In conclusion, it appears that chromophore regeneration is very slow in tadpoles before stage 53, but this regeneration becomes much more efficient at stage 53. A similar delay in the maturity of chromophore regeneration may partially underlie the low sensitivity of rods observed in newborn mammals, including human infants.

KEY WORDS: retinaldehyde • rat • vision

INTRODUCTION

Much is known about the development of retinal rods and cones in *Xenopus laevis*, a favorable animal for studying vertebrate development because its developmental stages have been well characterized (Nieuwkoop and Faber, 1956). During *Xenopus* development, the optic vesicle appears at Nieuwkoop-Faber stage 26 and the optic cup is formed at stages 27/28. At stages 37/38, rudimentary photoreceptor outer segments with membranous discs begin to appear (Witkovsky et al., 1976; Kinney and Fisher, 1978a). At stage 40, rods can be morphologically distinguished from cones and, at stages 44–46, the rods lose their oil droplets and their outer segments acquire the mature cylindrical form (Witkovsky et al., 1976; Kinney and Fisher, 1978a; Chang and Harris, 1998). During this period (stages 40–46), the incorporation of membrane into the outer segment and the increase in outer-segment volume both proceed at roughly uniform rates (Kinney and Fisher, 1978a; Hollyfield and Rayborn, 1979). At stages 53/54, the rod outer segment reaches adult length, but

at stages 57–66, which constitute the metamorphic climax (peaking at stage 62), there is, surprisingly, a reported shortening of the rod outer segment, attributed to an increase in disk shedding/phagocytosis rather than a decrease in membrane synthesis; after metamorphosis, the rod outer segment relengthens (Kinney and Fisher, 1978b).

Opsin, the protein moiety of visual pigment, is expressed in parallel to the development of the rod outer segment. Rod opsin mRNA can be detected at stages 32/34, after which the transcript level increases until stage 40, and remains steady thereafter at least through stage 46 (Stiemke et al., 1994). Rod opsin protein becomes observable at stages 37/38, in concert with the appearance of outer-segment disks; its level increases rapidly through stages 43/44, and continues to increase thereafter, albeit slowly, at least through stage 46 (Stiemke et al., 1994). By using the aspartate-isolated P_{III} component of the electroretinogram (ERG)* as an indicator, Witkovsky et al. (1976) have found that the rod photoresponse first appears at stages 37/39. Thereafter, the absolute threshold of the rod response decreases rapidly through stage 42, and then continues to decrease, though more slowly, through stage 59 (Wit-

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Abbreviation used in this paper: ERG, electroretinogram.

kovsky et al., 1976). This increase in rod photosensitivity was attributed purely to growth of the rod outer segment, because visual pigment concentration was found to remain constant based on extraction studies (Witkovsky et al., 1976). In rat, there was likewise a large increase in rod sensitivity during the first month of life, but this sensitivity change was attributed to a dramatic increase in the percentage of rod opsin with chromophore (Ratto et al., 1991). Considering the apparent difference in findings between the two species, we ask in this paper whether, as in rat, there are developmental changes in the rod sensitivity of *Xenopus* that reflect chromophore availability. We examined this question by carrying out recordings from single *Xenopus* rods at various stages of development.

Photoreceptors in adult *Xenopus* consist of "red" rods, "green" rods, and three classes of cones (Wilhelm and Gábriel, 1999; Röhlich and Szél, 2000; Witkovsky, 2000). Most photoreceptors are rods, of which the great majority (97–98%) are "red" rods (Röhlich and Szél, 2000). With dehydro-retinal (vitamin A_2) being the predominant, if not exclusive, chromophore in both tadpole and adult (Bridges et al., 1977; see also Palacios et al., 1998), the wavelength of maximal absorption (λ_{max}) for the "red" rod pigment is $\sim$523nm (Witkovsky et al., 1981; Witkovsky, 2000; see also Palacios et al., 1998). The "green" rods have outer segments narrower and shorter than "red" rod outer segments, and they express a pigment with λ_{max} at $\sim$445 nm (Witkovsky et al., 1981; Witkovsky, 2000). "Red" rods were used exclusively for this study. They were distinguished from "green" rods by their larger size, and confirmed by their high sensitivity to 520-nm light.

MATERIALS AND METHODS

Preparation

Xenopus laevis (African Clawed Frog Colony) tadpoles (pigmented) were purchased from Nasco and kept under a 12 h dark/12 h light cycle (ordinary ceiling white fluorescence light: depending on viewing direction, irradiance at the level of the animals was between 10 and 80 μW/cm^2, equivalent to $\sim$10^5 to 10^6 photons μm^{-2} s^{-1} if all energy were in the visible spectrum) in continuously aerated, 0.1$\times$ Gerhardt's Ringer (in mM: 10 NaCl, 0.15 KCl, 0.2 CaCl$_2$, and 0.1 MgCl$_2$) at room temperature. They were fed twice weekly with ground Nasco frog brittle. After dark-adaptation overnight, a tadpole was pithed and the eyes were removed under infrared illumination. The retinae were isolated and stored in normal Ringer solution (in mM: 90 NaCl, 5 KCl, 3.2 MgCl$_2$, 2 CaCl$_2$, 3 Na$_2$-Succinate, 3 Na-Glutamate, 10 Glucose, 10 HEPES, 0.02 EDTA and 20 NaHCO$_3$, pH 7.4) on ice for up to several hours in darkness until use. After isolation of the retina, the tadpole was staged according to the Normal Table of *Xenopus laevis* (Nieuwkoop and Faber, 1956). Developmental stage instead of age was used because the time to reach a given stage varied considerably even among tadpoles of the same batch and raised under identical conditions. For the same reason, it was rather difficult to determine precisely the stage of an animal before sacrifice, which explains a degree of randomness in the de-

velopmental stages of the animals used for experiments. When needed, a retina was finely chopped with a razor blade under normal Ringer solution in infrared light, then transferred to the recording chamber (Xiong et al., 1997).

Recordings and Optics

Membrane current was recorded from a single rod outer segment projecting from a fragment of retina with the suction-pipette technique at room temperature, 20–24°C (Baylor et al., 1979a). All signals were low-pass filtered at 100 Hz (8-pole Bessel) during recording and at 30 Hz during analysis. The traces shown in the figures are averages of multiple trials. Diffuse, unpolarized light flashes at 520 nm and of 8-ms duration were used in all experiments except when the action spectrum was measured. After recording, a video image of each outer segment (while still in the suction pipette) was taken in order to measure its diameter and length.

The effective collecting area, A_e, of an outer segment for incident light normal to the longitudinal axis of the outer segment is given by $A_e = 2.303\pi r^2 lQ\alpha f$ (Baylor et al., 1979b), where r and l are the radius and length of the rod outer segment, Q is the quantum efficiency of isomerization, α is the transverse specific optical density of the outer segment, and f is a factor that depends on the polarization of the incident light. For unpolarized light, f is 0.5 (Baylor et al., 1979b; Xiong et al., 1997). We have adopted $\alpha = 0.012$ μm^{-1} at λ_{max} (the value for the red rods of aquatic salamander, Harosi, 1975; see also Witkovsky et al., 1981) and Q = 0.67 (Dartnall, 1972). The above expression for A_e is a linear approximation of an exponential function for absorption, and holds when $2.303\alpha f(2r) << 1$, true for *Xenopus* tadpole rods ($r < 4$ μm).

Solutions and Chemicals

Normal Ringer solution was used extracellularly. Pipette solution was identical to normal Ringer except for the 20 mM NaHCO$_3$ replaced with 20 mM NaCl. Crystalline 11-cis-retinal was obtained from Dr. Rosalie Crouch (Medical University of South Carolina) and was stored under argon at -80°C in a light-tight container. A stock solution was prepared by adding just-sufficient ethanol to dissolve a small amount (microgram-range) of the 11-cis-retinal crystals, and the chromophore concentration in this stock solution was determined by spectrophotometry after a 2,000-fold dilution in ethanol, using a molar extinction coefficient for 11-cis-retinal of 25,000 M^{-1} cm^{-1} at 380 nm (Jones et al., 1989). The stock solution was stored at -20°C and used within a week. When needed, the appropriate amount of stock solution was diluted with normal Ringer to give 1 ml of final solution with a chromophore concentration of 100 μM (final ethanol concentration under 0.1% vol/vol), in which an isolated retina was incubated for $\sim$40 min in darkness. Afterwards, the retina was transferred into 0.4 ml of normal Ringer and finely chopped as described above.

Data Analysis

The plots of peak amplitude of the flash response against light intensity were fitted with the Michaelis function:

$$\frac{r}{r_{max}} = \frac{I_F}{I_F + \sigma_F}, \qquad (1)$$

where r is peak response amplitude, r_{max} is maximum response amplitude, I_F is flash intensity, and σ_F is half-saturating flash intensity. The dim-flash sensitivity, S_F, is given by:

$$S_F \equiv \frac{r}{I_F} \approx \frac{r_{max}}{\sigma_F}, \text{ for small } I_F. \qquad (2)$$

For most of the experiments, it makes more sense to remove any change in S_F due to change in r_{max} by simply dividing with r_{max}. The normalized S_F is thus equal to $1/\sigma_F$.

As another parameter of the rod response, we measured the time-to-peak of the half-saturated flash response. Traditionally, the time-to-peak of the linear, dim-flash response, instead of the half-saturated flash response, is used as a response parameter. However, the dark current of the early-stage rods in this work was often too small to define the linear, dim-flash response. For uniformity, then, the half-saturated flash response was used instead. In experiments where no elicited flash response was exactly half-saturated, the time-to-peak was calculated as the average of the times-to-peak of the two flash responses bracketing the half-saturation point mostly closely.

In bleach experiments, with a bleaching light step of intensity I_S (photons.$\mu m^{-2}s^{-1}$ at 520 nm) and duration T seconds, the number of photoisomerizations (Rh^*) is $2.303\pi r^2 lQ\alpha fI_S T$. With a visual-pigment density in the outer segment of ~ 3.5 mM (Harosi, 1975), which corresponds to $\sim 2 \times 10^6$ pigment molecules/μm^3 (this in turn agrees quite well with that predicted from a generally accepted surface density of 30,000 pigment molecules/μm^2 of disk membrane, given a disc-center-to-disc-center spacing of ~ 0.03 μm [Fig. 7.3 of Dowling, 1987]), the total number of visual pigment (porphyropsin) molecules is $\pi r^2 l \times 2.1 \times 10^6$. Thus, for low bleaches (i.e., assuming linearity), the fractional bleach is $4.4 \times 10^{-9} \cdot I_S T$, using the above Q, α, and f values.

The total change in sensitivity at different fractional levels of free opsin (i.e., without chromophore), F, due to bleaching can be calculated from the following equation (Jones et al., 1996):

$$\frac{S_F}{S_F^D} = \frac{(1-F)}{(1+k.F)}, \quad (3)$$

where S_F is the sensitivity for nonzero F, S_F^D is the sensitivity for $F = 0$, and k is a constant. In this model, the desensitization due to a bleach is caused by a combination of reduction in quantum catch (numerator) and an additional factor that increases linearly with free opsin (denominator), the latter now being thought to arise from a weak ability of free opsin to activate phototransduction (Cornwall and Fain, 1994).

The action spectrum (plot of dim-flash sensitivity against wavelength) is fitted with the following function (Govardovskii et al., 2000; see also Lamb, 1995):

$$S(x) = \frac{1}{\exp[A(a-x)] + \exp[B(b-x)] + \exp[C(c-x)] + D}, \quad (4)$$

where $x = \lambda_{max}/\lambda$, $B = 20.85$, $b = 0.9101$, $C = -10.37$, $c = 1.1123$, $D = 0.5343$, $A = 62.7 + 1.834\exp[(\lambda_{max} - 625)/54.2]$, and $a = 0.875 + 0.0268\exp[(\lambda_{max} - 665)/40.7]$.

All fittings were made with Microcal Origin 6.0.

RESULTS

Action Spectrum

In this work, we recorded from "red" rods of stage 46–66 tadpoles (stage 66 being the end of metamorphosis) and young adults. The outer segments of rods from tadpoles younger than stage 46 were too fragile for recording. The action spectra of "red" rods, obtained by comparing dim-flash sensitivities (see Eq. 2 and related text) at different wavelengths, measured at several developmental stages are shown in Fig. 1. For each cell, the measurements have been normalized to unity at,
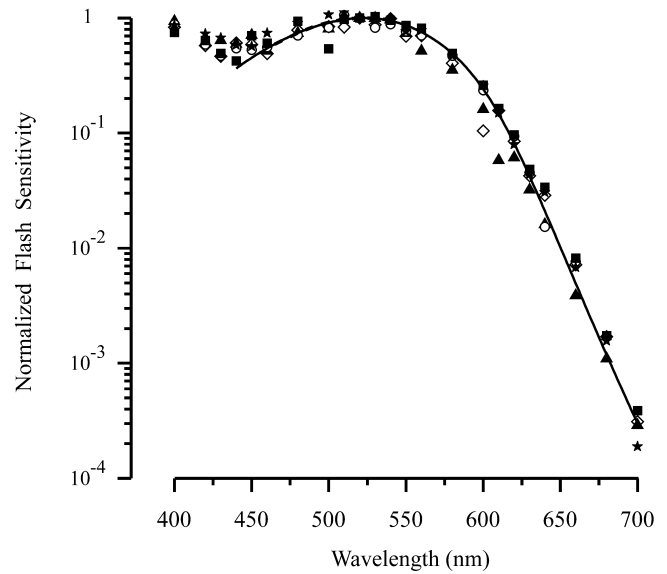

FIGURE 1. Action spectra of *Xenopus* rods at different developmental stages. The normalized dim-flash sensitivity was calculated from r/I_F (see text), and the sensitivity at 520 nm was then arbitrarily renormalized to unity. Data from one rod at each stage. Symbols: $\bigcirc$, stage 48; $\blacktriangle$, stage 50; $\diamondsuit$, stage 55; $\blacksquare$, stage 62; $\bigstar$, frog at size of 2.5–5cm. Curve is drawn from Eq. 4.

somewhat arbitrarily, 520 nm. The major component (α-band) of all action spectra can be well fitted by the template function, Eq. 4, with a λ_{max} of 523 nm (solid curve), in agreement with earlier work (Witkovsky et al., 1981) and consistent with the presence of dehydroretinal in *Xenopus* pigments throughout development and in adult (Bridges et al., 1977). Obviously, the presence of a small amount of A_1 pigment (i.e., with retinal instead of dehydro-retinal as chromophore) in the *Xenopus* retina (Azuma et al., 1988; Palacios et al., 1998) would not have been readily detected if it were present in minute amounts in each "red" rod or in a minority of these photoreceptors.

Dark Current and Physical Dimensions of Rod Outer Segment During Development

The maximum light response of a rod, reflecting the dark-current amplitude, increased steadily from stage 46 to about stage 57 (Fig. 2 A). Thereafter, the recorded dark-current amplitude was more variable, possibly reflecting a genuine decline before metamorphosis. The period of apparent current decline we observed, from stage 57 to perhaps stage 66, coincided with the period previously found by others to show a progressive shortening of the rod outer segment (Kinney and Fisher, 1978b). However, we did not observe any obvious correlative shortening of the recorded rod outer segments during this period (Fig. 2 B). The rod outer segments chosen for recording might not have been a true representation of the bulk population be-
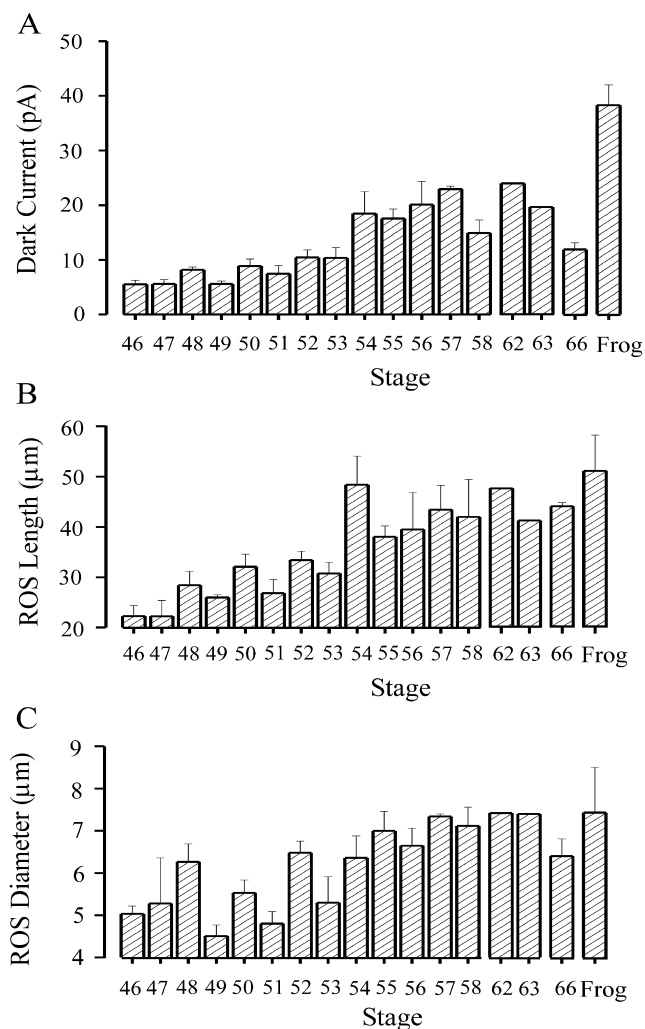
FIGURE 2. Dark currents and physical dimensions of recorded rod outer segments (ROS) at different developmental stages. Results from tadpoles at stages 46–66 and frogs at sizes of 2.5–5cm. The histograms indicate averaged data and SEM. The number of rods studied at each stage were: 6(stage 46), 2(47), 5(48), 2(49), 10(50), 5(51), 9(52), 3(53), 4(54), 10(55), 5(56), 3(57), 4(58), 1(62), 1(63), 5(66) and 2(frog), respectively.
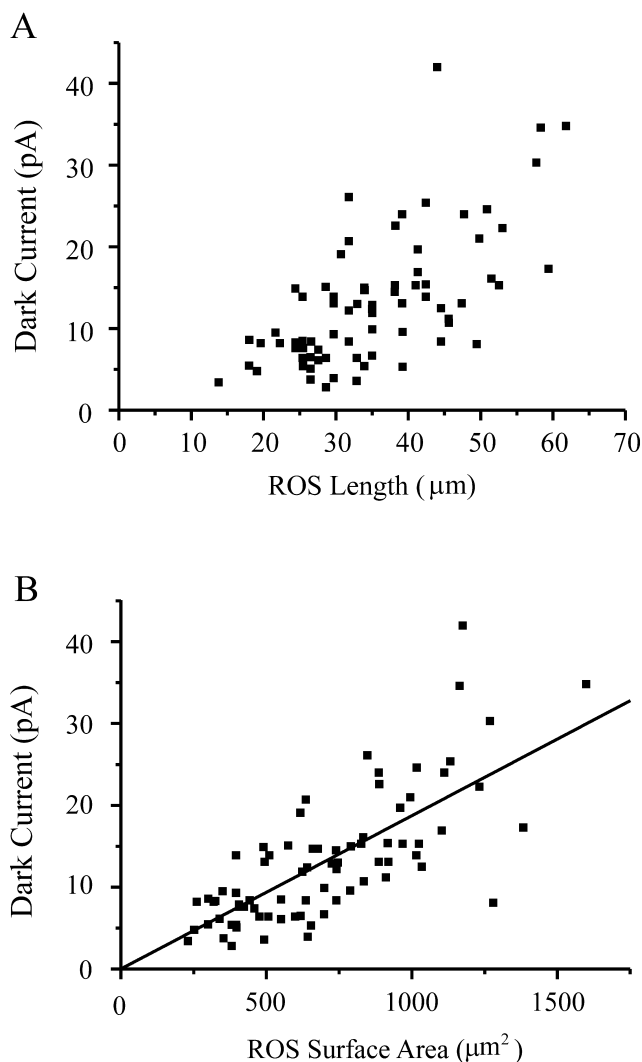
FIGURE 3. Variation of dark current with rod outer segment length and surface area. Altogether, 77 cells from stage 46–66 tadpoles and young adult frogs. Solid line in B is a linear-regression line, with a slope of 0.0188 pA/μm^2.

cause we tended to select the longer and larger rod outer segments, in order to avoid the "green" rods. Therefore, an unknown biological reason may underlie the apparent decline in current at stages 57–66 in Fig. 2 A, or the decline could be a serendipitous artifact due to some of the recorded outer segments being not in the best of conditions. From stage 46 to stage 66, the average diameter of the recorded rod outer segments likewise increased, from 5 to 7 μm (Fig. 2 C), a trend consistent with morphological studies (Witkovsky et al., 1976; Kinney and Fisher, 1978a). In Fig. 3, the dark-current amplitude is plotted against the length (Fig. 3 A) or the surface area (Fig. 3 B) of individual rod outer segments throughout this period. The clustering of data around a linear-regression line in Fig. 3 B suggests

an approximately uniform membrane-current density, possibly reflecting a constant density of cyclic GMP–gated channels per unit area on the plasma membrane through the developmental stages.

Rod Sensitivity and Time-to-peak of Half-saturating Flash Response During Development

A more dramatic developmental change is a sharp increase in light sensitivity of the rod at stage 53. In Fig. 4, the flash-response families from two overnight-dark-adapted rods, at stages 51 and 56, respectively, are compared. The rod at stage 51 was considerably less sensitive, and had faster response kinetics (note difference in time scale between Fig. 4, A and B) than the rod at stage 56. From the collected results of Fig. 5 A, it is clear that the flash sensitivity increased sharply at stages 52–54. The
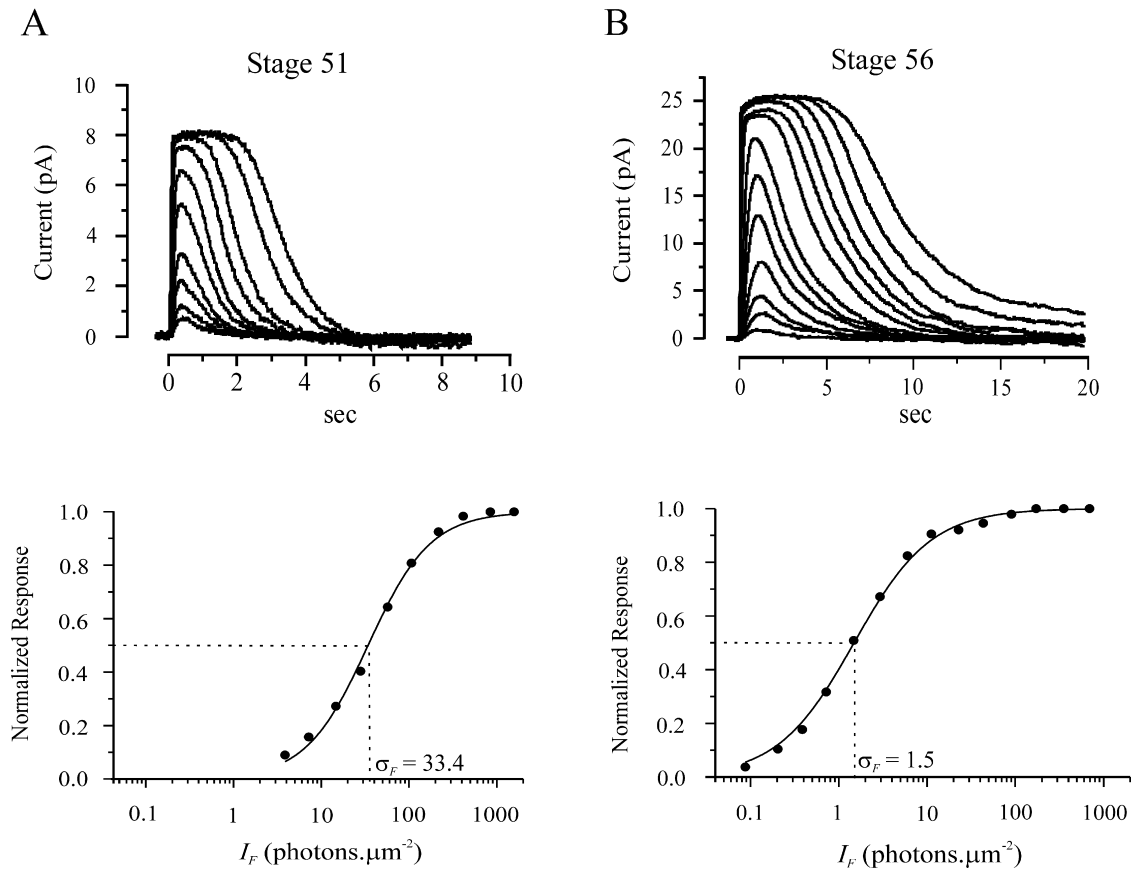
FIGURE 4. Rod response properties at stages 51 and 56. In each case, top panel indicates flash response-intensity family from an overnight-dark-adapted rod, and bottom panel shows the relation between peak amplitude and flash intensity of the same cell. (A) Flash intensities were 3.86, 7.18, 14.67, 27.95, 57.07, 106.3, 216.9, 413.4, 844.1, and 1571.7 photons.μm^{-2}, respectively. Light flash was delivered at time 0. The solid curve is Eq. 1 with σ_F equal 33.4 photons.μm^{-2}. (B) Flash intensities were 0.09, 0.20, 0.39, 0.72, 1.47, 2.93, 5.99, 11.2, 22.8, 43.4, 90.5, 172.0, 352.9, and 693.7 photons.μm^{-2}, respectively. The solid curve is Eq. 1 with σ_F equal 1.5 photons.μm^{-2}.

parameter of normalized flash sensitivity, S_F/r_{max} (equal to $1/\sigma_F$, from Eq. 2), offers the advantage of removing any change in S_F associated with the increase in dark current due to physical growth of the rod outer segment. This parameter showed a sharp, ~10-fold increase at stage 53 (Fig. 5 B). Associated with this large increase in sensitivity was an equally sharp increase in the time-to-peak of the half-saturated flash response (Fig. 5 C; see also Data Analysis in MATERIALS AND METHODS). In addition to the steep change at stage 53, there appeared to be smaller and more gradual decreases in sensitivity and time-to-peak before stage 53 and after stage 57.

Effect of Chromophore Treatment

A large increase in flash sensitivity was similarly observed in rat rods during the first month of life (P13-P35), attributed to an increase in the availability of chromophore (Ratto et al., 1991). Accordingly, we checked for the same mechanism in *Xenopus* rods. Fig. 6 A shows the flash intensity-response family of a rod from a stage 52 retina preincubated with 100 μM 11-cis-

retinal for 40 min in darkness. Dehydro-retinal was not used because of its instability and unavailability. Indeed, even though the dark current of the treated rod remained small, its flash sensitivity and the time-to-peak of its half-saturated flash response had become comparable to those of untreated rods at stage 53 or after (compare Fig. 4 B). Fig. 6 B shows another rod, from a stage 55 retina also pretreated with chromophore. In this case, however, the flash sensitivity and the time-to-peak of half-saturated flash response remained similar to those of untreated rods at the same developmental stage. In these chromophore-pretreatment experiments, the use of 11-cis retinal instead of dehydro-retinal to regenerate any free opsin would have shifted the λ_{max} of any regenerated pigment to around 500 nm, even though we still used 520-nm light for testing sensitivity. However, as shown in Fig. 1, the flash sensitivity was relatively unchanged whether measured at λ_{max} or at 20 nm from λ_{max} of an action spectrum. In actuality, the difference would be even smaller because most of the native pigment already had dehydro-retinal (see
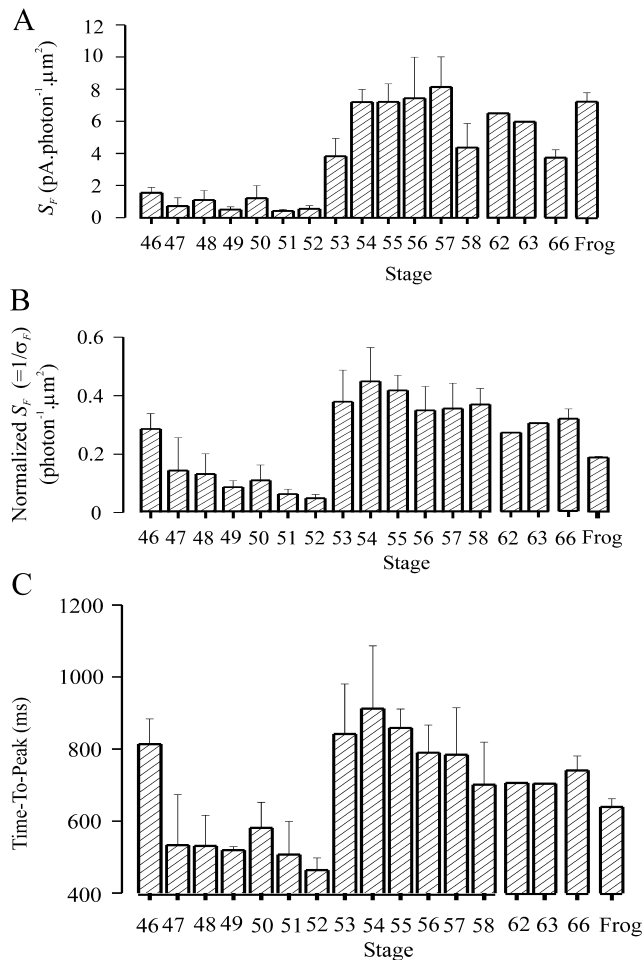
A

B

C

FIGURE 5. Change in flash sensitivity and in time-to-peak of half-saturated flash response during development. Collected results from rods of overnight-dark-adapted tadpoles at stages 46–66 and frogs at sizes of 2.5–5 cm. Data are average, with SEM shown. The number of rods measured at each stage is as in Fig. 2.

steady illumination. The short time-to-peak, a general sign of light adaptation, of the rod response at stage 52 or earlier is consistent with the rods being in a "light-adapted" state at these early developmental stages. It is surprising that Ratto et al. (1991) did not report, nor did their data show, any acceleration in response kinetics for rat rods from P13 to P35 which should accompany the lower flash sensitivity. The reason for this discordance between sensitivity and time-to-peak in rat rods is unclear, but may have to do with the difficulty of working with mammalian rods, which tend to be more labile in their conditions (and possibly aggravated by the use of HEPES- instead of bicarbonate-buffered Ringer by Ratto et al. [1991]). In Fig. 7 A, the pretreatment with 11-cis retinal was able to bring the flash sensitivity at stages 50 and 52 to levels as high as those of treated/untreated cells at or after stage 53. Thus, pigment regeneration by the chromophore treatment appeared to have reached completion. Further, the apparent lack of effect of chromophore on rods after stage 53 suggests that all of their opsin already has chromophore. A fractional increase in dark current is expected to accompany the chromophore treatment because of the removal of free opsin and therefore of some constitutive level of phototransduction activity (see text related to Fig. 9). The apparent lack of difference in dark current between control and chromophore-treated rods in Fig. 7 C may just reflect an insufficient sample size for the comparison.

Quantitative Equivalence between Early Developmental Stages and Photobleaching

In Fig. 8, the normalized flash sensitivity is plotted against the time-to-peak of the half-saturated flash response at different developmental stages, in double-logarithmic coordinates (open squares). The data points corresponding to low sensitivity/fast response kinetics generally came from rods of early-stage tadpoles, whereas those corresponding to higher sensitivity/longer time-to-peak generally came from rods of late-stage tadpoles. A linear fit to the points gives a slope of 2.84, not too different from the relation between flash sensitivity and time-to-peak of dim-flash response obtained at different levels of adaptation to background light (2.52; Baylor et al., 1980). Perhaps most revealing are the measurements from late-stage rods (i.e., after stage 53) obtained both before (upright triangles) and after (inverted triangles) bleaching light (see figure legend and below). These two sets of data lie along the same straight line, but segregate at the two ends of the line as expected. This plot strongly suggests that the developmental changes in flash sensitivity and time-to-peak involve similar mechanisms that underlie the changes due to bleaching, which produces free opsin.

later) and therefore its λ_{max} would not be shifted by 11-cis-retinal. Accordingly, we have not corrected for the very small change in spectral sensitivity. Collected results on flash sensitivity and time-to-peak from this chromophore-pretreatment experiment are shown in Fig. 7, A and B, in which the dark bars represent data from chromophore-treated rods and the hatched bars are redrawn from Fig. 5, B and C. Similar to flash sensitivity, the response time-to-peak at stage 52 or earlier became comparable to that at stage 53 or later after chromophore treatment. These results are consistent with the idea that, as in P13-P35 rat, some of the rod opsin is devoid of chromophore at *Xenopus* stage 52 or earlier, a condition that can be reversed by incubation with chromophore. Because free opsin is now known to activate phototransduction (Cornwall and Fain, 1994; Jones et al., 1996), a rod containing free opsin would enter into an adapted state much as it would under
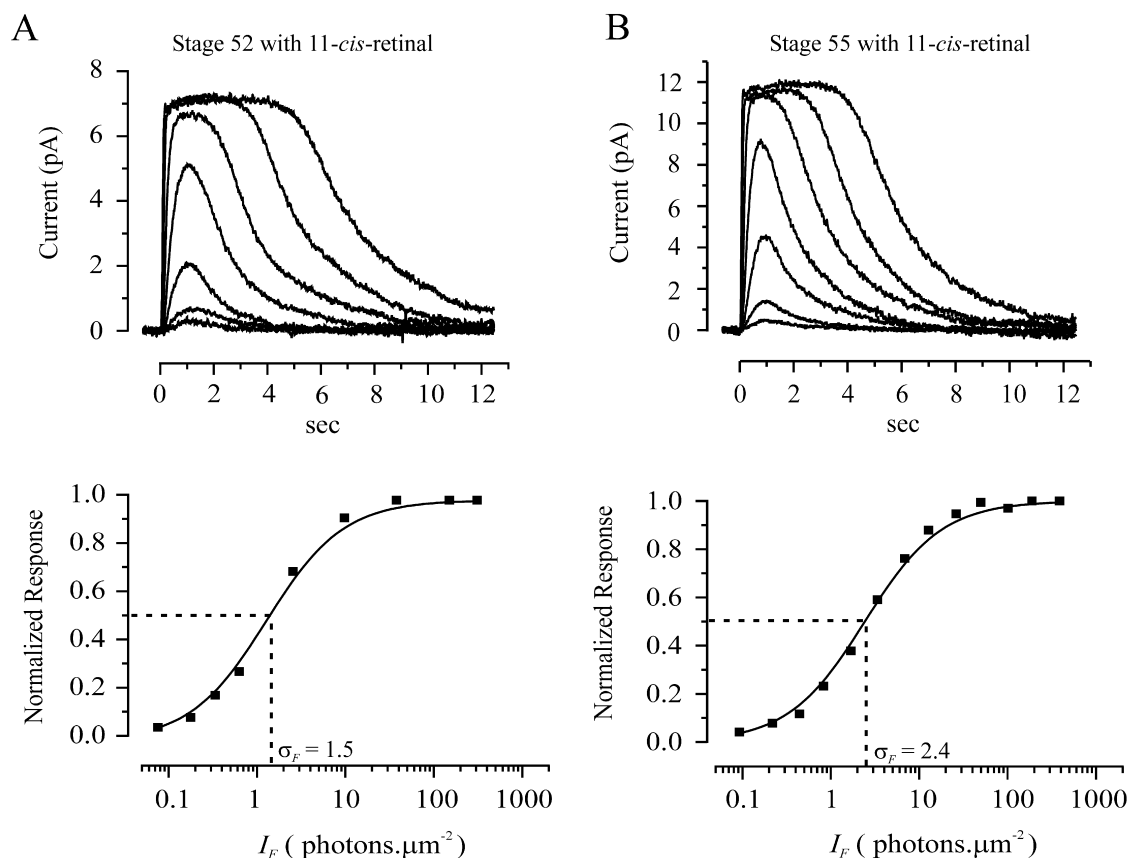
FIGURE 6. Rod response properties at stages 52 and 56, after incubation with 11-cis retinal. In each case, top panel indicates flash response-intensity family from an overnight-dark-adapted rod, and bottom panel shows the relation between peak amplitude and flash intensity of the same cell. Preincubation of isolated retina with 100 μM 11-cis-retinal in normal Ringer for 40 min in darkness. (A) Flash intensities were 0.09, 0.20, 0.72, 2.93, 11.2, 43.4, and 172.0 photons.μm^{-2}, respectively. Light flash was delivered at time 0. The solid curve is Eq. 1 with σ_F equal 1.5 photons.μm^{-2}. (B) Flash intensities were 0.09, 0.44, 1.68, 6.86, 26.1, 101.5, and 385.7 photons.μm^{-2}, respectively. The solid curve is Eq. 1 with σ_F equal 2.4 photons.μm^{-2}. Additional flash intensities not shown in the top panels are included in the bottom panels.

Percentage of Free Opsin in Early Development

It is possible to estimate the percentage of free opsin in rods before stage 53 by matching their lower sensitivity to the level of desensitization in later-stage rods produced by standard light bleaches. In these experiments, we avoided comparing control sensitivity and chromophore-pretreated sensitivity in separate animals by using one retina from each animal for control sensitivity measurements and the other retina for sensitivity measurements after pretreatment with 11-cis-retinal. The collected results shown in the inset of Fig. 9 indicate that the normalized dim-flash sensitivity of rods before stage 53 (stages 50–52) increased 8.5-fold on the average after chromophore treatment, whereas that of rods after stage 53 was practically unchanged by the treatment, confirming the results shown in Fig. 7 A.

Next, we performed bleaching experiments on post-stage 53 rods in which controlled amounts of pigment were bleached (see MATERIALS AND METHODS) and the resulting decreases in flash sensitivity were measured after the dark current had recovered to a steady level over a period of 30 min or longer, depending on the bleaching light intensity. The overall desensitization (represented as $S_F/S_F^D = \sigma_F^D/\sigma_F$, with S_F^D being the flash sensitivity in the absence of free opsin and a correspondent meaning for σ_F^D) is due to both a decrease in quantum catch and a desensitizing effect caused by a weak ability of free opsin to activate phototransduction. It can be expressed by Eq. 3 (Jones et al., 1996). In Fig. 9, the two continuous curves are fits of Eq. 3 to the data obtained from five rods (same as the five cells shown by triangles in Fig. 8), using either a linear fit (Curve 1) or a logarithmic fit (Curve 2). The constant k in Eq. 3 is 17.8 for Curve 1 and 25 for Curve 2, both not far from the value of 16.2 obtained for salamander rods (Jones et al., 1996). With these curves as standards, a sensitivity ratio of 8.5 from the inset gives a free opsin percentage of 28% (Curve 1) and 22% (Curve 2), respectively, at stages 50–52. With a more simplistic straight-line interpolation of the data points, the estimated value would
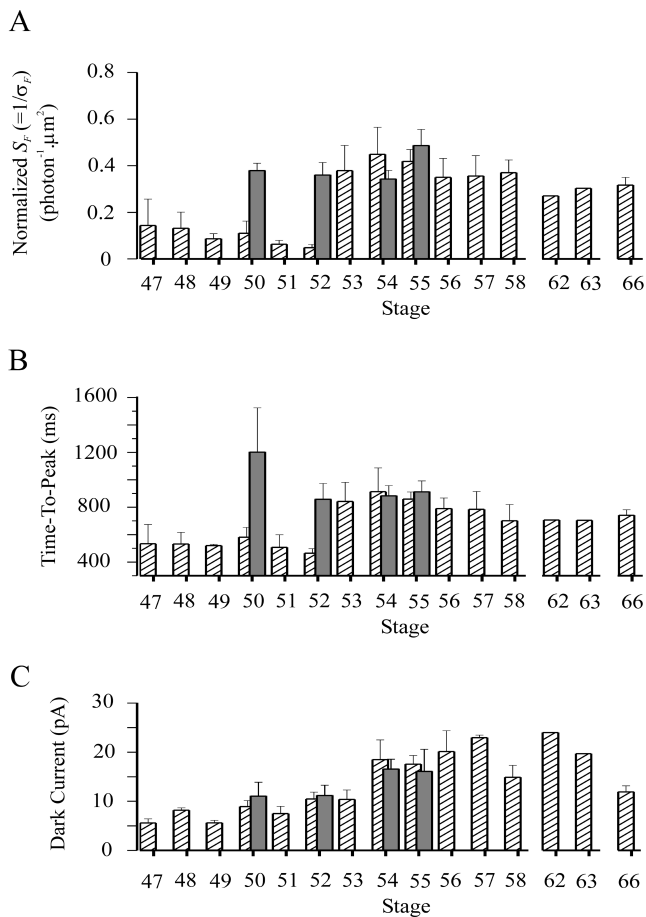
A

B

C

FIGURE 7. Normalized flash sensitivity, time-to-peak of half-saturated flash response, and dark current at different developmental stages, after pretreatment of the isolated retina with 100 µM 11-cis-retinal for 40 min in darkness. Experiments on rods of overnight-dark-adapted tadpoles at stages 46–66 and frogs at sizes of 2.5–5 cm, as in Fig. 5. Dark bars indicate the collected chromophore-pretreatment data, showing average ± SEM. The number of rods measured at each stage are 4(stage 50), 6(52), 5(54) and 2(55). The hatch bars are redrawn from Figs. 5, B and C, and 2 A.

have been also ∼22% (not depicted). In the above bleaching experiments, after a 10–27% bleach, the fractional reduction in dark current in "steady-state" due to free-opsin activity was ∼0.3–0.4 (see Fig. 9 legend). The true reduction could be smaller if the post-bleach rod had not yet reached true steady-state in these experiments. In the above experiments generating the standard bleaching curve, we have not corrected for any potential pigment regeneration after a bleach due to endogenous stores of chromophore, because such regeneration has been reported to be minimal (Cornwall et al., 1983, 1990).

Effect of Prolonged Dark Adaptation

Finally, we asked whether the deficit of chromophore in prestage 53 retina was possibly due to an immature machinery for the recycling of chromophore. Even
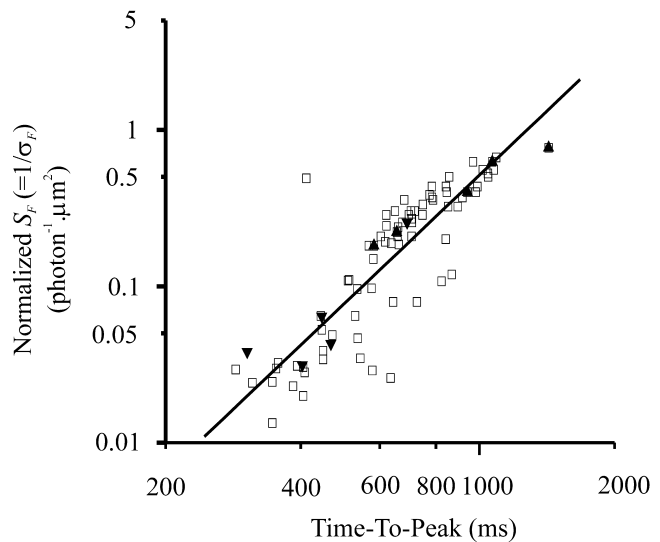

FIGURE 8. Same power-law relation between normalized flash sensitivity and response time-to-peak during development as with light bleaches. Log-log coordinates. □, control, dark-adapted rods from different developmental stages; ▲, rods at stage 53 or later before bleaching (3 cells at stage 54, 1 at stage 56, and 1 at stage 58); ▼, the same cells after bleaching. The straight line fitted to the control rod data has a slope of 2.84. The cells marked by triangles are the same cells as those marked by filled squares in Fig. 9 (see Fig. 9 legend).

though all forgoing experiments were done on rods from animals that had been dark-adapted overnight (10–12 h), the pigment regeneration could be so slow that any pigment bleached during daytime might not have fully regenerated during night time. To address this question, we dark-adapted tadpoles for 7 d or longer before measuring rod sensitivity. Although the number of measurements were limited, the flash sensitivities of rods at stages 50 and 52 (which probably could be combined to give better reliability, considering that these stages showed similar sensitivities in control conditions) under these conditions indeed approached those of rods at stage 53 and after, whereas the sensitivity of these latter rods was hardly affected by such prolonged dark adaptation (Fig. 10 A, dark bars). As expected, the time-to-peak of the half-saturated flash response behaved likewise (Fig. 10 B, dark bars). Although we cannot rule out such possibilities as a difference in feeding behavior of the tadpoles in light versus darkness, a parsimonious interpretation of these results is that the pigment-regeneration mechanism is inefficient before stage 53. We have not yet attempted to follow the pigment-regeneration time course with intermediate dark-adaptation periods.

DISCUSSION

We have described here experiments in which the flash sensitivity of overnight-dark–adapted *Xenopus* rods was
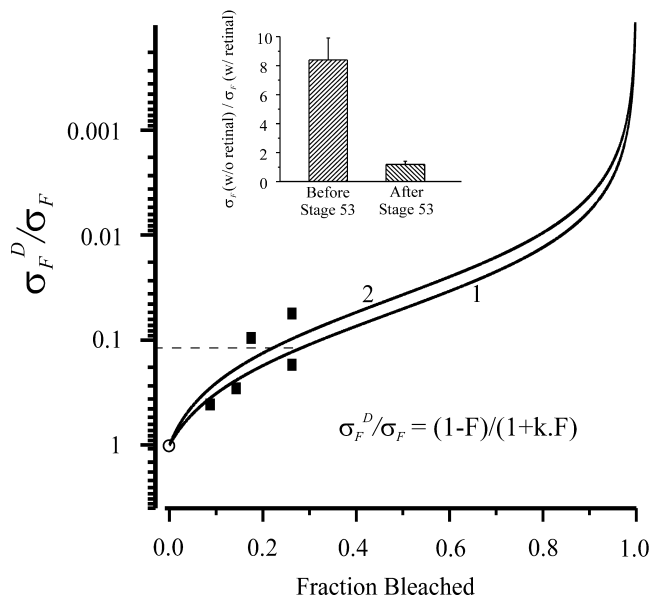
FIGURE 9. Calculation of fractional free opsin in prestage 53 rods. The ratio of normalized flash sensitivity pre and postbleach, represented by σ_F^D/σ_F, is plotted against fraction bleached and used as a standard, obtained from five poststage 53 cells indicated by filled squares. Same cells as those marked by triangles in Fig. 8; the point at zero fractional bleach (open circle) is simply a constraint for the curve fit. The stage, % bleach, and "steady-state" dark current before and after bleach of each cell were: stage 53, 17.5%, 13.9 pA, 7.5 pA; stage 54, 26.2%, 12.2 pA, 4.1 pA; stage 54, 14.3%, 30.3 pA, 17.1 pA; stage 55, 8.7%, 15.3 pA, 10.9 pA; stage 55, 26.2%, 15.3 pA, 10.4 pA. Continuous curves are Eq. 3 with k = 17.8 (Curve 1) and 25 (Curve 2). (Inset) Ratio of flash sensitivity with and without 11-cis-retinal treatment. 10 cells in total before stage 53 (4 at stage 50, 1 at stage 51, and 5 at stage 52) and 5 cells in total after stage 53 (3 at stage 54, 1 at stage 56, and 1 at stage 58). Sensitivity ratio before stage 53 being ~8.5 and close to unity after stage 53. The reciprocal of 8.5 (horizontal dotted line) is used for estimating percentage of free opsin in prestage 53 rods, which gives 28% from Curve 1 and 22% from Curve 2.
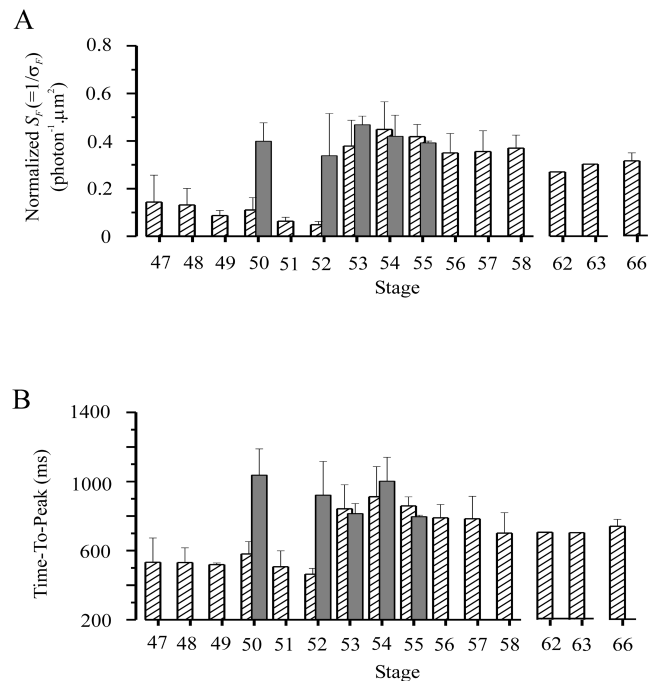

FIGURE 10. Collected results on normalized flash sensitivity and time-to-peak of half-saturated flash response of rods after prolonged dark adaptation at various developmental stages. Dark bars indicate the collected results. The number of rods studied at each stage was: 3(stage 50), 2(stage 52), 3(stage 53), 5(stage 54) and 2(stage 55). Hatched bars are redrawn from Fig. 5, B and C.

followed through stages 46–66 (stage 66 being the end of metamorphosis), the developmental window for which suction-pipette recording from single rods is possible. We have found that, at stage 53, there is a very steep (~10-fold) increase in the flash sensitivity (normalized by dark current) of *Xenopus* rods. Accompanying this steep rise in sensitivity is a steep prolongation of the time-to-peak of the flash response.

Prior work with steady illumination or short bleaches has indicated that background and bleaching adaptations are both characterized by codecreases in flash sensitivity and response time-to-peak (e.g., Baylor et al., 1979a, 1980; Cornwall et al., 1983, 1990). Indeed, after pretreatment with 11-cis-retinal, the flash sensitivity and response time-to-peak of prestage 53 rods became similar to those of poststage 53 rods, indicating that prestage 53 rods, despite overnight dark adaptation, remained light adapted due to the presence of free opsin,

which is known to activate phototransduction weakly (Cornwall and Fain, 1994). The percentage of free opsin at stages 50–52 is estimated to be 22–28%. The simplest explanation for the presence of free opsin is that the mechanism for pigment regeneration is very inefficient before stage 53, so that even overnight dark adaptation is insufficient for fully regenerating the pigment bleached during daytime. This notion is supported by the observation that the signs of light adaptation disappeared in prestage 53 animals after they had been continuously dark adapted for many days. Presumably, the pigment-regeneration system matures rapidly at stage 53. Nonetheless, we cannot conclude that it has reached the fully-mature state by stage 53, because we have not yet examined for any change in rod sensitivity with shorter-than-overnight dark adaptation during the stage 53-to-adult period. In principle, the slow step that rate-limits pigment regeneration at early developmental stages can reside anywhere in the following steps: retrieval of photoisomerized chromophore from rods, reisomerization of chromophore to the 11-cis-state, and its delivery back to bleached opsin.

In addition to the dramatic changes at stage 53, we also observed smaller and more gradual decreases in sensitivity and response time-to-peak during the period of stages 46–52 and perhaps the period of stage 57

adult. The gradual, prestage 53 changes may be due to the maturation of the pigment-regeneration system generally lagging behind the physical growth of the rod outer segment and the accompanying increase in opsin content, which would impose a larger demand on pigment regeneration. On the other hand, the apparent decrease in sensitivity from stage 57 into adulthood is more difficult to understand. Even though stages 57–66 represent the metamorphic climax, to be followed by adulthood, it seems rather unlikely that the pigment-regeneration system in the eye would change under these circumstances. To better understand this question, it would be necessary to carry out chromophore-pretreatment or prolonged dark adaptation experiments in poststage 57 tadpoles and young adults. Alternatively, the twofold change in sensitivity (and less than twofold corresponding change in response time-to-peak) at and after stage 57 may be an artifact arising from the limited sampling of rods at each stage. Finally, it should be mentioned that Witkovsky et al. (1976) have not observed any steep change in *Xenopus* rod sensitivity at stage 53 in their ERG study (see their Fig. 6), even though the overall sensitivity change (∼10-fold) they observed from stage 46 to 62 is comparable to what we found. This group did not report how long their animals were dark-adapted before ERG measurements, but it would seem unlikely that these animals were dark adapted for more than a day. Thus, the reason for this difference in observation between the two studies remains unknown.

In any case, it appears that, as in rat retina (Ratto et al., 1991), the low availability of chromophore in *Xenopus* retina is a major factor in the poor sensitivity of rods during early life of the animal. Ratto et al. (1991) have observed a drastic 50-fold increase in flash sensitivity from P12/P13 (when visual function begins) to P35. At the time, their observation would imply that at most 2% of rat rhodopsin had chromophore at P13, based solely on the reduction in quantum catch. Now that free opsin is known to activate phototransduction weakly (and that bleaching can have additional effects; see Sokolov et al., 2002), the amount of bleached pigment required for this level of desensitization need not be this high. For example, in our experiments, an 8.5-fold decrease in sensitivity results from a mere 22–28% bleach, instead of an 80–90% bleach as would be implicated if quantum-catch were the only factor. The actual percentage of free opsin in young rat can only be estimated from a standard bleaching curve similar to that shown in Fig. 9, but this is not available at present. Also, the period of dark adaptation was not specified sufficiently clearly in Ratto et al. (1991)—described merely as "dark-adapted for at least 1 h"—for their measurements to be quantitatively compared across animals. Nonetheless, their data can qualitatively be explained by an immaturity of the pigment-regeneration system. For example, the age period corresponding to the steepest increase in rat-rod sensitivity (P13-P18) in Ratto et al. (1991) parallels that for the rapid increase in 11-cis-retinyl esters in the retina (Carter-Dawson et al., 1986). Also, more recent work by Dodge et al. (1996) has shown that, when rats are dark-adapted for 12–18 h, there is no evidence of free rod opsin in the eyes of 13-, 19-, 34-d-old or adult animals. Insufficient dark adaptation would not invalidate the overall conclusion of a developmental change in rod sensitivity in rat, but would exaggerate the phenomenon.

In humans, there are likewise reports of a considerably higher threshold for detecting light for newborn/young infants than adults (Powers et al., 1981; Hamer and Schneck, 1984; Brown, 1986, 1990; Brown et al., 1987; Banks and Bennett, 1988). Although optical factors as well as postphotoreceptor elements in the visual pathway are likely to contribute to this age-dependent sensitivity change (Brown, 1986, 1990; Banks and Bennett, 1988), there is now strong evidence that at least part of this change originates from rod photoreceptors (Fulton and Hansen, 1992, 2000; Breton et al., 1995; Nusinowitz et al., 1998; Fulton et al., 1999; Hansen and Fulton, 1999, 2000). Furthermore, judging from Table I of Fulton et al. (1999), chromophore treatment could increase the amount of rhodopsin extracted biochemically from the eyes of <12-mo-old infants. Although extraneous factors for the loss of holopigment in these experiments cannot be ruled out, one possibility would be an immature pigment-regeneration system in months old infants. One way to address this question would be to test the behavioral light threshold of infants after different periods of dark-adaptation.

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