Electrophysiological Changes During Early Steps of Retinitis Pigmentosa

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PURPOSE. The rhodopsin mutation P23H is responsible for a significant portion of autosomal-dominant retinitis pigmentosa, a disorder characterized by rod photoreceptor death. The mechanisms of toxicity remain unclear; previous studies implicate destabilization of P23H rhodopsin during light exposure, causing decreased endoplasmic reticulum (ER) exit and ER stress responses. Here, we probed phototransduction in Xenopus laevis rods expressing bovine P23H rhodopsin, in which retinal degeneration is inducible by light exposure, in order to examine early physiological changes that occur during retinal degeneration.

METHODS. We recorded single-cell and whole-retina responses to light stimuli using electrophysiology. Moreover, we monitored morphologic changes in rods after different periods of light exposure.

RESULTS. Initially, P23H rods had almost normal photoresponses, but following a brief light exposure varying from 4 to 32 photoisomerizations per disc, photoresponses became irreversibly prolonged. In intact retinas, rods began to shed OS fragments after a rod-saturating exposure of 12 minutes, corresponding to approximately 10 to 100 times more photoisomerizations.

CONCLUSIONS. Our results indicate that in P23H rods light-induced degeneration occurs in at least two stages, the first involving impairment of phototransduction and the second involving initiation of morphologic changes.

Keywords: retinitis pigmentosa, phototransduction, photoresponse, rhodopsin, photoreceptor

Rod outer segments (OSs) are comprise thousands of stacked discs containing photon-absorbing rhodopsin molecules packed in an almost crystalline fashion.1–5 Photo-excited rhodopsins activate G-proteins, each of which activates one phosphodiesterase (PDE) molecule.6–8 Activated PDEs hydrolyze cyclic guanosine monophosphate, thereby closing cyclic nucleotide-gated channels9,10 to produce well-characterized photoresponses.2,11,12

Retinitis pigmentosa (RP), a disease of progressive rod photoreceptor degeneration,13 is caused by mutations in many genes. However, the rhodopsin mutation P23H is the most common cause of autosomal-dominant RP in North America.14,15 In Xenopus laevis tadpoles expressing the bovine form of P23H rhodopsin (hereafter named bP23H), photoreceptors degenerate rapidly when exposed to cyclic light.16–18 However, when reared in darkness, degeneration is prevented. Therefore, these animals have been used as a model for RP16,17,19,20 particularly the light-exacerbated retinal degeneration (RD) that likely occurs in cases of sector RP21–23 The exact mechanisms of toxicity remain unclear.

The P23H mutation promotes structural instability.24 Previous studies suggest a mechanism involving destabilization of bP23H opsin by light, decreasing its exit from the ER.25,26 In dark-reared X. laevis, expression levels of bP23H rhodopsin are low relative to the endogenous (wild type [WT]) rhodopsin, likely due to ER quality control. However, a significant quantity reaches the OS, resulting in expression levels of 7.5% relative to endogenous rhodopsin16,26 (re-assessed for this study, see Supplemental Fig. S1). In dark-reared animals subsequently exposed to light, inner segment (IS) and OS membrane abnormalities rapidly develop.18 Similarly, low basal expression, aggregation, and proteolytic degradation of P23H rod opsin expressed in the Caenorhabditis elegans neurons can be partially reversed by providing 9-cis retinal.27

P23H mutant mice have been examined using electroretinography (ERG), and showed abnormal a-waves.28 At P40 they have abnormal ERGs and reduced rod nuclei numbers.29 In the present manuscript, for the first time to our knowledge, we were able to probe phototransduction in intact retina and isolated rods from transgenic X. laevis expressing bP23H rhodopsin that were bred and housed in continuous darkness. We find that under these circumstances, unexpectedly bP23H-expressing rods do not degenerate and have almost normal photoresponses. In this way, we determined two early steps of the onset of RP and we quantified the number of photoisomerizations initiating these steps. The first step is caused by an exposure to light causing just a few photoisomerizations per disc and impairs phototransduction, although rods retain their integrity and morphology; the second step is initiated by a light exposure of 12 minutes causing rods to shed OS, lose their morphology, and degenerate. Therefore, RP in mutant rods is initiated by a very limited number of photoisomerizations.
Materials and Methods

Breeding and Rearing of Transgenic X. Laevis

X. laevis tadpoles carrying the bP23H transgene and expressing bP23H rhodopsin were generated by mating heterozygous or homozygous transgenic males with WT females.\textsuperscript{16,17,19} WT tadpoles were derived from separate matings, or were siblings of transgenic tadpoles. Tadpoles were transferred to an 18°C to 21°C incubator that maintained 24-hr/d constant darkness. Expression levels were determined by dot blot assay as previously described\textsuperscript{30} by determining the ratio of mAb 1D4 labelling (anti-mammalian rhodopsin, MA8556; Millipore Sigma, Burlington, MA, USA) to B630N labeling (recognizes both mammalian and frog rhodopsin, gift of W. Clay Smith, University of Florida, Gainesville, FL, USA) relative to a standard containing both antibody epitopes and to nontransgenic retinas (Supplemental Fig. S1). All experiments adhered to the ARVO statement for the Use of Animals in Ophthalmic and Vision Research.

Isolation of Photoreceptors

Tadpoles and froglets were killed as previously described.\textsuperscript{30} The developmental stage of each animal was determined (in the public domain, http://www.xenbase.org/anatomy/alldev.do). Eyes were enucleated and hemisected under infrared 820-nm illumination. Dissociated rods were obtained as reported.\textsuperscript{31,32} Isolated intact rods obtained by mechanical dissociation were immersed in Ringer solution containing (in mM) 110 NaCl, 2.5 KCl, 1 CaCl\textsubscript{2}, 1.6 MgCl\textsubscript{2}, and 3 HEPES-NaOH, 0.01 EDTA, and 10 glucose (pH 7.7–7.8 buffered with NaOH). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). All experiments were performed at 22°C to 24°C. Images were acquired using HCImage software 4.3.1.33 (Hamamatsu Corporation, Bridgewater, NJ, USA).

Single-Cell Photoresponse Recordings

After mechanical isolation, the IS of an isolated intact rod was drawn\textsuperscript{33} into a silane-coated\textsuperscript{34} borosilicate electrode (Blaubrand, intramark micropipette; BRAND GMBH + CO KG, Wertheim, Germany) (internal diameter of 4–6 μm) filled with Ringer’s solution. Rods were viewed under 900-nm light using two cameras (Hamamatsu ORCA-Flash 4.0; Hamamatsu Corporation, Bridgewater, NJ, USA; and Jenoptik ProgRes MF; JENOPTIK 1 Optical Systems, Goesswizter, Jena, Germany) at two magnifications and stimulated with 491-nm diffuse light (Rapp OptoElectron, Hamburg, Germany) from the ×10 objective of an inverted microscope (Olympus IX71; Olympus Corporation, Tokyo, Japan). To stimulate the entire OS, we used a stable continuous 491-nm laser module (for details see Mazzolini et al.\textsuperscript{33}). Photoresponses were recorded as previously described\textsuperscript{31} using an Axopatch 200A (Molecular Devices, LLC., San Jose, CA, USA) in voltage clamp-mode. The current was low-pass filtered at 20 Hz and digitized at 100 Hz. All recordings were processed, analyzed, and baselines corrected with Clampfit 10.3 (Molecular Devices). Data are expressed as mean ± standard error of the mean; single points represent single experiments.

Single-Photon Response Analysis

Single-photon responses were evoked by a series of 20 to 99 dim flashes delivered every 5 seconds, with duration of 10 ms. The data were fitted with the equations from reference from Baylor et al.\textsuperscript{2} Threshold light intensities were tested on three bP23H and four WT rods at stages 55 to 59, one bP23H and two WT rods derived from froglets and on three WT rods derived from adults.

ERG Recordings

Dark-reared tadpoles (age 10 weeks, stage 56; offspring of a heterozygous transgenic and WT animal) or siblings exposed to cyclic light for 1 week, were anesthetized in 0.01% Tricaine in 0.1X MMR. ERG recordings to a series of 448-nm blue light flashes of increasing intensity from a light-emitting diode source were obtained as described by Vent-Schmidt et al.\textsuperscript{35} The recording equipment incorporated a 1-Hz low-frequency cut-off filter and a 300-Hz high-frequency cut-off filter. Following ERG analysis tadpoles were genotyped to distinguish bP23H and WT animals as previously described.\textsuperscript{35}

Confocal Microscopy

Eyes were fixed overnight at 4°C in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4, and processed, cryosectioned, and labeled for confocal microscopy using Alexa488-conjugated wheat germ agglutinin (Invitrogen, Waltham, MA, USA) and Hoechst 33342 (Sigma, St. Louis, MO, USA) as previously described.\textsuperscript{30} For transducin labeling, samples were fixed in 3.7% formaldehyde in 75% methanol. Polyclonal anti-alpha-transducin (gift of W. Clay Smith) and monoclonal anti-rhodopsin mAb 1D4 were used at 1:1000 dilution and detected with Cy3-conjugated anti-rabbit and anti-mouse secondary antibodies, respectively (Jackson Research, West Grove, PA, USA), as previously described.\textsuperscript{30} Images were obtained using a Zeiss 510 meta confocal microscope equipped with a ×40 NA 1.2 water-immersion objective (Carl Zeiss, Oberkochen, Baden-Württemberg, Germany). Phagosomes were identified by morphology with a blinded lab member. Phagosome area was quantified using Imagej software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Estimation of the Number of Photoactivated bP23H Rhodopsins (Rh\textsuperscript{*}) on a Disc

Assuming 25,000 Rh/μm\textsuperscript{2} one bP23H disc has an area of 12.6 μm\textsuperscript{2} (based on a diameter of 4 μm) giving 25,000 Rh/μm\textsuperscript{2} × 12.6 μm\textsuperscript{2} = 3.15 × 10\textsuperscript{5} Rh/disc.\textsuperscript{11} There are 2100 discs/rod in toad\textsuperscript{11} with a length of 60 μm. Our observed bP23H OS length is 53 μm, therefore 1155 discs. The total Rh/rod = 1155 × 3.15 × 10\textsuperscript{5} = 3.6 × 10\textsuperscript{8}. In dark-reared animals, bP23H Rh is 7.5% of the total\textsuperscript{11} giving an estimated number of bP23H Rh per rod of 2.7 × 10\textsuperscript{7} (bP23H Rh 2.36 × 10\textsuperscript{7} per disc).

Considering our lower limit of photoisomerization to see an effect on phototransduction of 60,000 Rh\textsuperscript{*} in 30 to 60 minutes, we have 60,000/3.6 × 10\textsuperscript{8} = 0.00017 Rh/rod (2 Rh\textsuperscript{*} every 10,000 Rh); considering our upper limit of 500,000 Rh\textsuperscript{*} in approximately 6 minutes we have the following: 500,000/3.6 × 10\textsuperscript{8} = 0.00014 Rh/rod (1.4 Rh\textsuperscript{*} every 1000 Rh). Therefore, we obtain in the former case 4 bP23H Rh\textsuperscript{*} per disc (0.00017 Rh/rod × 2.3 × 10\textsuperscript{7} bP23H Rh/disc), in the latter 32 bP23H Rh\textsuperscript{*} per disc (0.00014 Rh/rod × 2.3 × 10\textsuperscript{7} bP23H Rh/disc).

Results

X. laevis Carrying the bP23H Transgene Have a Rapid Light-Induced RD

As previously reported,\textsuperscript{16–18,26} X. laevis tadpoles expressing bP23H rhodopsin show no detectable RD when reared in complete darkness for up to 10 weeks. However, when exposed to 1700-lux cyclic light for 1 week, RD occurs rapidly (Fig. 1A). Our transgenic line expresses bP23H rhodopsin in rods uniformly throughout the retina at 7.5% of the level of...
endogenous rhodopsin\textsuperscript{16} (Supplemental Figs. S1 and S2). Therefore, these animals present a unique opportunity to examine the electrophysiology of nondegenerating rods expressing relatively large quantities of a disease-causing rhodopsin mutant, as well as the initial physiological alterations of rods undergoing RD induced by light.

Dark-Reared \textit{X. laevis} Carrying the \textit{bp23H} Transgene Have Relatively Normal ERG Responses

We compared ERG recordings from \textit{bp23H} and WT tadpoles (Fig. 1B). For animals reared 10 weeks in darkness, \textit{bp23H} recordings (red traces) were similar to WT (black traces). Although B-wave amplitude appeared slightly lower in \textit{bp23H} tadpoles, we found no statistically significant effect of genotype. A-wave amplitudes were similar between the two groups, but smaller in \textit{bp23H} animals at high flash intensities (2-way ANOVA, \(P = 0.006\) for effect of genotype). In contrast, after 1 week in cyclic light, A- and B-wave amplitudes were markedly different between genotypes (effect of genotype for B-wave, \(P = 1.2 \times 10^{-12}\), A-wave, \(P = 5.6 \times 10^{-8}\) consistent with significant RD.

Conversion From Arbitrary Units to Photoisomerizations Through Single-Photon Analysis

For single-cell photoresponse recordings, we calibrated our laser illumination from arbitrary units (AU) to induced photoisomerizations (Rh\textsuperscript{+}) per rod by performing single-photon analysis\textsuperscript{2} of mutant (Figs. 2A–C) and WT rods (Figs. 2D–F) obtained from tadpoles and froglets kept in constant darkness and WT rods obtained from adults kept in cyclic light (Figs. 2G–I) and estimated the number of rhodopsin molecules activated by a given flash intensity.\textsuperscript{4} We established that our dimmest flash of 50 AU, with a duration of 10 ms, generated 1.5 to 2.5 photoisomerizations with a mean response amplitude of approximately 0.5 pA. We use this conversion throughout the manuscript (see Methods). Our \textit{X. laevis} single-photon response is slightly lower than previously reported and both estimates are lower than reported for adult \textit{Bufo} rods.\textsuperscript{2,37,38}

Transgenic \textit{X. laevis} Reared in Darkness Have Almost Normal Phototransduction Machinery

We carried out single-cell suction-electrode recordings from isolated rods from WT (Fig. 3A) and \textit{bp23H} (Fig. 3B) tadpoles reared in darkness.

Isolated WT and \textit{bp23H} rods responded to flashes of increasing intensity with a decrease of the circulating current where the maximal amplitude reflects the size of the dark current (Figs. 3C, 3D) as previously described for adult \textit{X. laevis} rods.\textsuperscript{31} The maximal amplitude (I\textsubscript{max}) varied between developmental stages (Figs. 3C, 3D and the Table) as previously observed in WT \textit{X. laevis} rods\textsuperscript{37,38} and between WT and \textit{bp23H} rods. For a given developmental stage, \textit{bp23H} rods produced 35% lower maximal photoresponses than WT rods. When normalized to their maximal amplitude (Fig. 2E), the rising phases of WT and \textit{bp23H} photoresponses (black versus red traces) were superimposable (red traces) and had a similar time course and time to peak (Fig. 3F and the Table).

An analysis of WT and mutant rod geometry showed that WT OS were 11 \textmu m longer and 0.5 \textmu m wider than \textit{bp23H} OS (Table). Therefore, the ratio of the OS surface area to \textit{bp23H} and WT rods is approximately 0.6, in good agreement with the maximal photoresponse ratio. We also compared the relation between the normalized photoresponse amplitude (R/R\textsubscript{max}) and light intensity (Fig. 3H), fitted by a Michaelis-Menten equation (see the Table). The I\textsubscript{1/2} value for \textit{bp23H} was half that of WT, indicating reduced sensitivity. The relation between the maximal amplitude R\textsubscript{max} and OS length was approximately linear for both \textit{bp23H} and WT rods (Fig. 3H).
We also recorded photoresponses from tadpoles maintained in a single 12-hour light/12-hour dark cycle. WT rods displayed normal morphology and responses (Figs. 3I, 3K), whereas the responses and morphology of the (very few) remaining bP23H rods were altered (Figs. 3J, 3L).

We investigated the rising (Fig. 4A) and falling phases (Fig. 4B) of photoresponses from WT and bP23H rods (black versus red traces) following normalization to the maximal photoresponse. The photocurrent rapidly decreased (rising phase) after the application of bright flashes, with matching kinetics for WT and bP23H (Fig. 4A). In addition, the time course of the falling phase for WT and bP23H was very similar (Fig. 4B). We also compared the initial phase of light adaptation during exposure to either repetitive flashes or longer pulses.39 Time courses of photoresponses to 10 consecutive flashes separated by 1 and 2 seconds was remarkably alike (Figs. 4C, 4D). Complementary results were observed when 20-second pulses were considered (Fig. 4E). Additionally, we observed a delayed photoresponse decline associated with the onset of light adaptation in both WT and bP23H rods, indicating the initial steps of light adaptation were congruent (Fig. 4G).

The Phototransduction Machinery in bP23H Rods is Impaired Following Saturating Light Exposures

We know that RD occurs if bP23H transgenic animals are reared in light, but how many photons are necessary to trigger degeneration? Is the phototransduction machinery impaired before rod collapse? We exposed WT and bP23H rods to light and estimated the number of Rh* while recording photoresponses. In the majority of WT recordings, photoresponse amplitudes were stable over 30 to 60 minutes. In some cases, it was possible to record photoresponses with the same maximal amplitude for 1 hour or longer. Figure 5A shows an initial WT photoresponse to a bright flash (black trace) and responses after 47, 74, and 128 minutes (gray and pale gray traces). The morphology of the rod did not change significantly during recording (see inset of Fig. 5A and Figs. 6A–D). Figure 5B shows a similar experiment with a bP23H rod: in contrast to WT, the photoresponse was prolonged after approximately 60 minutes of continuous recording (compare red and dark red traces). With time, the photoresponse amplitude declined and the time course was further prolonged (red and pale red traces in Fig. 5B). Again, the morphology did not change appreciably (see Figs. 5B, inset,
FIGURE 3. Comparison of photoresponses of WT and bP23H rods. (A, B) Isolated rods from a WT and a bP23H X. laevis tadpole, respectively. (C) A family of photoresponses to diffuse light of increasing intensity for the WT rod in (A). The maximal amplitude of current is 8.7 pA for a flash inducing 150 to 250 Rh*. The flashes were applied at the time indicated by the black bar and had a duration of 10 ms. The light intensity produced by the laser was equivalent to 6 to 10, 15 to 25, 30 to 50, 60 to 100, and 150 to 250 Rh*, respectively. The photoresponses of WTs' rod had a maximal amplitude of $6.5 \pm 0.5$ pA ($n = 2$) at stage 49 to 54, $7.0 \pm 0.7$ pA ($n = 7$) at stage 55 to 59 and $13.4 \pm 1.1$ pA ($n = 6$) starting from the stage...
and 6E–H). We compared variation in amplitude (Fig. 5C) and duration (Fig. 5D) of the maximal response with time for WT (black dots, n = 5) and bP23H rods (red triangles, n = 3): bP23H photoresponses declined and were prolonged after approximately 60 minutes. We also averaged photoresponses (n = 7) from different bP23H rods at the beginning of recording (dark red) and after tens of minutes (red trace in Fig. 5E). This averaging confirmed decreased amplitude and prolongation of the photoresponse.

We observed prolongation of bP23H rod photoresponses after tens of minutes and the occurrence of between 6 ± 0.2 pA (n = 3) at stage 49 to 54, 4.9 ± 0.5 pA (n = 4) at stage 55 to 59 and increased to 7 ± 1.2 pA (n = 7) pA starting from the stage 66 (froglets-adult stage) of development. (E) Superimposition of normalized photoresponses in (C) (black) and in (D) (red). (F) Superimposition of normalized dim flashes, eliciting 6 to 10 Rh*, from 10 WT and 8 bP23H rods (developmental stage 49–60). (G) Comparison of the relation between the normalized amplitude of photoresponses (R/R_{max}) and light intensity (I) for WT (black circle: n = 3–10) and bP23H (red triangles: n = 3–8). The data were fitted with the equation R/R_{max} = I/(I + I_{1/2}), where I_{1/2} is equivalent to 10 to 16 Rh* ± 1 for WT (black line) and to 16 to 27 Rh* ± 2 for mutants (red line). (H) Relation between OS length and saturation current for WT (black circles; n = 10) and bP23H (red triangles; n = 8). The data were fitted with a linear regression R_{sat} = c*I, with a value of 0.13 and 0.11 of c for WT and bP23H rods, respectively. (I, J) Isolated rods from a WT and a bP23H X. laevis tadpoles that were exposed to a single 12-hour light/12-hur dark cycle. (K) Family of photoresponses from the rod in (I) elicited by 6 to 10, 15 to 25, 30 to 50, 60 to 100, and 150 to 250 Rh*, respectively. The maximal amplitude of current is 6.9 pA for a flash inducing 150 to 250 Rh*. In (A, B, I, J) the scale bars represent 10 μm.

Figure 4. Comparison of rising/falling phases of bP23H and WT photoresponses. Analysis of light-adaptation response to repetitive flashes and step of light, WT and bP23H rods. (A) Expanded scale for families of selected photoresponses to normalized diffuse light (Fig. 1E) of increasing intensity of a WT (black) and bP23H rods (red) showing the rising phase. The histograms represent the collective data for the activation rate of the saturating responses of bP23H (n = 4) and WT (n = 7) tadpoles at stages 55 to 59. (B) The same as in (A) but for the falling phase. Black and red continuous lines are exponential decay fittings with the equation y = ae^{-bs}. Coefficient a was 3.1 for the bP23H fitting and 2.3 for the WT. (C) Averaged photoresponses evoked from 10 identical nonsaturating light flashes with a duration of 10 ms, equivalent to 150 to 250 Rh* applied with a time interval (Δt) of 1 s for the WT (black traces n = 7) and for the bP23H (red traces n = 3). (D) As in (C) but for photoresponses evoked with a Δt of 2 s (black traces n = 6 and red traces n = 7). (E) Example of photopresponse to a step of light with a duration of 20 s in nonsaturating light conditions for WT (black traces) and bP23H (red traces) equivalent to 15 to 25 Rh*/sec and 30 to 50 Rh*/sec, respectively.
Early Electrophysiological Changes in RP

### TABLE

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Condition</th>
<th>OS, μm</th>
<th>Rod Length, μm</th>
<th>Diameter, μm</th>
<th>T &lt;sub&gt;peak&lt;/sub&gt;, ms</th>
<th>T &lt;sub&gt;longer&lt;/sub&gt;, ms</th>
<th>S &lt;sub&gt;dark&lt;/sub&gt; 1/Rh&lt;sup&gt;*&lt;/sup&gt;</th>
<th>I &lt;sub&gt;1/2&lt;/sub&gt;, ph</th>
<th>I &lt;sub&gt;dark&lt;/sub&gt;, ph</th>
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<tbody>
<tr>
<td>WT</td>
<td>Rod saturating</td>
<td>51.4 ± 4.7 (2)</td>
<td>53.1 ± 4.2 (3)</td>
<td>34.2 ± 4.2 (2)</td>
<td>25.3 ± 5.1 (2)</td>
<td>23 ± 3 (6)</td>
<td>10 to 16 (7)</td>
<td>5.7 to 10 (7)</td>
<td>10.6 ± 2 (7)</td>
</tr>
<tr>
<td>bP23H</td>
<td>Rod saturating</td>
<td>40.2 ± 1.2 (3)</td>
<td>33.6 ± 1.2 (3)</td>
<td>4.2 ± 0.4 (3)</td>
<td>4.2 ± 0.4 (2)</td>
<td>4.2 ± 0.4 (2)</td>
<td>16 to 27 (6)</td>
<td>2.1 to 3 (6)</td>
<td>3.5 ± 10 (7)</td>
</tr>
<tr>
<td>WT</td>
<td>Cyclic light</td>
<td>51.4 ± 4.7 (2)</td>
<td>53.1 ± 4.2 (3)</td>
<td>34.2 ± 4.2 (2)</td>
<td>25.3 ± 5.1 (2)</td>
<td>23 ± 3 (6)</td>
<td>10 to 16 (7)</td>
<td>5.7 to 10 (7)</td>
<td>10.6 ± 2 (7)</td>
</tr>
<tr>
<td>bP23H</td>
<td>Cyclic light</td>
<td>40.2 ± 1.2 (3)</td>
<td>33.6 ± 1.2 (3)</td>
<td>4.2 ± 0.4 (3)</td>
<td>4.2 ± 0.4 (2)</td>
<td>4.2 ± 0.4 (2)</td>
<td>16 to 27 (6)</td>
<td>2.1 to 3 (6)</td>
<td>3.5 ± 10 (7)</td>
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Idark, light-saturated response; I <sub>1/2</sub>, light intensity necessary to obtain a response that is half of the saturating response; S <sub>dark</sub> is the fractional sensitivity of the normalized dim flash response with an amplitude of approximately 0.2 of the I <sub>dark</sub> response, calculated as the amplitude of the dim flash response divided by its strength and then normalized for the dim flash amplitude (with amplitudes of ~0.2 I <sub>dark</sub>). NSF, not significant.

* <i>p</i> < 0.001, compared with WT values.
† <i>p</i> < 0.05, compared with WT values.
‡ <i>p</i> < 0.01, compared with WT values.

### DISCUSSION

The present manuscript reveals three major and novel findings in rods bearing the P23H mutation, the leading cause of RP. First, if X. laevis tadpoles carrying the rhodopsin P23H mutation are reared in darkness, mutant rods have functional and almost normal phototransduction machinery. Second, when exposed to a bright light lasting 1 second (Fig. 4), their photoreponses are irreversibly prolonged. Third, when light exposure is maintained for 12 minutes (Fig. 5), significant OS shedding occurs and RD is initiated. Therefore, the first step leading to RD is the prolongation of photoreponses associated with the photoisomerization of 6 × 10<sup>4</sup> to 5 × 10<sup>5</sup> Rh<sup>*</sup>, equivalent to 4 to 32 P23H rhodopsins per disc. However, initiation of dramatic morphologic changes identifiable by light microscopy, such as OS shedding, requires 1 to 2 orders of magnitude greater Rh<sup>*</sup>. How can we account for this extreme sensitivity? While previous phototransduction models were based on freely diffusing rhodopsin, recent studies indicate that rhodopsins—and presumably bP23H rhodopsin—are organized in discs along parallel tracks of dimers.12-15 Signal amplification requires multiple diffusion encounters, likely involving rhodopsins anchored in tracks of dimers while other phototransduction components diffuse more freely.12,46 The photoreponse prolongation observed in bP23H rods is...
Figure 5. Comparison of the photoresponses of WT and bP23H rods to prolonged or acute exposure of saturating light (developmental stage 60-66). (A) Examples of photoresponses evoked by identical saturating flashes (100k AU, equivalent to $3 \times 10^3$ to $5 \times 10^3$ Rh*) with a duration of 10 ms applied at the beginning of the experiments (black traces) and at specific time interval (gray traces) for a WT rod. In the inset, the correspondent isolated rod from WT X. laevis froglet at the beginning (0 min) and at the end (128 min) of the experiment. (B) Examples of photoresponses evoked by identical saturating flashes as in (A), applied at the beginning of the experiments (dark red traces) and at specific time interval (red traces) for a bP23H rod. In the inset, the correspondent isolated rod from bP23H rod X. laevis froglet at the beginning (0 min) and at the end (96 min) of the experiment. (C) Plot representing the variation of maximal current amplitude (in pA) in relation to different time along the experiment (WT and bP23H rods, $n = 3$). (D) Plot representing the elongation of saturation time (in pA) in relation to different time along the experiment (WT and bP23H rods, $n = 3$). (E) Averaged photoresponses for the bP23H rods at the beginning (dark red trace, $n = 7$) and at the end (light red trace, $n = 7$) of the experiment. (F) Example of photoresponses of WT rods evoked by identical saturating flashes (equivalent to $3 \times 10^3$ to $5 \times 10^3$ Rh*) with a duration of 10 ms applied before (pre) and after (post) a step (in the middle) with a duration of 1 second (equivalent to $3 \times 10^5$ to $5 \times 10^5$ Rh*). (G) Superimposition of photoresponses of WT rods evoked by identical saturating flashes (equivalent to $3 \times 10^3$ to $5 \times 10^3$ Rh*) with a duration flash of 10 ms applied at the beginning (pre), in the middle (post) and at the end of the experiment (last). (H) The same as in (F) but for a bP23H rod. (I) The same as in (G) but for bP23H rod. In all insets scale bar, 10 μm.

Figure 6. bP23H rods display the same morphology during multiple saturating light stimulations. (A–D) Monitoring of a WT rod morphology through time (same rod as in Fig. 5A). The traces represent examples of photoresponses to saturating flashes of light equivalent to $3 \times 10^3$ to $5 \times 10^3$ Rh*, shortly before/after the associated pictures. (E–H) Monitoring of a bP23H rod morphology through time (same rod as in Fig. 5B). The traces represent examples of photoresponses to saturating flashes of light equivalent to $3 \times 10^3$ to $5 \times 10^3$ Rh*, shortly before/after the associated pictures.
consistent with impaired rhodopsin shutoff. Transducin GTPase, arrestin, rhodopsin kinase, and RGS9 are involved in phototransduction shutoff and could have roles in this impairment.

The residue P23 is located on the intradiscal face of rhodopsin in the N-terminal domain of the protein. The majority of the bP23H rhodopsin that evades quality control and reaches the OS is proteolytically processed to remove a portion of the N-terminal domain. This portion includes the N-terminal glycosylation sites, (most likely) the P23H residue itself, and a hydrophobic cluster that is essential for the structural stability of the entire protein. Therefore, OS bP23H rhodopsin is structurally destabilized, either by insertion of a missense amino acid, or removal of the N-terminus.

Structural destabilization of bP23H rhodopsin could result in aggregation or alterations of track structures that trap activated transducin, or prevent normal interactions of cascade shutoff proteins, effectively impairing not only transduction initiated by bP23H rhodopsin, but also transduction initiated by WT rhodopsins in the same or adjacent tracks. Another possibility is that an alternate bP23H rhodopsin structure is induced by interaction with WT meta-II rhodopsin. Thus, it is possible that upon light exposure, bP23H rhodopsin adopts an alternate conformation that alters not only the structure of the N-terminus, but also the domains that interact with signal transduction components. Under these conditions, a small impairment of the shutoff of bleached mutant P23H rhodopsins could lead to a noticeable prolongation of the photoreponse as experimentally observed (Fig. 4).

A rod-saturating exposure lasting 12 minutes induces OS shedding in mutant bP23H rods but not in WT rods (Fig. 5). Based on two different calculations, the number of Rh* leading to OS shedding in bP23H rods ranges from $7.2 \times 10^6$ to $2.7 \times 10^7$ per rod, or 460 to 1610 bleached bP23H rhodopsins per...
disc, which could aggregate with other rhodopsins, possibly altering local membrane fluidity and slowing local phototransduction shut off. Indeed, the specific organization of rhodopsins in tracks of dimers is likely to be compromised by the misfolding of very few, and possibly even 4 to 52, bleached bP23H rhodopsin, while the presence of some hundreds to thousands of misfolded P23H rhodopsins dramatically disrupts OS architecture and could lead to OS shedding.

Single-cell recordings from photoreceptors in animal models of RD are rare because of difficulty in acquiring the data. In 2-week-old mutant P23H mice reared in a standard light cycle, it is difficult to identify rod OS even at the retinal margin because of severe RD. However, X. laevis models of RP are well suited to this purpose due to the larger size of their rods, and the availability of several RP models in which RD can be dramatically limited by dark rearing.

Light-exacerbated RD has been reported in P23H rats and fruit flies, and is strongly suggested by the sector RP phenotype reported in human patients. Similar to P23H X. laevis, expression levels of P23H rhodopsin in knockin mice is low due to a biosynthetic defect, and the accumulation of mutated rhodopsin in the ER has been associated with cell stress, activation of the unfolded protein response, the activation of the ubiquitination system and ER-associated degradation. While it is generally thought that ER stress-based mechanisms are responsible for the resulting RD, exacerbating ER stress by light exposure should require timeframes that would allow biosynthesis of significant quantities of rhodopsin, rather than the rapid timeframes of seconds to minutes seen in our study. Interestingly, pharmacologic treatment of P23H mice with metformin, which based mechanisms are responsible for the resulting RD, exacerbating ER stress by light exposure should require timeframes that would allow biosynthesis of significant quantities of rhodopsin, rather than the rapid timeframes of seconds to minutes seen in our study. Interestingly, pharmacologic treatment of P23H mice with metformin, which promotes delivery of P23H rhodopsin to the OS, exacerbates RD. Therefore, OS-based mechanisms may contribute to cell death in both X. laevis and other species, and may be favored under certain conditions. Moreover, results consistent with abnormal rod responses have been observed in RP patients carrying the P23H mutation or other mutations causing sector RP, including an altered and prolonged dark-adaptation phase after the light stimuli, suggesting an altered recovery phase of rod response. Similar findings were reported regarding ERG measurements obtained from P23H (VPP) transgenic mice. It will be interesting to determine whether the effects we observed are common to multiple mutations or unique to P23H rhodopsin.

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