Molecular Properties of Rhodopsin and Rod Function*

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Hiroo Imai†1,2, Vladimir Kefalov3,1,2, Keisuke Sakurai†1, Osamu Chisaka†, Yoshiki Ueda†4, Akishi Onishi†5, Takefumi Morizumi†, Yingbin Fu§, Kazuhisa Ichikawa**, Kei Nakatani‡6, Yoshito Honda**6, Jeannie Chen§§, King-Wai Yau†, and Yoshinori Shichida†7

From the †Department of Biophysics, Graduate School of Science, Kyoto University and Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Kyoto 606-8502, Japan, the ‡Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, the Departments of §Cell and Developmental Biology, Graduate School of Biostudies, and ††Ophthalmology and Visual Science, Graduate School of Medicine, Kyoto University, Kyoto 606-8502, Japan, the **Department of Brain and Bioinformation Science, Kanazawa Institute of Technology, Ishikawa 924-0838, Japan, the ‡‡Graduate School of Life and Environmental Sciences, University of Tsukuba and Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Ibaraki 305-8572, Japan, and the §§The Mary D. Allen Laboratory for Vision Research, Doheny Eye Institute, Keck School of Medicine, University of Southern California, Los Angeles, California 90033

Signal transduction in rod cells begins with photon absorption by rhodopsin and leads to the generation of an electrical response. The response profile is determined by the molecular properties of the phototransduction components. To examine how the molecular properties of rhodopsin correlate with the rod-response profile, we have generated a knock-in mouse with rhodopsin replaced by its E122Q mutant, which exhibits properties different from those of wild-type (WT) rhodopsin. Knock-in mouse rods with E122Q rhodopsin exhibited a photosensitivity about 70% of WT. Correspondingly, their single-photon response had an amplitude about 80% of WT, and a rate of decline from peak about 1.3 times of WT. The overall 30% lower photosensitivity of mutant rods can be explained by a lower pigment photosensitivity (0.9) and the smaller single-photon response (0.8). The slower decline of the response, however, did not correlate with the 10-fold shorter lifetime of the meta-II state of E122Q rhodopsin. This shorter lifetime became evident in the recovery phase of rod cells only when arrestin was absent. Simulation analysis of the photoresponse profile indicated that the slower decline and the smaller amplitude of the single-photon response can both be explained by the shift in the meta-I/meta-II equilibrium of E122Q rhodopsin toward meta-I. The difference in meta-III lifetime between WT and E122Q mutant became obvious in the recovery phase of the dark current after moderate photobleaching of rod cells. Thus, the present study clearly reveals how the molecular properties of rhodopsin affect the amplitude, shape, and kinetics of the rod response.

Light absorption by rhodopsin in rod photoreceptor cells results in the activation of a G protein-mediated signal transduction cascade that eventually generates an electrical response (1). The key proteins in this cascade have been identified, and their molecular properties as well as interactions with each other have been extensively investigated (2, 3). A current question is how well these properties and interactions correlate with the response profile of the photoreceptor cells. Although the gene knock-out approach has been very useful in addressing this question for the proteins rhodopsin kinase and arrestin (4, 5), this strategy is less appropriate for rhodopsin and G protein, because the deletions of these proteins eliminated the light response (6, 7). The gene knock-in approach is an alternative way, with a mutant protein replacing the wild-type (WT) version. The maintenance of the same expression level of the protein in this procedure is important, because the interpretation can be difficult otherwise. For example, the photoresponse profile is altered when the rhodopsin content in rods is halved (8, 9).

Our past work on comparing rhodopsin and cone pigments (10) has shown that their photosensitivities are not so different, but the meta-II (the G protein-activating state), as well as the subsequent meta-III, intermediates of cone pigments exhibit faster decay than those of rhodopsin. In addition, the equilibrium between meta-II and its precursor, meta-I, is different between rhodopsin and cone pigments (11). Finally, we have found that the amino acid residues at positions 122 and 189 underlie these differences in molecular properties (12, 13). Thus, to obtain insight into how the molecular properties of rhodopsin correlate with the photoresponse profile of rod cells, and to elucidate the differences in response properties between rods and cones, we have generated a knock-in mouse line in

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†† These authors contributed equally to this work.

‡‡ Present address: Dept. of Cellular and Molecular Biology, Primate Research Institute, Kyoto University, Aichi 484-8506, Japan.

§§ Present address: Dept. of Ophthalmology and Visual Sciences, Washington University School of Medicine, St. Louis, MO 63110.

### References

1. These authors contributed equally to this work.

2. Present address: Dept. of Cellular and Molecular Biology, Primate Research Institute, Kyoto University, Aichi 484-8506, Japan.

3. Present address: Dept. of Biophysics, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan.

4. Present address: Dept. of Ophthalmology and Visual Sciences, Washington University School of Medicine, St. Louis, MO 63110.

5. Present address: Dept. of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD 21205.

6. Present address: Osaka Red Cross Hospital, Osaka 543-5111, Japan.

7. To whom correspondence should be addressed. Tel.: 81-75-753-4213; Fax: 81-75-753-4210; E-mail: shichida@vision-kyoto-u.jp.

8. The abbreviations used are: WT, wild type; ROS, rod outer segment.
which E122Q rhodopsin replaced WT rhodopsin in the rod cells.

Mutation of the Glu122 residue in chicken rhodopsin into the corresponding residue in cone pigments results in a faster decay of meta-II, a shift of the meta-I/meta-II equilibrium toward meta-I (12, 14), and a faster regeneration of the pigment (15). Thus, this point mutant recapitulates certain cone-pigment-like properties in rhodopsin. Residue 122 is located near the β-ionone ring of the chromophore in the helical core of the visual pigment (16), and its substitution has little effect on the interaction of the pigment with other proteins, such as G protein. More importantly, the replacement of only one codon in the native rhodopsin gene without disturbing the exon-intron arrangement should give similar transcription and translation as WT. This knock-in mouse allows us to compare the molecular properties of WT and E122Q rhodopsin in the same native environment, i.e. membranes of the rod outer segment (ROS). Our findings reported here clearly show that several of the rhodopsin molecular properties, such as absorption spectrum, photosensitivity, shift in the meta-I/meta-II equilibrium, and meta-III decay, do correlate well with the rod response profile and the recovery of rod sensitivity after a bleaching light. The meta-II decay also correlates well with the decline phase of the rod response when arrestin is absent.

**EXPERIMENTAL PROCEDURES**

**Generation of Rhodopsin-mutant Mouse**—Mutant mice with E122Q rhodopsin were generated as previously reported (17, 18). Briefly, the mouse rhodopsin genomic clones were modified by replacing Glu122 (GAA) with Gln (GAG) in the second exon and introducing the positive selection marker (PGKneoA) into the first intron in the reverse direction (Fig. 1A). The E122Q-positive ES cells were screened from electroporated R1 ES cells derived from the 129/Sv x 129Sv-CP mouse line, and chimeric mice were generated by injecting them into C57BL/6J blastocysts, which were subsequently implanted into the uteri of pseudo-pregnant foster females. The chimeras were mated with C57BL/6J mice to generate heterozygous mice that were then inbred to generate homozygous mice. The targeted allele has been maintained in both 129/SvJ and C57BL/6J backgrounds according to the guidelines for animal experiments at Kyoto University.

**Estimation of Protein Expression Level**—Protein expression levels were estimated by spectroscopy and Western blotting. Retinas were isolated from mouse eyes, homogenized, and extracted by buffer A (1% dodecylmaltoside, 50 mM HEPES, 140 mM NaCl, 3 mM MgCl2, pH 6.5) for spectroscopy or by SDS-PAGE sample buffer (8% SDS, 0.125 M Tris/Cl (pH 6.8), 20% glycerol, 5% mercaptoethanol) for Western blotting. The concentration of pigment in the extract solubilized by buffer A was estimated from the maximum absorbance in the difference spectrum before and after irradiation with >480 nm light in the presence of 100 mM hydroxylamine. For calculations, we used molar extinction coefficients of both rhodopsin and E122Q rhodopsin. Molar extinction coefficients of rhodopsin and E122Q rhodopsin at their absorption maxima (502 and 487 nm, respectively) are 40,200 ± 2,100 and 44,800 ± 2,200 M$^{-1}$ cm$^{-1}$, respectively, which were estimated using a method previously described (19) and the acid-denaturing method (20). These values are in good agreement with those estimated previously for bovine rhodopsin (21, 22). The expression levels for WT and E122Q rhodopsins, transducin, and rhodopsin kinase were also estimated by Western blotting with antibodies against rhodopsin (1D4), transducin (23), and rhodopsin kinase (sc-561, Santa Cruz).

**Histology**—Eyes were removed and fixed for 4 h at 4 °C in 4% paraformaldehyde in phosphate-buffered saline. After cryoprotection in cold 30% sucrose, the tissue was mounted with Tissue-Tek OCT and sectioned at 10-μm thickness along the vertical meridian passing through the optic nerve. Staining was with 1% toluidine blue O.

**Spectroscopy of Pigments in Native ROS Membranes**—ROS membranes were isolated by a conventional sucrose flotation method (24) from retinas of mice dark-adapted for 12 h. The membrane fractions were suspended in buffer B (112.5 mM NaCl, 3.6 mM KCl, 2.4 mM MgCl2, 1.2 mM CaCl2, 10 mM HEPES (pH 7.4)) and sonicated. Absorption spectroscopy was performed as previously reported on a Shimadzu MPS-2000 or UV-2400 spectrophotometer (25), and a specially constructed CCD spectrophotometer (26). The sample temperature was regulated by thermostat to within 0.1 °C in the cell holder of the spectrophotometer. Light sources for sample irradiation were a 1-kW tungsten halogen lamp (Rikagaku Seiki) and a short arc power flash (Nissin Electronic Co., pulse duration = 350 μs), which were attached in the Shimadzu spectrophotometer and the CCD spectrophotometer, respectively. A glass cut-off (VY50, VY52, or VY54; Toshiba) or an interference (500 nm; Nihon Shinku) filter was used for selection of the irradiation wavelengths. Details of procedures for specific measurements including irradiation conditions are described in the figure legends.

**Single-cell Recordings**—WT and homozygous E122Q mice were housed in a 12/12-h light-dark cycle. Mice lacking arrestin (Arr$^{−/−}$) (4), including E122Q, Arr$^{−/−}$ mice, were reared in constant darkness. Animals raised in a light-dark cycle were dark-adapted overnight before the experiment. Tissue preparation and recording techniques followed procedures described elsewhere (27). Briefly, an animal was euthanized by CO2 asphyxiation/cervical dislocation and the eyes were removed under dim red light. All further manipulations were performed under infrared light. The retinas removed from the eyes were chopped into small pieces with a razor blade. Small pieces of the retina were placed in the experimental chamber on the stage of an inverted microscope and perfused with bicarbonate-buffered solution (112.5 mM NaCl, 3.6 mM KCl, 2.4 mM MgCl2, 1.2 mM CaCl2, 10 mM HEPES (pH 7.4), 20 mM NaHCO3, 3 mM sodium succinate, 0.5 mM sodium glutamate, 0.02 mM EDTA, and 10 mM glucose). The solution was bubbled with 95% O2, 5% CO2, and warmed to 36–38 °C in a flow heater (28) before it entered the experimental chamber. Membrane current was recorded with a suction electrode from an ROS projecting from a piece of retina. The recording electrode was filled with 140...
RESULTS AND DISCUSSION

Generation of E122Q Knock-in Mice—WT rhodopsin was replaced by its E122Q mutant in mouse by using gene targeting (Fig. 1, A and B). The homozygous animals were viable and fertile. The expression level of E122Q rhodopsin in these animals was indistinguishable from that of WT rhodopsin (Fig. 1C), and the levels of transducin, rhodopsin kinase, and green-sensitive cone opsin were also indistinguishable (data not shown). The homozygous animals with the positive selection marker (PGKneopA) still in the genome had an expression level of visual pigment about 72% of that of WT animals (Fig. 1C) but their overall retinal structure, especially that of ROS, appeared to be normal under light microscopy (Fig. 1D). The numbers of photoreceptor nuclei in WT and mutant mice were almost identical. That is, rows of photoreceptor nuclei in WT and mutant mice were 10.8 ± 1.1 and 10.2 ± 0.9, respectively, at 10 weeks of age (n = 20 from 3 animals for each genotype). The lengths of outer segment were also indistinguishable between WT (18 ± 0.8 μm) and E122Q mutants (19 ± 1.6 μm).

Molecular Properties of WT and E122Q Rhodopsins—We first asked whether the E122Q mutant rhodopsin in mouse ROS exhibited molecular properties different from those of WT, as might be expected from our previous heterologous expression experiments with the same mutation in chicken rhodopsin (12).

The results are summarized in Fig. 2 and Table 1. The absorption maximum of E122Q rhodopsin extracted from ROS with buffer A was about 15 nm blue-shifted from that of the corresponding WT rhodopsin (Fig. 2A), similar to the findings with bovine (14, 21, 22, 34, 35), chicken (15), and mouse (11) E122Q rhodopsins expressed in culture cells. The photosensitivity of E122Q rhodopsin at 500 nm was 0.92 ± 0.01 of WT (Fig. 2B, Table 1). Whereas the molecular extinction coefficient at λmax was higher for E122Q rhodopsin compared with WT rhodopsin, at 500 nm the two pigments had very similar extinction coefficients (Fig. 2A and see “Experimental Procedures”). Thus, the smaller photosensitivity of E122Q rhodopsin at 500 nm indicates that E122Q rhodopsin had a quantum yield lower than that of WT rhodopsin. We next investigated the thermal equilibrium between meta-I and meta-II after irradiating WT and E122Q rhodopsins in ROS membranes. We found that the equilibrium was significantly shifted toward meta-I in E122Q rhodopsin compared with WT (Fig. 2, C and D). Under the conditions described in the legends of Fig. 2, C and D, the ratio of meta-I to meta-II in E122Q rhodopsin was 34:66, while that in WT was 18:82. These values were estimated by fitting the respective spectrum obtained at 100 ms after 500 nm irradiation of each pigment with a linear combination of the spectra.

mm NaCl, 3.6 mm KCl, 2.4 mm MgCl₂, 1.2 mm CaCl₂, 3 mm HEPES (pH 7.4), 0.02 mm EDTA, and 10 mm glucose. 20-ms flashes were delivered from a calibrated light source via computer-controlled shutters. Light intensity and wavelength were set by using calibrated neutral density and interference filters. The current was amplified, low-pass filtered at 30 Hz, digitized at 1 kHz, and stored on a computer for subsequent analysis.

Simulation of Rod Photoresponses—The rod-response profile was simulated by the described method previously (29) with some modifications. First, the reaction scheme was modified so that transducin can bind to phosphodiesterase at the mole ratio of 1:1. Second, parameters such as rod outer segment morphology (30) and phosphodiesterase deactivation rate constant (31) appropriate for mouse rods were substituted for those for amphibian rods used in the previous model. The reaction model was constructed with the A-cell 5.1 software (32, 33).
Spectroscopic characteristics of WT rhodopsin and E122Q mutant. A, absorption spectra of WT and E122Q rhodopsins. WT and E122Q opsins (25 μM) were prepared in the respective ROS membranes suspended with buffer B (pH 7.4) and reacted with the same amounts of 11-cis-retinal (final concentration of 2.2 μM). After the reactions were completed, the pigments were bleached by a 10-min irradiation of yellow (>480 nm) light by using a Y-50 cut-off filter in the presence of 100 mM hydroxylamine. The difference spectra before and after the irradiation were then calculated. The negative peak at 360–370 nm represents the λ\textsubscript{max} of free all-trans-retinal oxime produced by bleaching. All experiments were performed at 2 °C. B, photosensitivity of wild-type (open squares) and E122Q (closed triangles) rhodopsins extracted from respective ROS membranes with buffer A (pH 6.5). The pigments were then irradiated with a green light (500 nm) at 37 °C for adequate time in the presence of 50 mM hydroxylamine. The amounts of remaining pigments were plotted as a function of total photons exposed. C and D, estimation of the relative amount of meta-I and meta-II in the equilibrium mixtures produced by irradiation of WT (C) and E122Q (D) rhodopsins at 37 °C. WT and E122Q rhodopsins in ROS membranes (curves 1 in C and D) were suspended to buffer B (pH 7.4) and irradiated with a 500-nm light pulse and the spectra 100 ms after the irradiation of the pigments were recorded by the CCD spectrophotometer. Then the spectra (curves 2, dotted curve) in C and D containing only meta-I and meta-II were calculated by subtracting the spectra of residual 11-cis-pigments and photo-regenerated 9-cis-pigments from the spectra recorded. The amounts of 11-cis- and 9-cis-pigments were estimated by simulation of the measured spectra at the wavelength length regions longer than 580 nm (or 560 nm in E122Q sample) with those of 11-cis- and 9-cis-pigments (26, 51). The amounts of 11-cis- and 9-cis-pigments present in the illuminated WT sample were 41.8 and 4.0%, respectively. Those in the illuminated E122Q sample were 41.6 and 4.5%, respectively. The amounts of meta-I and meta-II in the mixtures were estimated by simulating the spectra with template spectra of meta-I and meta-II (26). Solid spectra in C and D are the simulated spectra of meta-I and meta-II present in the mixtures. Individual spectra of meta-I or meta-II in the mixture are also shown in C or D as broken curves. E and F, spectral changes observed in the conversion process of meta-II to meta-III in WT (E) and E122Q (F) rhodopsins. WT and E122Q rhodopsins in ROS membranes were irradiated with a 500-nm light pulse and subsequent spectral changes were monitored by the CCD spectrophotometer. Spectra were recorded 10 and 100 ms, 1, 2, 4, 8, 15, and 30 s, 1, 2, 4, 8, and 15 min after irradiation of the sample in E, and 10 and 100 ms, 1, 2, 4, 8, 15, and 30 s, and 1 min after irradiation of the sample in F. G and H, spectral changes observed in the conversion process of meta-III to retinal plus opsin in WT for meta-I and meta-II obtained independently (26). Within a few seconds after irradiation, E122Q meta-II decayed with a time constant about 10 times faster than that of WT meta-II (Fig. 2, E and F). The decay of E122Q meta-III was also 2.4-fold as fast as WT meta-III (Fig. 2, G and H, see also Fig. 5C).

**TABLE 1**

Parameters for the molecular properties of mouse rhodopsin

| Parameter                      | Wild type | E122Q |
|-------------------------------|-----------|-------|
| λ\textsubscript{max} (nm)      | 502 ± 1.3 (7) | 487 ± 1.4 (7) |
| Rel. photosensitivity at 500 nm | 1.0 | 0.92 ± 0.01 (5) |
| Meta-II decay time constants (s) | 95 ± 15 (7) | 7.7 ± 3.5 (8) |
| Meta-III decay time constants (min) | 17.7 ± 3.0 (3) | 7.4 ± 3.7 (3) |
| Regeneration time constants    | 177 ± 32 (3) | 47 ± 10 (3) |

Proteins were detergent-extracted from dark-adapted eyes and measured at 2 °C. † Proteins remained in ROS membranes that were suspended in buffer B (pH 7.4) at 37 °C. ‡ Proteins remained in ROS membranes that were suspended in buffer B (pH 7.4) at 2 °C.

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**Rhodopsin E122Q Knock-in Mouse**

The action spectra of WT and E122Q rods (Fig. 3) were best fit by modified A1 visual-pigment templates (36) with λ\textsubscript{max} = 496 ± 3 nm (n = 10) for WT and 480 ± 3 nm (n = 6) for E122Q rods, in good agreement with the absorption maxima of the corresponding rhodopsins described earlier (Fig. 2A). At 500 nm, the flash sensitivity, defined as the reciprocal of half-maximal flash strength (1/\text{I}\textsubscript{0}), was lower for E122Q rods, being 0.72 that of WT (Table 2). To derive the single-photon response amplitude, we stimulated a cell with a series of 30–100 identical dim flashes. According to Poisson statistics, the amplitude of the single-photon response can be estimated from the ensemble variance to mean ratio of the response amplitude. The single-photon response amplitude (\text{S}\textsubscript{p}) derived in this way was 0.45 ± 0.03 pA (n = 26) for mutant rods versus 0.56 ± 0.04 pA (n = 16) for WT rods (Fig. 3D and Table 2). The rate of activation of the single-photon response of the E122Q mutant rod is slightly smaller than that of WT. The decline phase of the dim-flash response was notably slower in mutant rods, as reflected by the longer integration time (Fig. 3D and Table 2). These results would be due to the altered molecular properties of rhodopsin by the mutation of E122Q. Thus we tried to simulate the rod response by altering the molecular properties of rhodopsin.

**Simulation of Photoresponse by Chemical Reaction Model**

Can we explain the differences in response characteristics between WT and E122Q rods by the differences between WT and E122Q rhodopsins? E122Q rods exhibited a flash sensitivity at 500 nm about 0.7 times that of WT. This difference can be accounted for by the combined difference in pigment sensitivity at 500 nm (0.9) and difference in the magnitude of the single-photon response (0.8) (0.9 × 0.8 = 0.72). Interestingly, because (G) and E122Q (H) rhodopsins. WT and E122Q rhodopsins in ROS membranes were irradiated with a 500-nm light pulse and subsequent spectral changes were monitored by the CCD spectrophotometer. Spectra were recorded 4, 8, 15, and 30 min and 1 h after irradiation of the sample in G, and 1, 2, 4, 8, 15, 30 min and 1 h after irradiation of the sample in H.
journals/2007/282/i9/pr26680a-02.jpg

![Image of a molecular structure with labels](http://jbc.asm.org/content/282/9/6680/f1.large.jpg)

**FIGURE 3.** Electrophysiological recordings with a suction pipette from single rods of WT and E122Q mice. *A* and *B*, responses from WT (*A*) and homozygous E122Q (*B*) rods. In both cases, 20-ms flashes of a 500-nm light delivering 5.9, 11, 26, 46, 100, 190, 400, and 770 photons μm⁻² were given at the time instant of 0 s. Each trace is the averaged response from multiple flash trials. The records were low-pass filtered at 30 Hz. The maximal response was 12.3 pA for the WT rod and 14.9 pA for the E122Q rod. The E122Q rods were from E122Q mice without the positive selection marker (PKG neo) in the genome. *C*, collected action spectra of WT and E122Q rods. Sensitivity was calculated by dividing the dim-flash response amplitude by the flash intensity. Measurements were made at 450, 460, 480, 500, 510, 530, 550, 600, 650, and 680 nm. Averaged data (± S.E.) for WT (*open squares*; *n* = 9) and E122Q (*closed squares*; *n* = 6) rods. The data for E122Q rods were obtained from the rods of mice with and without the positive selection marker (PKGneoA) in the genome. For data from mice still retaining the marker, the average spectral sensitivity at 500 nm has been scaled to the same value as that for mice without the marker. *D*, single-photon responses of WT and E122Q rods. Thin trace is the averaged single-photon response of WT rods and the thick trace is that of E122Q rods. Averaged amplitude of the single-photon response was 0.56 pA (*n* = 16) for WT rods and 0.45 pA (*n* = 26) for E122Q rods. The amplitude of the single-photon response can be estimated from the ensemble variance-to-mean ratio of the response amplitude (see text). The E122Q rods were from the mice without the positive selection marker in the genome.

**TABLE 2** Parameters for the single-cell response of mouse rods

|                | WT                 | E122Q              |
|----------------|--------------------|--------------------|
| *Iₚₓ* (pA)     | 10.8 ± 0.4 (23)    | 9.8 ± 0.4 (19)     |
| *Iₚₛ* (photons μm⁻²) | 33.9 ± 1.6 (23)    | 46.6 ± 1.6 (19)*   |
| *Sₓ* (pA)      | 0.56 ± 0.04 (16)   | 0.45 ± 0.03 (26)*  |
| *tₚ* (ms)      | 160 ± 3 (16)       | 175 ± 3 (26)*      |
| *tₚ* (ms)      | 309 ± 16 (16)      | 379 ± 22 (26)*     |

*a* *Iₚₓ*, dark current.  
*b* *Iₚₛ* flash strength at 500 nm that gives the half-maximal response.  
*c* Difference from WT being statistically significant, *p* < 0.005.  
*d* *Sₓ*, single-photon response.  
*e* Difference from WT being statistically significant, *p* < 0.05.  
*f* *tₚ*, time to peak.  
*g* *tₚ*, integration time defined as the area of the dim flash response divided by its peak amplitude.

the size of the single-photon response in E122Q mutant was about 0.8 times that of wild-type, whereas the integration time recorded in E122Q mutant was longer (about 1.3 times), the total area under the response (amplitude × time) was quite similar in both cases. Simulation analysis based on a model previously described (29) clearly showed that these differences in the single-photon response between E122Q and WT rods could be accounted for by a change in the meta-I/meta-II equi-librium. In addition, the shift in the meta-I to meta-II equilib-rium used in our simulation correctly predicted the small difference in the rates of activation of the single photon response between WT and E122Q rods (Fig. 3D). The simulation was able to reproduce these observed differences in amplitude and kinetics when meta-II was assumed to be the only intermediate that can be phosphorylated by rhodopsin kinase (Fig. 4, main panel at bottom), but not when only meta-I is phosphorylated or when both meta-I and II can be phosphorylated (Fig. 4, insets A and B at bottom). Indeed, it can be seen that the time to peak and the response recovery are delayed in the E122Q mutant when only meta-II is phosphorylated. Otherwise, they are unchanged when both intermediates are phosphorylated, and are accelerated when only meta-I is phosphorylated.

Based on the simulation, the mutant rhodopsin could be phosphorylated slightly slower than WT due to the small amount of meta-II in the equilibrium state. Therefore, the shut-off of rhodopsin activation by phosphorylation would be less in the mutant. We tried to experimentally assess the phosphorylation efficiency of the E122Q mutant rhodopsin in knock-in mouse ROS, because phosphorylation efficiency affects the rod photosensitivity (37). Preliminary results by using the methods described in Ref. 38 indicated that the pigment was phosphorylated similarly to the WT rhodopsin. However, the difference in kinetics was too small to resolve under our experimental conditions. Although further biochemical experiments with high time resolution (39, 40) would be necessary, it is clear that the difference in the single photon response between WT and E122Q rods can be accounted for by the difference in the meta-I/meta-II equilibrium between WT and E122Q rhodopsins.
Rhodopsin E122Q Knock-in Mouse

Correlation between Meta-II Decay and the Slow Decline of Photoresponse on the Arrestin Knock-out Background—Our experiments and simulation indicated that the slower decline of the dim-flash response of E122Q than WT rods is due to a shift in the meta-I/meta-II equilibrium toward meta-I in E122Q rods. The same experiments also indicated that there is no correlation between the time course of the dim-flash response and the 10-fold faster decay of meta-II of E122Q rhodopsin. This observation is in good agreement with previous results from Xenopus rods expressing transgenic cone pigment (41). The explanation is that the shutoff of active pigment by phosphorylation and subsequent binding of arrestin is fast enough that it precedes meta-II decay even when the latter is accelerated 10-fold by the E122Q mutation. In rods lacking arrestin, on the other hand, the lifetime of meta-II should become visible in the dim-flash response because in this case meta-II decay would be rate-limiting (4). In Fig. 5B, the dim-flash response decline of Arr−/− and E122Q Arr−/− rods had two phases: a fast decline reflecting pigment phosphorylation (4) that was identical in both genotypes, and a slow phase that was indeed 10-fold longer for Arr−/− rods (45.8 ± 3.9 s, n = 3; 38.1 s for the cell in Fig. 5B) as for E122Q, Arr−/− rods (4.9 ± 0.4 s, n = 14; 6.6 s for the cell in Fig. 5B). This good correlation in the WT/E122Q speed ratio between the slow phase of response decline in the arrestin−/− background and the time course of meta-II decay measured spectroscopically (Fig. 5A) provides conclusive evidence that the prolonged decay of the response in arrestin−/− rods does reflect meta-II decay. The 2-fold slower absolute rate of decay of meta-II in ROS membrane compared with that obtained by single-cell recordings, especially evident for WT rhodopsin (83.3-s time constant for isolated membrane in Fig. 5A, 95 ± 15 s, n = 7, versus the above 45.8 s in intact cell), suggests that the decay is more rapid when the pigment is phosphorylated. Indeed, we have previously found that phosphorylation of rhodopsin in vitro does accelerate meta-II decay.10

Similar Time Courses between Meta-III Decay and Dark Current Recovery after a Bleach—The rates of meta-III decay we measured spectroscopically from both WT and mutant pigments also correlated well with the rate of recovery of the post-bleach dark current of the corresponding isolated rod photoreceptors. Fig. 5C shows the time courses of formation and decay of meta-III for WT and E122Q rhodopsins in ROS membrane. The time courses were simulated by sequential two-exponential curves and the decay time constants of meta-III were estimated to be 14.3 min in WT rhodopsin and 6.3 min in E122Q rhodopsin, respectively; collected experiments gave time constants of 17.7 ± 3.0 min (n = 5) for WT and 7.4 ± 3.7 min (n = 3) for mutant. Fig. 5, D and E, show the dark-current recovery from a WT rod and an E122Q rod following essentially identical bleaches (20%). The recovery time courses in both WT and E122Q rods were roughly exponential, with a time constant of 19.5 and 6.8 min, respectively; collected experiments gave time constants of 16.8 ± 2.1 min (n = 5) for WT and 8.3 ± 1.0 min (n = 9) for mutant. Thus, the E122Q mutation resulted in a 2-fold acceleration of the dark-current recovery after a 20% bleach. The similar time courses measured by absorption spectroscopy and single cell recording, whether for WT or mutant rhodopsins, suggest that the dark-current recovery in isolated cells indeed reflects the decay of meta-III.

This observation supports the idea that the decay of photoactivated rhodopsin is one of the mechanisms of photoresponse recovery with meta-III as the key intermediate. There is an equilibrium between meta-II and meta-III (42), so that a certain amount of meta-II is still present before the complete decay of meta-III into opsin and all-trans-retinal. In fact, transducin is still activated even after the complete shift from meta-II to meta-III, measured using spectroscopy (15). A possible mechanism by which the physiologically inactive meta-III could be

10S. Tachibanaki, H. Imai, A. Terakita, and Y. Shichida, unpublished observation.
affecting the phototransduction cascade is by converting back to the physiologically active meta-II form. The resulting equilibrium between meta-II and meta-III would dictate that the activity of the phototransduction cascade would follow the slow decay of meta-III. In addition, recent investigations showed that meta-III can be converted back to meta-II by interaction with transducin and arrestin in the ROS membrane (43, 44). Therefore, a certain amount of meta-III can convert to meta-II (active state) in the photoactivated cells, sustaining activity by competition between transducin and arrestin (45). Because the decay of the meta-III is about 100 times faster for cone pigments than for rhodopsin (46), it is not surprising that cones also recover from a bleach hundreds of times faster than rods (47, 48). In other words, the lifetime of meta-III could govern the difference in kinetics of recovery from bleach between isolated rods and cones.

We also measured the in situ rate of dark adaptation in the animal by exposing mice to intense light that bleached 75% of pigment and then measuring the amount of regenerated rhodopsin extracted from the animals after a variable period in darkness. Interestingly, we found no difference between WT and E122Q mice. They gave time constants of 40 ± 11.4 (WT, n = 4) and 40.4 ± 9.0 min (E122Q, n = 4), both considerably slower than in vitro regeneration (wild-type rhodopsin, 177 ± 32 s (n = 3); E122Q rhodopsin, 47 ± 10 s (n = 3); see Table 1). The regeneration of rhodopsin is closely correlated to the slow phase of dark adaptation, whereas the fast phase is independent of the bleaching of rhodopsin (49).

Thus, the rate-limiting step in dark adaptation after a bright bleaching light appears to reside in the availability of 11-cis-retinal to the rods, due either to the regeneration mechanism in the pigment epithelium or to the delivery of the regenerated chromophore to the rods (45, 50).

In conclusion, we generated a knock-in mouse line whose rod photoreceptors expressed mutant instead of WT rhodopsin, and demonstrated clear correlations between the molecular properties of rhodopsin and the characteristics of the rod response. We found that the shift in the meta-I/meta-II equilibrium affects the peak amplitude of single-photon response and its decay time course, and that the decay rate of meta-II does not limit the termination of the response. Also, the decay of meta-III dominates the recovery of the rod after a bleach. The knock-in mouse represents a new tool to correlate the biophysical properties of the phototransduction proteins and the photoreceptor response properties.

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