Signaling States of Rhodopsin in Rod Disk Membranes Lacking Transducin $\beta\gamma$-Complex

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Purpose. To characterize the possible role of transducin Gt$\beta\gamma$-complex in modulating the signaling properties of photoactivated rhodopsin and its lifetime in rod disc membranes and intact rods.

Methods. Rhodopsin photolysis was studied using UV-visible spectroscopy and rapid scanning spectroscopy in the presence of hydroxylamine in highly purified wild-type and Gtγ-deficient mouse rod disc membranes. Complex formation between photoactivated rhodopsin and transducin was measured by extra-metarhodopsin (meta) II assay. Recovery of dark current and flash sensitivity in individual intact wild-type and Gtγ-deficient mouse rods was measured by single-cell suction recordings.

Results. Photoconversion of rhodopsin to meta I/meta II equilibrium proceeds normally after elimination of the Gt$\beta\gamma$-complex. The meta I/meta II ratio, the rate of meta II decay, the reactivity of meta II toward hydroxylamine, and the rate of meta III formation in Gtγ-deficient rod disc membranes were identical with those observed in wild-type samples. Under low-intensity illumination, the amount of extra–meta II in Gtγ-deficient discs was significantly reduced. The initial rate of dark current recovery after 12% rhodopsin bleach was three times faster in Gtγ-deficient rods, whereas the rate of the late current recovery was largely unchanged. Mutant rods also exhibited faster postbleach recovery of flash sensitivity.

Conclusions. Photoactivation and thermal decay of rhodopsin proceed similarly in wild-type and Gtγ-deficient mouse rods, but the complex formation between photoactivated rhodopsin and transducin is severely compromised in the absence of Gt$\beta\gamma$. The resultant lower transduction activation contributes to faster photoresponse recovery after a moderate pigment bleach in Gtγ-deficient rods. (*Invest Ophthalmol Vis Sci.* 2012; 53:1225–1233) DOI:10.1167/iovs.11-9350

Rhodopsin (Rh), a prototypical member of the G-protein–coupled receptor (GPCR) superfamily, is the visual pigment in rod photoreceptor cells of the retina responsible for dim light vision. Upon light excitation and *cis-trans* isomerization of its chromophore, 11-*cis*-retinal, rhodopsin undergoes several conformational changes leading to the formation of its active state, metarhodopsin (meta) II (meta II, or R*). Meta II interacts with and activates the heterotrimeric G-protein transducin (Gt) by catalyzing the GDP/GTP exchange on Gt α-subunit (Gtα). In turn, Gtα-GTP activates the downstream effector enzyme 3′,5′-cGMP phosphodiesterase (PDE6) by relieving the inhibitory effect of its PDEγ subunit. Activated PDE6 reduces the concentration of the second messenger cGMP in rods, thus causing the closure of cyclic nucleotide–gated channels on the outer segment plasma membrane, cell hyperpolarization, and photoresponse (see Refs. 1–3 for reviews).

Gt is a key component in the light-induced signaling cascade of rod photoreceptors (see Refs. 4, 5 for reviews). It is a peripheral membrane protein complex formed by the association of three subunits—Gtα, Gtβ (Gβ1), and Gtγ (Gγ1). Gtβ and Gtγ exist as a heterodimer, which is nondissociable under physiological conditions, with its two subunits depending on each other for proper folding and stability.[6] A Gtα knockout mouse model has demonstrated the essential role of Gtα in phototransduction: rods lacking Gtα are completely insensitive to light.[7] The role of Gt$\beta\gamma$ in native rods is less clear. There is evidence that Gt$\beta\gamma$ dimer is an inactive partner of Gtα in phototransduction, acting as a scaffold protein responsible for the proper targeting of Gtα toward the disc membranes in which rhodopsin is located.[8,9] In contrast, several other studies suggest a direct role of Gt$\beta\gamma$ in the activation of Gt by R*,[10–14] probably by aiding conformational changes on Gtα to open the nucleotide-binding site, but the exact mechanisms are unknown. Therefore, it is possible that Gt$\beta\gamma$ may regulate the functions of Gt at two different stages, first by stabilizing the interaction of the Gtα subunit with R* and second by stimulating the guanine nucleotide exchange on Gtα. Various Gt$\beta\gamma$ complexes have been shown to increase the affinity of GPCRs similar to rhodopsin to their Gα signaling partners and to help GPCRs further increase the rate of G-protein activation.[15–18]

Several long-living intermediates (meta I, meta II, and meta III) are formed during the process of rhodopsin photobleaching. They exist in G-protein–dependent equilibria[19] that are also pH and temperature dependent.[20–23] Among these metaproducts, the active state meta II is the most important for Gt activation.[3,19] Under physiological conditions, meta II (380 nm) either decays thermally on the minute time scale into the apoprotein opsin and all-*trans*-retinal by hydrolysis of its deprotonated retinal Schiff base or converts to a meta III byproduct that has a protonated retinal Schiff base and absorbance maximum at 465 nm.[22,24] The two processes take place in parallel. In the absence of peripheral regulatory proteins, mammalian meta III is formed spontaneously from meta II within minutes[24,25] and then slowly decays into free retinal and opsin on a significantly longer time scale. This process is highly temperature and pH dependent.[26] Pioneering work of Emeis and Hofmann[27] and subsequent in vitro studies[28,29] established

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that Gt modulates the meta I/meta II ratio after bleaching and shifts this equilibrium toward meta II. Similarly, Gt, through its direct interaction with meta III, induces the transition of meta III into meta II-like species.[30] The thermal decay of all rhodopsin metaproducts is a physiologically important process that is crucial for the timely recovery of photosensitivity of photoreceptors during their dark adaptation.[31] Meta III can also serve as an intermediate "storage" form for all-*trans*-retinal that is toxic in its free form.[26,32–34]

Early studies on interactions between R* and Gt revealed that only heterotrimeric Gt, but not individual Gt subunits, binds with high affinity to rod outer segment (ROS) membranes in a light-dependent manner.[35] Indeed, structural studies of Gt and rhodopsin have identified potential points of contacts between them; sites on both Gtα and Gt$\beta\gamma$ subunits form a continuous receptor-binding surface.[14,36] Although previous nuclear magnetic resonance NMR[37,38] and recent x-ray crystallographic studies[39,40] have shed more light on the structural organization of the meta II–Gt complex, the possible physiological contribution of the Gt$\beta\gamma$ complex for modulating the signaling properties of R* and its lifetime remains largely unknown.

We therefore investigated this issue by using highly purified ROS disc membranes and intact rods from mice lacking the Gtγ subunit. This mouse model is advantageous because it is more physiologically relevant than traditional in vitro assays in elucidating the contribution of the Gt$\beta\gamma$ complex in visual signal transduction. Elimination of Gtγ expression in mouse rods results in the dramatic loss of visual signal amplification and the accompanying reduction of visual sensitivity at all functional levels.[41] Here we measured the rates of formation and thermal decay and the relative ratios of all rhodopsin signaling states (meta I, meta II, and meta III) by UV-visible spectroscopy at various levels of bleaching and pH conditions. Rhodopsin and meta II were also studied by the conformation-sensitive chemical probe hydroxylamine using rapid scanning spectroscopy. Spectroscopic assays measuring R*-Gt complex formation were complemented with physiological experiments that followed the recovery of rod dark current and photosensitivity after moderate bleaches to trace the quenching of residual activation of phototransduction by metarhodopsins. Overall, experiments described here extend our understanding of the functioning of the Gt$\beta\gamma$ complex in mouse rods.

Materials and Methods

Generation of *Gngt1* Knockout Mice

All experiments were performed in accordance with the policy on the Use of Animals in Neuroscience Research and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Saint Louis University Institutional Animal Care and Use Committee and the Washington University Animal Studies Committee.

Generation of the rod Gtγ gene (*Gngt1*) knockout mouse (Gtγ-KO) was described recently.[41] Briefly, the targeting construct was designed to replace the coding region of *Gngt1* with the Neo cassette. The construct was electroporated into embryonic stem cells, and G418-resistant clones were identified by PCR, DNA sequencing, and Southern blot analysis. Positive clones were injected into blastocysts to generate chimeric mice. Germline transmission in F1 and in subsequent generations derived by crossings with C57BL/6 was confirmed by PCR. All animals were 6 to 8 weeks old, were maintained under standard 12-hour light/12-hour dark cycles, and were dark-adapted for at least 12 hours before experiments.

Preparation and Characterization of ROS Disc Membranes

Native ROS disc membranes (RDM) were isolated from 50 to 150 frozen wild-type or Gtγ-KO mouse retinas by the discontinuous sucrose gradient method, as described.[42,43] All reported results were averages of three to six measurements from two separate RDM preparations. Total RDM protein and rhodopsin concentrations were measured as described.[44] The molar extinction coefficient of rhodopsin at 500 nm (ε_{500}) used for rhodopsin concentration calculations was 40,600 M/cm.[45] Membrane suspensions were stored at −80°C in isotonic buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride).

UV-Visible Spectroscopy

All measurements were performed on a UV-visible spectrophotometer (Cary-50; Varian, Palo Alto, CA). Specific temperatures were maintained with a Peltier-controlled cuvette holder. The sample compartment was continuously infused with dry air.

Near-complete photoactivation of rhodopsin was achieved by illumination of samples for 20 seconds with a 150-W fiberoptic light source passed through a 490 ± 5 nm bandwidth interference filter. Bleaching of 5% rhodopsin was performed with a 505-nm light-emitting diode (LED) flash controlled by a high-power LED driver (pulse duration, 50 ms). For analysis of meta I/meta II equilibrium, membrane suspensions were irradiated as described at 20°C in 20 mM BTP buffer (20 mM Bis-tris-propane, 130 mM NaCl, 1 mM $MgCl_2$) at the indicated pH. The difference spectrum (photoproduct minus dark rhodopsin) was determined immediately after the bleach, and absorbance differences at 380 nm (meta II absorbance peak) were calculated. For analysis of meta I, the difference spectrum (photoproduct minus spectrum obtained after incubation with hydroxylamine for 15 minutes at 20°C after near-complete bleach) was determined, and absorbance differences at 480 nm were measured. All data were normalized to dark rhodopsin.

For analysis of the rate of thermal meta II decay, a series of postbleach spectra was recorded. Then difference spectra were obtained by subtracting the dark state spectrum from sequential postbleach spectra recorded at indicated times. The time-dependent change of absorbance at 380 nm was used to calculate the time course of meta II decay. Data were normalized to dark rhodopsin absorbance. The rate of meta II decay was calculated by fitting the data with a single exponential function in KaleidaGraph 3.6.4.

The assay of meta II formation and decay in the presence of hydroxylamine was performed using rapid scanning spectroscopy, as described.[44]

Meta III formation was monitored by analyzing time-dependent changes of absorbance at 465 nm derived from a series of difference spectra between postbleach spectra and the first light spectrum recorded at time 0, as described.[30,46,47]

Stability of Rhodopsin in RDM Samples in Presence of Hydroxylamine

Stability of rhodopsin dark state in the presence of hydroxylamine in wild-type and Gtγ-KO RDM was estimated by monitoring the rate of 500-nm absorbance decrease after the addition of freshly neutralized hydroxylamine to RDM samples in 20 mM BTP buffer, pH 7.2, to a final concentration of 50 mM.[48] The reaction was carried out in darkness at 20°C. Successive spectra were recorded at predetermined time intervals, to monitor the loss of pigment at 500 nm. Data were calculated by using the following equation: $\Delta A = (A_i - A_f)/(A_0 - A_f)$, where A_i is the absorbance recorded at any particular time point, A_f is the absorbance at the final postbleach time (180 minutes), and A_0 is the absorbance at postbleach time 0, and they were fitted with a single exponential function. At the end of each experiment, the sample was fully bleached to determine the total rhodopsin absorbance for normalization. Base-

line drift in all spectra was corrected to zero pigment absorbance at 750 nm.

Assay of Extra–Meta II

The amount of extra–meta II was measured in meta II buffer (20 mM BTP, pH 8.0, 130 mM NaCl, 1 mM $MgCl_2$) at 4°C, by UV-visible spectroscopy.[14] After recording the dark rhodopsin spectrum, a single flash of light (duration, 50 ms; bleaching, ~5% rhodopsin) was applied, and an immediate postbleach spectrum was recorded. From the difference between the two spectra, meta II absorbance was calculated at 380 nm and then normalized to absorbance of the total rhodopsin present in the sample. To calculate the background level of meta II under the assay conditions, wild-type RDM were treated with pertussis toxin (PT; preactivated with 20 mM DTT and 2 mM adenosine triphosphate for 40 minutes, at 37°C) before the extra–meta II assay. Because efficient adenosine diphosphate (ADP) ribosylation of Gtα requires Gtβγ,[49] background meta II was not measured in Gtγ-KO RDM and was considered to be comparable to that in wild-type. The reaction was carried out in darkness in 20 mM BTP buffer, pH 8.0, containing 1 mM nicotinamide adenine dinucleotide and 0.1 μg/μL activated PT for 60 minutes, at 30°C.[49] Background meta II measured after the treatment with PT was subtracted from meta II obtained in the absence of PT to calculate Gt-dependent extra–meta II.

Single-Cell Suction Recordings

Animals were dark-adapted overnight, and their retinas were removed under infrared illumination, chopped into small pieces, and transferred to a perfusion chamber on the stage of an inverted microscope. A single rod outer segment was drawn into a glass microelectrode filled with solution containing 140 mM NaCl, 3.6 mM KCl, 2.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 3 mM HEPES (pH 7.4), 0.02 mM EDTA, and 10 mM glucose. The perfusion solution contained 112.5 mM NaCl, 3.6 mM KCl, 2.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 mM HEPES (pH 7.4), 20 mM $NaHCO_3$, 3 mM Na succinate, 0.5 mM Na glutamate, 0.02 mM EDTA, and 10 mM glucose. The perfusion solution was bubbled with 95% O_2/5% CO_2 mixture and heated to 37°C.

Light stimulation was applied by 20-ms test flashes of calibrated 500-nm light. The stimulating light intensity was controlled by neutral-density filters. Saturating test flashes were used to monitor the recovery of rod dark current (I_{dark}) after bleaching 12% of rhodopsin with a 3.4-second step of 500-nm light. Rod flash sensitivity (S_f) was estimated as the ratio of the amplitude of dim flash responses from the linear range and their test flash intensity. The bleach fraction was estimated from the relation: $F = 1 - \exp(-IPt)$, where F is the fraction of pigment bleached, I is the bleaching light intensity of not-attenuated 500-nm light (6.4×10^6 photons μm^2/s), and P is the photosensitivity of mouse rod at the wavelength of peak absorbance (5.7×10^{-9} μm^2), from Woodruff et al.[50] Photoresponses were amplified, low-pass filtered (30 Hz, 8-pole Bessel), and digitized (1 kHz). Data were analyzed using Clampfit 10.2 and Origin 8.5 software.

Results

Meta I–Meta II Equilibrium in Wild-type and Gtγ-KO RDM

To test for the possible contribution of Gtβγ in rhodopsin photolysis, we obtained highly purified RDM from wild-type and Gtγ-KO retinas. We first recorded a full UV-visible spectrum immediately after rhodopsin photoactivation with near-complete bleach (Fig. 1, Table 1) and analyzed the equilibrium between metarhodopsins (meta I, λ_{max} = 480 nm; meta II, λ_{max} = 380 nm). Under the conditions of this experiment (pH 7.2, 20°C, isotonic buffer), the total absorbance of wild-type RDM consisted of 37% ± 9% meta I and 63% ± 9% meta II. The data are in good agreement with previous results estimating 68% of meta II formation in washed bovine ROS reconstituted with purified Gt (pH 7.2, 17°C).[51] Under the same conditions, meta I/meta II mixture in Gtγ-KO RDM was shifted only slightly toward meta I, resulting in 44% ± 8% meta I and 56% ± 8% meta II (Table 1). At alkaline pH (8.0), there was an expected shift in the equilibrium toward meta I (meta I/meta II = 81%/19% for wild-type RDM and 79%/21% for Gtγ-KO RDM), whereas at acidic pH (5.5), we observed the opposite shift toward meta II (meta I/meta II = 10%/90% for wild-type RDM and 9%/91% for Gtγ-KO RDM). Notably, meta I/meta II ratios in wild-type and Gt-KO RDM were similar under the same pH conditions (Table 1). Thus, our results indicate that in the absence of the Gtβγ complex, the photoactivation of rhodopsin results in a typical generation of meta I/ meta II equilibrium, with the meta I/meta II ratio similar to that in wild-type RDM.

Decay of Meta II and Formation of Meta III in Wild-type and Gtγ-KO RDM

To address the possible regulation of meta II stability and its conversion to meta III by the Gtβγ complex, we monitored the decay of meta II in isolated RDM after near-complete rhodopsin bleach. The kinetics of meta II decay in wild-type and Gtγ-KO RDM was determined by recording a series of postbleach spectra over a period of 30 minutes at 20°C and pH 7.2. The difference spectra from wild-type and Gtγ-KO RDM demonstrated similar decreases in the absorbance at 380 nm due to the decay of meta II and a simultaneous increase in the absorbance at 465 nm due to the formation

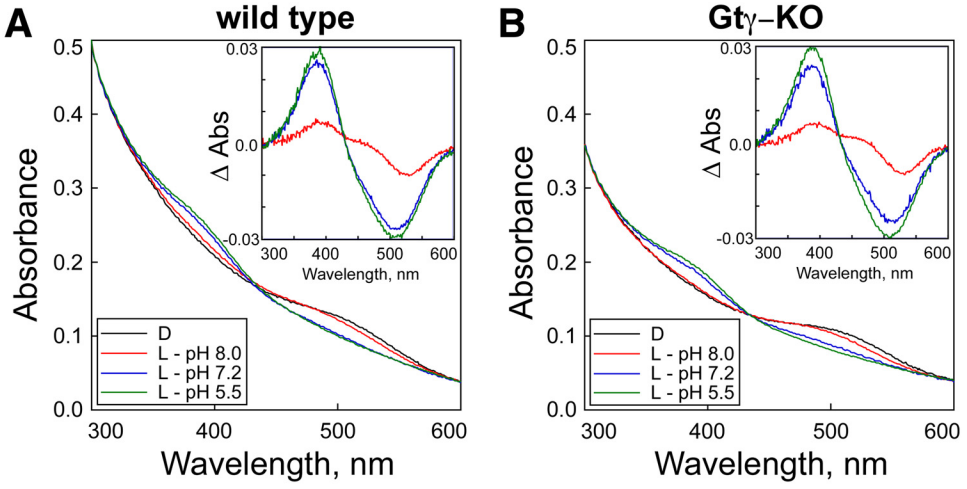

Figure 1. UV-visible absorbance spectra of rhodopsin in wild-type (**A**) and Gtγ-KO (**B**) RDM. Both dark (D) and light (L) spectra (recorded immediately after near-complete bleaching of wild-type and Gtγ-KO RDM) are shown (20°C, at indicated pH). *Insets*: corresponding L-D difference spectra.

Table 1. Ratios of Rhodopsin Photointermediates Meta I and Meta II in Wild-type and Gtγ-KO RDM under Various pH Conditions

	Wild-type		Gtγ-KO	
	Meta I	**Meta II**	**Meta I**	**Meta II**
pH 7.2*	0.37 ± 0.09 (6)	0.63 ± 0.09 (6)	0.44 ± 0.08 (10)	0.56 ± 0.08 (10)
pH 8.0†	0.81 ± 0.02 (2)	0.19 ± 0.02 (2)	0.79 ± 0.06 (6)	0.21 ± 0.06 (6)
pH 5.5‡	0.10 ± 0.01 (5)	0.90 ± 0.01 (5)	0.09 ± 0.10 (6)	0.91 ± 0.10 (6)

Values are mean ± SD (*n*).

* Determined after near-complete photobleaching of RDM suspended in 20 mM BTP buffer, pH 7.2, at 20°C.

† Determined after near-complete photobleaching of RDM suspended in 20 mM BTP buffer, pH 8.0, at 4°C.

‡ Determined after near-complete photobleaching of RDM suspended in 50 mM MEM buffer, pH 5.5, at 20°C.

of meta III (Fig. 2A). In both cases the conversion of meta II to meta III involved only the two photoproducts, as evidenced by the well-defined isosbestic point at 422 nm, indicating that the alternative pathway of meta II decay to opsin and all-*trans*-retinal is negligible. The time courses of meta II decay in wild-type and Gtγ-KO RDM are shown in Figure 2B. The absorbance change at 380 nm was normalized to the absorbance of dark rhodopsin and fitted with single exponential functions that yielded time constants of meta II decay of 10.5 ± 1.1 minutes for wild-type and 10.1 ± 1.7 minutes for Gtγ-KO RDM. Meta II decay half-times ($t_{1/2}$) in wild-type and Gtγ-KO RDM were 7.1 ± 0.7 minutes and 6.7 ± 1.2 minutes, respectively. These half-time values are in good agreement with published results of 8.2 ± 0.5 minutes for bovine rod disc membranes[32] and for purified mouse rhodopsin.[52]

The time courses of meta III formation in wild-type and Gtγ-KO RDM are shown in Figure 3B. The data were fitted with single exponential functions that yielded time constants of meta III formation of 10.1 ± 0.6 minutes and 9.5 ± 0.7 minutes for wild-type and Gtγ-KO RDM, correspondingly. The $t_{1/2}$ values of meta III production were 7.0 ± 0.3 minutes in wild-type and 7.0 ± 1.9 minutes in Gtγ-KO RDM, closely matching the rates of meta II decay. Thus, our results indicate that the lack of Gtβγ complex has no effect on both meta II decay and meta III formation in mouse RDM after near complete rhodopsin bleaching.

Interaction of Rhodopsin with Hydroxylamine in Wild-type and Gtγ-KO RDM

Hydroxylamine is frequently used in structure-function studies of rhodopsin because it interacts with the retinal Schiff base of metaproducts to form retinal oxime.[20,44,53,54] The retinal Schiff base in ground-state rhodopsin is stable in the presence of hydroxylamine, consistent with its well-protected chromophore binding site, as determined by x-ray crystallography.[55] In contrast, after rhodopsin photoactivation, all-*trans*-retinal oxime with sharp absorption peak at 365 nm is formed readily upon the decay of metarhodopsins, making hydroxylamine a useful rhodopsin conformation-sensitive chemical probe.[45]

Taking into account that proper folding and stability of membrane proteins are typically provided by multiple factors, we hypothesized that the lack of farnesylated Gtβγ as the major peripheral membrane-bound protein might influence, directly or indirectly, the protein-lipid microenvironment of rhodopsin retinal Schiff base in Gtγ-KO disc membranes and its accessibility to hydroxylamine in the dark rhodopsin state. To evaluate this hypothesis, we measured rhodopsin stability in the presence of hydroxylamine in wild-type and Gtγ-KO RDM. Absorbance of the dark rhodopsin state (500 nm) was monitored at 20°C over 180 minutes after the addition of 50 mM hydroxylamine. As expected, in wild-type RDM, dark rhodopsin was reacting poorly with hydroxylamine, with 80% of it

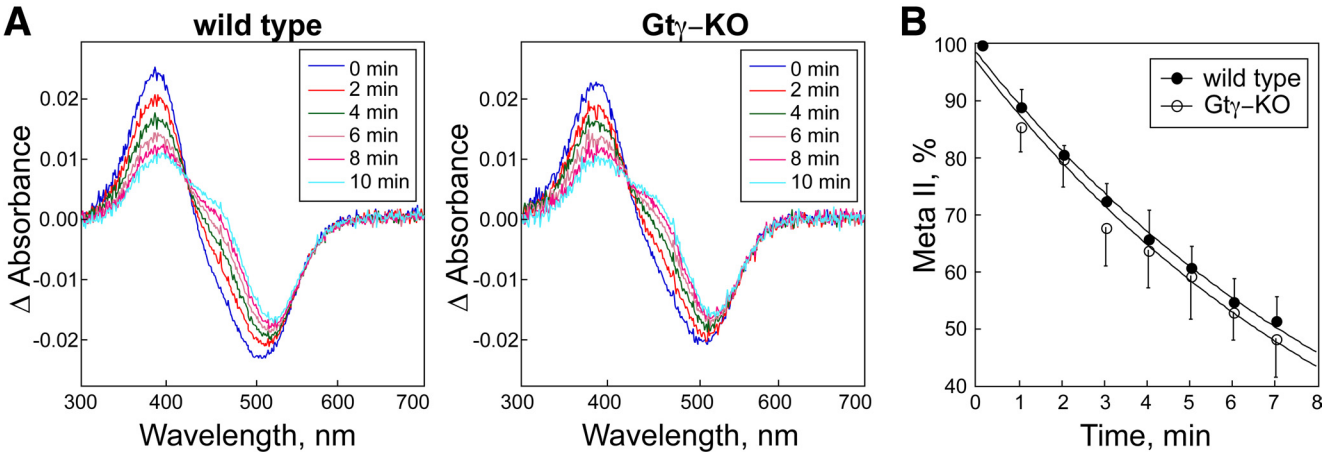

Figure 2. Comparison of meta II decay rates in wild-type and Gtγ-KO RDM. (**A**) Spectrophotometric analysis of changes in UV-visible absorption spectra after photobleaching of wild-type (*left*) and Gtγ-KO (*right*) RDM. Difference spectra were obtained by subtracting the spectrum recorded before the irradiation from those recorded at selected times after irradiation. (**B**) The loss of absorbance at 380 nm is plotted as a function of time after rhodopsin photoactivation. *Solid lines*: single exponential fits to the data. All fits showed $r^2 > 0.95$.

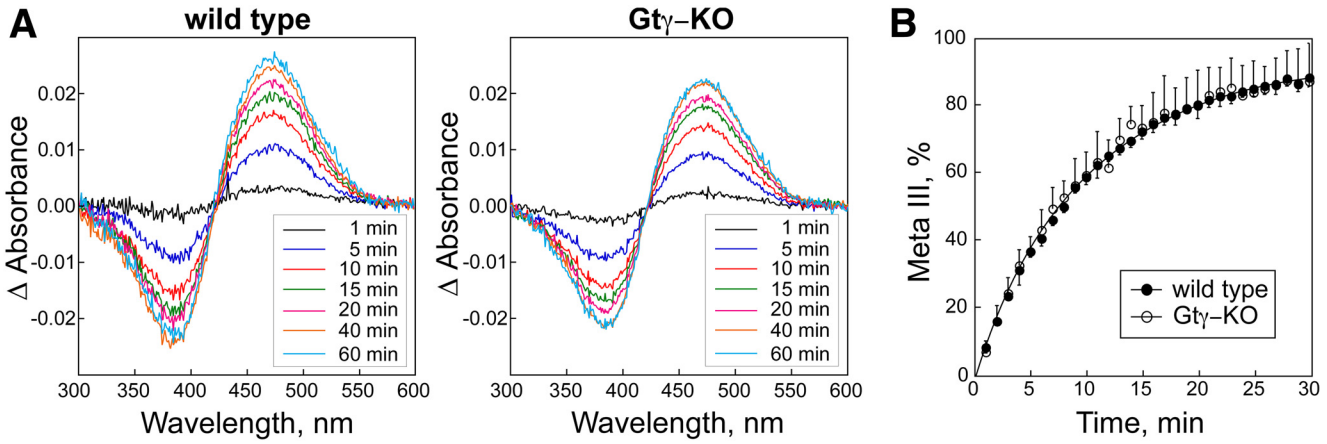

Figure 3. Comparison of meta III formation rates in wild-type and Gtγ-KO RDM. (**A**) Series of difference spectra after photoactivation of rhodopsin. The spectra were taken at 20°C, pH 7.2, at the times indicated. The spectrum obtained immediately after RDM bleaching was subtracted from postbleach spectra after extensive photoactivation of the membranes. (**B**) Rates of meta III formation. Normalized changes of absorption at 465 nm were plotted as a function of time. *Error bars* represent SD ($n = 5$ for wild-type; $n = 9$ for Gtγ-KO RDM). *Solid lines*: single exponential fits to the data. All fits showed $r^2 > 0.95$.

remaining intact by 180 minutes (Fig. 4A). In Gtγ-KO RDM, rhodopsin stability in the presence of hydroxylamine was identical with that in wild-type samples, indicating that the retinal Schiff base of dark rhodopsin in these membranes has no increased exposure to bulk solvent. This result implies that the retinal Schiff base conformation of dark rhodopsin in wild-type and Gtγ-KO RDM is the same.

Next, we examined whether the lack of Gtβγ affects the Schiff base conformation of R*. We reasoned that if meta II in wild-type and Gtγ-KO RDM were structurally similar, the metaproducts would have similar reactivity toward hydroxylamine. We used rapid scanning spectroscopy to measure the rates of meta II formation and decay at physiological pH in wild-type and Gtγ-KO RDM in the presence of hydroxylamine.[44] Because of the spectral absorbance overlap between meta II (380 nm) and retinal oxime (365 nm), the meta II data were collected at 405 nm, where the overlap was minimal. The A_{405} changes obtained from wild-type and Gtγ-KO RDM in the presence of hydroxylamine exhibited no differences in the rates of meta II formation and decay (Fig. 4B). The initial fast increase in absorbance caused by transient meta II formation was also identical. Similarly, we detected no difference in the slower phase of hydroxylamine-induced meta II decay. These observations imply that structural changes related to the retinal Schiff base solvent accessibility that accompany meta II formation in wild-type and Gtγ-KO RDM are essentially identical and confirm our conclusion that activation of rhodopsin proceeds similarly in wild-type and Gtγ-KO RDM.

Formation of Extra–Meta II in Wild-type and Gtγ-KO RDM

Binding of Gt to meta II shifts the meta I/meta II equilibrium toward meta II.[27,29,56] Thus, the assay of extra–meta II (or Gt-stabilized meta II) allows measurement of the amount of meta II–Gt complex. The formation of extra–meta II can be measured spectrophotometrically under conditions of slightly alkaline pH and low temperatures. Because the molar ratio of Rh/Gt in native disks is approximately 10:1, the use of less intense flashes photoactivating <10% of rhodopsin molecules is advantageous compared with near-complete bleach because it produces a higher Gt:R* stoichiometric ratio and improves the extra–meta II signal-to-noise ratio.[27] Typically, most of the meta II observed under these experimental conditions is caused by meta II–Gt complex formation. We tested the possible importance of Gtβγ in R*-Gt complex formation by comparing the amounts of extra–meta II in wild-type and Gtγ-KO RDM after applying a 505-nm light flash that bleached 5% of

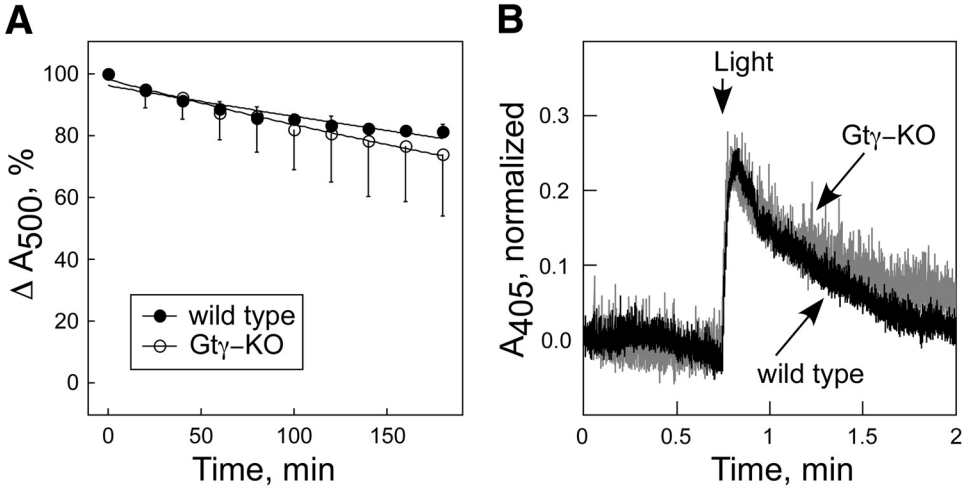

Figure 4. Rhodopsin stability in the presence of hydroxylamine in wild-type and Gtγ-KO RDM. (**A**) Time course of absorbance decrease at 500 nm in the presence of 50 mM hydroxylamine, at pH 7.2 and 20°C, in darkness. Normalized data points (*solid circles*) were fitted with single exponential functions (*solid lines*). Data are mean ± SD of three independent experiments. (**B**) Reactivity of meta II from wild-type and Gtγ-KO RDM toward hydroxylamine. Representative A_{405} time-resolved traces of meta II formation and decay in the presence of 50 mM hydroxylamine in wild-type and Gtγ-KO RDM in 20 mM BTP buffer, pH 7.2, at 20°C.

rhodopsin at 4°C and pH 8.0. The background (Gt-independent) level of meta II was measured by preincubating dark wild-type RDM with pertussis toxin, which catalyzes the ADP-ribosylation of Cys347 in Gtα and renders it incapable of interacting with R* and stabilizing extra–meta II.[57] Remarkably, the deletion of Gtβγ resulted in a drastic (~22-fold) reduction of the amount of extra–meta II formation in Gtγ-KO RDM compared with wild-type RDM (Fig. 5). Thus, the generation of meta II–Gt complex is significantly reduced in mouse RDM lacking Gtβγ.

Recovery of Dark Current and Flash Sensitivity after a Moderate Bleach in Wild-type and Gtγ-KO Rods

Next, we investigated whether the compromised Rh-Gt interactions in Gtγ-deficient mouse rods affect the residual ability of metaproducts to activate the phototransduction cascade in physiological conditions, by performing single-cell suction recordings. We compared rates of recovery of dark current (I_{dark}) and flash sensitivity (S_f) after bleaching a moderate fraction (12%) of the pigment in isolated wild-type and Gtγ-KO rods (Fig. 6). In the absence of RPE-driven rhodopsin regeneration under these conditions, this recovery would reflect the gradual quenching of the phototransduction cascade after the decay of formed meta I–III (partially inactivated by rhodopsin kinase and arrestin) to free opsin and all-*trans*-retinal.[58–61] Initially, the bleach completely suppressed the dark current, leaving the rods saturated before their current gradually and largely recovered (Fig. 6A). We found that the initial dark current recovery following 12% rhodopsin bleach was substantially (~3 times) faster in Gtγ-KO mouse rods (Figs. 6A, 6B). This result is consistent with the reduced affinity of Gt toward R* and presumably its long-living photointermediates in the absence of Gtβγ complex in mouse rods. Thus, despite the equal levels of rhodopsin activation in wild-type and Gtγ-KO rods, the lower overall Gt/PDE activation in Gtγ-deficient rods resulted in shorter time in saturation compared with wild-type cells. We also observed faster postbleach recovery of flash sensitivity in the same Gtγ-KO rods (Fig. 6C) whose dark sensitivity was approximately 2 log units lower than that of wild-type photoreceptors, as determined previously.[41] However, the rate of the late dark current recovery was largely unchanged in Gtγ-KO rods (Fig. 6B), in agreement with the unaltered rate of meta II decay in the absence of the Gtβγ complex (Fig. 2B).

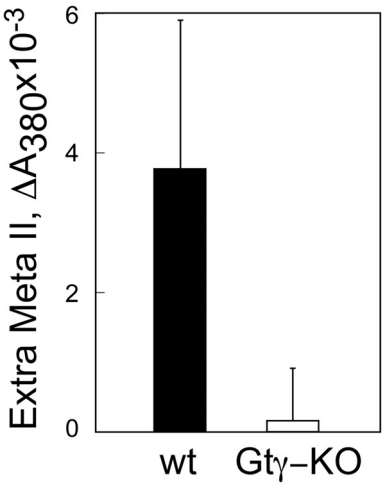

Figure 5. Formation of extra–meta II in wild-type and Gtγ-KO RDM. Wild-type and Gtγ-KO RDM were incubated in meta II buffer (20 mM BTP, pH 8.0, 130 mM NaCl, 1 mM $MgCl_2$) at 4°C, and extra–meta II was measured as described in Materials and Methods. Data are mean ± SEM of three independent experiments.

Discussion

Despite the key role of the Gt activation by meta II in phototransduction, the possible physiological role of Gtβγ complex in modulating the signaling properties of rhodopsin and its photolysis has not been tested. Experiments using rod disc membranes or proteins isolated from wild-type rods to address this question carry the danger of potential contamination with endogenous Gtβγ. To exclude this possibility, we investigated rhodopsin photointermediates in purified RDM derived from genetically engineered mice lacking the Gtγ subunit.[41] This Gtγ-KO model allowed us to study the possible contribution of the Gtβγ complex to establishing the meta I/meta II equilibrium and its pH dependence under close to physiological conditions. We found that the deletion of Gtβγ had no effect on the amounts of meta I and meta II in mouse RDM and that the normal pH dependence of their equilibrium was preserved (Fig. 1, Table 1).

The Gtβγ complex facilitates interactions between Gtα and R*[8] and is crucial for visual signal amplification in intact mouse rods.[41] The Gtγ subunit contributes to the binding of Gt to R*, and both its carboxyl-terminal sequence and its farnesylated Cys71 confer Gt selectivity toward rhodopsin.[10,17,62–64] It has been also shown that the farnesylated C-terminal peptide Gtγ (60-71) specifically stabilizes the meta II active photointermediate.[65,66] Thus, it is conceivable that Gtβγ may control not only the primary step of Gt interaction with meta II but also its decay kinetics. However, our data argue against the latter possibility and demonstrate that the lack of Gtγ subunit in mouse RDM has no effect on the thermal decay of meta II after near-complete rhodopsin bleach (Fig. 2) or on its tautomeric transition to meta III (Fig. 3). Given that previous studies have failed to identify any possible G-protein subunit substitutions for Gtβγ in Gtγ-KO rods[41,67] that could have mimicked the effect of Gtβγ, it is likely that the rate of meta II decay is independent of the presence of Gtβγ in mouse rods, at least at high bleaching levels.

In addition, consistent with the normal ability of rhodopsin to form meta II and meta III and their unaffected thermal decay rates in Gtγ-KO RDM, our results on the susceptibility of ground-state rhodopsin and R* toward hydroxylamine argue against any appreciable effect of Gtβγ on specific retinal Schiff base conformational changes in R* (Fig. 4).

Based on mathematical modeling of dim flash photoresponses from intact mouse rods, it has been suggested recently that a 3–5 times shorter lifetime of unphosphorylated R* could be the major factor contributing to the faster response inactivation of Gtγ-deficient rods compared with wild-type cells.[41] Several factors, including potentially more rapid R* phosphorylation and arrestin binding in Gtγ-KO rods, may explain this finding. In vertebrate rods, the thermal decay of metaproducts is too slow under physiological temperatures (on the minute timescale) to affect the inactivation kinetics of their dim flash photoresponses.[68] To have a physiologically meaningful effect, the decay of metaproducts in the absence of Gtβγ must be on the millisecond timescale, comparable to the inactivation time of a typical rod dim flash response (50–100 ms[69]). This scenario is unlikely, and our data on the meta II decay in wild-type and Gtγ-KO RDM demonstrate that in the absence of Gtβγ, meta II decays normally (Fig. 2). The possible contribution of phosphorylation and arrestin binding to faster inactivation of dim flash responses in Gtγ-KO rods is a subject of future research.

By measuring the level of extra–meta II formation (Fig. 5), we observed drastic reduction in effectiveness of R*-Gtα inter-

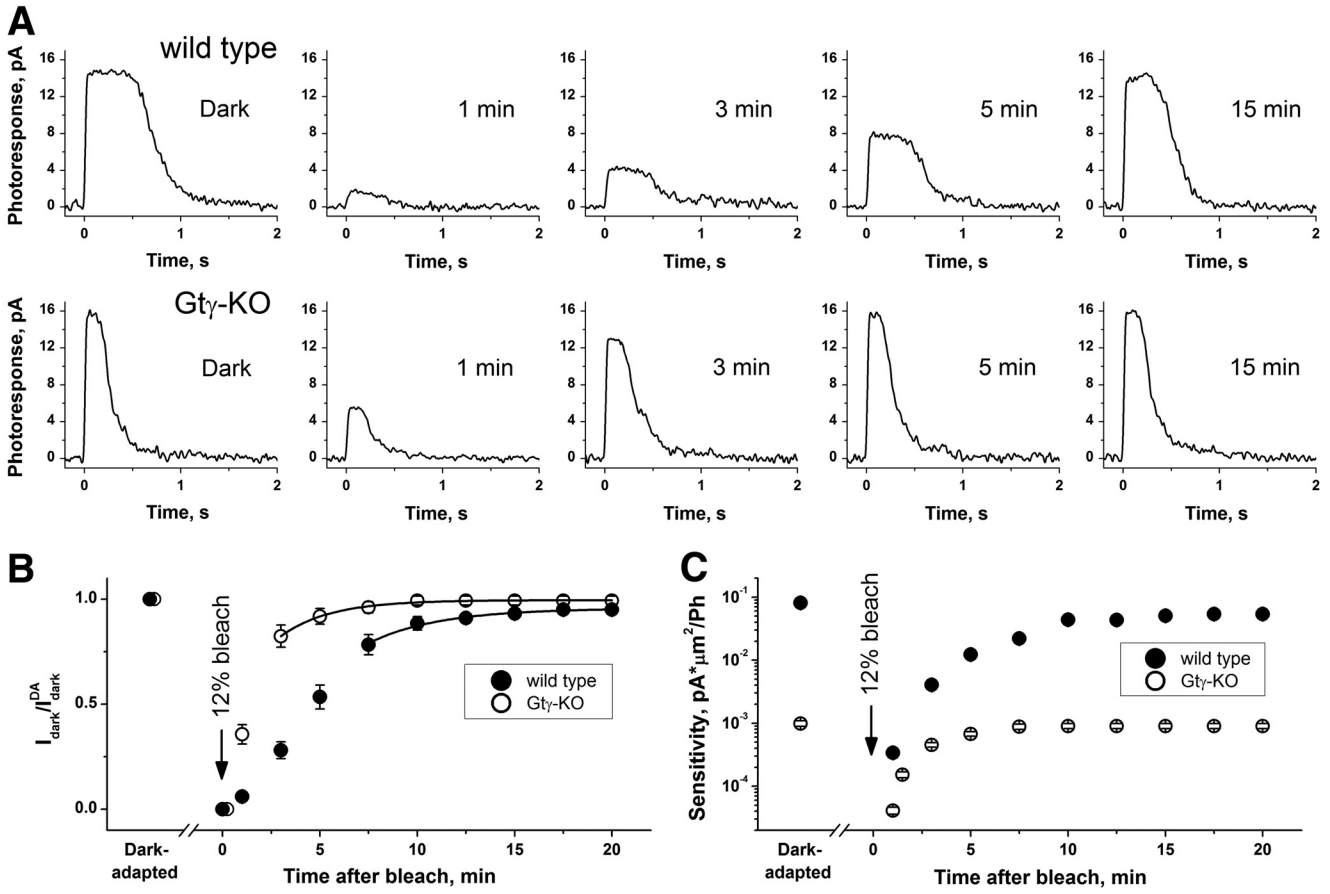

FIGURE 6. Recovery of maximal photoresponse amplitude (I_{dark}) (**A**, **B**) and flash sensitivity (S_f) (**C**) after bleaching 12% rhodopsin (at time 0) in wild-type (n = 14) and Gtγ-KO (n = 18) solitary mouse rods. Data were derived from suction electrode recordings. (**A**) Representative saturated photoresponses from dark-adapted and subsequently bleached rods of wild-type (*upper row*) and Gtγ-KO (*lower row*) mice. Postbleach times are indicated. (**B**) Maximal response amplitudes were normalized to their dark-adapted values (DA). Values are mean ± SEM. (*solid lines*) Single-exponential fits to late data points (starting at 80% of dark current recovery), with time constants of 3.6 minutes for wild-type rods and 2.5 minutes for Gtγ-KO rods. (**C**) Recovery of rod flash sensitivity (S_f) after bleaching 12% rhodopsin (same cells as in **A**). S_f was calculated from the linear region of the intensity-response curve as the ratio of response amplitude and flash strength. Values are mean ± SEM.

actions in Gtγ-KO RDM. Part of this reduction was likely due to the lower concentration of Gtα in Gtγ-KO rods.[41] More important, the lower amount of extra–meta II in Gtγ-KO RDM indicates a direct role of the Gtβγ complex in meta II stabilization and the efficiency of R*-Gt coupling. Indeed, previous biochemical studies demonstrated that both Gtα and Gtβγ subunits are required for efficient Gt binding to R*.[8,35] In agreement with these findings, later studies using purified Gt subunits confirmed that the affinity of Gtα toward R* is significantly increased by Gtβγ.[70,71] Other studies suggested that the Gtγ subunit contributes to the specificity of R*-Gt interactions.[62,63] Finally, it has been recently demonstrated that Gtα has a significantly reduced ability to bind to R* in purified Gtγ-KO RDM.[41] Therefore, the ~22-fold reduced amount of extra–meta II found in Gtγ-KO RDM compared with wild-type RDM preparations (Fig. 5) can be explained by a combination of the two factors: approximately sixfold lower concentration of Gtα[41] and fourfold lower affinity of Gtα to R* in the absence of Gtβγ under the specific experimental conditions. Interestingly, a recent physiological study revealed significant reductions of signal amplification in rods lacking Gtγ,[41] resulting in decline in the sensitivity of scotopic vision in mice.[41,67] Thus, our present findings are in line with the substantial desensitization of Gtγ-KO rods revealed in physiological experiments.

Gtγ-deficient rods displayed substantially accelerated recovery of their maximal response amplitude and flash sensitivity after a moderate (12%) rhodopsin bleach compared with wild-type cells (Fig. 6). In intact amphibian rods, the recovery of photocurrent after moderate bleaches can be well explained by the slow decay of all metaproducts (already partially inactivated by rhodopsin kinase and arrestin binding) and the gradual decrease of their ability to activate phototransduction.[61] Our data indicate that the photolysis of R* in wild-type and Gtγ-KO RDM proceeds similarly. However, if the efficiency to activate Gt in the absence of Gtβγ were reduced in mutant rods because of the combined effect of lower Gtα concentration and its less efficient interaction with R*, this would have resulted in lower total phototransduction activation by all metaproducts and, therefore, faster recovery of dark current from its saturation. Indeed, we observed that the dark current escaped from saturation earlier and had a substantially faster initial recovery in Gtγ-KO rods compared with wild-type rods after identical 12% rhodopsin bleaches. We also found that despite the grossly different levels of phototransduction activation in wild-type and Gtγ-KO rods after an identical 12% bleach, the kinetics of the late recovery of their dark currents were comparable (Fig. 6B), indicating that this recovery is driven by the decay of metaproducts. In this regard, it is important to emphasize that the fine balance between the efficient visual signal amplification mediated by Rh-Gt interactions on the one hand and the timely recovery of rods from

saturation on the other hand is required for proper functioning of rod photoreceptors in dim light conditions.

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