Phosducin Regulates the Expression of Transducin βγ Subunits in Rod Photoreceptors and Does Not Contribute to Phototransduction Adaptation

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For over a decade, phosducin's interaction with the βγ subunits of the G protein, transducin, has been thought to contribute to light adaptation by dynamically controlling the amount of transducin heterotrimer available for activation by photoexcited rhodopsin. In this study we directly tested this hypothesis by characterizing the dark- and light-adapted response properties of phosducin knockout (Pd−/−) rods. Pd−/− rods were notably less sensitive to light than wild-type (WT) rods. The gain of transduction, as measured by the amplification constant using the Lamb-Pugh model of activation, was 32% lower in Pd−/− rods than in WT rods. This reduced amplification correlated with a 36% reduction in the level of transducin βγ-subunit expression, and thus available heterotrimer in Pd−/− rods. However, commonly studied forms of light adaptation were normal in the absence of phosducin. Thus, phosducin does not appear to contribute to adaptation mechanisms of the outer segment by dynamically controlling heterotrimer availability, but rather is necessary for maintaining normal transducin expression and therefore normal flash sensitivity in rods.

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INTRODUCTION

Phosducin is one of the least understood signaling proteins of photoreceptor cells. It was originally identified as a phosphoprotein interacting with the βγ subunits of transducin in vitro (Lee et al., 1984, 1987; Gaudet et al., 1996; Muller et al., 1996; Schulz et al., 1996). This interaction with βγ led to the attractive hypothesis that phosducin contributed to photoreceptor adaptation to steady light by sequestering the βγ subunits of transducin from the α-subunit in a light- and phosphorylation-dependent manner (Lee et al., 1992; Yoshida et al., 1994; Wilkins et al., 1996; Willardson et al., 1996). Because transducin can be efficiently activated by photoexcited rhodopsin only in its heterotrimeric αβγ form (Fung, 1983), the sequestration of βγ by phosducin was suggested to reduce the rate of transducin activation, thus yielding photoresponses of reduced amplitude characteristic of the light-adapted cells. This idea was subsequently challenged by several reports indicating that most phosducin in the rod cell is located outside the outer segment, a photoreceptor's organelle where the visual signal transduction takes place (Lee et al., 1988; Gropp et al., 1997; Thulin et al., 1999; Nakano et al., 2001; Sokolov et al., 2004). Yet no direct evidence supporting or rejecting this putative mechanism has been reported so far and the hypothesis is still commonly discussed (e.g., Klenk et al., 2006; Partridge et al., 2006). On the other hand, phosducin was demonstrated to participate in another cellular function by assisting transducin βγ subunits in their light-driven translocation from rod outer segments (Sokolov et al., 2004), a process that takes place after prolonged exposure of rods to very bright light bleaching at least 4,000-6,000 rhodopsin molecules per rod per second (Sokolov et al., 2002; Lobanova et al., 2007; see Calvert et al., 2006 for a recent review).

In this study, we used the phosducin knockout mouse to evaluate the original hypothesis that phosducin contributes to adaptation to low and moderate levels of illumination by dynamically controlling the availability of transducin heterotrimer for activation.

MATERIALS AND METHODS

Animal Care and Use

Mice were cared for and handled following an approved protocol from the Institutional Animal Care and Use Committees of our respective universities and in compliance with National Institutes of Health guidelines for the care and use of experimental animals. A colony of phosducin knockout mice (Sokolov et al., 2004) and

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Abbreviation used in this paper: WT, wild-type.

wild-type (WT) mice C-57Bl/6 and 129SV (Charles River) were maintained and confirmed by genotyping and Western blotting, and maintained at normal diurnal cycle.

Measurements of Rhodopsin Content in the Retinas and Preparation of Retina Samples for SDS-PAGE

7–8-wk-old mice were dark-adapted overnight and killed, and their retinas were harvested and placed into freshly prepared 10 mM hydroxylamine (titrated to pH 7.4–7.6 with NaOH) containing 2.5% *n*-octyl-β-D-glucopyranoside (100 μl per retina). Retinas were homogenized by a brief 1–2-s burst of ultrasound using Microson ultrasonic cell disruptor equipped with a 3-mm probe (Misonix Inc.). The extracts were cleared by a 2-min centrifugation at 16,000 rpm using a table top centrifuge 5415D (Eppendorf). Rhodopsin concentration in the supernatant was determined by difference spectrometry as described previously (Sokolov et al., 2002). The whole procedure was performed under the dim red light to avoid rhodopsin bleaching. After rhodopsin concentration in the original retina extract was determined, it was diluted ~80 times with the sample buffer containing 250 mM Tris-HCl, pH 6.8, 6 M Urea, 4% SDS, 10 mg/ml DTT, and bromophenol blue to yield the final rhodopsin concentration of 50 fmol/μl and stored in 50-μl aliquots at −80°C.

Quantitative Western Blot Analysis of the Proteins from Retina Extracts

For quantification of transducin subunits, 10 μl of the retina extract containing 500 fmol of rhodopsin was subjected to SDS-PAGE alongside with the 10-μl samples containing 25, 50, 80, 100, and 150 fmol of bovine rod transducin α and βγ subunits purified according to Heck and Hofmann (2001). Proteins were separated on 15-well 10–20% Tris-HCl gel (Bio-Rad Laboratories), transferred to Immobilon-FL PVDF membrane (Millipore) in Towbin buffer containing 25 mM Tris, 192 mM glycine, 10% (vol/vol) methanol, for 1.5 h at 0.25 A. The detection of individual protein bands was conducted using the Odyssey Infrared Imaging System (LI-COR Biosciences) according to the manufacturer's manual. Fluorescence of the specific bands was determined using the Odyssey software.

Blots were probed with sc-389 antibody against QYGDSARQDDARKL sequence within $G\alpha_{t1}$, sc-373 antibody against VINIEDLTEKDK sequence of $G\gamma_1$ (Santa Cruz Biotechnology), and PA1-725 antibody against RQEAEQLKNQIRDARKAC sequence within $G\beta_1$ (Affinity BioReagent) to simultaneously detect all three subunits of rod transducin. All three epitopes are conserved between *Bos taurus* and *Mus musculus*. Affinity-purified sheep polyclonal antibody against phosducin was as in Sokolov et al. (2004). Anti–rabbit and anti–sheep Alexa Fluor 680–conjugated secondary antibodies were purchased from Invitrogen.

Determination of Phosducin Phosphorylation under Different Levels of Illumination

Retinas were harvested from mice dark adapted overnight under dim red light and stored on ice in L-15 media (Invitrogen) supplemented with 10 mM glucose and 0.1 mg/ml BSA (Sigma-Aldrich), for 1–2 h. Light conditioning of the retinas was performed in the custom-made transparent flowthrough cell, at 5 ml/min flow of bicarbonate-buffered Locke's solution (pH 7.4) supplemented with 10 mM glucose, which was constantly aerated with 95% CO_2/5% O_2 gas mixture and heated to maintain 35–37°C in the cell. The cell was positioned on a light diffuser illuminated from behind from an adjustable tungsten-halide lamp. The flux of photons that activate rhodopsin was determined on the surface of the diffuser using a calibrated photodiode (PDA-750, Terahertz Technologies) covered by a glass filter with a spectral sensitivity closely matching that of rhodopsin (BG 39, Newport Franklin Inc.). The photon flux (F, units: photons/μm^2 s) was then calculated from the equation $F = W/(E_{500} \cdot A)$, where W is the lamp power in watts; E_{500}, energy of a 500-nm photon, $hc/\lambda = 3.98 \times 10^{-19}$ J; A, the photodiode's detecting area = 1 cm^2 = 10^8 μm^2. Following light conditioning, the retina was transferred into 0.2 ml of buffer containing 125 mM Tris/HCl, pH 6.8, 4% SDS, 6 M urea, and 10 mg/ml DTT and homogenized by short ultrasonic pulses. The extract was cleared by centrifugation. 15 μl aliquots were separated on 18-well 10% Tris-HCl gels (Bio-Rad Laboratories), transferred to PVDF membrane Immobilon FL (Millipore). To determine the degree of phosducin phosphorylation, the blots were double labeled with phosphospecific Pdc54p and Pdc71p rabbit antibodies (Lobanova et al., 2007), followed by pan-specific sheep antibodies against phosducin (Sokolov et al., 2004). Quantification of the specific bands was performed on an Odyssey Infrared Imaging System (LI-COR Biosciences) according to the manufacturer's protocols, using secondary anti–rabbit antibody conjugated to Alexa Fluor 680 and anti–sheep antibody conjugated to Alexa Fluor 800 (Invitrogen). Fluorescence values of phosphorylated phosducin bands were divided by those of total phosducin bands, and then the amounts of phosphorylated phosducin in the light-conditioned samples were normalized to those in the dark-adapted samples on the same gel.

Suction Electrode Recordings

Adult mice dark adapted overnight were killed and their retinas dissected and stored on ice in L-15 media (Invitrogen) supplemented with 10 mM glucose and 0.1 mg/ml BSA (Sigma-Aldrich). WT animals used in this study included Pd+/+ littermates, as well as C57BL/6J and 129SV mice from Charles River. No significant differences in rod physiology or Western blotting (see below) were observed between individual groups, and thus all three control populations were pooled for comparison to the Pd−/− rods, which were of mixed C57BL/6J and 129SV backgrounds. Suction electrode recordings were performed as previously described (Krispel et al., 2003). In brief, the retina was chopped into small pieces in L-15 solution supplemented with DNase I (~25 units/ml; Amersham Biosciences) and placed in a recording chamber perfused with bicarbonate-buffered Locke's solution (pH 7.4) supplemented with 10 mM glucose and maintained at 35–37°C. Retinal pieces were visualized under infrared light using a CCD camera (Stanford Photonics), and an individual rod outer segment was gently drawn into a suction electrode containing 140 mM NaCl, 3.6 mM KCl, 2.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 3 mM HEPES, 0.02 mM EDTA, 10 mM glucose (pH 7.4). Membrane currents were recorded using a current-to-voltage converter (Axopatch 1B; Axon Instruments, Inc.) and low-pass filtered (8-pole Bessel; Frequency Devices) using 30-Hz corner frequency. Membrane current and photodiode voltage were digitized and recorded at 200 Hz using IGOR-National Instruments acquisition software (IgorPro for NIDAQ for Windows; Wavemetrics). Brief flashes (10 ms) of 500-nm light were used for stimulation in darkness or in the presence of steady 520-nm light. Light intensities were controlled by calibrated neutral density filters, and the power of the tungsten-iodide lamp was measured after each day of recording using a silicon photodiode (Graseby Optronics).

Bright steady light (520 nm; 3.6×10^6 photons/μm^{-2} s) was used to drive phototransduction strongly for a short period of time (Fig. 3). To compensate for the ~30% reduction in sensitivity between WT and Pd−/− rods (Table I), this bleaching light was applied to WT rods for 1.4 s and to Pd−/− rods for 2.0 s. Assuming an effective collecting area of 0.36 μm^2 (Krispel et al., 2006) and 7×10^7 rhodopsins per rod (Lyubarsky et al., 2004), this corresponds to a 2.6 and 3.7% bleach in WT and Pd−/− rods, respectively. The recovery of the circulating current following this bleaching light was followed by delivering saturating flashes every 10 s (which produced an additional cumulative bleach of

TABLE I

Characteristics of Dark-adapted Mouse Rods with and without Phosducin

	Dark current	Time to peak	τ_{rec}	Integration time	Elementary amplitude	Normalized flash sensitivity	I_o
	pA	*ms*	*ms*	*ms*	*pA*	*photons*$^{-1}$ μm^2	*photons*/μm^2
WT	13.9 ± 0.6 (27)	110 ± 5 (24)	196 ± 11 (24)	247 ± 13 (24)	0.64 ± 0.08 (19)	0.0127 ± 0.0009 (24)	58.9 ± 4.5 (19)
Pd−/−	13.1 ± 0.5 (30)	93 ± 3 (25)	187 ± 14 (25)	248 ± 22 (26)	0.25 ± 0.01 (17)	0.00854 ± 0.0006 (23)	87.9 ± 6.2 (21)

Normalized flash sensitivity was determined as the peak amplitude of dim flash response amplitude, normalized by the dark current and divided by the flash strength. I_o is the flash strength that elicited a half-maximal response.

<0.1%) and comparing the maximal amplitude to that before the bleaching light.

The form of each rod's single photon response was estimated by variance-to-mean squared analysis as previously described (Mendez et al., 2000). The dark-adapted flash sensitivity (in photons$^{-1}\mu m^2$) was calculated as the normalized response amplitude (peak amplitude of the dim flash response divided by the maximal response amplitude) divided by the flash strength. The integration time of the average response to a dim flash was determined by dividing the time integral of the response by the peak amplitude (Baylor and Hodgkin, 1973). The time constant of recovery (τ_{rec}; Table I) was determined by fitting a single exponential function to the final falling phase of the average dim flash response. To determine the dominant time constant of recovery from saturating flashes, a straight line was fitted to the relationship between the time in saturation and the natural log of the flash strength (in units of photons μm^{-2}) up to ln i = 9. The time that a bright flash response remained in saturation was plotted defined as the time interval between the midpoint of the flash and the time at which the response recovered by 10%.

Light-dependent modulation of flash sensitivity was assessed by plotting the log of the normalized flash sensitivity as a function of the log of the background intensity in photons/μm^2/s. In practice, the dark-adapted flash sensitivity and dark current level was determined before and after each background intensity. Following the onset of background light, a waiting period of 30–60 s allowed the current to reach equilibrium before test flashes were given atop the steady background light to elicit just-detectable incremental responses. For comparison of WT and Pd−/− rods, background intensities for each cell were scaled so that the cell's Io value coincided with the mean Io value for the population (see values given in Table I). The curve in Fig. 6 was drawn according to the Weber-Fechner relation:

$$\frac{S_F}{S_F^D} = \frac{1}{1 + I / I_o^B},$$

where I is the background light intensity and I_o^B is the background intensity that decreased the incremental flash sensitivity to one half its dark value.

The earliest rising phases of the population average single photon responses, $r(t)$, were fitted with the activation model of Pugh and Lamb (1993):

$$\frac{r(t)}{r_{max}} = 1 - \exp\left[-\frac{1}{2} A\phi (t - t_{eff})^2\right],$$

where r_{max} is the saturating amplitude, A is the amplification constant in units of s^{-2}, ϕ is the average number of photoexcited rhodopsins ($\phi = 1$) and t_{eff} is the effective delay, which we assumed to be 2 ms.

RESULTS

Dark-adapted Pd−/− Rods Show Reduced Sensitivity

To test the idea that phosducin regulates light sensitivity of the rod by controlling the availability of transducin heterotrimer, we used suction electrodes to record from individual rods of dark-adapted WT and Pd−/− mice. Generally speaking, dark-adapted responses of Pd−/− rods were similar to those of WT rods (Fig. 1 A). The dark currents, measured by the maximal response amplitudes, were not significantly different in rods lacking phosducin

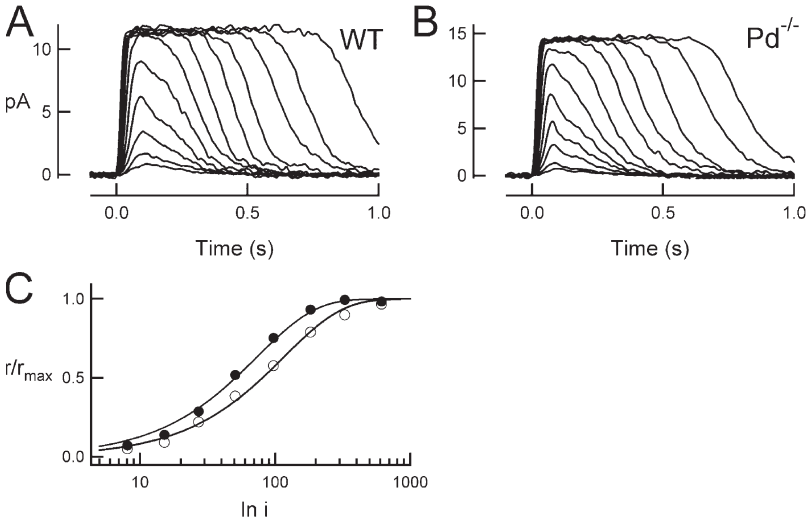

Figure 1. Families of flash responses from representative rods of WT (A) and phosducin knockout (B) mice. Flash strengths ranged from 8.05 to 6654 photons/μm^2 by factors of 2. (C) Normalized response amplitudes as a function of flash strength for the rods shown in A and B. Points were fitted by saturating exponential functions, with I_o values of 50.8 (WT) and 80.5 (Pd−/−) photons/μm^2.

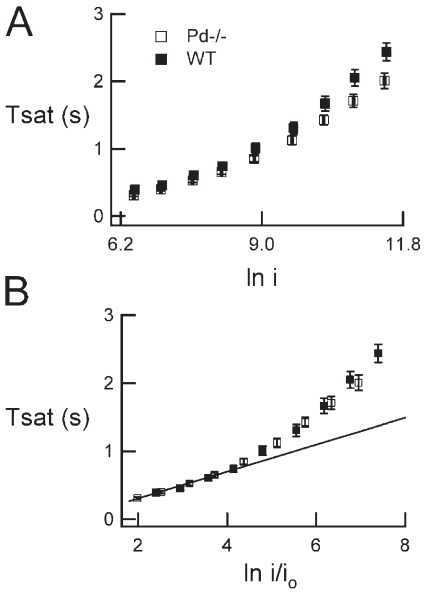

Figure 2. Saturating flash responses of phosducin knockout rods show normal recovery kinetics. (A) The time that a bright flash response remained in saturation was plotted as a function of the natural log of the normalized flash strength (in photons/μm^2). In mouse rods, this relation is linear up to ln i ~9 (Chen et al., 2000), with the slope equal to the dominant time constant of response recovery (Pepperberg et al., 1992; Nikonov et al., 1998). (B) Normalizing for the difference in sensitivity between Pd−/− and WT rods (dividing the x-axis by the Io values for each individual cell) underscores the similarity of Pd−/− and WT recovery time constants across the entire range of tested flash strengths. The straight line, which has been fitted to the Pd−/− data points, has a slope of 0.198 s.

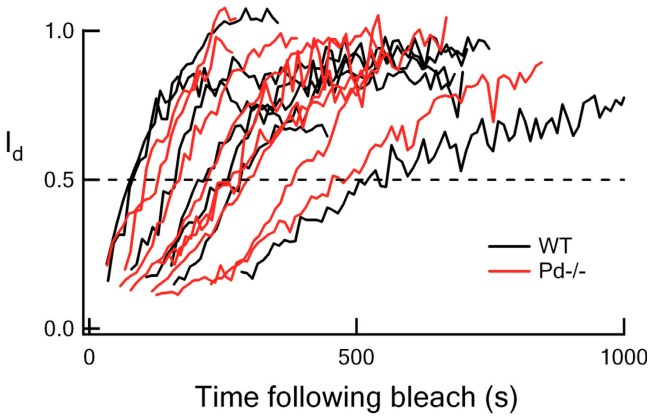

Figure 3. Phosducin knockout rods show normal recovery from larger bleaches. Recovery of the dark current (I_d) as a function of time following substantial bleaching exposures in different WT (black, $n = 7$) and Pd−/− (red, $n = 9$) rods. The brief (1.4 s) light exposure that bleached an average of 2.6% of the rhodopsin in WT rods suppressed I_d strongly, and it recovered to its dark value slowly, over a period of hundreds of seconds (see Materials and methods). Bleaches that drove transduction to a similar extent in Pd−/− rods (3.7% bleach) yielded responses that recovered along a similar time course.

(Table I; Student's two-tailed t test; P = 0.30), consistent with a grossly normal outer segment length and retinal morphology reported previously for Pd−/− retinas (Sokolov et al., 2004). The recovery of dim flash responses (Table I) and responses to saturating flashes (Fig. 2) were indistinguishable in the two types of rods. We also attempted to analyze the recovery of the dark current following short steps that should activate all transducin present. Although individual cells recovered along variable time courses (Fig. 3), the average times required for half-maximal recovery of the dark current following a 3% bleach were indistinguishable between the two populations (228 ± 62 s, $n = 7$ for WT rods, and 255 ± 51 s, $n = 9$ for Pd−/− rods). However, Pd−/− and WT rods did show a clear difference in their overall sensitivity to light. The flash strength required to elicit a half-maximal response (I_o) was 49% greater (P = 0.0005) than that needed for eliciting half-maximal responses from WT rods (Fig. 1 B; Table I). Likewise, the flash sensitivity (S_f^D), measured as the normalized dim flash response amplitude per flash strength (photons^{-1} μm^2), was 33% lower in Pd−/− rods than WT rods (Table I). These measures of sensitivity (S_f^D and I_o) are both dependent on the outer segment collecting area as well as changes in phototransduction gain (e.g., the number of transducin heterotrimers activated by a given photoexcited rhodopsin). One measure of sensitivity that is independent of effective collecting area is the single photon response amplitude. We calculated the amplitude of the single photon response in Pd−/− and WT rods using variance-to-mean analysis (Rieke and Baylor, 1998; Mendez et al., 2000). On average, the amplitude of the single photon response was more than twofold smaller in Pd−/− rods than in WT rods (P = 4×10^{-5}; Fig. 4 A; Table I), and reached a peak slightly earlier (P = 0.005; Table I).

The number of transducin molecules activated per photoexcited rhodopsin determines how steeply the dim flash response rises from baseline (Pugh and Lamb, 1993) and is dependent upon transducin concentration (Pugh and Lamb, 1993; Sokolov et al., 2002). We compared the rising phases of average dim flash responses from WT and Pd−/− rods and found that the single photon responses of Pd−/− rods rose more gradually than those of WT rods. Fitting the Lamb-Pugh model of phototransduction activation (Pugh and Lamb, 1993) to the initial rising phases of the population average single photon responses revealed that the amplification constant was reduced by 32% (Fig. 4 B).

Phosducin Knockout Results in Reduced Expression of Transducin βγ-Subunit in Rods

One possible explanation for the reduced amplification constant in Pd−/− rods is that loss of phosducin causes transducin mislocalization and/or reduced heterotrimer expression. Indeed, ~1/3 reduction in the expression of transducin β-subunit was noted in the original paper

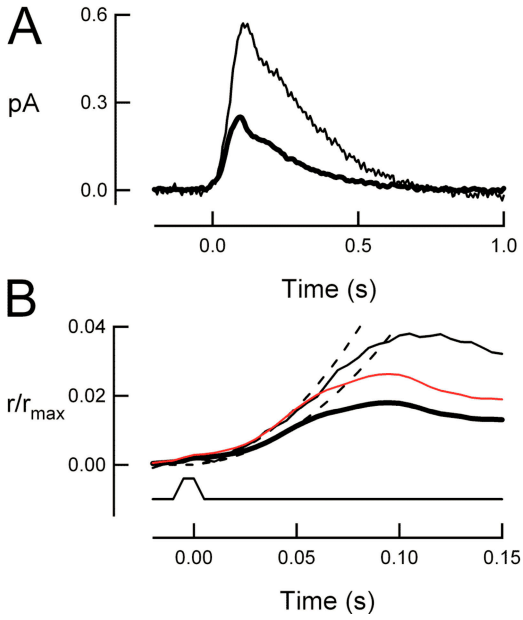

Figure 4. Single photon responses of Pd−/− rods are smaller than normal. (A) Population average single photon responses from 19 WT (solid) rods and 18 Pd−/− rods (bold). (B) Same traces as in A on an expanded time scale and normalized by the average dark currents for the population of rods (14.8 pA, WT; 13.9 pA, Pd−/−). Dashed lines are parabolic fits of the Lamb-Pugh model of phototransduction activation (see Materials and methods), resulting in amplification constants of 11.1 s^{-2} (WT) and 7.6 s^{-2} (Pd−/−). Red trace is the Pd−/− response multiplied by 1.46, compensating for the difference in the amplification constants. The scaled Pd−/− trace reaches a smaller peak amplitude than the WT response, highlighting an effect on the response unexplained by the 32% reduction in amplification constant.

describing the Pd−/− phenotype (Sokolov et al., 2004). We therefore determined the expression levels of all α, β, and γ transducin subunits in the retinas of WT and Pd−/− mice by quantitative Western blotting. Retinas were obtained from age-matched WT and Pd−/− mice that were maintained in the adjacent cages, under 12 h light/12 h dark conditions in a standard cage room. Due to the mixed C57BL/6J and 129SV genetic background of Pd−/− mice (Sokolov et al., 2004), we first compared the levels of transducin subunits in C57BL/6J and 129SV mice and found no difference (unpublished data). Aliquots of the retina extracts containing identical amounts of rhodopsin were separated side by side on SDS-PAGE, together with samples containing various amounts of transducin subunit standards, and analyzed by Western blotting using specific antibodies against rod transducin α, β, and γ subunits. Immunofluorescence intensities of the specific bands were measured using the Odyssey Infrared Imaging System (LI-COR Biosciences). The fluorescence signal from each transducin subunit band in retina extracts was compared with those in the transducin standards (Fig. 5), and presented as a percent fraction of rhodopsin (Table II). We found that retinas of Pd−/− mice contained reduced amounts of all three transducin subunits compared with WT, with the expression of transducin β and γ subunits being affected the most. Interestingly, in both WT and phosducin knockout mice the overall amount of transducin αβγ heterotrimer appeared to be limited by the amount of synthesized transducin γ-subunit, which was estimated to be equal to 14.1 ± 0.7% of the rhodopsin in WT and 9.0 ± 0.4% of the rhodopsin, in Pd−/− retinas. The observed reduction in transducin αβγ heterotrimer in Pd−/− retinas is consistent with the decrease in amplification constant observed in the single cell recordings.

Pd−/− Rods Show Normal Light-dependent Modulation of Flash Sensitivity

To test whether Pd plays a dynamic role in controlling the availability of transducin in the presence of steady

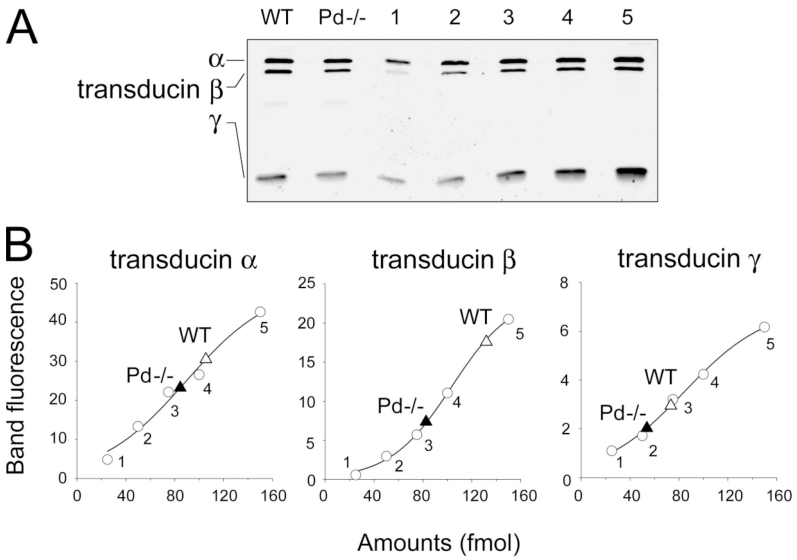

Figure 5. Quantitative Western blotting of Pd−/− retinal extracts reveals decrease in transducin subunits expression. (A) Whole retinal extracts containing 100 fmol rhodopsin from WT and Pd−/− mice were separated on 10–20% polyacrylamide gels alongside 25, 50, 75, 100, 150 fmol purified transducin standard (1–5), and probed in Western blot analysis with antibodies against rod transducin subunits. (B) Fluorescence of the corresponding transducin standards bands from the top panel were plotted against the amounts of transducin in samples 1–5 (open circles), and fitted with sigmoid curves. Triangles represent the fluorescence of transducin bands from retina extracts. The number of determinations for each subunit is indicated in Table II.

TABLE II

Comparison of Transducin Subunit Abundance in the Retinas of Phosducin Knockout and WT Mice

	$G_{\alpha t1}$	$G\beta_1$	$G\gamma_1$
Pd−/−	14.5 ± 0.5 (15)	14.4 ± 0.7 (17)	9.0 ± 0.4 (17)
WT	17.8 ± 0.8 (16)	20.9 ± 1.3 (16)	14.1 ± 0.7 (17)
Reduction, %	18.5	31.1	36.1
P value	0.001	0.00009	0.000007

The abundance of each transducin subunit is represented as a percent of rhodopsin content. Error indicates SEM, number of experiments is given in parentheses. Pd−/−, phosducin knockout mice.

light, we measured the flash sensitivities of 7 WT and 10 Pd−/− rods in the presence of background lights of varying intensities. In WT rods, background light causes a reduction in the amplitude of the incremental flash response, as well as a speeding of its time course (Fig. 6 A). Rods lacking phosducin displayed the same behavior in the presence of steady light (Fig. 6). In both WT and Pd−/− rods, the incremental flash sensitivity varied with background intensity according to the Weber-Fechner relation (see Materials and methods), although the background light intensity needed to reduce the dark-adapted sensitivity to half (I_o^B) was brighter than in WT rods (1352 for Pd−/− rods, 899 for WT rods, in photons μm^{-2} s). The higher I_o^B value for Pd−/− rods is consistent with the notion that Pd−/− rods are less sensitive than WT rods due to the lower transducin expression. When the background light intensity was normalized by the dark-adapted I_o values, the light-induced decline in incremental flash sensitivities of Pd−/− and WT rods were indistinguishable (Fig. 6 B). Thus, although dark-adapted Pd−/− rods contain less transducin heterotrimer and therefore are less sensitive to steady background light, the lack of Pd had no effect on the ability of the dark-adapted rods to adjust their incremental flash sensitivity in the presence of background light.

Adaptive Acceleration Is Normal in Phosducin Knockout Rods

Several long-lasting forms of light adaptation have been described that either reduce sensitivity (Calvert et al., 2002) or speed response kinetics (Krispel et al., 2003) after several minutes of just-saturating light. The mechanisms for these long-lasting forms of adaptation have not been identified, but may involve the availability of heterotrimer. To test for Pd's involvement in these other forms of adaptation, we determined the magnitude and extent of the shortening of the time that a bright flash response remained in saturating following 3 min of just-saturating steady light (Fig. 7).

In WT rods, this stimulus induced a 36 ± 3% shortening of the time in saturation that decayed back to its dark-adapted value with a time constant (τ_{offset}) of 81 ± 15 s (Krispel et al., 2003). Rods lacking phosducin likewise showed a 38 ± 9% shortening (n = 4; P = 0.73) and τ_{offset} of 65 ± 17 s (n = 3; P = 0.54), indicating that phosducin does not play an essential role in the induction or fading of this adaptation mechanism.

Light-dependent Dephosphorylation of Phosducin in WT Mouse Retina

Because Pd−/− rods showed no physiological changes other than that attributable to decreased transducin levels, we sought to determine whether light-dependent dephosphorylation of phosducin normally occurs in WT rods under conditions of our physiological recordings, including the range of light intensities, solutions, and

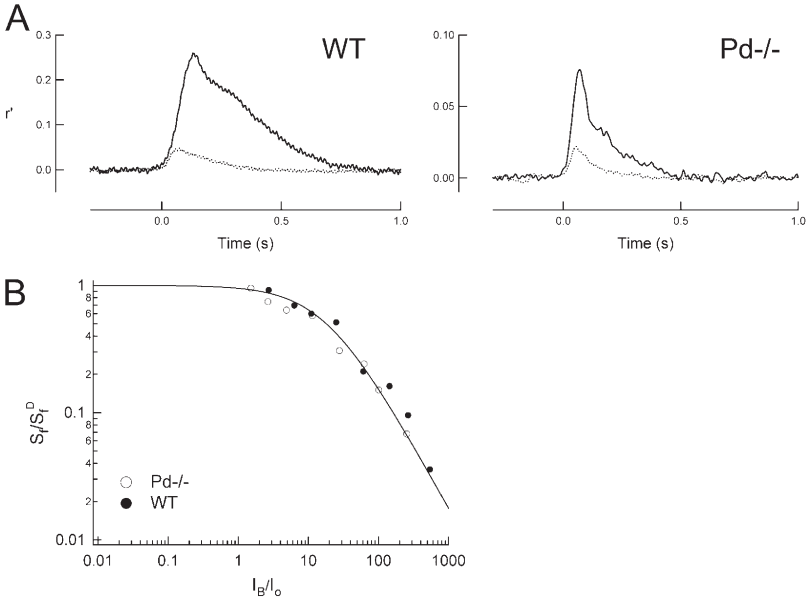

Figure 6. Adaptation of incremental flash sensitivity is normal in Pd−/− rods. (A) Average dim flash responses (r′; in pA) delivered in darkness (solid) or in the presence of steady light (dash) that decreased the circulating (dark) current by ~75%. In both WT and Pd−/− rods, steady light decreased the response amplitude and slightly accelerated the response time course as previously described (e.g., Krispel et al., 2003). Dark currents (in pA) were 16.2 (WT) and 16.1 (Pd−/−). (B) Flash sensitivity in background light (S_f) was normalized by the dark-adapted flash sensitivity (S_f^D), measured before and after each background light intensity. The x-axis was normalized by the average I_o values for WT and Pd−/− rods, which caused the two relations to superimpose, indicating that Pd does not contribute directly to the changes in flash sensitivity that accompany background illumination. Points reflect mean values, with the number of cells varying between 2 and 7 for WT and 2 and 10 for Pd−/−.

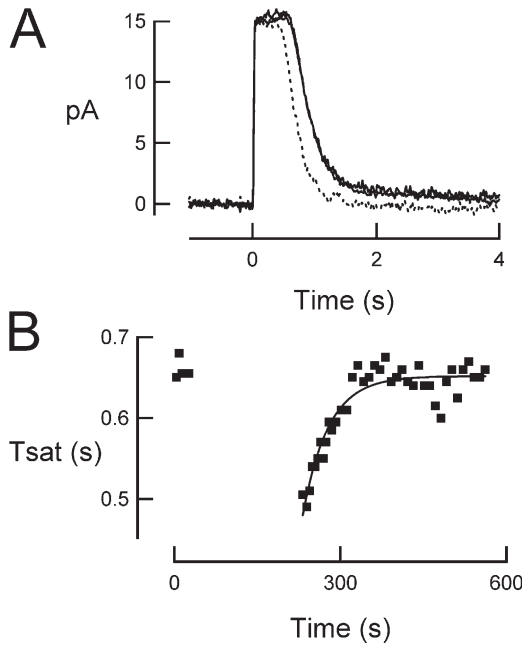

Figure 7. Adaptive acceleration is normal in Pd−/− rods. Prolonged (3 min) exposure to just-saturating light induced a shortening of the time in saturation that persisted for tens of seconds following the adapting light offset (Krispel et al., 2003). (A) Responses of a Pd−/− rod to a test flash (1945 photons/μm^2) before, immediately after (dashed), and 100 s after a saturating light that produced a 0.86% cumulative bleach, assuming 7×10^7 rhodopsins per rod (Lyubarsky et al., 2004) and an effective collecting area of 0.36 μm^2 (Krispel et al., 2006). (B) The shortening of the time in saturation was reversible, decaying exponentially back to the dark-adapted value with a time constant of 45 s.

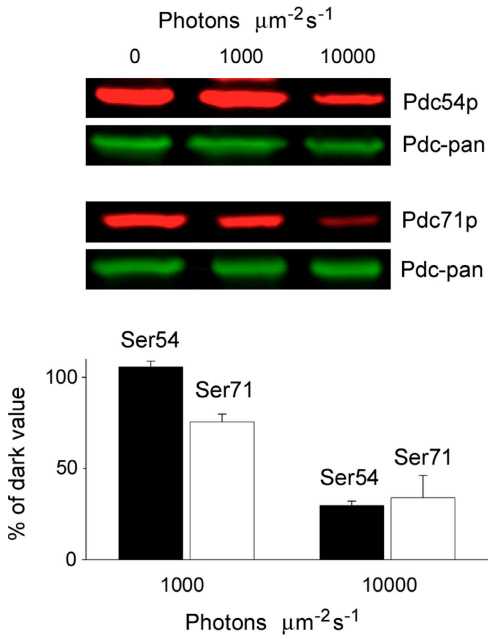

Figure 8. Light-dependent dephosphorylation of phosducin in WT mouse retinas. Dark-adapted retinas were exposed to the steady light of indicated intensities, under conditions closely matching those in the experiments described in Figs. 3 and 6. After 2 min of exposure, the retinas were homogenized in SDS-PAGE sample buffer, and the degree of phosducin phosphorylation was determined by Western blotting. (A) Phosducin bands were double labeled with either phosphospecific Pdc54p or Pdc71p antibody (red) and Pdc-pan antibody (green). (B) The fluorescence values of each red band was divided by the fluorescence value of the corresponding green band, and then the amount of phosphorylated phosducin in the light was normalized to the dark-adapted value (SEM, $n = 3$).

temperature (see Materials and methods). We analyzed the status of phosducin phosphorylation in retinas following 2-min exposures to two different light intensities, which represented the middle and high end of intensities used in our adaptation experiments (Fig. 8). Thus, the light adaptation protocols used in our studies of light adaptation should have been sufficient to induce changes in phosducin's phosphorylation state in WT rods.

DISCUSSION

For years, phosducin has been thought to regulate the availability of transducin heterotrimer for activation through the interaction of dephosphorylated phosducin with the G protein's βγ subunits. However, a direct physiological test of this hypothesis has never been performed. We have found that the dark- and light-adapted response properties of Pd−/− rods were indistinguishable from those of WT rods in every way except one: Pd−/− rods were less sensitive than normal both in the dark-adapted and light-adapted states. The gain of transduction, as measured by the amplification constant using the Lamb-Pugh model of activation, was 32% lower in Pd−/− rods than in WT rods. The amplification constant quantitatively reflects several different gain parameters, including the rate of G protein activation by rhodopsin, the coupling efficiency for the activation of PDE by transducin, the rate constant of cGMP hydrolysis, and the cooperativity of the cGMP-gated channels (Pugh and Lamb, 1993). Because there was a 36% reduction in the level of transducin βγ-subunit expression (this study), and over 20% of the remaining transducin was mislocalized from the outer segments (Sokolov et al., 2004), the simplest explanation for the reduced amplification constant in Pd−/− rods is a reduced rate of transducin activation resulting from its reduced concentration. Note that it is not unexpected that the reduction in the rod outer segment transducin concentration may be larger than the reduction in the amplification constant because the dependency of the rate of transducin activation on its concentration is hyperbolic rather than linear (e.g., Heck and Hofmann, 2001). The effect on the amplification constant was not detected in the initial characterization of Pd−/− mice by ERG (Sokolov et al., 2004); presumably, suction electrode recording is more sensitive or the delivered light intensities more accurately controlled in single cell experiments.

It is notable that the single photon response amplitude was roughly 2.5-fold less in Pd−/− rods, whereas the amplification constant was reduced by only ∼32%. Scaling the average Pd−/− response to match the WT amplification constant (Fig. 4 B, red trace), brings the earliest rising phase of the response into alignment with that of the WT response (until ∼50 ms after the flash), but also reveals another factor that contributes to the reduced amplitude, a small but reliable decrease in the overall time to peak. This decrease is rather unexpected because in WT rods, responses of smaller amplitude have longer time to peak than responses of larger amplitudes. These results suggest that in addition to a decrease in the amplification, the loss of phosducin was also accompanied by some other change affecting the response amplitude, which could include cascade deactivation mechanisms or calcium feedback (Nikonov et al., 1998). Because transducin, rhodopsin kinase, and arrestin bind to the same sites on rhodopsin (Kuhn et al., 1984; Schleicher et al., 1989; Langlois et al., 1996; Krupnick et al., 1997; Thurmond et al., 1997; Pulvermuller et al., 2000), it seems plausible that reduced transducin concentration could be accompanied by increased rates of rhodopsin phosphorylation and arrestin binding and ultimately more rapid rhodopsin deactivation. This is consistent with rhodopsin deactivation beginning on the time scale of the rising phase of the response (Chen et al., 1995, 1999; Krispel et al., 2006).

The reduced amplitude and faster kinetics observed in the Pd−/− responses are superficially similar to the reduced amplitude and faster kinetics of light-adapted flash responses of normal rods. However, every experimental examination of light adaptation in Pd−/− rods failed to reveal even subtle defects in the ability of Pd−/− rods to adapt to light. Therefore, Pd−/− rods are not simply constituitively light adapted.

Our quantitative analysis of the transducin subunits contents in Pd−/− mice confirmed the initial report that the expression level of transducin β-subunit is reduced (Sokolov et al., 2004) and demonstrated that the content of the γ-subunit is decreased to a similar degree. Furthermore, the superior linearity of the infrared fluorescence–based Western blot detection and utilization of a calibration curve for each individual transducin subunit allowed us to reveal that the content of the α-subunit is also reduced, although to a lesser degree (Table II). The mechanism by which loss of phosducin leads to changes in heterotrimer expression is completely unknown. Furthermore, regulation of phosducin expression itself seems complex, as abolishing expression of the α-subunit (Calvert et al., 2000) or expression of only one copy of the rhodopsin gene (Calvert et al., 2001) leads to increases in phosducin expression. The reduction of transducin expression in the absence of phosducin may occur posttranslationally, as phosducin can protect βγ subunits from proteolysis (Obin et al., 1996, 2002), or it may occur at the level of transcription (Zhu and Craft, 2000).

Interestingly, the expression level of transducin determined in this study for WT rods is a bit higher than previously reported based on the maximal level of light-dependent GTPγS binding in osmotically intact mouse rod outer segments (Tsang et al., 1998). This could be explained in part by a fraction of transducin localized outside the rod outer segment even in the completely dark-adapted rods (e.g., Zhang et al., 2003; Sokolov et al., 2004; Kerov et al., 2005). On the other hand, our data do not support a recent report that dark-adapted rod outer segments contain three to four times more transducin βγ subunits than α subunits (Clack et al., 2006). One explanation for the difference may be that the outer segments used in their study were purified on a sucrose gradient. High sucrose concentrations are documented to induce hyperosmotic shock, which may cause protein leakage from the rods (Schnetkamp et al., 1979). Because transducin βγ-subunit has higher membrane affinity than α-subunit (e.g., Seitz et al., 1999), it may be better retained in the rod outer segments subjected to such shock.

In summary, the complete lack of phosducin does not affect a rod's ability to adapt to steady, even fairly bright light that remains on sufficiently long for light-dependent dephosphorylation (Lee et al., 1984; Song et al., 2007). These results suggest that phosducin does not dynamically regulate the availability of transducin heterotrimer and therefore does not control the light sensitivity of rods. It is more likely that phosducin's role in photoreceptor physiology is geared toward longer-term regulatory functions rather than short-term signaling. One such function is setting the transducin expression level, which is consistent with the fact that the majority of phosducin in rods resides in the inner segment, where protein synthesis and degradation take place. Another function is the facilitation of transducin translocation (Sokolov et al., 2004), which takes place at light intensities brighter than can be used in single cell recordings. Transducin translocation may contribute to the global readjustment of rod light sensitivity during the normal diurnal cycle and may also serve to optimize the rod outer segment protein composition for energy conservation and protection of the cell from light-induced damage (Burns and Arshavsky, 2005; Fain, 2006).

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