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Cone Phosphodiesterase-6α′ Restores Rod Function and Confers Distinct Physiological Properties in the Rod Phosphodiesterase-6β-Deficient rd10 Mouse

Wen-Tao Deng, Keisuke Sakurai, Saravanaan Kolandaivelu, Alexander V. Kolesnikov, Astra Dinculescu, Jie Li, Ping Zhu, Xuan Liu, Jiijing Pang, Vince A. Chiiodo, Sanford L. Boye, Bo Chang, Visvanathan Ramamurthy, Vladimir J. Kefalov, and William W. Hauswirth

Phosphodiesterase-6 (PDE6) is the key effector enzyme of the vertebrate phototransduction pathway in rods and cones. Rod PDE6 catalytic core is composed of two distinct subunits, PDE6α and PDE6β, whereas two identical PDE6α′ subunits form the cone PDE6 catalytic core. It is not known whether this difference in PDE6 catalytic subunit identity contributes to the functional differences between rods and cones. To address this question, we expressed cone PDE6α′ in the photoreceptor cells of the retinal degeneration 10 (rd10) mouse that carries a mutation in rod PDE6 subunit. We show that adenovirus-associated virus-mediated subretinal delivery of PDE6α′ rescues rod electroretinogram responses and preserves retinal structure, indicating that cone PDE6α′ can couple effectively to the rod phototransduction pathway. We also show that restoration of light sensitivity in rd10 rods is attributable to assembly of PDE6α′ with rod PDE6γ. Single-cell recordings revealed that, surprisingly, rods expressing cone PDE6α′ are twofold more sensitive to light than wild-type rods, most likely because of the slower shutoff of their light responses. Unlike in wild-type rods, the response kinetics in PDE6α′-treated rd10 rods accelerated with increasing flash intensity, indicating a possible direct modulatory function of cone PDE6α′ activity. Together, these results demonstrate that cone PDE6α′ can functionally substitute for rod PDE6β in vivo, conferring treated rods with distinct physiological properties.

Introduction
Rod and cone photoreceptor cells share a similar phototransduction pathway but exhibit strikingly different physiological properties. Rods, responsible for scotopic vision, are highly light sensitive. Cones, responsible for photopic vision, are intrinsically less sensitive, have faster response kinetics, and adapt to a wider range of light intensities (Pugh and Cobbs, 1986; Fu and Yau, 2007). One of the key unresolved questions is how the physiological differences between rods and cones can be correlated with the distinctive properties of their phototransduction proteins. Previous studies have shown that the lower thermal stability of cone pigments is likely to contribute to the lower sensitivity of cones but, once activated, rod and cone pigments can couple equally efficiently to rod or cone transducin (Kefalov et al., 2003, 2005; Shi et al., 2005, 2007; Fu et al., 2008). Thus, consistent with our previous research (Deng et al., 2009) and other studies (Ma et al., 2001), the signaling properties of rod and cone transducin α-subunit do not contribute to the difference in light sensitivity between rods and cones (but see Chen et al., 2010). As a result, the expression levels and molecular properties of phototransduction components downstream of transducin are likely to play an important role in defining the distinctive physiological properties of rods and cones.

The photoreceptor cyclic nucleotide phosphodiesterase-6 (PDE6) plays an essential role in phototransduction by regulating the GMP levels in rods and cones (Fu and Yau, 2007). The most obvious distinction between rod and cone PDE6 is that rod PDE6 is composed of two distinct catalytic subunits α, β (PDE6A, PDE6B) and two inhibitory subunits γ (PDE6G), whereas cone PDE6 is composed of two identical catalytic subunits α′ (PDE6C) plus two cone-specific inhibitory subunits γ′ (PDE6H) (Gillespie...
and Beavo, 1988; Hamilton and Hurley, 1990; Li et al., 1990). Each of the catalytic subunits of PDE6 consists of two N-terminal regulatory cGMP-binding GAF (for cGMP-specific phosphodiesterases, adenylyl cyclases, and FlhA) domains (GAFa and GAFb) and a catalytic domain located in the C-terminal region. The catalytic domains are highly conserved among rod and cone PDE6 subunits and exhibit equivalent enzymatic activities (Mun and Cote, 2001; Muradov et al., 2010). Among the GAF domains, rod PDE6G displays a higher affinity toward cGMP than cone PDE6 (Gillespie and Beavo, 1989). It has been suggested that these differences in GAF binding affinities toward cGMP and PDE6γ might contribute to the higher efficiency of cone PDE6 activation by transducin α-subunit (Muradov et al., 2010).

In this study, we tested whether PDE6 catalytic subunit identity contributes to the functional differences between rods and cones by expressing cone PDE6α in the retinal degeneration 10 (rd10) photoreceptor cells, which carry a mutation in the β-subunit of rod PDE6 (Chang et al., 2007). We show that cone PDE6α can restore rd10 rod function by assembling with rod PDE6γ. Furthermore, it confers rods with distinct physiological properties.

Materials and Methods

Animals. rd10 mice and wild-type (WT) C57BL/6j controls were obtained from The Jackson Laboratory. The mice of either sex were bred and maintained in the University of Florida Health Science Center Animal Care Services Facilities in a continuously dark room, except for husbandry at ∼400 lux illuminance. All experiments were approved by the local Institutional Animal Care and Use Committee at the University of Florida and Washington University and conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and National Institutes of Health regulations.

Construction and packaging of adenovirus vectors. PDE6α and PDE6γ cDNAs were purchased from Invitrogen. The adenovirus vector (AAV) vector containing murine PDE6α or PDE6γ cDNA under the control of small chicken β-actin (smCBA) promoter was packaged in AAV serotype 8 (AAV8) Y733F by transfection of HEK293 cells according to previously published methods (Zolotukhin et al., 1999).

Subretinal injections. Postnatal day 14 (P14) rd10 pups raised in the dark were brought to a normal illuminated room for injection and then returned to the dark. A total volume of 1 μl of AAV8 Y733F-smCBA-PDE6α vector (4.25 × 10^12 vector genomes/ml) was injected subretinally into the left eyes, and the right contralateral eyes served as untreated controls. Subretinal injections were performed as described previously (Pang et al., 2006, 2008). Briefly, a 33 gauge blunt needle mounted on a 5 μl Hamilton syringe was introduced through the corneal opening made by 30 gauge needle, and injections were visualized by fluorescein-positive subretinal bleb. One percent propylene glycol (PEG) ethanolamine ophthalmic ointment was given after injection.

Electroretinogram analyses. At 5 weeks after injection, rod- and cone-mediated electroretinograms (ERGs) were recorded separately using a UTAS Visual Diagnostic System equipped with Big Shot Ganzfeld (LKC Technologies) according to protocols described previously with minor modifications (Pang et al., 2010). Scotopic rod recordings were performed with three increasing light intensities at −1.6, −0.6, and 0.4 log cd/s/m^2. Ten responses were recorded and averaged at each light intensity. Photopic cone recording were taken after mice were adapted to a white background light of 30 cd/m^2 for 5 min. Recordings were performed with four flash intensities at 0, 1, 2, 7, 8, and 1.4 log cd/m^2 in the presence of 30 cd/m^2 background light. Fifty responses were recorded and averaged at each intensity. Scotopic and photopic b-wave amplitudes from untreated, treated rd10, and WT controls at each intensity were averaged and used to generate an SD. The differences between recordings from untreated and treated eyes were analyzed by the paired t test.

Morphology and immunohistochemistry. Treated rd10 mice were killed and enucleated 24 h after ERG recordings for morphological and immunohistochemical analysis. The eyes were fixed in a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde for 3 h at room temperature and then paraffin embedded and sectioned at 4 μm through the optic nerve for hematoxylin and eosin (H&E) staining. Retinal sections for immunohistochemistry were prepared according to previously described methods (Deng et al., 2009, 2012). Briefly, eyes were fixed in 4% paraformaldehyde. Cornea, lens, and vitreous were removed from each eye without disturbing the retina. The remaining eyecups were rinsed with PBS and then cryoprotected by placing it in 30% sucrose in PBS for 4 h at 4°C. Eyecups were then embedded in cryostat compound (Tissue Tek OCT; Sakura Finetek) and frozen at −80°C. Retinal tissue cryosections were sectioned at 12 μm thickness, rinsed in PBS, and blocked in 2% normal goat serum and 0.3% Triton X-100 in 1% BSA in PBS for 1 h at room temperature. Anti-PDE6α (5848P) (Kirschmann et al., 2010), rhodopsin, or red/green-cone opsin (Millipore Bioscience Research Reagents) antibodies (all 1:1000 dilutions) were diluted in 0.1% Triton X-100 and 1% BSA in PBS and incubated with sections overnight at 4°C. The sections were then washed three times with PBS, incubated with IgG secondary antibody tagged with Alexa Fluor-594 (Invitrogen) at 1:500 dilution and lectin peanut agglutinin (PNA) conjugated to Alexa Fluor-488 (Invitrogen) at 1:200 dilution in PBS at room temperature for 1 h, and washed with PBS. Sections were mounted with Vectashield Mounting Medium for Fluorescence (H-1006; Vector Laboratories) and covered slipped. Sections were analyzed with a Carl Zeiss CD25 microscope fitted with Axiosview release 6.0 software.

Western blot analysis. Untreated, AAV8 Y733F-smCBA-PDE6α-treated rd10 and WT eyes (five eyes each) were carefully dissected, and the eyecups were pooled and homogenized by sonication in a buffer containing 0.23 M sucrose, 5 mM HCl, pH 7.5, and protease inhibitors (Roche Complete). After centrifugation, aliquots of the ex-
**Immunoprecipitation.** Frozen retinal eye cups from untreated, AA.VV Y733F-smCBA-PDE6α–treated rd10 and WT (five each) were homogenized in 400 μl of immunoprecipitation (IP) buffer (in mM: 10 Tris-HCl, pH 7.5, 100 KCl, 20 NaCl, and 1 MgCl2) containing protease and phosphatase inhibitors and 10 μl iodoacetamide using a pellet pestle (VWR) in a 1.5 ml Eppendorf tube on ice (15 s for three times). After homogenization, Triton X-100 was added to a final concentration of 1% (500 μl total volume). The homogenized retinal extracts were preclarified by addition of 10 μl of immunopure immobilized Protein A plus beads (Thermo Fisher Scientific) by incubating at 4°C for 1 h. Supernatants were collected by centrifuging at 10,000 × g (Eppendorf 5414) for 5 min at 4°C. IP was performed with supernatants (400 μl) using mouse monoclonal ROS-1 antibodies. We used l.5 μg of ROS-1 antibody for each pull-down experiment. Bound proteins were eluted by boiling with 50 μl of 1X Laemmli’s sample buffer and separated by 4-20% SDS-polyacrylamide gel (Bio-Rad) and transferred to Immuno-Blot LF PVDF membrane (Bio-Rad). Immunoblot analyses were performed with individual rod PDE6α, PDE6β, PDE6δ, and cone PDE6α (3184p)-specific primary antibodies according to our previously published method (Kolandaivelu et al., 2011).

**Single-cell recordings.** Mice kept in darkness for at least 12 h were killed by CO2, and the eyes were removed under dim red light. Under infrared light, the retina was cut into small pieces and then finely chopped. Isolated pieces of retina were stored in Locke’s solution at 4°C until use. The perfusion Locke’s solution (in mM: 112 NaCl, 3.6 KCl, 2.4 MgCl2, 1.2 CaCl2, 10 HEPES, 20 NaHCO3, 3 Na2-Ascorbate, 0.5 Na-glutamate, and 10 glucose, pH 7.4) was equilibrated with 95% O2/5% CO2, bubbled, and heated to 34–37°C. Glass capillaries were pulled and heat polished to fit the rod outer segment (ROS) diameter and then filled with electrode solution containing the following (in mM): 140 NaCl, 3.6 KCl, 2.4 MgCl2, 1.2 CaCl2, 10 HEPES, and 10 glucose, pH 7.4. A rod photoreceptor was drawn into the electrode to record the inward current of the outer segment (OS). The dark current was amplified by a current-to-voltage converter (Axopatch 200B, Molecular Devices), low-pass filtered by an eight-pole Bessel filter with a cutoff frequency of 30 Hz (Kreon-Bit-Tec), digitized at 1 kHz, and recorded with pClamp 8.2 software (Molecular Devices). Ten-millisecond flashes were delivered from a calibrated light source via computer-controlled shutters. Light intensity and wavelength were changed with neutral density and interference (λmax = 500 nm) filters (Edmund Optics).

**Results.** The scotopic visual acuity of 2-month-old mice were determined using a two-alternative forced-choice protocol (Unino et al., 2011).

**Figure 2.** ERG responses, retinal morphology, immunohistochemistry, and scotopic visual acuity of rd10 mice after AA.VV Y733F-smCBA-PDE6α delivery at 5 weeks after injection (5-wk-pj). A, B, Representative examples of dark-adapted ERG traces (A) and light-adapted ERG traces (B) from an rd10 mouse at 5 weeks after injection. C, D, Dark-adapted ERG was partially restored in injected rd10 eyes. Statistical analysis demonstrated a significant difference between control and treated eyes for dark-adapted b-waves at −1.6, −0.6, and 0.4 log cd/1000° (p < 0.01). E, Light-adapted ERG responses were improved in treated rd10 eyes compared with their untreated controls as a result of a functional rescue and rod cell survival (p < 0.02). Error bars are mean ± SEM. E, Comparison of ERG responses between PDE6β–treated and PDE6α–treated rd10 eyes 5 weeks after injection. There were no significant differences in dark-adapted b-wave amplitudes at three light intensities tested between PDE6β–treated and PDE6α–treated rd10 eyes (p > 0.1). Error bars represent the mean ± SEM. F, Scotopic visual acuity in PDE6α–treated rd10 mice 5 weeks after injection. Data were derived from mouse optomotor responses to rotating gratings under background monitor luminance of −4.45 log cd/m2. Bar graphs are mean ± SEM.
2008). The Optomotry system (Cerebral Mechanics) consisted of a square array of four computer monitors with a pedestal in the center where the mouse was placed. An infrared-sensitive television camera and a round array of six infrared light-emitting diodes mounted above the animal were used to observe the mouse but not the monitors. Using a staircase paradigm, rotating sine-wave vertical gratings were applied on the monitors where they formed a virtual cylinder around the animal (Furukawa et al., 2006). The mice responded to the stimuli by reflexively rotating their head in either clockwise or counterclockwise direction. Optomotor responses were measured under monitor background illumination of -4.45 log cd/m², which was set by neutral density film filters.

Visual acuity was defined as the threshold for spatial frequency (F) of gratings with 100% contrast and measured at the speed (S) of 6.0%/s, F was gradually altered by the computer protocol until its combined threshold for both stimulus directions was determined. Temporal frequency (F) was automatically adjusted by the computer software, based on the following equation: F = S × F (Umino et al., 2008). Data were analyzed using independent two-tailed Student’s t-test, with an accepted significance level of p < 0.05.

Results
Expression of cone PDE6a in rd10 mouse retinas
An AAV8 Y733F capsid-tyrosine mutant vector containing the mouse PDE6a' cDNA driven by a ubiquitous smCBA promoter was delivered subretinally to one eye of rd10 mice at P14, whereas the contralateral eyes remained un.injected and served as controls. PDE6a' expression in treated retinas was analyzed by immunostaining (Fig. 1A) and Western blot analysis (Fig. 1B) 5 weeks after injection. PDE6a' expression was found in both rods and cones of treated rd10 mice after immunostaining with a cone-specific PDE6a' antibody, whereas it was found exclusively in the cones of WT control mouse retinas based on colocalization with a cone OS sheath-specific PNA marker. Photoreceptor cells in untreated retinas were significantly degenerated at this age, and only a weak spotty staining was detected for residual cones (Fig. 1A). Low levels of expression were also observed in the inner retina most likely as a result of nonspecificity of the PDE6a' antibody because similar labeling was observed in untreated and treated rd10, as well as in the WT sections (Fig. 1A). Western blot analysis using the same antibody (Fig. 1B) detected abundant PDE6a' expression in injected rd10 retinas compared with WT controls in which PDE6a' was expressed predominantly in cones. This result provides evidence that PDE6a' is robustly expressed in rd10 rods after AAV8 treatment because rods comprise the majority (97%) of photoreceptor cells in the mouse retina. PDE6a' expression was reduced to almost undetectable levels in retina from un.injected rd10 animals (Fig. 1B), presumably because of the degeneration of cones caused by the loss of PDE6B-deficient rods.

Functional and structural retinal preservation in PDE6a' treated rd10 eyes
To determine whether exogenously expressed cone PDE6a' can rescue rod function in rd10 mice, full-field scotopic and photopic ERG responses were recorded from un injected rd10 mice, injected rd10 mice 5 weeks after injection, and age-matched WT controls. Rod-mediated ERG responses were undetectable in rd10 mice at this age (7 weeks old), whereas vector delivery of PDE6a' to rd10 rods led to significant restoration of rod-driven ERG responses (Fig. 2A, C). The average rod-mediated b-wave amplitude at a flash intensity of -1.0 log cd/m² in treated eyes was 109 ± 39 μV (mean ± SD), whereas it was undetectable in contralateral untreated eyes (n = 3, p < 0.01). The untreated eye rod ERG b-wave amplitude was ~35% of the WT level. Cone-mediated ERG amplitudes in injected eyes also showed some improvement compared with un injected controls (Fig. 2B, D), presumably as a result of better preservation of the cones after restoration of rod function and rod survival (Fig. 3C). The average cone b-wave amplitude was 44 ± 8 μV (mean ± SD) in injected eyes versus 27 ± 10 μV in contralateral untreated eyes at 1.4 log cd/m² (n = 3, p < 0.02). We also recorded ERG responses
from some rd10 mice injected with vector expressing PDE6β and observed no significant differences between PDE6β and PDE6α treatments (Fig. 1E), suggesting that therapy in the rd10 mouse was equivalent whether we used the heterologous rod subunit or the homologous cone subunit. Finally, the scotopic visual acuity of PDE6α-treated rd10 mice improved significantly (0.349 ± 0.088, n = 8) over that of untreated controls (0.069 ± 0.024, n = 5) (Fig. 2F), although rod visual performance still remained subpar compared with WT mice (0.776 ± 0.072).

Three rd10 mice exhibiting significant ERG rescue were killed 2 d after the recordings, and retinal morphology was analyzed by H&E staining (Fig. 3A). Only one layer of photoreceptor nuclei remained in the outer nuclear layer of untreated rd10 retinas with no evident outer or inner segments. In contrast, retinal structure was partially preserved in injected eyes, with five to seven layers of nuclei remaining compared with 12 layers in WT controls. Additionally, treated retinas retained ~20–50% of the normal OS length. Uninjected, injected, and WT retinal sections were also stained with rhodopsin antibody (Fig. 3B) and cone opsin-specific (Fig. 3C) antibody to further confirm the morphological rescue. Expression of both rhodopsin and cone opsin was evident and much more abundant in treated rd10 eyes compared with their spotty staining observed in uninjected controls.

We also recorded ERG responses from rd10 mice at 5 months after treatment (Fig. 4A,B). The average rod-driven ERG b-wave amplitude (Fig. 4A) at a flash intensity of −1.6 log cd/s/m² was 81 ± 15 μV (mean ± SD), and it was significantly higher than the undetectable ERGs in untreated eyes (μ = 3, p < 0.005). The cone-mediated ERG responses (Fig. 4B) were also undetectable in untreated eyes at this age, whereas the average b-wave amplitude in treated eyes at 1.0 log cd/s/m² was 51 ± 13 μV (mean ± SD) (μ = 3, p < 0.03). PDE6α expression was still evident in both rods and cones in treated eyes at 5 months after injection (Fig. 4C). Transducin was strongly expressed in the treated eyes as determined by immunostaining, whereas it was undetectable in untreated eyes at 5 months after injection (Fig. 4D). Thus, PDE6α-mediated rescue of rd10 rod structure and function persisted even months after the AAV injection.

**Figure 4.** ERG rescue and immunohistochemistry of rd10 mice 5 months after injection. **A,** Dark-adapted ERG from uninjected, contralateral injected rd10, and WT control eyes at light intensity of 0.4 log cd/s/m² (p < 0.005). **B,** Light-adapted ERG from the same mice at light intensity of 1.0 log cd/s/m² (p < 0.05). Error bars are mean ± SD. C, PDE6α expression 5 months after delivering AAV8.CMV.CAG-fabo.PDE6α in rd10 retinas. Immunofluorescence of PDE6α (labeled as red) can be detected in both rods (arrow) and cones (arrowheads) in injected rd10 retinas. Only spotty staining was found in untreated eyes as a result of significant retinal degeneration. Cones were labeled by PNA (green). D, Immunostaining of uninjected, injected rd10, and WT retinas with transducin antibody (red). Green, Cone OS sheath-specific PNA. Scale bar, 20 μm. INL, Inner nuclear layer; ODL, outer nuclear layer.
PDE6 catalytic subunits (Fig. 5A). Compared with age-matched WT controls, minor amounts of PDE6α or PDE6β were expressed in total retinal extracts from injected animals. In contrast, there was a dramatic upregulation in PDE6α' expression in these retina. Although lower than in WT controls, we observed robust expression of rod PDE6γ in treated animals likely as a result of complex formation with the virally introduced PDE6α'. To directly test whether the formation of a complex between cone PDE6α' and rod PDE6γ existed, we performed IP with a monoclonal antibody, ROS-1, that exclusively recognizes assembled and functional PDE6 complex from both rods and cones (Kola and et al., 2009, 2011). As expected, we observed assembled rod and cone PDE6 subunits in ROS-1 pull-downs from WT controls (Fig. 5B). Assembled PDE6α' was also observed from surviving cones in uninjected animals. In treated rd10 animals, we detected a complex of PDE6α' and PDE6γ indicating that the restoration of light sensitivity in rd10 rods is attributable to the function of cone PDE6α' assembles with rod PDE6γ (Fig. 5B).

**Figure 5.** Cone PDE6α' subunit expressed in rods associates with rod PDE6γ inhibitory subunit. A Immunoblot analysis with indicated antibodies shows the total level of PDE6 subunits immunoprecipitated from WT control, rd10 injected (R10 inj), and rd10 injected (R10 inj) animals. B IP of assembled PDE6 subunits with ROS-1 antibody from retinal extracts of WT control, uninjected rd10, and injected rd10 animals. After ROS-1 IP, immunoblots were probed with indicated antibodies. Compared with total extracts (T), IP samples (I) were 10 times more concentrated.

**Single-cell recordings from injected rd10 rods**

To gain additional insight into the light responses generated by rods expressing cone PDE6α', we performed single-cell recordings from injected rd10 rods and WT controls. For comparison, we also obtained responses from rd10 rods treated with vector expressing rod PDE6β. Although all retinas of PDE6α' and PDE6β-treated rd10 mice were still subjected to some level of degeneration, we were able to find areas with healthy rods in portions of the retina in which AAV vectors seemed to have been successfully delivered. We obtained photoreponses from 14 PDE6α'-treated rods (from two animals) and 22 PDE6β-treatment rod (from three animals). No significant differences were found between the photoreponses of WT and PDE6β-treated rd10 rods (compare with Fig. 6A, C, Table 1), indicating that the exogenous expression of PDE6β by AAV injection into rods of rd10 mice successfully rescued rod photoreceptor functions. The dark currents, measured from saturated photoreponses, were comparable among WT, PDE6β-treated, and PDE6α'-treated rods (Table 1). Thus, PDE6α' ectopically expressed in rod photoreceptors could form a functional complex with rod PDE6γ and maintain normal spontaneous activity and dark cGMP levels. However, we also observed several unusual features in the responses of PDE6α'-treated rods. First, PDE6α'-treated rods had higher intensity and produced larger single-photon responses than WT rods (Fig. 7A, Table 1). Consistent with this result, intensity-response relationships of dark-adapted rods showed that the flash intensity required for half-saturating response of the PDE6α'-treated rods was approximately twofold lower than that of WT rods (Fig. 6D, inset, Table 1). Second, the time-to-peak and integration time of dim-flash response were substantially prolonged in PDE6α'-treated rd10 rods (Fig. 7A, Table 1). The rising phase of dim-flash response was similar among WT, PDE6β-treated, and PDE6α'-treated rd10 rods (Fig. 7A), which indicates that light-induced cGMP hydrolysis activated by PDE occurred at comparable rates. However, the response recovery phase was substantially delayed in PDE6α'-treated rods, indicating that the deactivation of cone PDE6α' was less effective. This slower than normal response shuttoshould potentially explain the increased sensitivity and single-photon response amplitude in PDE6α'-treated rd10 rods. Third, unlike in WT and PDE6β-treated rd10 rods, the response kinetics in PDE6α'-treated rods accelerated substantially with increasing flash strength (Fig. 6D), and the resulting intensity-response curves appeared shallower than those of WT and control PDE6β-treated rd10 rods. Both of

**Figure 6.** Typical flash response families from single-cell recordings obtained from WT (A), PDE6α' treated (B), and PDE6β-treated (C) rd10 rods. Flashes of intensities increasing in 0.5 log unit steps were delivered at a time interval of 10 ms. Diminishing flash intensities were 3.6, 0.61, and 1.8 photons/μm² in the WT, PDE6α' treated, and PDE6β treated rods, respectively. D Fractional response (R/R₀) of individual rods as a function of flash intensity (I) normalized for half-saturating flux intensity (J). Data from WT rods (black circles, n = 10), PDE6β-treated rd10 rods (blue circles, n = 22), and PDE6α' treated rd10 rods (red circles, n = 14) are well fit by saturating-exponential function and by Hill equation with n = 0.95, respectively. Inset: Cumulative data of sensitivity (I) from individual rods (open circles). Mean values are represented as filled circles. Error bars are ± SEM.
Table 1. Rod response parameters of single-cell recordings

<table>
<thead>
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<th>WT (n = 10)</th>
<th>PDE6αα (n = 14)</th>
<th>PDE6ββ (n = 22)</th>
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<td>Dark current (pA)</td>
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<td>14.8 ± 1.1</td>
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<td>Sensitivity (photons μm−2)</td>
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<td>Time-to-peak (ms)</td>
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<td>178 ± 7</td>
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<td>Integration time (ms)</td>
<td>448 ± 34</td>
<td>790 ± 35</td>
<td>495 ± 29</td>
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<td>Single-photon response (pA)</td>
<td>0.71 ± 0.08</td>
<td>1.55 ± 0.22*</td>
<td>0.76 ± 0.09</td>
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*Estimated using a procedure that measures the dim-flash responses evoked by constant light stimuli. One-way ANOVA with the post hoc Tukey's HSD test determined significant differences at $p < 0.05$ for WT and PDE6ββ. No significant differences were found between WT and PDE6αα.

Discussion

In this study, we expressed cone PDE6αα subunit exogenously in the retina of rd10 mice to investigate its biochemical and light signaling properties in a rod cell environment. Our results demonstrate that cone PDE6αα can functionally substitute for rod PDE6αβ to mediate light signaling in rods, as shown by full-field ERG analysis, behavioral experiments, and single-cell recordings. Rod PDE6α catalytic subunits are destabilized in PDE6αα-injected retinas despite the functional and morphological rescue of rods, and restoration of rod light sensitivity is mediated by assembly of cone PDE6αα with rod PDE6γ. Rods with cone PDE6αα are approximately two times more sensitive than WT cells, and this difference is likely the result of the slower shut-off of their light responses. The slower rate of deactivation indicates that inhibition by rod PDE6γ or the hydrolysis of Taα−GTP on PDE6αα-transducin α complex by regulator of G-protein signaling-9 (RGS9) is less efficient than normal.

We demonstrated previously that AAV-mediated subretinal delivery of rod PDE6β transgene confers long-term rescue of visual function and morphological preservation of the rd10 retinas (Pang et al., 2011). In the present study, AAV8 Y733F cone PDE6αα-treated rd10 retinas showed comparable levels of rescue in gross morphology, amplitudes of rod-driven full-field ERG signals, and the maximal amplitude of single-photon responses, clearly demonstrating that cone PDE6αα can couple effectively to the rod visual signaling pathway in response to light. Our work complements the previous finding of the ability of rod PDE6 to substitute for cone PDE6 to mediate visual signaling in Nlα− cpfl1 mouse model (Kolandaivelu et al., 2011). Although the PDE6αα transgene was driven by an smCBA ubiquitous promoter, we detected most PDE6αα in photoreceptor cells in which it is normally expressed. We observed similar phenomena of endogenous cell-specific expression in the cases of PDE6α (Pang et al., 2006), transducin (Deng et al., 2009), and PDE6βα (Pang et al., 2011) proteins when using the ubiquitous smCBA promoter. The significant scatter in the sensitivity of the AAV-treated rods (Fig. 6D, inset) most likely reflects the variability of AAV-mediated PDE6 expression.

We also showed that vector-expressed cone PDE6αα localized properly to ROS membranes is essential for rapid activation by transducin (Leibman et al., 1987). Cone PDE6αα ectopically expressed in rods of X. laevis was shown to colocalize with endogenous PDE6αα on disc rim regions in rods (Muravov et al., 2009). The similar rising phases of dim-flash responses between PDE6αα−treated rd10 and WT rods as shown with single-cell recordings suggest that the activation rate of the catalytic cone PDE6 subunit in the rod environment is comparable with that of rod PDE6 and that exogenously expressed cone PDE6αα is appropriately localized to ROS disk membranes.

We further show that the restoration of light sensitivity in rd10 rods is attributable to the assembly of cone PDE6αα with rod PDE6γ. Robust expression of PDE6γ was observed in injected retinas, most likely as a result of complex formation with the virus-introduced PDE6αα. The presence of cone PDE6αα or restoration of rod cells did not help in preserving rod PDE6α, which was degraded without its PDE6β partner. It appears that, regardless of cell type, cone PDE6αα forms homodimers to be functional in vivo. The same holds true for rod PDE6 in the sense that PDE6αα and PDE6β are obligated to function as heterodimers (Kolandaivelu et al., 2011). Apparently, the state of association is determined by the properties of the subunits rather than the photoreceptor cell type. All families of vertebrate cyclic nucleotide phosphodiesterases function as homodimers, and, although the reason behind the heterodimerization of rod PDE6 is not known, it presumably exists as a mechanism to control the amount of functional PDE6 enzyme present in rods (Kolandaivelu et al., 2011).

The equivalent rate of activation between WT rods and rd10 rods expressing cone PDE6αα or PDE6β suggests that activated Taα−GTP can effectively release the inhibitory constraint of rod PDE6γ from cone PDE6αα catalytic domain. However, the slower shut-off of PDE6αα−treated rods indicates that deactivation of cone PDE6αα by inhibitory rod PDE6γ or the hydrolysis of α-subunit-bound GTP on PDE6αα−transducin complex is less efficient. The GAF δ domains also bind to the inhibitory γ subunits and play a role in the dimerization of the PDE6 catalytic subunits (Muravov et al., 2004). The strength of interaction between PDE6γ and GAF domains is modulated by cGMP binding to GAF domain. cGMP binding induces an allosteric GAF conformational change and enhances PDE6γ binding affinity, and, in a reciprocal manner, binding of γ-subunit to PDE6 catalytic dimer increases the binding affinity of cGMP to the GAF domains (Yamazaki et al., 1982; Cote et al., 1994). Accordingly, dissociation of either one weakens the binding of the other. Based on a structural study of PDE6γ (Barren et al., 2009), it has been suggested that the interaction between Taα−GTP and PDE6γ induces a hinge-like movement of the last 10 residues away from...
the enzyme active site without the TacR–GTP/PDE6γ complex completely disassociating from the PDE6 holoenzyme. The inactivation of TacR–GTP by its intrinsic GTPase activity is the rate-limiting step to restore the photoreceptor to a dark-adapted state and its regulator RGS9-1 associates with PDE6γ to accelerate the GTPase activity of TacR–GTP (Arashaky and Burns, 2012). The multiple interactions of PDE6γ with PDE6αβ, TacR–GTP, and RGS9-1 complex are likely to occur in a precisely controlled temporal sequence that coordinates the activation and deactivation of PDE6 (Zhang et al., 2012). The major sequence difference between cone and rod PDE6 resides in the GAF domains, with cone PDE6 displaying a lower affinity toward cGMP. The relative affinity of rod PDE6β binding to TacR–GTP versus the PDE6 catalytic subunits may be defined by the state of cGMP occupancy on the GAF domains of PDE6αβ. Likewise, the affinity of rod PDE6γ for vector-expressed cone PDE6α may be lower than that for the rod PDE6αβ. These differences may contribute to the slower inactivation of the cone PDE6α expressed in rods. It would be interesting to study the effects of replacing rod PDE6γ with cone PDE6γ or the entire rod PDE holoenzyme with cone PDE6, because PDE6γ critically regulates phototransduction through on and off interactions with PDE6αβ. TacR–GTP, and RGS9-1. Overall, it is difficult from our results to gain a clear view of the role of PDE in the differences in sensitivity or kinetics between rods and cones. Interestingly, although rods and cones share the same GAP complex, cones express RGS9 at higher levels (Zhang et al., 2003). This observation, together with the slow inactivation of cone PDE6α in rods observed by us indicate that, perhaps, the timely TacR–GTP/PDE complex inactivation in cones requires higher GAF activity than in rods.

Finally, our single-cell recordings from cone PDE6αβ-treated rd10 rods demonstrated an unusual response activation with increasing flash strength. This, together with a shallower intensity–response curve for these rods indicates a potential acceleration of cone PDE inactivation with increased phototransduction activation. A direct modulation of PDE activity was recently suggested as an additional adaptation mechanism in mouse rods (Chen et al., 2012), although it has not been directly demonstrated. Notably, however, we did not observe substantial response acceleration in WT or PDE6β-treated rd10 rods, suggesting that this is a cone PDE-specific phenomenon. Such a negative feedback modulation of cone PDE6 is an exciting novel concept and represents a potential mechanism for extending the functional range of cones. Future studies should help elucidate the mechanisms that regulate cone PDE6 activity and how this phenomenon affects cone light adaptation.

References