PKC α Is Essential for the Proper Activation and Termination of Rod Bipolar Cell Response

Klaus Ruether,¹ Andreas Feigenspan,² Judith Pirngruber,^{3,4} Michael Leitges,⁵ Wolfgang Baehr,^{6,7,8} and Olaf Strauss⁹

PURPOSE. Protein kinase (PKC)- α is abundant in retinal bipolar cells. This study was performed to explore its role in visual processing.

METHODS. PKC α -knockout (*Prkca*^{-/-}) mice and control animals were examined by using electroretinography (ERG), light microscopy, and immunocytochemistry.

RESULTS. The *Prkca*^{-/-} mice showed no signs of retinal degeneration up to 12 months of age, but ERG measurements indicated a decelerated increase in the ascending limb of the scotopic (rod-sensitive) b-wave as well as a delayed return to baseline. These results suggest that PKC α is an important modulator that affects bipolar cell signal transduction and termination. Confocal microscopy of retinal sections showed that PKC α co-localized with calbindin, which indicates a PKC α localization in close proximity to the horizontal cell terminals. In addition, the implicit time of the ERG c-wave originating from the retinal pigment epithelium (RPE) and the recovery of photoreceptors from bleaching conditions were substantially faster in the knockout mice than in the wild-type control animals.

CONCLUSIONS. These results suggest that PKC α is a modulator of rod-bipolar cell function by accelerating glutamate-driven signal transduction and termination. This modulation is of importance in the switch between scotopic and photopic vision. Furthermore, PKC α seems to play a role in RPE function. (*Invest Ophthalmol Vis Sci.* 2010;51:6051–6058) DOI:10.1167/iovs.09-4704

The visual process starts with the absorption of light by rods and cones in the retina. The second-order retinal neurons of both types of photoreceptors are the bipolar cells and horizontal cells. Rods are directly connected to ON-bipolar

cells, which depolarize after the hyperpolarization of the rod photoreceptors in response to light. Cones transmit their signals to depolarizing ON- and hyperpolarizing OFF-bipolar cells. Rod bipolar cells receive input from rod photoreceptors, as light-dependent stimulation of rods reduces their release of the synaptic transmitter glutamate.¹ This deactivates an ON-bipolar cell-specific metabotropic glutamate receptor, mGluR6, on the postsynaptic membrane, leading to depolarization of the bipolar cell^{2–5} and to an excitation of AII amacrine cells.⁶ The depolarization in light results from opening of cation channels by an unknown mechanism. The nature of the cation channel has long been elusive, but recent evidence suggests that a TRP-like channel, TRPM1, mediates synaptic function in ON-bipolar cells.^{7,8} In the dark, mGluR6 activates G α_o proteins with high affinity.^{9–11} Thus, the activation of cation channels results from a reduction of levels of the active form of the G α_o subunit, most likely the G α_o -1 splice variant. However, the target protein of G α_o -1 has not been identified, and the pathway leading to closure of cation channels is unknown.¹²

In the retina, PKC isoforms are found in bipolar,¹³ ganglion,^{14,15} amacrine,¹⁶ and pigment epithelial¹⁷ cells. PKC α is mainly expressed by rod bipolar cells and, to a lesser extent, by amacrine cells and cones.^{16,18–22} The role of PKC α , a Ser/Thr-specific protein kinase, in bipolar cell function is not understood. Its activation requires an increase in intracellular free Ca²⁺ and binding to diacylglycerol (DAG), which leads to a transfer of PKC α to the cell membrane. Immunohistochemical staining of rod bipolar cells has shown that PKC α is not only localized in the cell body but also in the dendrites and the synaptic terminals.^{18,23,24} Activation of PKC α by different phorbol esters potentiates the pool of releasable neurotransmitter vesicles at the synaptic site of the cell by regulation of the actin cytoskeleton.^{24–29} At the dendritic site, PKC α activation is known to reduce GABA-A-induced currents and to increase the run-down of GABA-A-induced currents.^{30–32} PKC α may provide a link between the stimulation of the mGluR6 receptor and the modulation of GABA-A receptors at the dendritic site of the bipolar cell.³³ To establish the light-dependent physiological role of PKC α in bipolar cells, we investigated the consequence of PKC α deletion in knockout mice by means of immunocytochemistry and by ERG measurements.

METHODS

Mice

PKC Mutant Mice. The knockout mice investigated in this study were originally generated to clarify the role of diacylglycerol-sensitive PKC isoforms such as PKC α in insulin-dependent glucose transport.³⁴ Knockout mice were genotyped by Southern blot analysis and PCR, as described elsewhere.³⁴

Experimental Mice. Heterozygous *Prkca*^{+/-} chimeric germ line-transmitting mice, crossed into wild-type 129/SV mice, and male littermate offspring, homozygous for either knockout (-/-) or full

From the ¹Charité- Eye-Hospital, Campus Virchow-Klinikum, Universitätsmedizin Berlin, Berlin, Germany; the ²Department of Biology, University of Erlangen-Nuernberg, Erlangen, Germany; the ³Department of Neurobiology, University of Oldenburg, Oldenburg, Germany; ⁴The Biotechnology Centre of Oslo, University of Oslo, Oslo, Norway; the ⁵Moran Eye Center and the ⁶Department of Neurobiology and Anatomy, University of Utah Health Science Center, Salt Lake City, Utah; the ⁷Department of Biology, University of Utah, Salt Lake City, Utah; and the ⁸Department of Experimental Ophthalmology, University Eye-Hospital, University Medical Center, Regensburg, Germany.

⁹Present affiliation: Department of Molecular Oncology, University of Göttingen, Göttingen, Germany.

Supported by Deutsche Forschungsgemeinschaft (DFG) Grant Ru 457/7-1.

Submitted for publication September 28, 2009; revised January 22, March 27, and May 2, 2010; accepted May 14, 2010.

Disclosure: K. Ruether, None; A. Feigenspan, None; J. Pirngruber, None; M. Leitges, None; W. Baehr, None; O. Strauss, None
Corresponding author: Klaus Ruether, Charité-Augenklinik Campus Virchow-Klinikum, Augustenburger Platz 1, 13353 Berlin, Germany; ruether@freenet.de.

retention (+/+) of the PKC α gene, were selected for experimental use. Note that the reporter gene construct and embryonic stem (ES) cells used for targeting were derived from the 129/SV mice; thus, crossing chimeras into the 129/SV mice yielded F1 heterozygous offspring on a pure 129/SV background. Consequently, the wild-type *Prkca*^{+/+} and the *Prkca*^{-/-} mice were genetically similar, except for the targeted gene.

The ERG measurements were subsequently performed in two groups of animals. For the basic ERG, 11 knockout mice were compared to 10 control animals. Some additional electroretinographic examinations were not performed in all animals. The respective number of animals investigated is indicated in the description of each subexperiment. All animals were between 6 and 11 weeks old at the time of examination. Some control and knockout animals were recorded at the ages of 4 and 6 months, to prove that the changes found were persistent, which was the case. The mice were held in an animal laboratory in a 12-hour-dark/12-hour-light cycle. All experiments were approved by the local animal use committee and were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Policies on the Use of Animals and Humans in Neuroscience Research.

Electroretinography

The methodology used to record the ERG is described elsewhere.³⁵ The mice were kept in darkness for at least 2 hours before examination. The pupils were dilated by tropicamide 0.5% and atropine 1%. Xylazine 15 mg/kg body weight and ketamine 100 mg/kg were injected subcutaneously for anesthesia. A monopolar contact lens electrode served as the recording electrode. Silver-needle electrodes, fixed subcutaneously, served as the reference and ground. While the ERG was recorded, the mouse was placed in a commercially available Ganzfeld bowl (Toennies Multiliner Vision, Höchberg, Germany). The signal was amplified by 10,000 with a band-pass filter, in the range of 1 to 300 Hz. A high-pass of 1 Hz is the standard used in our laboratory, as the frequency content of the ERG-b-wave is normally more than 10 Hz. With the high-pass filter set to lower levels, the b-wave changes observed in the *Prkca*^{-/-} mice were even more pronounced (see Fig. 4, c-wave recording, high-pass filter of 0.08 Hz).

Oscillatory potentials (OPs) were obtained by band-pass filtering from 100 to 300 Hz. In the dark-adapted state, a flash series consisting of eight steps starting at $-4.0 \log \text{cds} \cdot \text{m}^{-2}$ and reached $0.48 \log \text{cds} \cdot \text{m}^{-2}$. The first two responses were averaged three times (flash interval: 2 seconds), responses three and four were averaged twice, and the final four responses were not averaged. Subsequently, the OPs were recorded ($0.4 \log \text{cds} \cdot \text{m}^{-2}$, two responses, flash interval 15 seconds, rejection of the first response).

After the c-wave (see below) was recorded, the background light ($1.5 \log \text{cds} \cdot \text{m}^{-2}$) was turned on, and the photopic ERG was recorded (1.2 and $1.6 \log \text{cds} \cdot \text{m}^{-2}$, average of 10 recordings at 1.5 Hz). Subsequently, the animals were further light adapted for 10 minutes and the photopic ERG was recorded again.

The b-wave amplitude was determined from a-wave trough to b-wave peak, behind the last prominent OP. As the cone ERG is dominated by distinct OPs, a postrecording smoothing procedure was applied to assess amplitudes and implicit times.

ERG c-Wave

c-Wave recordings were taken with mice in the dark-adapted state, immediately after the scotopic ERG. A green light-emitting diode (LED) mounted in a Kooijman electrode (Roland Consult, Brandenburg, Germany) and located directly in front of the recording electrode served as the stimulus. The stimulus duration was 200 ms, and the stimulus energy was $1.8 \log \text{cds} \cdot \text{m}^{-2}$. As DC recording produced too many artifacts, a band-pass filter between 0.08 and 20 Hz was chosen. Beside this long-duration stimulus a brief LED stimulus (1 ms) was also delivered. This kind of stimulus evokes only a b-wave, not a c-wave. By

subtraction of both responses the c-wave could be better identified and analyzed.

ERG a-Wave Analysis

The high-intensity stimuli suitable for the analysis of the dark adapted a-wave were generated in the Ganzfeld globe, which was equipped with a photoflash for this purpose (Ganzfeld; Toennies Multiliner Vision). This equipment provides flashes ranging in strength from 0.4 to $2.7 \log \text{cds} \cdot \text{m}^{-2}$. Five knockout and four control animals were dark adapted for at least 2 hours. Six flash energies were applied ranging from 1.5 to $2.7 \log \text{cds} \cdot \text{m}^{-2}$. At higher energies, the scotopic a-wave amplitude saturated. To prevent rod adaptation, no averaging was performed. The interstimulus interval was at least 2 minutes in duration. The analysis of the a-wave data is related to the Lamb-Pugh model of phototransduction,³⁶⁻⁴⁰ rendering R_{max} as the saturated amplitude and S as the amplification constant of the rod photoreceptor.

Dark Adaptation

The ability to adapt the visual system to low light levels (dark adaptation) is mainly based on retinal processing.⁴¹ Adaptation from a rod-desensitizing background light can therefore be estimated by looking at the recovery of ERG responses. Since adaptation is due to receptor and postreceptor processes, b- and a-wave recovery was investigated. After a dark adaptation of 2 hours, an ERG response at $-2.0 \log \text{cds} \cdot \text{m}^{-2}$ was recorded. Subsequently, the mice were light adapted for 5 minutes at $1.85 \log \text{cds} \cdot \text{m}^{-2}$. To investigate the b-wave recovery, we delivered the $-2.0 \log \text{cds} \cdot \text{m}^{-2}$ stimulus 1 minute after turning off the light and every 2 minutes afterward. As soon as a b-wave could be identified, the amplitude was plotted up to 19 minutes after the background light was turned off. The identification of the first detectable b-wave was performed by two independent investigators. Twenty minutes after the light exposure, the scotopic mixed rod-cone response (stimulus $0.48 \log \text{cds} \cdot \text{m}^{-2}$) was recorded and compared between knockout (nine animals) and control mice (nine animals).

The investigation of the a-wave recovery as a parameter of photoreceptor adaptation was accomplished in an independent step. After dark adaptation, a $1.95 \log \text{cds} \cdot \text{m}^{-2}$ response was recorded. The bleaching was the same as with the b-wave ($1.85 \log \text{cds} \cdot \text{m}^{-2}$ for 5 minutes). Subsequently, the $1.95 \log \text{cds} \cdot \text{m}^{-2}$ stimulus was delivered immediately after the light was turned off and every 3 minutes thereafter up to 21 minutes. This longer interstimulus interval was chosen first to minimize rebleaching and second because the potential was clearly visible at the first measurement. For a- and b-wave recordings, statistical analysis was performed with repeated-measures ANOVA. In the case of the b-wave, the analysis started with the first detectable b-wave. The mixed cone-rod response recorded after 20 minutes was tested by the two-tailed *t*-test.

Preparation of Tissue and Immunocytochemistry

Adult wild-type (C57BL/6) and *Prkca*^{-/-} mice (129/SV) were deeply anesthetized by intraperitoneal injection of ketamine and xylazine. The procedures were approved by the local animal care committees and are in accordance with the German law for animal experiments (Tierschutzgesetz) and the ARVO Statement. The eyes were enucleated and transferred into a Petri dish containing carboxygenated Ames medium (Sigma, Taufkirchen, Germany). After removal of the cornea and lens, the posterior eyecups were immersion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) for 15 minutes. After fixation, the retinas were cryoprotected in 30% sucrose, embedded in gelatin, and sectioned vertically ($15 \mu\text{m}$) with a cryostat. After several washes in PB, vertical sections were incubated in a solution containing 10% normal goat serum (NGS), 1% bovine serum albumin (BSA), and 0.5% Triton X-100 in PB for 1 hour, and they were subsequently transferred for an overnight incubation to a solution containing primary rabbit anti-calbindin D28K (Swant, Bellinzona, Switzerland), rabbit anti-mGluR6 (Neuromics, Edina, MN), rabbit anti-Pcp2⁴² (Bradley M. Denker, Harvard Medical School, Boston, MA), mouse anti-kinesin II (clone

FIGURE 1. Localization of PKC α in the retina. (A) Light microscopy of 6-month-old retina sections. In some of the 6/6 examined animals, there was some minor atrophy of the IPL. OS, photoreceptor outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. (B) Wild-type retinal sections, and (C) *Prkca*^{-/-} retinal sections stained with anti-PKC α antibody and with anti-Pcp antibody. Rod bipolar cells in the control mouse (B) stained intensely for PKC α ; no labeling was found in the knockout retina (C). However, anti-Pcp2

staining revealed a normal number of rod ON-bipolar cells in the *Prkca*^{-/-} retinas. (D) Wild-type retinal sections and (E) *Prkca*^{-/-} retinal sections stained with anti-mGluR6 antibody and with anti-kinesin antibody, to show the synaptic connection between rod and bipolar cells. No differences between the control mouse (D) and the knockout retina (E) indicate no changes in the synaptic connections. Sections (A–D) were from 1- to 9-month-old retinas.

RESULTS

PKC α and Survival of Retinal Neurons

To generate a *Prkca*^{-/-} mouse, we replaced exon 2 of the PKC α gene with a neomycin cassette by using targeted deletion.³⁴ The *Prkca*^{-/-} mouse, originally generated to investigate the role of DAG/Ca²⁺-sensitive PKC α in insulin-stimulated glucose transport in adipocytes and skeletal muscle, is viable and propagates normally, and its life expectancy does not appear to be restricted. As expected, immunocytochemical staining of *Prkca*^{-/-} retinal sections with anti-PKC α antibody were negative (Fig. 1C), whereas wild-type retina showed prominent staining in bipolar cells and a subset of amacrine cells (Fig. 1B). Regarding the retinal architecture, the thickness of *Prkca*^{-/-} outer (ONL) and inner (INL) nuclear layers appeared normal (Fig. 1A). However, in some of the examined *Prkca*^{-/-} animals, mild atrophy was observed in the INL, but staining of the *Prkca*^{-/-} retinas for Pcp2 (Purkinje cell protein-2) showed no changes in the number of rod ON-bipolar cells in 9- to 12-month-old animals (Fig. 1C). In addition, staining of the *Prkca*^{-/-} retinas for mGluR6 and kinesin revealed no changes in the synaptic connections between the rods and bipolar cells (Figs. 1D, 1E). The *Prkca*^{-/-} photoreceptor function was not impeded (a-wave, described later), and the pupillary light reflex of knockout and control mice in the dark, determined by infrared pupillometry (AmTech, Dossenheim, Germany), was indistinguishable (data not shown).

Localization of PKC α

Tissues from PKC α -deficient mice offer the possibility of more detailed analysis of PKC α localization, because they can be used as a negative control that prevents positive staining due to nonspecific binding by antibodies. Vertical sections of retinas from wild-type mice were stained against PKC α (Fig. 2A) and double-labeled with antibodies against calbindin, which is a marker of horizontal cells (Fig. 2B). Analysis of the merged image revealed yellow dots, which indicate the localization of PKC α in horizontal cell synaptic endings. Quantification by pixel analysis (Fig. 2C) showed a significant localization of PKC α close to horizontal cell terminals. In addition, the high degree of localization of PKC α in dendrites with close contact to horizontal cells was quantified by Pearson's correlation coefficient of the merged image analysis (Fig. 2D).

staining revealed a normal number of rod ON-bipolar cells in the *Prkca*^{-/-} retinas. (D) Wild-type retinal sections and (E) *Prkca*^{-/-} retinal sections stained with anti-mGluR6 antibody and with anti-kinesin antibody, to show the synaptic connection between rod and bipolar cells. No differences between the control mouse (D) and the knockout retina (E) indicate no changes in the synaptic connections. Sections (A–D) were from 1- to 9-month-old retinas.

FIGURE 2. Co-localization of PKC α and calbindin in the dendrites of ON-bipolar cells. **(A)** Double staining of a retinal section with antibodies against PKC α and rod antibodies against calbindin (horizontal cell marker). In the merged image, areas of co-localization can be identified (yellow pixels). **(B)** Selection of margins for pixel analysis. *Left:* staining against PKC α ; *middle:* staining against calbindin; *right:* merged image. **(C)** *Left:* magnification of an area with merger of the images of anti-PKC α and anti-calbindin staining; *top right:* analysis of merged pixels on this area; *bottom right:* Pearson's correlation coefficient for the 10 selected margins. **(D)** Pearson's correlation coefficient of the merged image analysis. The coefficient of $82\% \pm 1.6\%$ (overlap coefficient 0.90 ± 0.019 ; Li's intensity correlation coefficient of 0.38 ± 0.0067) indicates a high degree of localization of PKC α in dendrites with close contact to horizontal cells.

The *Prkca*^{-/-} Rod Pathway Response

Scotopic ERG b-Wave. To correlate the loss of PKC α activity with functional defects in the bipolar cells, we first measured the scotopic (dark-adapted) ERG in normal and knockout mice. The scotopic ERG allows the analysis of the rod system where the leading edge of the a-wave arises from the light-induced hyperpolarization of rods, and the b-wave is primarily generated by the depolarization of rod bipolar cells. The scotopic ERGs of knockout and control mice show a typical a- and b-wave pattern (averaged data of 10 control and 11 *Prkca*^{-/-} mice; Figs. 3A, 3B), with very similar amplitudes at low stimulus levels. However, the time to reach the peak of the b-wave (peak time)—in particular the decline of the b-wave to the baseline—was prolonged in the *Prkca*^{-/-} retina. This effect was pronounced at high flash intensities (i.e., -1.0 , 0 , and $0.48 \log \text{cds} \cdot \text{m}^{-2}$), but a broadening of the b-wave was observed at all flash intensities.

Superimposing the averaged data recorded at a flash energy of $-2.4 \log \text{cds} \cdot \text{m}^{-2}$ and $0.48 \log \text{cds} \cdot \text{m}^{-2}$ evoking a rod response and a combined rod-cone response, respectively, highlighted the

difference in b-wave descent in the knockout and control animals, but also showed an altered ascending limb of the b-wave (Fig. 3A, Fig. 3F, insets) resulting in a different time to peak.

To quantify both, we analyzed the effects of the mainly rod-driven response at $-2.4 \log \text{cds} \cdot \text{m}^{-2}$ and the combined rod-cone response ($0.48 \log \text{cds} \cdot \text{m}^{-2}$). The differences between the peak times in the *Prkca*^{-/-} and *Prkca*^{+/+} mice were 22.8 ms (rod response) and 44.2 ms (rod-cone response), respectively (Table 1). The prolonged peak time of the *Prkca*^{-/-} rod response was mainly due to the later onset of the b-wave (42.4 ms, *Prkca*^{-/-}, and 28.6 ms, *Prkca*^{+/+}; Table 2). In contrast, in the combined rod-cone response, the shallower slope of the ascending limb of the b-wave prevailed (Fig. 3F). When the ascending limbs were divided into four proportions (25%, 50%, 75%, and 100%), the respective times to reach these points were significantly different between the *Prkca*^{-/-} and *Prkca*^{+/+} mice, in the case of the $0.48 \log \text{cds} \cdot \text{m}^{-2}$ response, but not with the $-2.4 \log \text{cds} \cdot \text{m}^{-2}$ response (Table 2, Fig. 3F). A comparison of the decline of the b-waves showed that the time to reach 50% of the maximum b-wave amplitude on the way to baseline differed between the *Prkca*^{-/-} and *Prkca*^{+/+} mice by 18.3 ms (rod response) and 121 ms (rod-cone response; Table 1). This result shows that the prolongation of b-wave deactivation prevails at higher stimulus energies, where rods and cones contribute to the response.

The OPs (low-amplitude oscillating waves on the rising phase of the b-wave) reflect neuronal synaptic activity in inhibitory feedback pathways initiated by the amacrine cells in the inner retina.⁴³ As the OPs are not independent of bipolar cell function, changes in them may be in part secondary to changes in bipolar cell activity. However, a change in OP may also be related to PKC α activity in amacrine cells, as it can be found at least in some of them. Highlighting the OP by setting the high-pass recording filter to 100 Hz showed that the amplitudes in the *Prkca*^{-/-} mice were lower and the implicit times were longer than in the control mice (Fig. 3C).

The Scotopic a-Wave. ERG a-wave analysis was performed in five knockout and four control mice. In figure 3D, the averaged data at one flash energy ($2.1 \log \text{cds} \cdot \text{m}^{-2}$) are shown. The mean values of $\log R_{\text{max}}$ and $\log S$ derived from the ensemble fit (all stimulus strengths integrated) were almost identical ($\log R_{\text{max}}$, 2.27 ± 0.86 and $2.27 \pm 0.95 \mu\text{V}$, and $\log S$, 2.04 ± 0.68 and $2.02 \pm 0.87 \text{ s}^{-2} [\text{td} \cdot \text{s}^{-1}]$, *Prkca*^{-/-} and *Prkca*^{+/+} mice, respectively). These data clearly indicate that deletion of PKC α had no effect on the a-wave parameter $\log S$, reflecting normal rod phototransduction, and on R_{mP_3} , reflecting unchanged rod disc area. An unchanged a-wave is consistent with the absence of a role of PKC α in the activation of phototransduction.

Alterations in the *Prkca*^{-/-} c-Wave

The c-wave arises from hyperpolarization of the RPE apical membrane due to a decrease in the subretinal K⁺ concentration resulting from reduction in the dark current of photoreceptors.⁴⁴ Figure 4A displays a typical c-wave recording in a control mouse. The top trace displays the original recording, with a long stimulus duration (200 ms), evoking the b- and c-wave. The second recording shows the b-wave evoked by a 1-ms stimulus evoking a b- but no c-wave. To isolate the c-wave, the second trace was subtracted from the first, rendering a c-wave without a b-wave (third curve). In contrast to wild-type recordings, the c-wave recording in the knockout mouse (Fig. 4B, top curve) showed the presence of a fusion-wave containing parts of b- and c-waves caused in part by a prolonged b-wave. Judging from the c-wave parameter, the fusion between the b- and c-wave was also due to a considerably shortened implicit time of the c-wave (Fig. 4B, bottom trace). The implicit time was determined by either the peak of the wave or, in the case of a plateau, by the center of the

FIGURE 3. Scotopic (A–D) and photopic (E) ERG recordings of the control and $Prkca^{-/-}$ mice. (A) Superimposed control recordings ($Prkca^{+/+}$), mean of 10, at 5 different flash intensities. (B) Superimposed recordings of $Prkca^{-/-}$ animals, mean of 11. At higher flash energies (traces 4 and 5), the prolongation of the b-wave was clearly visible. (A, inset) Direct comparison of $Prkca^{+/+}$ (black) and $Prkca^{-/-}$ (red) at -2.4 and 0.48 log cds $\cdot$ m⁻², respectively. (C) The OPs, the constitutive part of the ascending limb of the b-wave, were only slightly smaller in the knockout mice, but were considerably delayed. (D) The $Prkca^{+/+}$ and $Prkca^{-/-}$ a-waves evoked by high flash energies were indistinguishable (flash energy, 2.1 log cds $\cdot$ m⁻², mean of four control and five knockout mice). (E) Photopic ERG after 10 minutes of light adaptation (means of 10 $Prkca^{+/+}$ and 11 $Prkca^{-/-}$). (F) Course of the normalized latencies (i.e., the same starting point) at different positions of the ascending limb of the b-wave. Rod response (dashed lines): $P = 0.71$ (repeated-measures ANOVA); rod-cone response (solid line): $P = 0.013$ (repeated-measures ANOVA). y-axis: latencies in milliseconds.

plateau. The difference in amplitudes did not reach statistical significance. Since the RPE expresses PKC α , and PKC α is known to regulate K⁺ channels in the RPE,¹⁷ the changes in the c-wave in $Prkca^{-/-}$ mice most likely result from an altered regulation of apical K⁺ conductance in the RPE.

The Cone Pathway Response: Photopic ERG

The photopic ERG was generated in the presence of a rod-desensitizing adapting field (background light, 1.5 log cds $\cdot$ m⁻²). Under these conditions, the responses of the control and knock-

out mice were essentially identical (Fig. 3E). The rise in cone b-wave amplitude during light adaptation⁴⁵ showed no significant difference between control and knockout mice (mean amplitude gain, 58.3 and 53.0 μ V, respectively; $P = 0.72$, repeated-measures ANOVA). These results suggest that deletion of PKC α does not affect the cone pathway.

Dark Adaptation

The rod-dominated b-wave of knockout mice evoked by a -2.0 log cds $\cdot$ m⁻² stimulus seemed to recover faster from

TABLE 1. Time Characteristics of the b-Wave Decline and b-Wave Peak Time of the Scotopic ERG

Scotopic b-Wave Amplitude	Time at 50% Half Maximum Amplitude (ms $\pm$ SEM)	Peak Time (ms $\pm$ SEM)
Rod response <i>Prkca</i> ^{-/-} *	203.1 $\pm$ 3	153.3 $\pm$ 4
Rod response <i>Prkca</i> ^{+/+}	184.8 $\pm$ 5	130.5 $\pm$ 4
<i>P</i> value, <i>t</i> -test	0.0023	0.0004
Rod-cone response, -/-†	293.1 $\pm$ 8	140.9 $\pm$ 6
Rod-cone response, +/+†	172.1 $\pm$ 6	96.7 $\pm$ 6
<i>P</i> value, <i>t</i> -test	0.0001	0.0001

* -2.4 log cds $\cdot$ m⁻².† 0.48 log cds $\cdot$ m⁻².

bleaching than in the control mice (Fig. 5B), but this difference did not reach statistical significance (ANOVA, $P = 0.06$). However, the response to the 0.48 log cds $\cdot$ m⁻² stimulus (combined rod-cone response) after 20 minutes of dark adaptation was more distinctive as the b-wave amplitude of the knockout mice at that time was significantly larger (*t*-test, $P = 0.0016$, data not shown). In contrast, the recovery of the a-wave amplitude was nearly indistinguishable between the control and knockout mice (Fig. 5A). These results suggest that the faster recovery of *Prkca*^{-/-} mice is due to postsynaptic mechanisms.

DISCUSSION

Our ERG measurements in mice lacking PKC α revealed that this isoform plays an important role in shaping the rod bipolar cell response to light. Since PKC α was described in previous studies as only an enhancer of bipolar function, the data presented herein shed new light on the role of PKC α in rod bipolar cell function.

The *Prkca*^{-/-} retinal structure as well as the function of photoreceptors appeared unaltered until the age of 12 months. However, knockout of the PKC α gene led to specific changes in retinal function. Specifically, the rise of the b-wave was slower and the decline was substantially more prolonged in the *Prkca*^{-/-} mice than in the wild-type mice. Thus, PKC α shapes the bipolar light response by influencing the activation and the termination of the bipolar cell response. This function seems to be specific to the PKC α isoform, as PKC β -1, also expressed in bipolar cells, is obviously unable to compensate for the lack of PKC α function.¹⁶

Regulation of ON-Bipolar Cell Activity

Role of PKC α in the Activation of Rod Bipolar Cells.

Until now, the mechanisms of the ON-bipolar response have not been thoroughly understood.¹² However, there is evidence

FIGURE 4. ERG c-wave recordings in *Prkca*^{+/+} and *Prkca*^{-/-} mice. (A, B) Trace 1 is the original c-wave recording with a long-duration stimulus (200 ms). Trace 2, a short-duration stimulus (1 ms) evokes only a b-wave, but no c-wave. Subtracting traces 2 from 1 results in an isolated c-wave. The implicit time in knockout mice was significantly shorter than that in the control mice, whereas the amplitude did not show a significant difference.

that the cation channel is the TRP channel (transient receptor potential-like cation channel) TRPM1 (TRP subfamily M, member 1).^{7,8} Light-dependent deactivation of the mGluR6 receptor activates synaptic cation channels via a reduction in G α_o expression.^{9-11,46} Activation of this channel leads to an influx of extracellular Ca²⁺ into the cell.⁴ The later onset and the slower increase in the ERG b-wave in *Prkca*^{-/-} mice, together with the fact that PKC α belongs to the group of Ca²⁺-dependent PKC isoforms, point to a role in the mGluR6-induced signal transduction pathway. In this role, PKC α would be a cofactor of cGMP, which is crucial for threshold modulation and response amplification.⁴⁷

It may be that PKC α is part of the signal cascade of the ON-bipolar cell response by phosphorylation of the cation channel. This notion is in accordance with observations made in studies exploring the role of calcineurin in rod bipolar cells. It was concluded that phosphorylation is essential for maintaining the activity of the cation conductance in ON-bipolar cells.⁴

Proper Termination of Rod Bipolar Cell Activity by PKC α . At low light intensities at which rod bipolar cells are active, the time of onset of the b-wave was prolonged and the rise of the b-wave was slower in the *Prkca*^{-/-} mice. At higher stimulus intensities, the less steep slope of the b-wave prevailed. Thus, PKC α is necessary to accelerate the deactivation

TABLE 2. Time Characteristics of the Ascending Limb of the Scotopic ERG b-Wave

Proportion of Ascending Limb	Rod Response* <i>Prkca</i> ^{-/-}	Rod Response* <i>Prkca</i> ^{+/+}	Rod-Cone Response† <i>Prkca</i> ^{-/-}	Rod-Cone Response† <i>Prkca</i> ^{+/+}
Start	42.4 $\pm$ 7.1	28.6 $\pm$ 5.3	17.7 $\pm$ 1.4	16.3 $\pm$ 1.6
25%	64.4 $\pm$ 8.2	51.1 $\pm$ 5.1	28.0 $\pm$ 2.6	23.6 $\pm$ 2.4
50%	73.2 $\pm$ 9.3	59.0 $\pm$ 5.6	34.6 $\pm$ 5.2	27.9 $\pm$ 2.8
75%	83.2 $\pm$ 10.2	67.4 $\pm$ 6.5	40.8 $\pm$ 6.7	33.0 $\pm$ 3.3
100%	109.1 $\pm$ 11.4	91.3 $\pm$ 11.3	51.5 $\pm$ 8.9	42.8 $\pm$ 3.9

The table lists the latencies (in milliseconds $\pm$ SEM) of the ascending limb of the ERG b-wave at different positions. In the rod response, the starting points are significantly different between *Prkca*^{-/-} and *Prkca*^{+/+} ($P = 0.0001$, *t*-test). However, there is no significant difference between the latencies of the normalized ascending limb ($P = 0.71$; repeated-measures ANOVA). In contrast, the latencies at the start of the rod-cone response b-wave (a-wave trough) are not significantly different ($P = 0.056$, *t*-test), but the latencies at the different positions of the ascending limb are ($P = 0.013$, repeated-measures ANOVA).

* -2.4 log cds $\cdot$ m⁻².† 0.48 log cds $\cdot$ m⁻².

FIGURE 5. Recovery of the b- and a-wave amplitudes. (A) The averaged ERG responses in control (black) and *Prkca*^{-/-} mice (red) after prolonged light exposure (5 minutes, 1.85 log cds · m⁻²). There was essentially no difference between the a-wave recoveries in the two groups (stimulus energy 2.0 log cds · m⁻²). (B) The recordings display the b-wave recovery in the dark over time (recorded every 2 minutes, from 1 to 19 minutes, stimulus energy -2.0 log cds · m⁻²). The b-wave of the *Prkca*^{-/-} retina seemed to recover faster, but the difference did not reach statistical significance (ANOVA: $P = 0.06$). Error bars. SEM.

of the glutamate-driven signal transduction cascade in rod bipolar cells. The desensitization of ON-bipolar cells, which is essential for perceiving light signals on a steady background light, depends on the possible entry of Ca²⁺ into the cell through the cation channel.⁵ The time constant for the inactivation after delivery of a saturating light stimulus has been shown to be 60 to 70 ms in mouse retinal slice preparations.⁴⁸ A prolongation of this inactivation is most probably due to the Ca²⁺-dependent inactivation mechanism. However, the mode of Ca²⁺ action on the cation channel is still unknown, and so the possible roles of PKC α in this mechanism remain elusive. Of interest, a second Ca²⁺-dependent inactivation pathway depressing the mGluR6 cascade with a time constant of approximately 2 minutes has been described,^{4,5} but, in salamander retinal slices. One can speculate that PKC α plays a role in the fast, but not in the slow, component of the Ca²⁺-dependent mechanism of mGluR6-pathway inhibition. However, the existence of such a slow component in the mouse retina has still to be established.

Notably, the effect on b-wave termination became more pronounced at higher flash intensities, which indicates that at higher light intensities, PKC α activation leads to a more pronounced inhibition of the rod bipolar response, which in turn minimizes a rod input into photopic vision, in accordance with PKC α -dependent changes in the recovery of the scotopic b-wave after light adaption. The recovery of the scotopic b-wave after light adaptation was faster in the *Prkca*^{-/-} mice. Thus, in these mice the switch between photopic and scotopic vision is shifted toward higher rod-driven input. The almost identical a-wave adaptation implied that this phenomenon reflects either again a role in the signal cascade leading to activation of bipolar cells or an additional role in the control of transmitter release by bipolar cells.

In summary, two major pathways seem to be of importance in bipolar cell activation and deactivation. One is the basic mechanism by which the ON-bipolar cell is depolarized,^{7,8} by the opening of TRPM1 channels caused by the reduction of G α_o after deactivation of mGluR6. The other one is based on Ca²⁺-dependent phosphorylation and modulates the sensitivity and time dependence of the first pathway. The second pathway seems to involve dephosphorylation by calcineurin⁴ or phosphorylation by PKC α . An increased dephosphorylation leads to a slower rise in bipolar cell activation, prolonged

bipolar cell shut off, and decreased sensitivity of the mGluR6 pathway. The mechanism of this effect is not clear, because the targets of Ca²⁺-dependent phosphorylation are not known.

Bipolar cells are known to receive an inhibitory input from horizontal cells by modulation of postsynaptic Cl⁻ conductance through the likely activation of GABA receptors.⁴⁹ Since we found localization of PKC α in horizontal cell terminals, the prolonged b-wave in the *Prkca*^{-/-} mice may also have resulted from insufficient inhibitory input by horizontal cells, but this assumption cannot be proven at present. However, this hypothesis is in accordance with previous work suggesting a PKC α -dependent modulation of GABAergic input from horizontal cell currents.^{30–32}

PKC α and the RPE

The generation of the c-wave is also changed in PKC α -deficient mice. Thus, PKC α may also play a role in the regulation of ion channels in the RPE. The c-wave results from a light-induced reduction of subretinal potassium concentration leading to hyperpolarization of the apical membrane of the RPE. PKC α may be a regulator of inward rectifier potassium channels in the RPE, which are believed to be involved in the generation of the c-wave. However, a precise interpretation requires a careful investigation of RPE physiology in PKC α -deficient mice by single-cell recording.

References

- Vardi N, Dzingra A, Zhang L, Lyubarsky A, Wang TL, Morigiwa K. Neurochemical organization of the first visual synapse. *Keto J Med.* 2002;51:154–164.
- Nomura A, Shigemoto R, Nakamura Y, Okamoto N, Mizuno N, Nakanishi S. Developmentally regulated postsynaptic localization of a metabotropic glutamate receptor in rat rod bipolar cells. *Cell.* 1994;77:361–369.
- Vardi N, Duvoisin R, Wu G, Sterling P. Localization of mGluR6 to dendrites of ON bipolar cells in primate retina. *J Comp Neurol.* 2000;423:402–412.
- Snellman J, Nawy S. Regulation of the retinal bipolar cell mGluR6 pathway by calcineurin. *J Neurophysiol.* 2002;88:1088–1096.
- Nawy S. Regulation of the on bipolar cell mGluR6 pathway by Ca²⁺. *J Neurosci.* 2000;20:4471–4479.
- Ghosh KK, Haverkamp S, Wässle H. Glutamate receptors in the rod pathway of the mammalian retina. *J Neurosci.* 2001;21:8636–8647.
- Shen Y, Heimel JA, Kamermans M, Peachey NS, Gregg RG, Nawy S. A transient receptor potential-like channel mediates synaptic transmission in rod bipolar cells. *J Neurosci.* 2009;29:6088–6093.
- Koike C, Obara T, Uriu Y, et al. TRPM1 is a component of the retinal ON bipolar cell transduction channel in the mGluR6 cascade. *Proc Natl Acad Sci U S A.* 2010;107:332–337.
- Nawy S. The metabotropic receptor mGluR6 may signal through G(o), but not phosphodiesterase, in retinal bipolar cells. *J Neurosci.* 1999;19:2938–2944.
- Dzingra A, Lyubarsky A, Jiang M, et al. The light response of ON bipolar neurons requires G[alpha]o. *Neurosci.* 2000;20:9053–9058.
- Dzingra A, Jiang M, Wang TL, et al. N. Light response of retinal ON bipolar cells requires a specific splice variant of Galpha (o). *J Neurosci.* 2002;22:4878–4884.
- Snellman J, Kaur T, Shen Y, Nawy S. Regulation of ON bipolar cell activity. *Prog Retin Eye Res.* 2008;27:450–463.
- Greferath U, Grünert U, Wässle H. Rod bipolar cells in the mammalian retina show protein kinase C-like immunoreactivity. *J Comp Neurol.* 1990;301:433–442.
- Wang SW, Kim BS, Ding K, et al. Requirement for math5 in the development of retinal ganglion cells. *Genes Dev.* 2001;15:24–29.
- Wu DY, Zheng JQ, McDonald MA, Chang B, Twiss JL. PKC isozymes in the enhanced regrowth of retinal neurites after optic nerve injury. *Invest Ophthalmol Vis Sci.* 2003;44:2783–2790.

16. Fyk-Kolodziej B, Cai W, Pourcho RG. Distribution of protein kinase C isoforms in the cat retina. *Vis Neurosci*. 2002;19:549–562.
17. Strauss O, Rosenthal R, Dey D, et al. Effects of protein kinase C on delayed rectifier K⁺ channel regulation by tyrosine kinase in rat retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci*. 2002;43:1645–1654.
18. Kolb H, Zhang L, Dekorver L. Differential staining of neurons in the human retina with antibodies to protein kinase C isozymes. *Vis Neurosci*. 1993;10:341–351.
19. Fukuda K, Saito N, Yamamoto M, Tanaka C. Immunocytochemical localization of the alpha-, beta I-, beta II- and gamma-subspecies of protein kinase C in the monkey visual pathway. *Brain Res*. 1993;658:155–162.
20. Grunert U, Martin PR, Wassle H. Immunocytochemical analysis of bipolar cells in the macaque monkey retina. *J Comp Neurol*. 1994;348:607–627.
21. Koistinaho J, Sagar SM. Localization of protein kinase C subspecies in the rabbit retina. *Neurosci Lett*. 1994;177:15–18.
22. Haverkamp S, Wassle H. Immunocytochemical analysis of the mouse retina. *J Comp Neurol*. 2000;424:1–23.
23. Wassle H, Boycott BB. Functional architecture of the mammalian retina. *Physiol Rev*. 1991;71:447–380.
24. Vaquero CF, Velasco A, de la Villa P. Quantitative measurement of protein kinase C immunoreactivity in rod bipolar cells of the goldfish retina. *Brain Res*. 1997;773:208–212.
25. Berglund K, Midorikawa M, Tachibana M. Increase in the pool size releasable synaptic vesicles by the activation of protein kinase C in goldfish retinal bipolar cells. *J Neurosci*. 2002;22:4776–4785.
26. Tachibana M. Regulation of transmitter release from retinal bipolar cells. *Prog Biophys Mol Biol*. 1999;72:109–133.
27. Job C, Lagnado L. Calcium and protein kinase C regulate the actin cytoskeleton in the synaptic terminal of retinal bipolar cells. *J Cell Biol*. 1998;143:1661–1672.
28. Minami N, Berglund K, Sakaba T, Kohmoto H, Tachibana M. Potentiation of transmitter release by protein kinase C in goldfish retinal bipolar cells. *J Physiol*. 1998;512:219–225.
29. Osborne NN, Broyden NJ, Barnett NL, Morris NJ. Protein kinase C (alpha and beta) immunoreactivity in rabbit and rat retina: effect of phorbol esters and transmitter agonists on immunoreactivity and the translocation of the enzyme from cytosolic to membrane compartments. *J Neurochem*. 1991;57:594–604.
30. Karschin A, Wassle H. Voltage- and transmitter-gated currents in isolated rod bipolar cells of rat retina. *J Neurophysiol*. 1990;63:860–876.
31. Gillette MA, Dacheux RF. Protein kinase modulation of GABAA currents in rabbit retinal rod bipolar cells. *J Neurophysiol*. 1996;76:3070–3086.
32. Feigenspan A, Bormann J. Modulation of GABAC receptors in rat retinal bipolar cells by protein kinase C. *J Physiol*. 1994;481:325–330.
33. Hoffpauir BK, Gleason EL. Activation of mGluR5 modulates GABA(A) receptor function in retinal amacrine cells. *J Neurophysiol*. 2002;88:1766–1776.
34. Leitges M, Plomann M, Standaert ML, et al. Knockout of PKC alpha enhances insulin signaling through PI3K. *Mol Endocrinol*. 2002;16:847–858.
35. Ruether K, van de Pol D, Jaissle G, Berger W, Tornow RP, Zrenner E. Retinoschisislike alterations in the mouse eye caused by gene targeting of the Norrie disease gene. *Invest Ophthalmol Vis Sci*. 1997;38:710–718.
36. Lamb TD, Pugh EN. A quantitative account of the activation steps involved in phototransduction in amphibian photoreceptors. *J Physiol*. 1992;499:719–758.
37. Pugh EN, Lamb TD. Amplification and kinetics of the activation steps in phototransduction. *Biochim Biophys Acta*. 1993;1141:111–149.
38. Hood DC, Birch DG. Light adaptation of human rod receptors: the leading edge of the human a-wave and models of rod receptor activity. *Vision Res*. 1993;33:1605–1618.
39. Hood DC, Birch DG. Rod phototransduction in retinitis pigmentosa: estimation and interpretation of parameters derived from the rod a-wave. *Invest Ophthalmol Vis Sci*. 1994;35:2948–2961.
40. Goto Y, Peachey NS, Ziroli NE, et al. Rod phototransduction in transgenic mice expressing a mutant opsin gene. *J Opt Soc Am A*. 1996;13:577–585.
41. Birch DG. Visual adaptation. In: Kaufmann PL, Alm A, eds. *Adler's Physiology of the Eye*. Tenth ed. St. Louis: Mosby; 2003:586–600.
42. Guan J, Luo Y, Denker BM. Purkinje cell protein-2 (Pcp2) stimulates differentiation in PC12 cells by Gbetagamma-mediated activation of Ras and p38 MAPK. *Biochem J*. 2005;392:389–397.
43. Wachtmeister L. Oscillatory potentials in the retina: what do they reveal. *Prog Retina Eye Res*. 1998;17:485–521.
44. Steinberg RH. Interactions between the retinal pigment epithelium and the neuronal retina. *Doc Ophthalmol*. 1985;60:327–346.
45. Peachey NS, Goto Y, al-Ubaidi MR, Naash MI. Properties of the mouse cone-mediated electroretinogram during light adaptation. *Neurosci Lett*. 1993;162:9–11.
46. Dhingra A, Faurobert E, Dascal N, Sterling P, Vardi N. A retinal-specific regulator of G-protein signaling interacts with Galpha (o) and accelerates an expressed metabotropic glutamate receptor 6 cascade. *J Neurosci*. 2004;24:5684–5693.
47. Nawy S, Jahr CE. Suppression by glutamate of cGMP-activated conductance in retinal bipolar cells. *Nature*. 1990;346(6281):269–271.
48. Berntson A, Smith RG, Taylor WR. Postsynaptic calcium feedback between rods and rod bipolar cells in the mouse retina. *Vis Neurosci*. 2004;21:913–924.
49. Duebel J, Haverkamp S, Schleich W, et al. Two-photon imaging reveals somatodendritic chloride gradient in retinal ON-type bipolar cells expressing the biosensor Clomeleon. *Neuron*. 2006;49:81–94.