



Contribution of Kir4.1 to the mouse electroretinogram

Jiang Wu,¹ Alan D. Marmorstein,² Paulo Kofuji,³ Neal S. Peachey^{1,4}

¹Cole Eye Institute, Cleveland Clinic Foundation, Cleveland, OH; ²Department of Ophthalmology, University of Arizona, Tucson, AZ; ³Department of Neuroscience, University of Minnesota, Minneapolis, MN; ⁴Research Service, Cleveland VA Medical Center, Cleveland, OH

Purpose: The electroretinogram (ERG) represents the combination of several distinct cellular processes and conductances. Here, we define the contribution that K⁺ conductance through Kir4.1 channels makes to the mouse ERG.

Methods: To obtain mice expressing different levels of Kir4.1, we mated *Kir4.1*^{+/-} mice and used PCR to identify *Kir4.1*^{+/-} and *Kir4.1*^{+/+} littermates. In addition, we mated *Kir4.1*^{+/-} males with females homozygous for the *nob* (no b-wave) defect, which eliminates post-receptoral contributions to the ERG. After overnight dark adaptation, mice were anesthetized and ERGs were recorded to 7 min stimuli, to focus on slow ERG components, or to strobe flash stimuli, to examine earlier ERG components.

Results: The amplitudes of the ERG c-wave and the fast oscillation, measured from the c-wave peak, were significantly larger in *Kir4.1*^{+/-} mice than in *Kir4.1*^{+/+} littermates. In comparison, the amplitude of the light peak, the other main component generated by the retinal pigment epithelium in response to light, did not differ between *Kir4.1*^{+/-} and *Kir4.1*^{+/+} mice. The amplitude of slow PIII, revealed by the *nob* genetic background, was reduced in *Kir4.1*^{+/-} mice.

Conclusions: These results indicate that a cornea-negative potential, generated by Kir4.1, normally opposes a positive polarity conductance that is generated by the apical membrane of the retinal pigment epithelium to form the c-wave measured at the corneal surface.

The electroretinogram (ERG) has been widely used to measure retinal function in research and clinical applications [1-3]. While the ERG is comprised of a series of distinct waves that are amenable to quantitative measurement, each reflects interactions among more fundamental components generated by different classes of retinal cells that differ in their polarity, amplitude and kinetics [1,4,5]. In light of the utility of the ERG as an overall measure of retinal function, it is important to define the role of these cellular events in shaping the final ERG waveform.

In the present study, we focus on K⁺ conductance carried by the inward rectifying Kir4.1 channel. Study of *Kir4.1* mutant mice [6] indicates that Kir4.1 is not involved in b-wave generation, but does underlie slow PIII, an ERG component with a cornea-negative polarity that follows the a-wave and is generated by retinal Müller cells. While slow PIII, but not the b-wave, is abolished in *Kir4.1*^{-/-} mice [6], it is not clear how slow PIII influences later ERG waves. To address this issue, we recorded ERGs from the corneal surface of *Kir4.1* mutant mice. Although *Kir4.1*^{-/-} mice do not survive to a suitable age for testing, this approach is readily applied to *Kir4.1*^{+/-} heterozygotes, which are viable. Since these mice express only about 50% of the wild-type (WT) complement of Kir4.1 channels in retinal Müller cells [6], comparison of responses obtained from *Kir4.1*^{+/-} and *Kir4.1*^{+/+} mice provide an opportunity to evaluate the influence of Kir4.1 channel activity on

other components of the ERG. The results indicate that Kir4.1 conductance makes a substantial contribution to the slower waves of the ERG that are generated in response to neural activity by non-neuronal elements of the retina.

METHODS

Animals and anesthesia: *Kir4.1*^{+/-} mice obtained on a C57BL/6J background from a breeding colony at the University of Minnesota were mated to obtain the mice studied here. As control data, we used results obtained previously from C57BL/6J mice [7] and also tested *Kir4.1*^{+/+} littermates. *Kir4.1* genotyping was accomplished on tail DNA using a published PCR protocol [6]. To isolate slow PIII, we also used the *nob* (no b-wave) mutant mouse. The *nob* mouse is a spontaneous mutant that was originally identified by the lack of an ERG b-wave [8], and is caused by an 85 bp deletion in the nyctalopin gene [9]. To generate double mutant mice for this study, *nob/nob* females were mated to *Kir4.1*^{+/-} males. As *nob* is a X linked trait, male offspring were either *Kir4.1*^{+/-/nob} or *Kir4.1*^{+/+/nob}. All recordings were made between 5 and 12 weeks of age.

After overnight dark adaptation, mice were anesthetized with ketamine (80 mg/kg) and xylazine (16 mg/kg). Eye drops were used to anesthetize the cornea (1% proparacaine HCl) and to dilate the pupil (1% mydriacyl, 2.5% phenylephrine HCl, and 1% cyclopentolate HCl). Mice were placed on a temperature regulated heating pad throughout the recording session. All procedures involving animals were approved by the local institutional animal care and use committees and were in accordance with the Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals.

Correspondence to: Neal S. Peachey, Cole Eye Institute (I-31), Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH, 44195; Phone: (216) 445-1942; FAX: (216) 445-3670; email: peachen@ccf.org

Recording and stimulation: Two stimulation and recording systems were used. To measure slow ERG components, we used a technique described in detail elsewhere [7]. Briefly, responses were recorded from the corneal surface of the left eye using an unpulled 1 mm diameter glass capillary tube with filament (BF100-50-10; Sutter Instruments; Novato, CA) that was filled with Hanks balanced salt solution to make contact with an Ag/AgCl wire electrode with an attached connector. A similar electrode placed in contact with the right eye served as a reference lead. Responses were differentially amplified (DP-301; Warner Instruments; Hamden, CT; dc-100 Hz; gain=1000) digitized at 20 Hz and stored using LabScribe Data Recording Software (iWorx; Dover, NH).

White light stimuli were derived from an optical channel using a Leica microscope illuminator as the light source, and delivered to the test eye with a 1 cm diameter fiber optic bundle. The unattenuated stimulus luminance was 4.4 log cd/m². For the mouse eye, this luminance corresponds to 6.8 log photoisomerizations/rod/s, based on the assumption that 1 photopic cd/m² equals 1.4 scotopic cd/m² for the tungsten halogen light source [10] and that 1 scotopic cd/m² is equivalent to 100 photoisomerizations/rod/s [11]. Neutral density filters (Oriol Instruments; Stratford, CT) placed in the light path reduced stimulus luminance. Luminance calibrations were made with a LS-110 photometer (Minolta; Ramsey, NJ) focussed on the output of the fiber optic bundle. A Uniblitz shutter system was used to control stimulus duration at 7 min. On a given day, an individual mouse was tested using only a single stimulus condition. Intensity response functions were developed from recordings made in different recording sessions that were separated by at least two days.

A second stimulation and recording system was used to record conventional ERGs. The ERG was recorded using a stainless steel electrode that made contact with the corneal surface through a thin layer of 0.7% methylcellulose. Needle electrodes placed in the cheek and the tail served as reference and ground leads, respectively. Responses were differentially amplified (0.05-1500 Hz), averaged and stored using an UTAS E-2000 signal averaging system (LKC Technologies; Gaithersburg, MD). After overnight dark adaptation, ERGs were recorded to flash stimuli presented in a LKC ganzfeld. Stimulus flashes ranging from -3.6 to 2.1 log cd sec/m² were presented to the dark-adapted eye.

Data analysis: The major components of the response were measured as described elsewhere [7]. In brief, the amplitude of the c-wave was measured from the pre-stimulus baseline to the peak of the c-wave. The fast oscillation (FO) was measured from the c-wave peak to the trough of the FO. The amplitude of the light peak (LP) was measured from the FO trough to the asymptote of the LP. The amplitude of the off response was measured from the LP value just prior to flash offset to the peak of the initial component. Intensity response functions were analyzed using a two-way analysis of variance (ANOVA). Because the group-stimulus intensity interaction terms tended to be significant, group data were subsequently compared separately at each of the six flash intensities used.

RESULTS & DISCUSSION

Figure 1 presents responses recorded from *Kir4.1*^{+/+} and *Kir4.1*^{+/-} mice to three different stimulus intensities. Each waveform represents the grand average of the responses obtained from three *Kir4.1*^{+/+} or eight *Kir4.1*^{+/-} mice. The major response components are labeled for the intermediate stimulus condition. As noted previously [7], response amplitude initially increases with stimulus intensity, but then decreases as stimulus intensity is increased further. In agreement with prior results [7], the polarity of the off response is negative for low intensity stimuli (0.4 and 2.4 log cd/m²) and positive when higher stimulus intensities are used (e.g., 4.4 log cd/m²). For each stimulus condition shown, the amplitude of the c-wave appears larger in *Kir4.1*^{+/-} mice (thick red tracings) than in *Kir4.1*^{+/+} littermates (thin blue tracings). To evaluate this, we measured the amplitude of each ERG component for each mouse.

Figure 2 presents intensity response functions for each of the major ERG components. In addition to data obtained from *Kir4.1*^{+/-} and *Kir4.1*^{+/+} mice, each panel also re-plots data obtained from C57BL/6J mice in a previous study [7]. Overall, the results obtained from both sets of control mice, *Kir4.1*^{+/+} littermates and C57BL/6J mice, were comparable. In comparison to control results, the amplitude of the c-wave (Figure 2A) was significantly greater in *Kir4.1*^{+/-} mice at all but the lowest flash intensity. The amplitude of the FO was also enhanced in *Kir4.1*^{+/-} mice relative to *Kir4.1*^{+/+} littermates or C57BL/6J mice (Figure 2B); the increase observed in *Kir4.1*^{+/-} mice was significant at all but the lower two flash intensities.

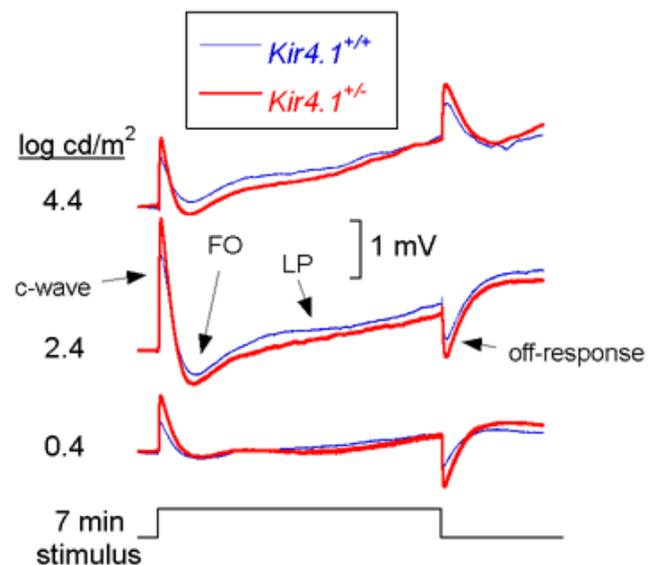


Figure 1. Comparison of ERGs obtained to 7 min stimuli from control and *Kir4.1* mutant mice. ERGs recorded to 7 min duration stimulus flashes from *Kir4.1*^{+/+} (thin blue tracings) or *Kir4.1*^{+/-} (thick red tracings) mice. Each record indicates the grand average of 3 *Kir4.1*^{+/+} or 8 *Kir4.1*^{+/-} mice. The stimulus intensity used to evoke these responses is indicated on the left. Stimulus presentation is represented by the lower trace. Amplitude calibration indicates 1 mV.

In comparison, there was no consistent difference between the LP amplitudes of *Kir4.1^{+/-}* and control mice under any stimulus condition. Finally, the amplitude of the off response was significantly larger in *Kir4.1^{+/-}* mice at three stimulus intensities (0.4, 1.4, 4.4 log cd/m²).

Since the c-waves measured here reflect the sum of a positive component generated from the apical retinal pigment epithelium (RPE) and slow PIII generated by Müller cells [12], a net increase in c-wave amplitude could reflect an increase in the apical RPE component or a reduction in slow PIII. In support of the latter interpretation, Kofuji et al. [6] provided in vitro evidence that the amplitude of slow PIII was reduced in *Kir4.1^{-/-}* mice. In that study, however, ERG data were not re-

ported from *Kir4.1^{+/-}* animals. To examine whether slow PIII was reduced in *Kir4.1^{+/-}* mice, we studied *Kir4.1/nob* double mutant mice, where the *nob* trait allows slow PIII to be directly evaluated [8].

Figure 3 compares average strobe flash ERGs obtained from *Kir4.1^{+/-/nob}* or *Kir4.1^{++/nob}* mice for one representative stimulus condition. To minimize individual variability, individual records were first normalized to the trough of the a-wave. Each plotted waveform represents the grand average of these normalized responses that were obtained from seven *Kir4.1^{+/-/nob}* (thick red tracing) or five *Kir4.1^{++/nob}* (thin blue tracing) mice. While the initial a-waves do not differ between *Kir4.1^{+/-/nob}* and *Kir4.1^{++/nob}* mice, the later portion of the

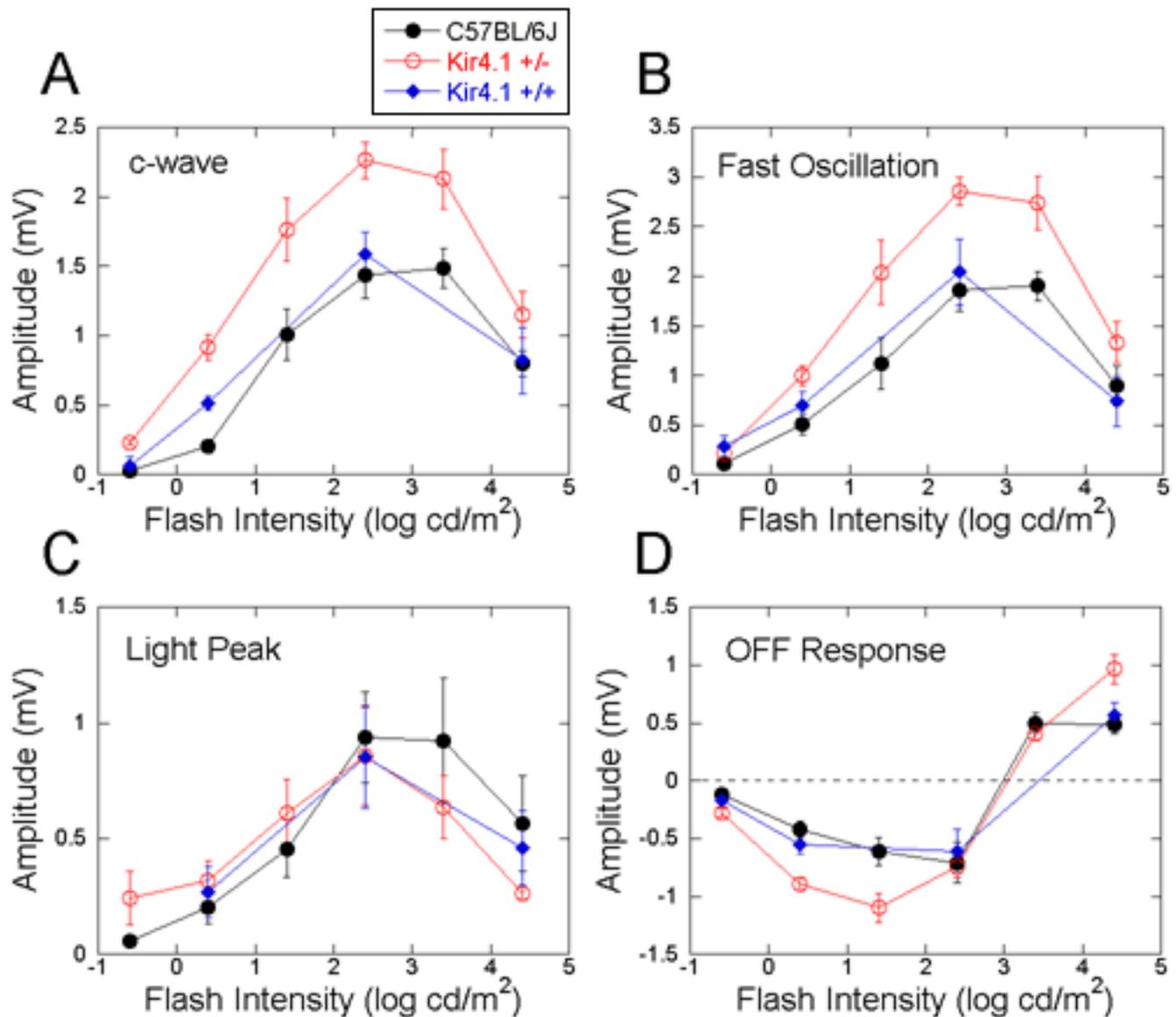


Figure 2. Intensity response functions for the amplitude of the major ERG components generated by the RPE in response to light. The different panels plot the amplitude of the c-wave (A), fast oscillation (B), light peak (C), or off response (D) as a function of flash intensity (black line, C57BL/6J; red line, *Kir4.1^{+/-}*; blue line, *Kir4.1^{+/+}*). Data points indicate the average of 3 to 8 responses obtained from different mice; the error bars represent the standard error of the mean. Note that *Kir4.1^{+/+}* mice were tested using only a subset of the stimulus intensities.

response, corresponding to slow PIII, is reduced in *Kir4.1^{+/-}/nob* mice. In fact, while the slow PIII responses of *Kir4.1^{+/-}/nob* mice maintain a stable negative plateau throughout the recording epoch, the peak amplitude of slow PIII is reduced in *Kir4.1^{+/-}/nob* mice and a negative plateau is not maintained. Similar differences between *Kir4.1^{+/-}/nob* and *Kir4.1^{+/+}/nob* responses were observed throughout the intensity range examined.

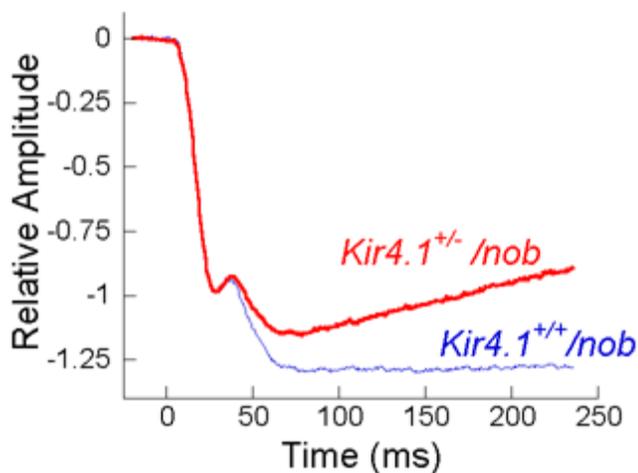


Figure 3. Comparison of strobe flash ERGs obtained from control and *Kir4.1* mutant mice. ERGs recorded to strobe flash stimuli from *Kir4.1^{+/+}/nob* (thin blue tracing) or *Kir4.1^{+/-}/nob* (thick red tracing) mice. Each record indicates the grand average of 5 *Kir4.1^{+/+}/nob* or 7 *Kir4.1^{+/-}/nob* mice. Stimulus intensity was $-0.03 \log \text{cd sec/m}^2$

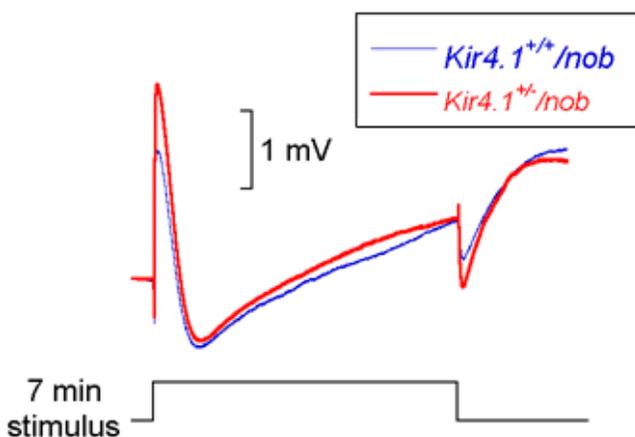


Figure 4. Comparison of ERGs obtained to 7 min stimuli from control and *Kir4.1* mutant mice. ERGs recorded to 7 min duration stimulus flashes from *Kir4.1^{+/+}/nob* (thin blue tracing) or *Kir4.1^{+/-}/nob* (thick red tracing) mice. Each record indicates the grand average of 3 *Kir4.1^{+/+}/nob* or 7 *Kir4.1^{+/-}/nob* mice. Stimulus intensity was 2.2 cd/m^2 . Stimulus presentation is represented by the lower trace. Amplitude calibration indicates 1 mV.

To follow up on these results, we also recorded dc-ERGs from *Kir4.1^{+/-}/nob* and *Kir4.1^{+/+}/nob* mice, using the stimulus condition that evokes the largest amplitude response ($2.2 \log \text{cd/m}^2$). Figure 4 compares grand average responses for 7 *Kir4.1^{+/-}/nob* (thick red tracing) or 3 *Kir4.1^{+/+}/nob* (thin blue tracing) mice. It is clear that the c-wave is again much larger in *Kir4.1^{+/-}/nob* than in *Kir4.1^{+/+}/nob* littermates. While the peak-to-trough amplitude of the FO is also greater in *Kir4.1^{+/-}/nob* mice, there is little difference in the position of the FO trough with respect to the initial pre-stimulus baseline nor in the LP components. At stimulus offset, the off response is again larger in *Kir4.1^{+/-}/nob* mice.

These results indicate that *Kir4.1* conductance influences the amplitude of the mouse ERG. In particular, the c-wave was enhanced in *Kir4.1^{+/-}* mice. The c-wave is known to represent the sum of two potentials that are generated in response to the light-induced decline in subretinal $[\text{K}^+]$. A positive potential, generated by hyperpolarization of the apical membrane of the RPE [13-15] is offset somewhat by slow PIII, a negative polarity signal that is generated by Müller cells [12]. The increase in c-wave amplitude of *Kir4.1^{+/-}* mice indicates that a *Kir4.1* conductance contributes to the negative polarity component. This agrees with the observation of Kofuji et al. [6] that slow PIII was eliminated in *Kir4.1^{-/-}* mice, and indicates that this conductance persists beyond the time frame analyzed in that study. An alternative explanation for the c-wave increase is that there might be an increased resistance across the retina/RPE. Evidence against this idea is provided by analysis of other ERG components such as the LP, which did not differ between *Kir4.1^{+/+}* and *Kir4.1^{+/-}* mice.

Our results also agree with the conclusion of Yang et al. [16] that the apical membrane of the RPE does not express *Kir4.1*, as suggested by earlier reports [17,18]. Instead, Yang et al. [16] found that the apical RPE expresses *Kir7.1*. It will be of interest to apply the ERG technique used here to mice lacking *Kir7.1* expression in the RPE to define the role of this channel in c-wave generation. In addition, study of other ion channel mutants should prove useful in continuing to evaluate the specific channels whose activity is reflected in the ERG.

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