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Functional abnormalities in the retinal pigment epithelium of CFTR mutant mice

Jiang Wu^a, Alan D. Marmorstein^{b,c}, and Neal S. Peachey^{a,d,*}

^a Cole Eye Institute, Cole Eye Institute (I-31), Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195, USA

^b Department of Ophthalmology and Vision Science, University of Arizona, Tucson, AZ 85711, USA

^c Optical Sciences Center, University of Arizona, Tucson, AZ 85711, USA

^d Research Service, Cleveland VA Medical Center, Cleveland, OH 44106, USA

Abstract

In response to light, the mouse retinal pigment epithelium (RPE) generates a series of slow changes in potential that are referred to as the c-wave, fast oscillation (FO) and light peak (LP) of the electroretinogram (ERG). While the FO is known to reflect a Cl^- conductance generated at the basal membrane of the RPE, the specific channel (s) underlying this potential has not been identified. In the present study we examined two strains of mice with *cftr* mutations to define the contribution that cystic fibrosis transmembrane regulator (CFTR)-mediated Cl^- conductance makes to the mouse ERG. Responses obtained from *cftr*^{Δ508/Δ508} mice exhibited an overall reduction in all components generated by the RPE in response to light without alteration of the luminance response function. Responses obtained from *cftr*^{-/-} mice were also reduced in amplitude. These results illustrate the usefulness of ERG analysis of mice deficient in ion channels that are expressed in the RPE, and indicate that CFTR contributes to the generation of RPE-driven ERG components, but that it is not the sole generator of any one of these components.

Keywords

cystic fibrosis transmembrane regulator; electroretinogram; fast oscillation; mouse; retinal pigment epithelium

1. Introduction

In response to a light stimulus, the first components of the electroretinogram (ERG) that can be recorded are generated by neuronal elements of the retina (Robson and Frishman, 1995; Kofuji et al., 2000; Robson et al., 2003). These initial components are then followed by a series of slow changes in potential that are generated by non-neuronal retinal cells (Steinberg et al., 1985). The positive polarity c-wave represents the sum of two components of opposite polarity that are generated in response to the decline in subretinal $[\text{K}^+]$ associated with the retinal response to light. A positive potential, generated by hyperpolarization of the apical membrane of the retinal pigment epithelium (RPE; Steinberg et al., 1970, 1980; Oakley and Green, 1976) is offset somewhat by slow PIII, a negative polarity signal that is generated by Müller glial cells (Witkovsky et al., 1975). In reptiles and higher vertebrates, the c-wave is followed

*Corresponding author. Cole Eye Institute, Cole Eye Institute (I-31), Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195, USA. peachen@ccf.org, neal.peachey@va.gov (N.S. Peachey).

by the negative polarity fast oscillation (FO) (Kikiwada, 1968; Griff and Steinberg, 1984; Linsenmeier and Steinberg, 1984). While the FO is generated in part by the recovery of slow PIII and the c-wave from their peaks, the major factor underlying the FO is a Cl^- induced hyperpolarization of the basal membrane of the RPE in response to the light-evoked decline in subretinal $[\text{K}^+]$ (Griff and Steinberg, 1984; Linsenmeier and Steinberg, 1984; Steinberg et al., 1985). The light peak (LP) follows the FO and reflects a Cl^- channel based depolarization of the basal membrane of the RPE (Linsenmeier and Steinberg, 1982; Steinberg et al., 1985).

Because all of these slow components are present in the mouse ERG (Wu et al., 2004b), the ERG provides a means to examine RPE function in mice with various gene defects. In the present study we have made recordings from mice with mutations in the gene for the cystic fibrosis transmembrane regulator (CFTR). CFTR is of interest because it is expressed in the RPE (Wills et al., 2000; Weng et al., 2002; Blaug et al., 2003), where it has the potential to operate as a cAMP-regulated Cl^- channel (Anderson et al., 1991), and because cAMP has been shown to modulate Cl^- conductances in multiple models of cultured RPE cells (Hughes and Segawa, 1993; Kuntz et al., 1994; Wills et al., 2000; Quinn et al., 2001; Loewen et al., 2003). In the present study, we report abnormalities in RPE function in *cfr* $^{\Delta 508/\Delta 508}$ mice, expressing the CFTR $\Delta 508$ mutation that is encountered most frequently in patients with cystic fibrosis (Bobadilla et al., 2002), and in *cfr* $^{-/-}$ mice, which lack CFTR altogether (Snouwaert et al., 1992).

2. Materials and methods

2.1. Animals and anesthesia

Adult *cfr* $^{\Delta 508/\Delta 508}$ mice and control littermates were obtained from a breeding colony established in the Cystic Fibrosis Animal Core facility of Case Western Reserve University. Mice lacking CFTR expression (Snouwaert et al., 1992) were obtained by breeding *cfr* $^{+/-}$ mice obtained from The Jackson Laboratory (Bar Harbor, ME) and were genotyped by PCR. Because *cfr* $^{-/-}$ mice have a short lifespan, and only a small number of *cfr* $^{-/-}$ mice were obtained in our breeding colony, *cfr* $^{-/-}$ mice and control littermates (i.e., *cfr* $^{+/-}$ or *cfr* $^{+/+}$) were tested at postnatal day (P) 25 under a restricted set of stimulus conditions.

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, 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 committee and were in accordance with the Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals.

2.2. Recording and stimulation

Two stimulation and recording systems and protocols were used. To measure ERG components generated by the RPE, 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 a Ag/AgCl wire electrode with an attached connector. A similar electrode placed in contact with the right eye served as a reference lead. Both electrodes were shielded in a black tube, and a baffle constructed from black electrical tape was used to shield the right eye from light stimulation. Responses were differentially amplified (DP-301; Warner Instruments, Hamden CT; dc-100 Hz; gain = 1000 $\times$) digitized at 20 Hz and stored using LabScribe Data Recording Software (iWorx; Dover, NH). After these initial setup procedures were finished, the stability of the recording was monitored for several minutes prior to stimulus presentation. Under these

conditions, mice did not usually develop reversible cataracts, probably because the corneal surface was moistened by the saline solution used to fill the capillary tube (Ridder et al., 2002).

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 and neutral density filters (Oriel Instruments, Stratford, CT) placed in the light path were used to reduce stimulus luminance. Luminance calibrations were made with a LS-110 photometer (Minolta, Ramsey, NJ) focused on the output of the fiber optic bundle. A Uniblitz shutter system was used to control stimulus duration at 7 min. Each mouse was tested only once on a given day, using only a single stimulus condition. While *cfr*^{-/-} mice and control littermates were tested using only a single stimulus condition run at P25, intensity–response functions were developed for *cfr*^{Δ508/Δ508} mice from recordings each made in a separate recording session (Wu et al., 2004a,b).

The amplitude of the c-wave was measured from the pre-stimulus baseline to the peak of the c-wave. The amplitude of the FO was measured from the c-wave peak to the trough of the FO. The amplitude of the LP was measured from the FO trough to the asymptotic value. The amplitude of the off-response was measured from the tail of the response to the peak of the off-response, which could be either negative or positive in polarity, depending on flash intensity (Wu et al., 2004a,b).

To record conventional ERGs, responses were 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. Under these conditions, mice typically develop reversible cataracts (Ridder et al., 2002). Responses were differentially amplified (0.3–1500 Hz), averaged, and stored using a UTAS E-3000 signal averaging system (LKC Technologies, Gaithersburg, MD). ERGs were recorded to flash stimuli presented in an LKC ganzfeld. Stimulus flash intensity ranged from −3.6 to 2.1 log cd s/m², and stimuli were presented in order of increasing intensity. The number of successive responses averaged together decreased from 20 for low-intensity flashes to 2 for the highest intensity stimuli. The duration of the interstimulus interval (ISI) increased from 4 s for low intensity flashes to 90 s for the highest intensity stimuli. The amplitude of the a-wave was measured from the pre-stimulus baseline to the value obtained 8 ms after stimulus presentation.

ERG intensity–response functions were analyzed using a two-way repeated measures analysis of variance.

3. Results

Fig. 1 compares average responses obtained from *cfr*^{Δ508/Δ508} mice and control animals for the stimulus conditions examined. The major response components generated by the RPE are labeled for one stimulus condition (2.4 log cd/m²). At each stimulus luminance, it appears that overall response amplitude was smaller in the responses of *cfr*^{Δ508/Δ508} mice than in those of controls. We developed intensity–response functions for each of the major components of the ERG studied here. As shown in Fig. 2A, c-waves of *cfr*^{Δ508/Δ508} mice were significantly ($P < 0.02$) smaller than those of controls at all flash intensities. The FO was also significantly ($P < 0.01$) reduced in amplitude at all stimulus conditions in *cfr*^{Δ508/Δ508} mice (Fig. 2B). LPs of *cfr*^{Δ508/Δ508} animals were also significantly ($P < 0.01$) reduced in amplitude (Fig. 2C). Finally, the amplitude of the off-response was significantly ($P < 0.01$) smaller in *cfr*^{Δ508/Δ508} mice (Fig. 2D).

The primary retinal activity that evokes ERG components generated by the RPE arises from the light response of rod photoreceptors (Wu et al., 2004b). To examine the possibility that these changes were secondary to abnormal rod function, we recorded dark-adapted ERG a-waves from control and *cftr*^{Δ508/Δ508} mice. Fig. 3 presents average (±SEM) intensity–response functions for the amplitude of the a-wave measured 8 ms after stimulus onset. There was no significant difference between a-wave amplitudes of control and *cftr*^{Δ508/Δ508} mice.

The Δ508 mutation promotes abnormal folding of the CFTR protein, which is subsequently retained in the endoplasmic reticulum (Cheng et al., 1990). Consequently, the Δ508 mutation results in abnormalities in cellular trafficking and in a loss of protein activity. We also studied *cftr*^{−/−} mice, which lack the CFTR protein (Snouwaert et al., 1992). Because *cftr*^{−/−} mice were obtained rarely and do not survive long enough to develop complete intensity–response functions these animals were examined at a single time point (P25), using a stimulus intensity (2.4 log cd/m²) that corresponds to the peak response in control mice (Figs. 1 and 2; Wu et al., 2004a,b). Fig. 4A compares the average response obtained from 4 *cftr*^{−/−} or 17 *cftr*^{+/+} control littermates. It is apparent that the response of *cftr*^{−/−} mice is reduced compared to WT littermates, although the overall waveform is generally normal. Peak-to-trough measures of each response component are plotted in Fig. 4B. In comparison to *cftr*^{+/−} or *cftr*^{+/+} littermates, the c-wave, FO and LP were each reduced in *cftr*^{−/−} mice. These reductions were not, however, statistically significant, perhaps reflecting the limited data available from *cftr*^{−/−} mice.

4. Discussion

Results obtained in *cftr*^{Δ508/Δ508} mice indicate that the Δ508 mutation alters overall RPE function, which is reflected as a reduction of all ERG components generated by the RPE. These changes are not secondary to a decrease in photoreceptor activity as ERG a-waves were indistinguishable between *cftr*^{Δ508/Δ508} and control mice. Instead, the c-wave, FO and LP reductions observed in *cftr*^{Δ508/Δ508} mice must reflect a change in RPE function that is associated with the Δ508 mutation. Because the Δ508 mutation results in abnormal cellular trafficking and in a loss of protein activity, we also examined *cftr*^{−/−} mice which lack CFTR altogether but do not have the Δ508-induced trafficking abnormalities. Although the ERG reductions noted in *cftr*^{−/−} mice were similar to those observed in *cftr*^{Δ508/Δ508} mice, it is important to bear in mind that our results were obtained in only a small number of *cftr*^{−/−} mice that were examined using a single stimulus condition. Further studies will be required to determine definitively whether the abnormalities noted in *cftr*^{Δ508/Δ508} mice reflect simply the lack of CFTR or some additional factor.

Many studies have addressed the fundamental mechanisms employed by the RPE cell to generate the various components of the ERG. The majority of these studies employed in vitro explants of bovine or human RPE/choroid, a preparation that allows a variety of manipulations to be conducted in a tightly controlled environment and that also has the advantage of isolating RPE-driven components from those of the neurosensory retina or other ocular and extraocular tissues. Through these experiments, it has been determined that the c-wave and FO are coupled, with the FO arising in response to the apical hyperpolarization of the RPE cell and the diminished activity of apical Cl[−] uptake mechanisms. It was using in vitro systems that the ability of cAMP to influence an FO like response was established, leading to the suggestion that CFTR might generate the FO response (Bialek et al., 1995; Quinn et al., 2001; Blaug et al., 2003). This conclusion was strengthened somewhat by two abstract reports of FO abnormalities in cystic fibrosis patients using electrooculogram testing (Miller et al., 1992; Lara et al., 2003). The data reported here, however, indicate that while CFTR activity may play a role in generating the FO, it is not the sole generator of that ERG component as this response component was retained in both *cftr*^{Δ508/Δ508} and *cftr*^{−/−} mice, and in *cftr*^{Δ508/Δ508} mice the FO reduction was comparable to that of the c-wave and was actually less severe than that of

the LP for high intensity stimulus conditions. The RPE is known, however, to express a number of other Cl^- channels that could contribute to generation of the FO, include CIC family members (Wills et al., 2000) and CLCA (Loewen et al., 2003).

There are a number of possible mechanisms by which a loss of CFTR activity could alter RPE function. Most likely, the loss of CFTR-mediated Cl^- conductances impacts upon the overall Cl^- flux of the RPE cell, which could manifest as a change in RPE plasma membrane resistance. That change alone could alter the observed amplitudes of the responses in question, as the amplitude of the c-wave is known to vary with the resistance of the basal RPE membrane (Linsenmeier and Steinberg, 1983). In the present study, the overall amplitude of the c-wave and FO were reduced in *cftr* ^{$\Delta 508/\Delta 508$} mice, but the position of the luminance-response function along the intensity axis was not altered, a finding consistent with this type of mechanism.

In summary, mice lacking normal CFTR exhibit a reduced ERG response from the RPE. Because all ERG components were reduced in parallel, but none were abolished, these results cast doubt on the hypothesis that CFTR is the sole or primary Cl^- conductance underlying the FO. Instead, these findings suggest that these responses are generated by the complex interplay of multiple ion channels. We suggest that ERG recordings will prove useful in the analysis of other lines of mutant mice lacking the normal complement of RPE ion channels as a means to dissect these phenomena.

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Fig. 1. ERGs recorded from control mice (thin blue tracings) or *cftr*^{Δ508/Δ508} littermates (thick red tracings) to 7-min stimulus flashes. Each waveform represents the grand average of responses obtained from 4–7 individual mice. Flash intensity is given in log cd/m². Time course of stimulus presentation is indicated by lower trace. Amplitude calibration indicates 1 mV.

Fig. 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 for adult mice ($\circ$: control; $\bullet$: *cftr*^{Δ508/Δ508}). Data points indicate the average ($\pm$ SEM) of responses obtained from 4–7 different mice.

Fig. 3. Intensity–response functions for the amplitude of the ERG a-wave measured 8 ms after stimulus onset in control (○) and *cftr*^{Δ508/Δ508} (●) mice. Data points indicate the average ($\pm$ SEM) of responses obtained from 7 different mice.

Fig. 4.

(A) ERGs recorded from *cftr*^{-/-} mice (thin blue tracing) or *cftr*^{+/+} littermates (thick red tracing) to 7-min stimulus flashes. Each waveform represents the grand average of responses obtained from 4 *cftr*^{-/-} mice or 17 *cftr*^{+/+} littermates tested at P25. Flash intensity was 2.4 log cd/m². Time course of stimulus presentation is indicated by lower trace. Amplitude calibration indicates 1 mV. (B) ERG amplitude measures. Bars indicate the average (±SEM) of 4 *cftr*^{-/-} mice (open bars), 15 *cftr*^{+/-} mice (hatched bars) or 17 *cftr*^{+/+} littermates (filled bars) tested at P25.