Voltage-Dependent Calcium Channel Ca_V1.3 Subunits Regulate the Light Peak of the Electroretinogram

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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). The LP is generated by a depolarization of the basolateral RPE plasma membrane by the activation of a calcium-sensitive chloride conductance. We have previously shown that the LP is reduced in both mice and rats by nimodipine, which blocks voltage-dependent calcium channels (VDCCs) and is abnormal in *lethargic* mice, carrying a null mutation in the calcium channel β_4 subunit. To define the α_1 subunit involved in this process, we examined mice lacking Ca_V1.3. In comparison with wild-type (WT) control littermates, LPs were reduced in $Ca_V1.3^{-/-}$ mice. This pattern matched closely with that previously noted in *lethargic* mice, confirming a role for VDCCs in regulating the signaling pathway that culminates in LP generation. These abnormalities do not reflect a defect in rod photoreceptor activity, which provides the input to the RPE to generate the c-wave, FO, and LP, because ERG a-waves were comparable in WT and $Ca_V1.3^{-/-}$ littermates. Our results identify Ca_V1.3 as the principal pore-forming subunit of VDCCs involved in stimulating the ERG LP.

INTRODUCTION

The light peak (LP) of the electroretinogram (ERG) is caused by a depolarization of the basolateral plasma membrane of the retinal pigment epithelium (RPE), resulting from changes in the activity of one or more calcium-sensitive chlo-ride channels (Gallemore and Steinberg 1989, 1993). This component is useful clinically in confirming the diagnosis of best vitelliform macular dystrophy (BVMD), where the LP is reduced in amplitude or absent, whereas other ERG components are spared. Because bestrophin (Best-1) mutations underlie BVMD (Petrukhin et al. 1998) and because bestrophin is localized to the basolateral RPE membrane (Marmorstein et al. 2000) and can function as a chloride channel when expressed in vitro (Sun et al. 2002), it has been proposed that bestrophin is the chloride channel that generates the LP.

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This proposal has been challenged by recent observations in mice lacking best-1, which retain the ERG LP (Marmorstein et al. 2006) and show an altered luminance response function suggesting that bestrophin functions to antagonize rather than generate the LP.

An alternative proposal for LP generation has been developed based on ERG LP recordings made after the systemic administration of nimodipine, an inhibitor of voltage-dependent calcium channels (VDCCs), and in *lethargic* mice, which carry a loss-of-function mutation in the VDCC β_4 subunit. In both cases, the LP was reduced in amplitude, and in *lethargic* mice, the luminance response function was 1 log unit less sensitive, whereas the response functions for other ERG components were less affected (Marmorstein et al. 2006; Rosenthal et al. 2006). In the arterially perfused cat eye model, Hofmann and Niemeyer (1985) reported that the LP was reversibly reduced when extracellular calcium concentrations were increased. Taken together with the results obtained in mice lacking best-1 and other results showing that bestrophin modulates the activity of VDCCs (Rosenthal et al. 2006), these data suggest that the LP is generated by a calcium-sensitive chloride channel, whose activity is modulated by VDCCs, which are in turn modulated by bestrophin (Marmorstein et al. 2006). This scenario indicates that similar results should be obtained from mice lacking the α_1 pore-forming subunit that pairs with β_4. Of the four Ca_V1 subunits, only $Ca_V1.3$ (CACNA1D) is known to be expressed in the RPE (Rosenthal et al. 2006). In this study, we report LP abnormalities in $Ca_V1.3^{-/-}$ mice that match those seen in *lethargic* mice, confirming a role for VDCCs in LP generation.

METHODS

Mice

$Ca_V1.3^{+/-}$ mice on a C57BL/6J background (Platzer et al. 2000) were obtained from a breeding colony located at Northwestern University and mated to generate the mice studied here. All procedures involving mice were approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic Foundation and were conducted in accordance with the Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals. Mice were genotyped using PCR and were tested between 6 and 16 wk of age.

Stimulation and recording

After overnight dark adaptation, mice were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (16 mg/kg) diluted in saline. The pupils were dilated with 1% mydriacyl, 1% cyclopentolate hydrochloride HCl, and 2.5% phenylephrine HCl, and the corneal surface was anesthetized with 0.5% proparacaine HCl. Mice were placed on a temperature-regulated heating pad during the ERG recording session.

To measure ERG components generated by the outer neural retina, responses were recorded from the corneal surface using a stainless steel electrode that was wetted with 0.7% methylcellulose. Needle electrodes placed in the cheek and the tail served as reference and ground leads, respectively. Responses were differentially amplified (0.3–1,500 Hz), averaged, and stored using a UTAS E-3000 signal averaging system (LKC Technologies, Gaithersburg, MD). Responses were obtained to stimuli presented in the dark. Flash stimuli were presented in an LKC ganzfeld and ranged in intensity from -3.6 to 2.1 log cd s/m^2; interstimulus intervals increased from 4 s at the lowest flash intensity to 61 s at the highest stimulus levels. Stimuli were presented in order of increasing intensity and at least two successive responses were averaged for each stimulus condition.

The amplitude of the a-wave was measured 8 ms after flash onset from the prestimulus baseline. The amplitude of the b-wave was measured from the a-wave trough to the peak of the b-wave

or, if no a-wave was present, from the prestimulus baseline. Implicit times of the a- and b-waves were measured from the time of flash onset to the a-wave trough or the b-wave peak.

To measure ERG components generated by the RPE, responses were recorded from the corneal surface of both eyes using a pair of 1-mm-diam glass capillary tubes with filament (BF100-50-10, Sutter Instruments, Novato, CA) that were filled with HBSS to make contact with a Ag/AgCl wire electrode with an attached connector. Both electrodes were shielded in a black tube, and responses were differentially amplified (DP-301, Warner Instruments, Hamden, CT; direct-current (dc)-100 Hz; gain = 1,000 times), 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 left eye with a 1-cm-diam fiber optic bundle; the right eye was shielded from light stimulation with a baffle constructed from black electrical tape. On a given day, mice were tested once using a single stimulus condition. Responses recorded to different intensity stimuli, used in recording sessions that were separated by several days, were used to construct intensity-response functions for each mouse. Maximum stimulus intensity (4.4 log cd/m^2; corresponding to 4.8 log photoisomerizations/rod/s) (Wu et al. 2004b) was reduced using neutral density filters (Oriel Instruments, Stratford, CT). Stimulus duration was controlled at 7 min using a Uniblitz shutter system.

The amplitude of the c-wave was measured from the prestimulus baseline to the peak of the c-wave. The amplitude of the fast oscillation (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 LP asymptote to the peak of the OFF-response, which in mice could be either negative or positive in polarity, depending on flash intensity (Wu et al. 2004a,b).

RESULTS

Figure 1 compares representative ERGs recorded from WT (*left*) and $Ca_V1.3^{-/-}$ mice (*right*) to two strobe flash stimulus intensities presented under dark-adapted conditions. Compared with WT mice, there was no difference apparent in the ERG waveforms obtained from $Ca_V1.3^{-/-}$ mice. This impression was examined quantitatively by plotting intensity-response functions for the amplitude (Fig. 2*A*) and implicit time (Fig. 2*B*) of the dark-adapted ERG a- and b-waves. For all measures, the WT and $Ca_V1.3^{-/-}$ data appeared in close agreement. This impression was confirmed statistically by two-way repeated-measures ANOVA, which indicated no significant difference in the a- or b-wave parameters of WT and $Ca_V1.3^{-/-}$ mice ($F_{1,4} < 1$). The components of the dc-ERG are primarily driven by rod photoreceptor activity (Wu et al. 2004a), and this input appears normal in $Ca_V1.3^{-/-}$ mice.

Figure 3 presents dc-ERGs recorded from WT (black tracings) and $Ca_V1.3^{-/-}$ (red tracings) mice to a 4 log unit range of flash intensities. Although all of the major components of the dc-ERG were present in the responses of WT and $Ca_V1.3^{-/-}$ mice, the LP components of $Ca_V1.3^{-/-}$ responses were smaller than those of WT.

Figure 4 presents intensity-response functions for the major components of the dc-ERG: the c-wave (Fig. 4*A*), the FO (Fig. 4*B*), the LP (Fig. 4*C*), and the OFF-response (Fig. 4*D*). The response functions of WT and $Ca_V1.3^{-/-}$ mice were comparable throughout the intensity range examined for the c-wave, FO, and OFF-response. Repeated-measures ANOVAs confirmed no difference ($F_{1,7} < 1$) between WT and $Ca_V1.3^{-/-}$ mice in the amplitudes of these components. In comparison, the LP component appeared consistently smaller in $Ca_V1.3^{-/-}$ than in WT mice. A repeated-measures ANOVA indicated that this reduction was statistically significant ($F_{1,7} = 9.64$; $P < 0.05$).

We have previously noted that *lethargic* mice lacking β_4 subunits have reduced LPs (Marmorstein et al. 2006). Figure 5 plots the WT and $Ca_V1.3^{-/-}$ LP data from Fig. 4*C* along with LP results obtained from *lethargic* mice and their control littermates. Although the overall LP response function is somewhat reduced in WT littermates of $Ca_V1.3^{-/-}$ mice in comparison with those of *lethargic* mice, the overall shape of the function is comparable. It was interesting to note that the deviations from the WT pattern are similar in both VDCC subunit mutants. In both cases, greatest reduction occurs at low and high stimulus intensities, with less of a reduction to the middle intensities used.

DISCUSSION

The LP component of the ERG was reduced in amplitude in $Ca_V1.3^{-/-}$ mice. This abnormality is not secondary to a defect in rod photoreceptor activity, which provides the initiating stimulus to the RPE to generate the LP (Wu et al. 2004b). This input was normal in $Ca_V1.3^{-/-}$ mice, based on analysis of the leading edge of the dark-adapted ERG a-wave. It is also unlikely that $Ca_V1.3$ deletion caused a change in the LP through indirect mechanisms, such as through cardiovascular effects. $Ca_V1.3$ is not involved in the regulation of vascular myogenic tone (Sinnegger-Brauns et al. 2004; Zhang et al. 2007), and the bradycardia present at rest in $Ca_V1.3^{-/-}$ mice is not observed under stressful conditions, like anesthesia (Platzer et al. 2000). Aside from congenital deafness, these mice do not have other neurological abnormalities (Clark et al. 2003). Instead, the selective reduction of the LP component is likely to reflect a defect in LP generation within RPE cells.

It is interesting to note that the abnormalities noted in $Ca_V1.3^{-/-}$ mice resemble closely those noted previously in *lethargic* mice (Marmorstein et al. 2006; Fig. 5), which carry a defect in the VDCC β_4 subunit (Burgess et al. 1997). Taken together, these results indicate that VDCCs play a role in generating the LP component. This conclusion is also supported by pharmacological studies where the VDCC blocker nimodipine was also found to reduce LP amplitude in WT mice (Marmorstein et al. 2006) and rats (Rosenthal et al. 2006). Because these studies examined only a limited range of stimulus conditions, it will be of interest to evaluate the effect of nimodipine and other agents that affect VDCC function across the intensity range examined here.

Because the LP is generated by the basolateral RPE membrane in response to light-evoked retinal activity, it is clear that LP generation needs an intracellular signaling pathway. Although this study contributes to our understanding of that pathway, further work will be needed to identify two key components. First, although the concept of an "LP substance," released by the neural retinal to a receptor on the apical membrane of the RPE, is generally accepted, the identity of this ligand has not been identified. Nevertheless, despite considerable effort to evaluate LP substance candidates (dopa-mine: Dawis and Niemeyer 1986; Gallemore and Steinberg 1990; epinephrine: Joseph and Miller 1992; adrenergic agents: Quinn et al. 2001; melatonin: Dawis and Niemeyer 1988), none of these has received unequivocal experimental support. In comparison, adenosine has been shown to reduce the LP in the arterially perfused cat eye (Blazynski et al. 1989), and adeno-sine receptors have been localized to the RPE (Blazynski 1993; Friedman et al. 1989). Although its role in LP generation remains to be determined, adenosine is known to regulate ATP-induced calcium-dependent intracellular signaling (Collison et al. 2005).

Second, although the LP generator is known to be a chloride channel (Gallemore and Steinberg 1989, 1993), the chloride channel that underlies the LP has yet to be identified. As noted above, mice lacking bestrophin generate LPs of normal or supernormal amplitude (Marmorstein et al. 2006), and rats overexpressing WT bestrophin have decreased sensitivity but no increase in LP amplitude (Marmorstein et al. 2004). Thus despite a substantial body of in vitro evidence

that bestrophin can form chloride channels (reviewed by Hartzell et al. 2005), such function is not needed to generate the LP, and overexpression of bestrophin does not translate simply to larger LPs. Instead, the observations that VDCCs are needed for LP generation, together with prior data showing that bestrophin modulates the activity of VDCCs (Marmorstein et al. 2006; Rosenthal et al. 2006), indicates that the signal pathway culminating in chloride channel activation and LP generation is modulated by VDCCs, which in turn are modulated by bestrophin. Although cystic fibrosis transmembrane regulator (CFTR) can form calcium-sensitive chloride channels (Anderson et al. 1991), LPs are retained in CFTR-mutant mice, albeit with reduced amplitude (Wu et al. 2006). Identification of the chloride channel(s) involved will be needed to complete a model of LP generation.

Finally, the body of evidence implicating VDCCs in generating the LP in rodents motivates the question of whether VDCCs play a similar role in the human RPE. It will be important to examine this question by, for example, using the electro-oculogram (Arden 1962; Arden and Kelsey 1962) to examine LPs in humans carrying VDCC mutations and/or after the administration of agents that alter VDCC function.

Acknowledgments

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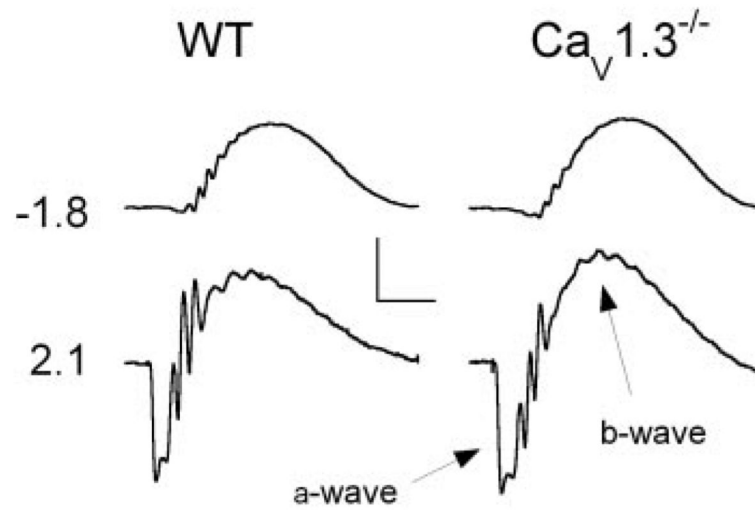
FIG. 1.
Representative dark-adapted ERGs obtained from strobe flash stimuli from WT (*left*) and
$Ca_V 1.3^{-/-}$ (*right*) littermates. Values to the *left* indicate flash intensity in log cd s/m^2. The major
components are indicated. Scale bars indicate 400 μV and 50 ms.
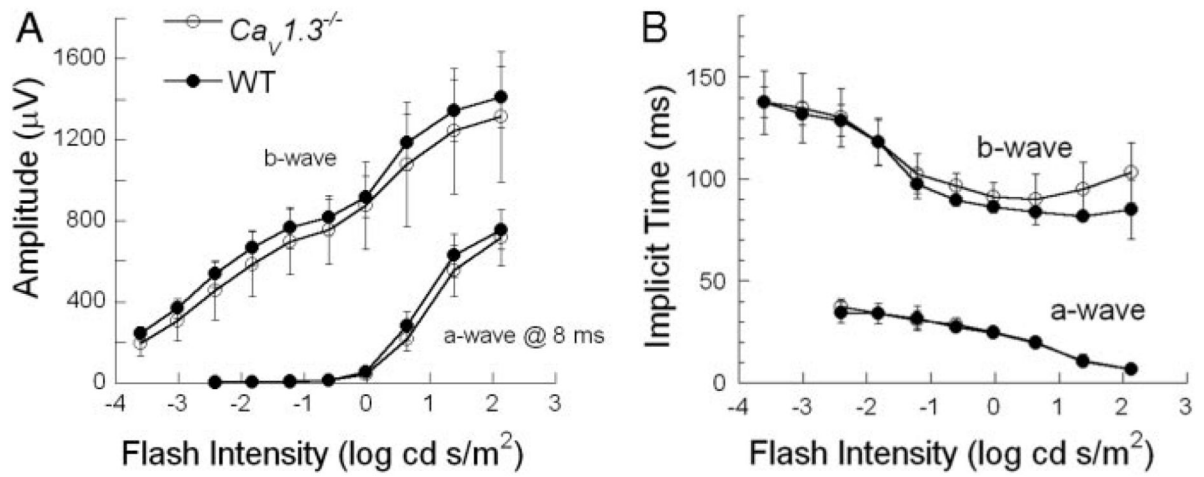
FIG. 2.
Intensity-response functions for the amplitude (*A*) and implicit time (*B*) of the major components of dark-adapted ERGs obtained from WT and *Ca 1.3*$^{-/-}$ littermates. Data points indicate average ± SD for ≥5 mice.
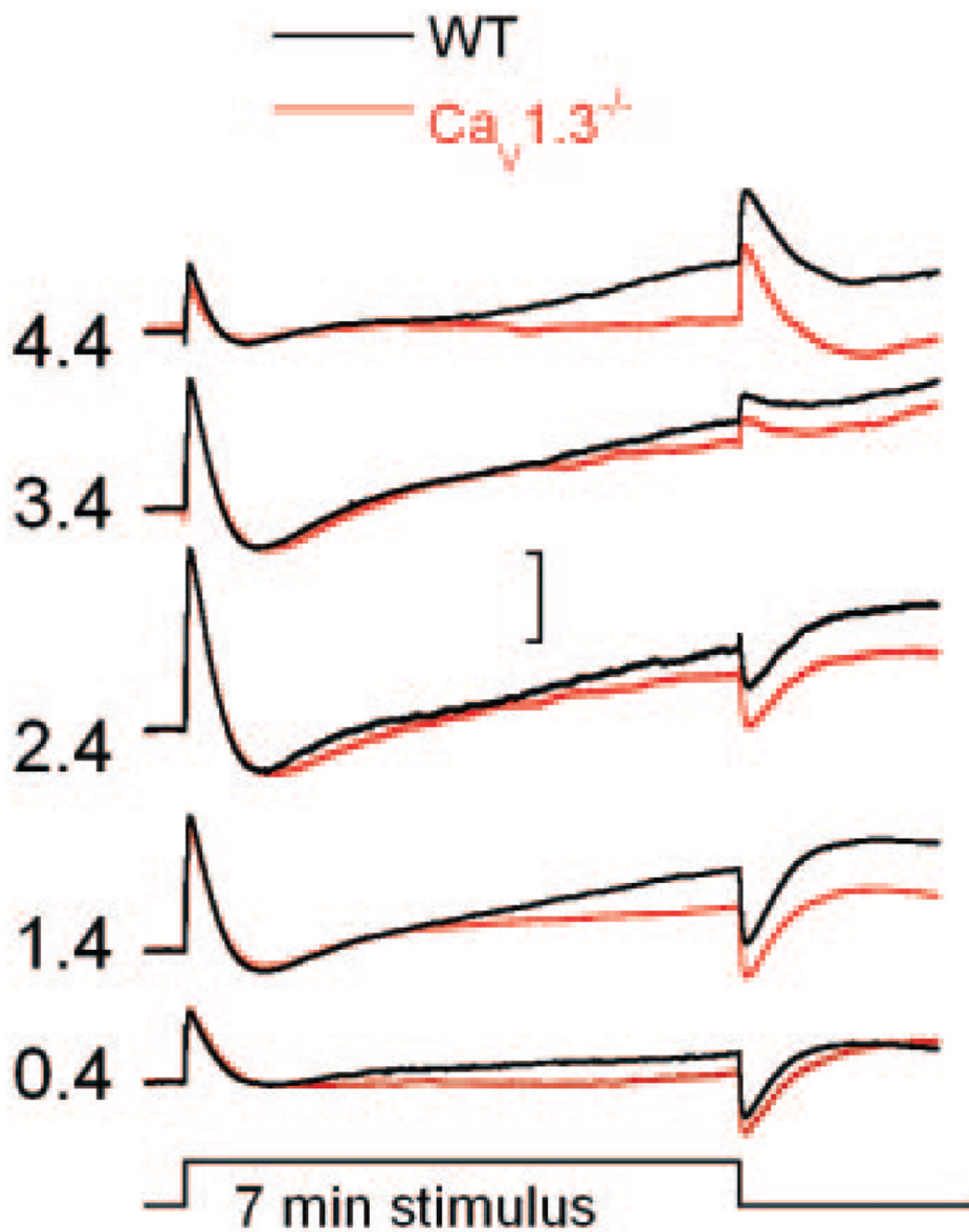
FIG. 3.
Representative dc-ERGs obtained from 7-min stimuli from WT (black tracings) and
$Ca_V1.3^{-/-}$ (red tracings) littermates. Values to the *left* indicate flash intensity in log cd/m^2.
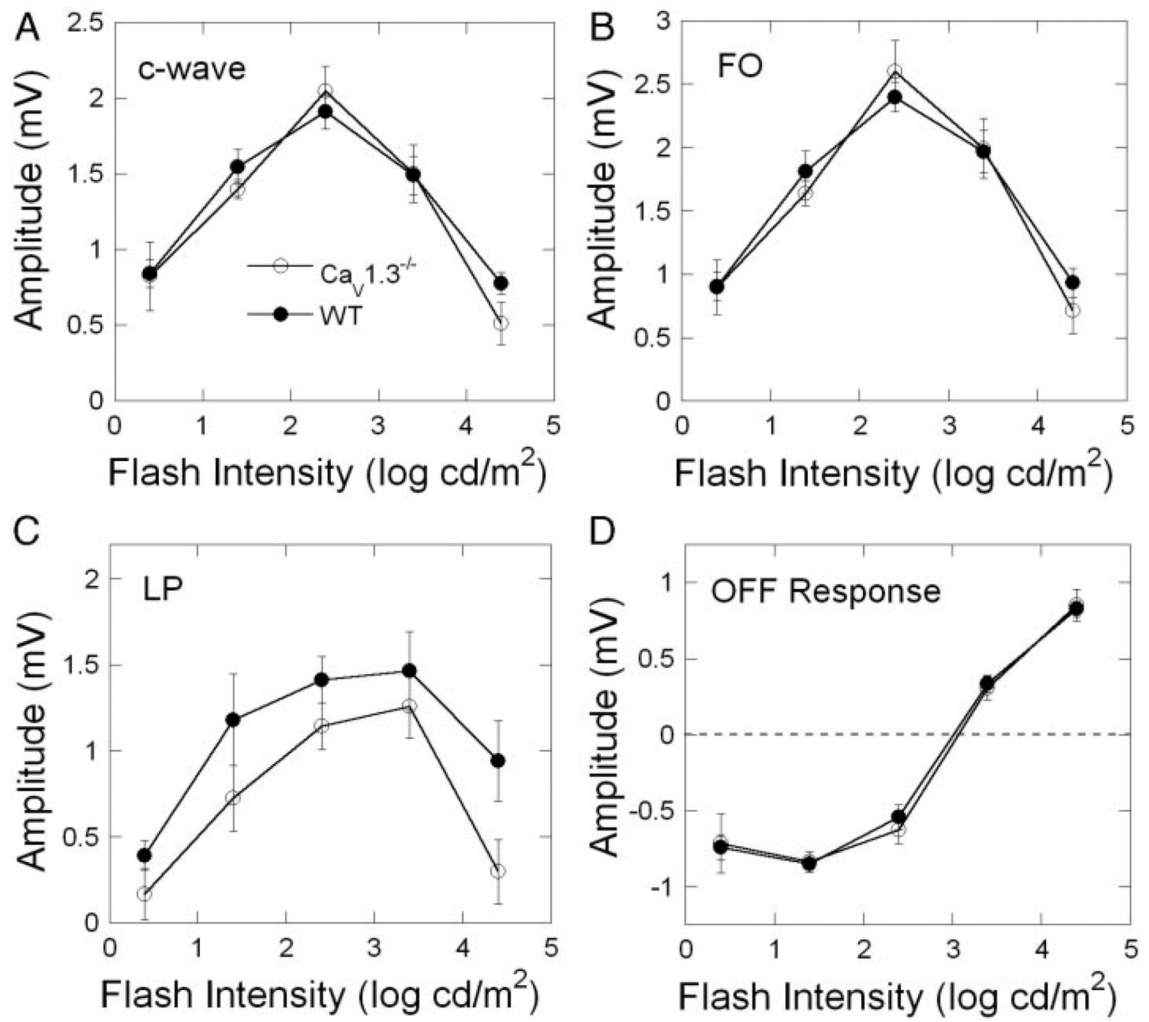Scale bar indicates 1 mV.

FIG. 4.
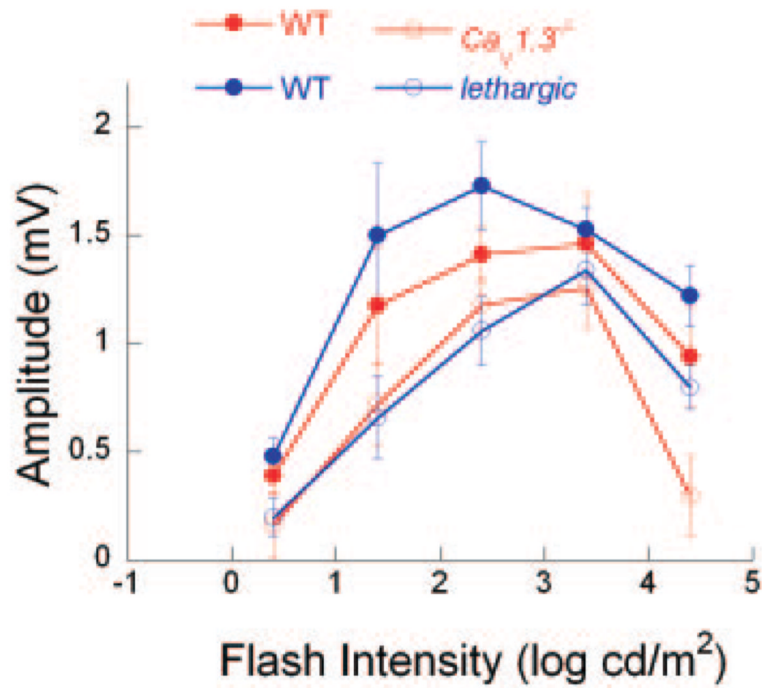Intensity-response functions for the major components of the dc-ERG. Data points indicate average ± SD for ≥5 mice.

FIG. 5.
Comparison of LP amplitude data obtained from VDCC mutant mice. Amplitude data from Fig. 4*C* for WT and $Ca_V1.3^{-/-}$ littermates (red filled and open circles, respectively) are plotted along with comparable data from Marmorstein et al. (2006) for WT and *lethargic* littermates (blue filled and open circles, respectively).