Harmonic analysis was used to characterize the rabbit flicker ERG elicited by sinusoidally modulated full-field stimuli under light-adapted conditions. The frequency-response function for fundamental amplitude, derived from Fourier analysis of the ERG waveforms, exhibited two limbs, with an amplitude minimum at approximately 30 Hz, and a high-frequency region peaking at around 45 Hz and extending to more than 100 Hz at higher adapting levels. At low frequencies (\(<=20\) Hz), the fundamental response amplitude was independent of mean luminance (Weber law behavior), whereas the response amplitude at high stimulus frequencies varied nonlinearly with mean luminance. At low frequencies, intravitreal administration of L-AP4, which blocks ON-pathway activity, reduced the fundamental response amplitude and produced a phase shift. On the other hand, PDA, which reduces OFF-pathway activity, had a minimal effect on both the response amplitude and phase at low frequencies. At high frequencies, L-AP4 increased the fundamental response amplitude at low mean luminances, whereas PDA had only a small effect on amplitude and phase. Both pharmacologic agents removed the minimum in the amplitude-frequency function as well as the abrupt change in phase at stimulus frequencies near 30 Hz. The results suggest that there is a nonlinear interaction between ON- and OFF-pathway activity over the entire stimulus frequency range examined in this study. These findings provide a basis for formulating protocols to evaluate the effect of pharmacologic agents and/or disease on the cone flicker ERG of rabbit.

1. Introduction

ERG recordings are used widely in the clinical evaluation of human retinal disorders (Fishman, 2001; Goodman and Ripps, 1960; Hood and Birch, 1997) and in animal models expressing mutations that result in retinal abnormalities (Peachey and Ball, 2003; Robson et al., 2004; Rosolen et al., 2005; Weymouth and Vingrys, 2008). Among various animal models, rodents, especially mouse, have increasingly become one of the main choices for study, owing to the large repositories of mouse lines with genetic defects, as well as advances in genetics and transgenic technology. However, although the rod pathway is very similar in rodent and primate retinas (Mills and Massey, 1999; Wässle, 2004), there are distinct differences in cone signal processing. For example, the rodent cone flicker ERG exhibits low temporal resolution (Ekesten et al., 1998; Krishna et al., 2002; Qian et al., 2008) compared to the cone flicker ERG response of monkey (Kondo and Sieving, 2001; Viswanathan et al., 2002) and human (Alexander et al., 2003; Hare and Ton, 2002; Kondo and Sieving, 2001; Viswanathan et al., 2002; Wu and Burns, 1996).

The rabbit is another animal species commonly used in studies of the visual system (Chen et al., 2010; Kondo et al., 2009; Perlman, 2009; Poznanski, 2005). In comparison to the rodent, the rabbit cone flicker ERG exhibits a robust response to light flickering at 30 Hz (Myers et al., 2009; Rosolen et al., 2005; Tsilimbaris et al., 2009), which is a temporal frequency adopted from the ISCEV standard for human testing (Marmor et al., 2009). However, the frequency-response characteristics of the rabbit cone flicker ERG have been largely unexplored. In order to provide a broader description of the cone flicker ERG of rabbit and to provide guidance on the appropriate ERG protocol for this species, we used harmonic analysis to characterize the rabbit flicker ERG elicited by sinusoidally modulated full-field stimuli under light-adapted conditions. This approach has been used previously to characterize the...
frequency-response characteristics of the cone flicker ERG of mouse (Krishna et al., 2002), rat (Qian et al., 2008), monkey (Kondo and Sieving, 2001; Viswanathan et al., 2002), and human (Alexander et al., 2003; Burns et al., 1992). Although the harmonic components of the cone flicker ERG do not as yet have clearly defined physiological generators, harmonic analysis provides a way of characterizing and quantifying ERG responses beyond the standard parameters of peak-to-trough amplitude and implicit time. That is, harmonic analysis provides information regarding the degree to which the response can be characterized by a fundamental (F) as well as the extent to which the waveform contains its various multiples (e.g., 2F, F/2, 3F, 3F/2).

2. Materials and methods

2.1. Animals

Adult male rabbits of both albino (New Zealand white) and pigmented (Dutch belted) strains were used for this study. No systematic differences were observed in the flicker ERG recordings from these animals under any of the test conditions, so the data were pooled. For each rabbit, ERGs from both eyes were recorded sequentially, with each eye randomly assigned either as a control or subject to injection with a pharmacological agent. All experimental procedures conformed to the statement on animal care of the Association for Research in Vision and Ophthalmology, and adhered to the Guidelines for the Care and Use of Laboratory Animals formulated by the Animal Care Committee of the University of Illinois at Chicago.

2.2. ERG recording

The instrumentation and recording procedures have been described previously (Qian et al., 2008). In brief, rabbits were anesthetized with ketamine and xylazine, and the pupils were dilated with topical phenylephrine and tropicamide. Body temperature was maintained at ~37 °C with a heating pad and monitored with a rectal thermometer. After applying a topical anesthetic (proparacaine, 0.5%), ERG responses were recorded from a chlorided silver wire electrode placed in the center of the cornea and connected to the input stage of a Grass AC amplifier (Model PS511; bandwidth = 0.3 to 300 Hz; without a 60 Hz notch filter); the sampling frequency was 2 kHz.

2.3. Light stimulation

Photic stimuli were delivered by multiple light-emitting diodes (LEDs) with a peak wavelength of 505 nm (Nichia NSPE590S, Tokushima, Japan) mounted in a small integrating sphere (Oriel 70500, Newport Corp., Stratford, CT) to provide a full-field stimulus. The current driving the LEDs was pulse-width-modulated under computer control, and the luminance was calibrated with a Minolta LS-100 photometer. Each sinusoidally modulated light stimulus was approximately 10 s in duration, with an even number of stimulus cycles in the waveform. Stimulus periods ranged from 250 ms to 10 ms, which corresponds to temporal frequencies of 4 to 100 Hz (note: stimulus frequencies in the text have been rounded to the nearest integer). Stimulus mean luminances were 25, 50, 100, 200, and 350 cd/m². At these luminance levels, rods are saturated, and ERG responses reflect the activity of cone-driven neural pathways in the retina. The stimulus contrast was defined as:

\[
(\text{L}_{\text{max}} - \text{L}_{\text{min}})/(\text{L}_{\text{max}} + \text{L}_{\text{min}})
\]

where \( \text{L}_{\text{max}} \) and \( \text{L}_{\text{min}} \) are the maximum and minimum stimulus luminances, respectively. In most experiments, the stimulus contrast was 90%. The eye from which the ERG recording was made was adapted to the mean luminance for approximately 3 min before data acquisition began.

2.4. Intravitreal injection

Pharmacologic agents were used to probe the contribution of ON and OFF retinal pathways to the rabbit flicker ERG, following a protocol published previously (Qian et al., 2008). In the present study, 2-amino-4-phosphonobutyric acid (L-AP4, a selective blocker of the ON pathway) and cis-2,3-piperidinedicarboxylic acid (PDA, which inhibits ionotropic glutamate receptors in the retina, including OFF bipolar cells and third order neurons) were used. Both chemicals were obtained from Sigma-Aldrich (St. Louis, MO). Solutions of these selective blockers were delivered to the anesthetized eye by intravitreal injection through a glass capillary needle introduced into the vitreous cavity by piercing the sclera 3 mm posterior to the temporal limbus at approximately a 45-degree angle to the optical axis. The injection site was monitored under a dissecting microscope, and a 50-μl aliquot of the test solution was injected into each eye. The final vitreal concentrations (1 mM L-AP4, 10 mM PDA) were derived by assuming complete mixing in the rabbit vitreous with an estimated volume of 1.2 ml (Dong and Hare, 2002).

2.5. Data analysis

Harmonic analysis was implemented with discrete Fourier transforms using the Matlab Signal Processing Toolbox (The Mathworks, Boston, MA). For each ERG recording, approximately 500 ms of data were omitted from the beginning and end of the response waveform to avoid onset and offset transients. The exact length that was omitted depended on the stimulus period and was an even number of cycles at both onset and offset. As a result, approximately 9 s of continuous data, consisting of an even number of cycles, were used for the harmonic analyses.

3. Results

3.1. ERG waveforms

Examples of typical ERG waveforms obtained from one control (untreated) rabbit eye, one L-AP4 injected eye, and one PDA injected eye at six temporal frequencies are shown in Fig. 1. The stimulus waveforms are shown below each ERG trace. For clarity of display, the 9 seconds of continuous data for each condition were divided into eighteen 500-ms segments, which were then averaged. The ERG waveforms in Fig. 1 represent these averages. It is apparent from this figure that the control rabbit retina responded well at high frequencies: an ERG response was clearly observable at a stimulus frequency of 83 Hz, and responses could also be detected at 100 Hz (data not shown). At low and high frequencies, the control ERG waveforms were approximately sinusoidal in shape. However, the response at 31 Hz was smaller than at the neighboring frequencies, and the waveform showed a double-peak shape. The waveforms of the rabbit flicker ERG obtained after L-AP4 or PDA injection are discussed in a later section.

3.2. Harmonic analysis

The amplitude spectra of the entire 9-s ERG responses of the control rabbit at these same stimulus frequencies are plotted in Fig. 2. For the most part, the amplitude spectra of the control rabbit ERG were dominated by the fundamental response (F). However, other harmonics were also present to varying degrees at the
different stimulus frequencies. For the response to the 8-Hz stimulus, a contribution from the second harmonic \((2F)\) was evident, as was a small contribution from the third harmonic \((3F)\). The \(3F\) component was slightly more prominent at a stimulus frequency of 16 Hz. At a stimulus frequency of 31 Hz, the \(F\) component was greatly reduced, and there was a relatively large contribution from the \(2F\) component. For waveforms at stimulus frequencies of 45 and 62 Hz, there were prominent contributions from the \(F\) component, as well as evidence of harmonic components at \(F/2\) and \(3F/2\); these latter components represent period doubling, as described in the Discussion. The response at a stimulus frequency of 83 Hz was basically sinusoidal, consisting almost exclusively of the \(F\) component.

Fig. 3 summarizes the mean amplitudes (Fig. 3A) and phases (Fig. 3B) of \(F\) and \(2F\) as a function of stimulus frequency for the control rabbit eyes \((n = 4)\) at a mean luminance of 100 cd/m². As per convention, the phase data are plotted in cosine phase. The amplitude function for \(F\) exhibited two limbs, with a prominent low-frequency region that was essentially low-pass in shape, and a high-frequency region peaking at approximately 45 Hz. The amplitude function for \(2F\) also showed two regions, with a peak at intermediate frequencies. In fact, at frequencies in the region of 30 Hz, the amplitudes of \(F\) and \(2F\) were approximately equal.

The phase function for \(F\) also exhibited two limbs: (1) a linear low-frequency region with a slope of \(-14.3\) Hz (corresponding to a response delay of 39.7 ms) and a y-intercept of \(-9.9\), and (2) a relatively linear high-frequency region with a shallower slope \((-7.0\) Hz, corresponding to a response delay of 19.2 ms). Between these two regions there was a relatively large phase shift of approximately 180°. The phase function for \(2F\) also showed two regions, with a discontinuity at approximately 30 Hz. At low frequencies, the slope of the phase-frequency function for \(2F\) \((-12.1\) Hz) was similar to that for \(F\). At high frequencies, however, the slope of the phase-frequency function for \(2F\) \((-14.2\) Hz) was approximately twice that for \(F\), as would be expected given that the phase data are plotted with respect to stimulus frequency.
3.3. Effect of mean luminance

Fig. 4 illustrates the effect of mean luminance on the amplitude (Fig. 4A) and phase (Fig. 4B) of \( F \). It is apparent that the two limbs of the amplitude function have different adaptational properties. For the low-frequency limb (<20 Hz), the amplitude of \( F \) was independent of mean luminance (Weber-law behavior). For the high-frequency limb, response amplitude increased systematically with increasing mean luminance, such that \( F \) was proportional to the log of the mean luminance.

The phase functions for \( F \) as a function of mean luminance (Fig. 4B) also showed two regions, with a distinct phase discontinuity at approximately 30 Hz. Each region could be well-described by linear functions. At low temporal frequencies (<30 Hz), the slopes of the least-squares regression lines decreased with increasing mean luminance, with slopes ranging from \(-17.2^\circ/\text{Hz}\) at 25 cd/m\(^2\) to \(-12.2^\circ/\text{Hz}\) at 350 cd/m\(^2\). On the other hand, the phases at high frequencies (>30 Hz) were relatively constant as a function of mean luminance, except at the lowest mean luminance.

3.4. Contribution of ON and OFF pathways

Administration of L-AP4 and PDA, which preferably reduce ON and OFF retinal pathway activity respectively, altered the flicker ERG responses elicited from the rabbit eye (Fig. 1, middle and right columns, respectively). For low-frequency stimuli (8 Hz), L-AP4 (1 mM) reduced the amplitude of the flicker ERG and altered the waveform shape, whereas PDA (10 mM) had only a relatively small effect on response amplitude and waveform shape. At high stimulus frequencies, both L-AP4 and PDA enhanced the flicker ERG amplitude.

![Fig. 2. Fourier spectra of the 9-s ERG waveforms at the stimulus frequencies shown in Fig. 1. Peaks of the various spectral components are indicated by the arrows.](image-url)

![Fig. 3. Mean values of \( F \) (squares) and \( 2F \) (triangles) for response amplitude (A) on log–log coordinates and phase (B) on linear coordinates (n = 4 eyes). The phase data were fit with linear functions using a least-squares procedure, with low- and high-frequency regions fit separately. In this and the following figures, error bars indicate ±1 standard error of the mean (SEM), and are only plotted when larger than the symbols.](image-url)
Fig. 5 shows the frequency-response relationship for \( F \) for eyes injected with L-AP4 or PDA at the different mean luminance levels. Reducing ON-pathway activity with L-AP4 (Fig. 5A) greatly reduced response amplitudes at low frequencies and increased response amplitude at high frequencies. Unlike the results for the control eyes (Fig. 4), the effect of mean luminance on amplitude was essentially constant across frequency for the L-AP4-injected eyes, with an overall deviation from Weber-law behavior. The primary effect of the PDA injection (Fig. 5B) was to eliminate the amplitude notch seen in the control eyes at approximately 30 Hz (Fig. 4A), as well as to reduce somewhat the Weber-law behavior at low frequencies. The effect of L-AP4 and PDA on the phase of \( F \) is shown in Fig. 5C and D. At low frequencies, L-AP4 induced a large phase change in \( F \) compared to the control eyes, with a flattening of the phase-frequency relation. In addition, after L-AP4 injection, the phase-frequency relationship was insensitive to luminance level at low and intermediate frequencies, unlike the case for the control eyes (Fig. 4B). PDA induced little change in phase values at low frequencies, but it removed the large phase shift around 30 Hz that was observed in the control eyes (Fig. 4B).

4. Discussion

In this study, we characterized the cone flicker ERG of rabbit in response to sinusoidally modulated light using harmonic analysis. The ERG response amplitudes of rabbit at low frequencies are much smaller than those of rodent (Krishna et al., 2002; Qian et al., 2008). However, the ERG responses of rabbit extend to much higher stimulus frequencies; i.e., responses were still detectable even with stimulus frequencies exceeding 80 Hz (Fig. 1), a feature that is similar to the cone flicker ERG of monkey (Viswanathan et al., 2002) and human (Alexander et al., 2003).

We observed period doubling in the rabbit cone flicker ERG in the frequency range between 45 and 62 Hz. Period doubling was manifested as harmonic components of \( F/2 \) and \( 3F/2 \) (Fig. 2). Typically, \( 3F/2 \) was larger than \( F/2 \), which is similar to period

Fig. 4. Mean fundamental response amplitude (A) and phase (B) (\( n = 4 \)) as a function of stimulus frequency at 5 different levels of stimulus mean luminance, indicated in the key.

Fig. 5. Effect of L-AP4 (A and C, \( n = 4 \)) and PDA (B and D, \( n = 5 \)) on mean response amplitude (A and B) and phase (C and D) of \( F \) at 5 different levels of stimulus mean luminance, indicated in the key.
doubling in the human cone flicker ERG (Alexander et al., 2008; Crevier and Meister, 1998) but different from that of rat, which shows a large F/2 component (Shah et al., 2010). However, period doubling was only observed in a subset of rabbit eyes, so this feature was not investigated further in this study.

We observed two distinct response regions of the rabbit cone flicker ERG at low and high frequencies. The amplitude–frequency function for F exhibited two limbs, with a minimum at approximately 30 Hz, and a second amplitude limb peaking at around 45 Hz (Fig. 3A). In addition, a phase shift was observed for responses in the range of 25 to 35 Hz. The phase values in both the low- and high-frequency regions could be described by linear functions, but with different slopes (Fig. 3B). For the low-frequency limb, the slope was much shallower than the value observed for the rodent flicker ERG (Krishna et al., 2002; Qian et al., 2008), which is consistent with faster kinetics (i.e., shorter response delay) of the rabbit retina. A discontinuity was also evident in the amplitude–frequency function for the F2 component. In particular, a large contribution from F2 was observed in the frequency range of 25 to 35 Hz (Fig. 3A), which is the region of the minimum in the amplitude–frequency function for F. Similarly, an abrupt phase change in F2 was also evident in this frequency region (Fig. 3B). The phase-frequency function (in cosine phase) for the rabbit cone flicker ERG was about −9.9° (Fig. 3B), which is approximately a 90° difference from the sine-phase stimulus. This indicates that the rabbit flicker ERG mainly represents a differential response to a light stimulus. In comparison, the rodent flicker ERG follows the stimulus waveform more directly, and the y-intercept of the phase-frequency function is close to the phase value of the light stimulus (Qian et al., 2008).

A difference between the low- and high-frequency regions of the rabbit cone flicker ERG was also observed in the effect of mean luminance (Fig. 4). For low-frequency responses, the amplitude of F varied little with the change in mean luminance, which corresponds to Weber-law behavior. In addition, the slope of the phase-frequency function decreased with increasing stimulus mean luminance, indicating faster response kinetics at higher mean luminances. At high frequencies, the amplitude of F increased nonlinearly with stimulus mean luminance, and there was little change in the slope of the phase-frequency function except at the lowest mean luminance. A differential effect of mean luminance on low- and high-frequency ERG responses has also been observed in the human cone flicker ERG (Wu and Burns, 1996). It is possible that similar retinal mechanisms underlie the response differences at low and high frequencies observed for both the human and rabbit flicker ERG. However, the two limbs in the human flicker ERG were more prominent when a low-contrast stimulus was used (Wu and Burns, 1996). We examined the effect of a low-contrast stimulus on the rabbit flicker ERG, but we did not observe a significant enhancement in the difference between the two frequency limbs (data not shown).

Using pharmacologic agents, we examined the rabbit cone flicker ERG at 5 different mean luminance levels after ON- or OFF-retinal activity was inhibited (Fig. 5). Administration of L-AP4 greatly reduced the response amplitude at low frequencies and produced a large shift in the phase values of F. On the other hand, low-frequency responses were only slightly altered by the administration of PDA. Both pharmacologic agents removed the minimum in the amplitude–frequency function of F and eliminated the abrupt change in phase at approximately 30 Hz, and they also induced a deviation from Weber-law behavior for low-frequency responses. These pharmacologic results indicate that the frequency-response function of F for the control rabbits is not simply the vector sum of the L-AP4-sensitive and PDA-sensitive components. For example, at stimulus frequencies of 30 Hz and above, the amplitude of F for the control animals was smaller than the amplitude of either the L-AP4-sensitive or the PDA-sensitive component, yet the phases of these two pharmacologically isolated components were essentially identical. Therefore, the amplitude minimum at 30 Hz cannot be due to a phase cancellation of the L-AP4-sensitive and PDA-sensitive components. At low stimulus frequencies, the phases of the two components were nearly 180° apart, but the amplitude of F was larger than the amplitude of either the L-AP4-sensitive or the PDA-sensitive component, indicating a positive rather than a negative combination. Therefore, there appears to be a nonlinear interaction between ON- and OFF-pathway activity in rabbit over the entire stimulus frequency range examined in this study.

A further consideration is that, while L-AP4 is thought to selectively isolate activity in the OFF pathway (photoreceptor to OFF bipolar cell to retinal ganglion cell), PDA not only blocks transmission from photoreceptors to OFF bipolar cells and horizontal cells, but it also blocks transmission from both ON and OFF bipolar cells to amacrine cells and ganglion cells. There is evidence for an inner retinal contribution to the ERG b-wave of rabbit (Dong and Hare, 2000) as well as to the monkey cone flicker ERG (Viswanathan et al., 2002). Therefore, it is possible that at least some of the differences between the pharmacologic results and the control results may result from differences in the contribution of inner retinal activity. Nevertheless, at least for the primate flicker ERG, the inner retinal component makes a greater contribution to F than to F (Viswanathan et al., 2002).

Distinctive response properties at low and high frequencies have been observed for the rat cone flicker ERG (Qian et al., 2008) as well as for the rabbit ERG. However, it is likely that the response properties observed in rabbit and rodent are mediated by different retinal mechanisms. For example, ERG responses at low and high frequencies in the two species differ in their contrast–response relationship, in the magnitude of the 2F contribution, in the effect of mean luminance, and in the relative contribution of the ON and OFF pathways. For rat, the contrast–response function is linear at low frequencies but saturating at high frequencies (Qian et al., 2008), whereas for rabbit, the contrast–response function is linear at all temporal frequencies (data not shown). For rat, the relative contribution of 2F to the flicker ERG is larger at high than at low frequencies (Qian et al., 2008), whereas for rabbit, the relative contribution of 2F is approximately the same at low and high frequencies (Fig. 3A). In addition, we did not observe any systematic difference in the effect of mean luminance on the rat flicker ERG (data not shown), whereas the low- and high-frequency limbs of the rabbit flicker ERG differ in their response changes to changes in mean luminance level (Fig. 4). Finally, the rat cone flicker ERG is dominated by ON-pathway activity at both low and high frequencies (Qian et al., 2008), whereas the rat cone flicker ERG appears to be governed by a nonlinear interaction between ON- and OFF-pathway activity. The cellular mechanisms that mediate these distinctive features in the rabbit and rat cone flicker ERG have yet to be elucidated.

Currently, there are no standard guidelines for recording the rabbit cone flicker ERG. Although the rabbit is a commonly used species for evaluating retinal function after various experimental procedures and/or drug treatment protocols (Myers et al., 2009; Rosolen et al., 2005; Tsilimbaris et al., 2009), most flicker ERG testing in rabbit has been performed at 30 Hz, a protocol adopted from the ISCEV standard for human ERG recordings (Marmor et al., 2009). This could be problematic, because the ERG fundamental response amplitude of rabbit is close to a minimum at 30 Hz and lies between two limbs of the amplitude–frequency function. A different stimulus frequency might provide a better choice for evaluating the effect of drugs and/or diseases on the rabbit retina. For example, a stimulus frequency of 20 Hz would produce responses that are relatively independent of mean luminance. On
the other hand, a stimulus frequency of 45 Hz would elicit responses that would lie close to the maximum for the high-frequency limb and would be sensitive to mean luminance. Depending on the functional defects induced by any disease condition or by an experimental treatment, and in consideration of the lack of a comprehensive understanding of the apparently nonlinear interaction between ON and OFF mechanisms over the entire response range tested here, particular drug/disease effects might be seen best at different frequencies in rabbit.

Acknowledgements

The authors thank Ms. Tara Nguyen for her excellent technical support. This work was supported by a grant from the Joyce Schroeder Fund (HQ), NIH research grant EY008301 (KRA), NIH core grant EY01792, RPB Senior Scientific Investigator Award (HR) and an unrestricted departmental award from Research to Prevent Blindness, Inc.

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Alexander, K.R., Barnes, C.S., Fishman, G.A., 2003. ON-pathway dysfunction and blindness, Inc. and an unrestricted departmental award from Research to Prevent Blindness, Inc. This work was supported by a grant from the Joyce Schroeder Fund (HQ), NIH research grant EY008301 (KRA), NIH core grant EY01792, RPB Senior Scientific Investigator Award (HR) and an unrestricted departmental award from Research to Prevent Blindness, Inc.

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