Temporal Properties of Cone ERGs of Pikachurin Null Mutant Mouse

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Purpose. Pikachurin is an extracellular matrix-like protein located in the synaptic cleft of photoreceptors. Pikachurin null mutant (Pika$^{-/-}$) mice have abnormal ON bipolar cell function. This study aimed to determine the contribution of the ON bipolar cell pathway (ON pathway) of Pika$^{-/-}$ mice to the flicker ERG response and, using vector analysis, identify how the contribution varies with the stimulus frequency.

Methods. Flicker ERGs were recorded from wild-type (WT), Pika$^{-/-}$, and mGluR6 null mutant (mGluR6$^{-/-}$) mice. The frequency of stimulation was 3.906 to 31.250 Hz. The amplitude and phase of the fundamental components were obtained by harmonic and vector model analysis. The mGluR6$^{-/-}$ mice were used as a model wherein the ON pathway is known to be absent.

Results. The amplitudes of the fundamental components of the Pika$^{-/-}$ mice were significantly smaller than those of WT mice for stimulation frequencies between 3.906 and 17.578 Hz. The phase of the fundamental components of the Pika$^{-/-}$ mice was between those of the WT and mGluR6$^{-/-}$ mice. Vector analyses showed that the functioning of the ON pathway of Pika$^{-/-}$ mice was 12% to 25% of that of the WT mice at low frequencies (i.e., $<$15.625 Hz); however, it was reduced to noise level at frequencies $>$17.578 Hz.

Conclusions. Vector model analysis can determine the degree of contribution of the ON pathway of Pika$^{-/-}$ mice to flicker ERG response and may be useful for determining the retinal function in mice models with abnormalities of the ON pathway.

Keywords: electororetinogram, flicker, pikachurin, on bipolar, harmonic analysis

Electroretinography (ERG) is used to study the physiological functions of the retina in humans and animals. Different stimuli are used to study specific properties of the retina. Some studies use intermittent stimuli to elicit ERG response and perform harmonic analysis to determine the temporal properties of the retina response.[1–7] In this technique, the amplitudes and phases of the fundamental components of the flicker ERG response are derived from a spectral analysis of the waveform at each stimulus frequency.[4,8] Analyzing flicker ERGs by harmonic analysis allows their characterization and quantification beyond the standard parameters of peak-to-trough amplitude and implicit time.

In primates, it has been reported that the depolarizing ON bipolar cell pathway (the ON pathway) and the hyperpolarizing OFF bipolar cell pathway (the OFF pathway) contribute nearly equally to photopic ERGs, with a small contribution coming from the photoreceptor component.[9–12] The "vector model analysis" method of Kondo and Sieving[8] permits evaluation of the respective ON and OFF pathways. They showed that the relative phases of the postsynaptic ON and OFF pathway components affect the flicker ERG and that, depending on the frequency, the cancelation and summation of these two pathway components contributes to the characteristic changes in the amplitudes of the flicker ERG.[8,13]

In rodents, the ON pathway dominates the cone ERG, with weak contributions coming from the OFF pathway and photoreceptor components.[14] Vector model analysis is useful in analyzing a mouse model with ON bipolar cell dysfunction.[4,15] Unfortunately, the use of this vector model analysis technique for characterization of abnormal retinal function in rodent models is limited because of the small number of suitable candidates with disorders in communication between the photoreceptors and the ON pathway or the ON bipolar cell itself.

Thus, this study aimed to determine whether vector model analysis can be used to evaluate partial impairment of the ON pathway. To accomplish this, we used Pikachurin null mutant (Pika$^{-/-}$) mice as a rodent model with abnormal ON bipolar cell function. Pikachurin is an extracellular matrix-like protein located mainly in the synaptic cleft of the photoreceptors adjacent to the ribbon synapses (the Pika$^{-/-}$ is not related to the small mammal known as the pika). Pika$^{-/-}$ mice are assumed to have abnormal ON bipolar cell function owing to a misalignment of the ON bipolar cell dendritic tips to the photoreceptor ribbon synapses.[16] For single-flash ERGs, the amplitude of the b-wave of the photopic ERG is reduced in Pika$^{-/-}$ mice and the implicit time of the scotopic and photopic flash ERG is prolonged. In addition, an earlier study using pharmacological blockade showed that Pika$^{-/-}$ mice have normal photoreceptor function.[17] These data indicated that Pika$^{-/-}$ mice have a partial disruption of the ON pathway with normal photoreceptor function. Thus, we assumed that Pika$^{-/-}$ mice should be a

suitable model for analyzing flicker ERGs by means of vector model analysis. For the analyses, we compared flicker ERGs recorded from Pika$^{-/-}$ mice with those recorded from wild-type (WT) and metabotropic glutamate receptor 6 null mutant (mGluR6$^{-/-}$) mice, which lack ON bipolar cell function. In mGluR6$^{-/-}$ mice, the mGluR6 protein is located at the synaptic terminal of the ON bipolar cells in both the rod and cone systems and the ON bipolar cells do not appear to contribute to the ERGs.[18] We show that vector model analysis reveals the contribution of the ON pathway to the flicker ERG response in Pika$^{-/-}$ mice.

Material and Methods

All experimental procedures adhered to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research and the guidelines for the Use of Animals of the Nagoya University School of Medicine. The Nagoya University Animal Experiment Committee approved this project (approval number 24317).

Twelve of each WT mice, Pika$^{-/-}$ mice, and mGluR6$^{-/-}$ mice were studied at 12 weeks of age. The Pika$^{-/-}$ mice were kindly provided by Takahisa Furukawa of the Institute for Protein Research of Osaka University. The mGluR6$^{-/-}$ mice were kindly provided by S. Nakanishi of Osaka Bioscience Institute. Both types of mice were maintained on a C57BL/6J background with more than five generations of backcrossing. The mice were maintained in a 12-hour light (<40 lux) and12-hour dark cycle.

ERG Recordings

The procedures used to record the ERGs were similar to those previously described in detail.[19,20] In brief, the mice were anesthetized with an intraperitoneal injection of ketamine and xylazine. The pupils were dilated to approximately 2 mm using topical 0.5% tropicamide and 0.5% phenylephrine HCl. The mice were placed on a heating pad for the duration of the ERG recordings. The ERGs were recorded with a gold wire loop electrode placed on the cornea and gold wire reference electrode placed in the conjunctival sac. Hydroxyethyl cellulose was used to keep the cornea and conjunctiva hydrated and ensure good electrical contact between the electrodes and the cornea and conjunctiva. The ground electrode was inserted into the tails of the mice.

Signals were amplified and band pass–filtered between 0.3 and 1000 Hz and digitized at 2000 Hz. A 60-Hz notch filter was used to reduce the contamination from stray line noise. The notch filter was second-ordered and provided approximately 32 dB of attenuation at 60 Hz, thereby reducing the effect of stray 60-Hz signals. Thirty ERGs were averaged with a computer-assisted signal averaging system (Power Lab; AD Instruments, Castle Hill, Australia).

Visual Stimulation

A Ganzfeld bowl (Model GS2000; LACE Electronica sel via Marmiccilo, Pisa, Italy) was used for stimulation. This stimulator was equipped with a xenon light source and the luminance of the strobe flash was measured with an integrating radiometer (40X-Spotmeter; United Detector Technology, Hawthorne, CA, USA). Photopic ERGs were recorded using four different stimulus intensities of –0.8 to 1.0 log cd-s/m^2 after 10 minutes of light-adaptation. For the flicker ERGs, the average luminance was 3.0 cd-s/m^2/flash. The stimulus was presented on a constant white background of 40 cd/m^2.

Harmonic Analysis

The fundamental and higher harmonic components in the ERGs elicited by each frequency of stimulation were assessed through harmonic analysis.[13,21,22] As there were 2048 Fast Fourier Transform (FFT) points, FFT could be performed using Excel (Excel 2013; Microsoft, Inc., Redmond, WA, USA). The sampling frequency (2000 Hz) was divided by the number of FFT points (2048) to produce a factor of approximately 0.977. Flicker ERGs were recorded for 15 frequencies ranging from 3.906 Hz (4 × 0.977) to 31.250 Hz (32 × 0.977) in steps of 1.953 Hz (2 × 0.977). Because of equipment limitations, we were not able to determine the stimulus frequencies beyond the third decimal place; therefore, the frequencies were rounded off accordingly.

The response spectra contained peaks at the stimulus frequency, the fundamental component (1F), and at the higher harmonics of 2F to 5F. Both the amplitudes and phases of the fundamental components and the amplitudes of the harmonic components from 2F to 5F were analyzed.

The phase lag of the fundamental component relative to the stimulus was determined and drawn on polar plots clockwise from the positive *x*-axis. Because FFT analysis yields phases of only between –360° and 360°, we extrapolated the absolute response phases beyond this limit through comparisons with phases of adjacent temporal stimulus frequencies, as shown in previous studies.[8] For example, if the true phase is 400°, Fourier analysis calculates a phase of 40°.

To obtain an estimate of the noise level at each temporal frequency, recordings were made without xenon stimuli in seven mice. The response to flash stimulus at each temporal frequency was considered to represent the actual ERG response if the amplitude exceeded three times the noise amplitude at the same temporal frequency.[4]

Direct Waveform Subtraction

We subtracted the waveforms of the averaged responses of mGluR6$^{-/-}$ mice ($n = 12$) from the waveforms of the averaged response of WT mice ($n = 12$) and Pika$^{-/-}$ mice ($n = 12$). We then measured the amplitude of the obtained waveforms (ON pathway) at each frequency.

Statistics

Statistical analysis of multiple groups was performed by using two-factor factorial ANOVA followed by the Tukey-Kramer test; *P* less than 0.05 was considered statistically significant.

Results

Representative ERGs of WT, Pika$^{-/-}$, and mGluR6$^{-/-}$ Mice

Representative ERGs, elicited by stimuli of four different intensities (–0.80 to 1.0 log cd-s/m^2) recorded under photopic conditions (10-minute exposure to 40 cd/m^2), are shown in Supplementary Figure S1. The amplitudes of the photopic b-wave in the Pika$^{-/-}$ mice were significantly smaller than those of the WT mice but larger than those of the mGluR6$^{-/-}$ mice. At the maximum stimulus intensity of 1.0 log cd-s/m^2, the b-wave amplitude of the Pika$^{-/-}$ mice was only 25% of that of the WT mice. The implicit times of the photopic b-waves were also delayed at all intensities in the Pika$^{-/-}$ mice. Although the amplitudes of the photopic b-waves were severely reduced in the Pika$^{-/-}$ mice, the oscillatory potentials were still present. In contrast, the photopic b-waves of the mGluR6$^{-/-}$ mice were abolished (Supplementary Fig. S1). These results are consistent

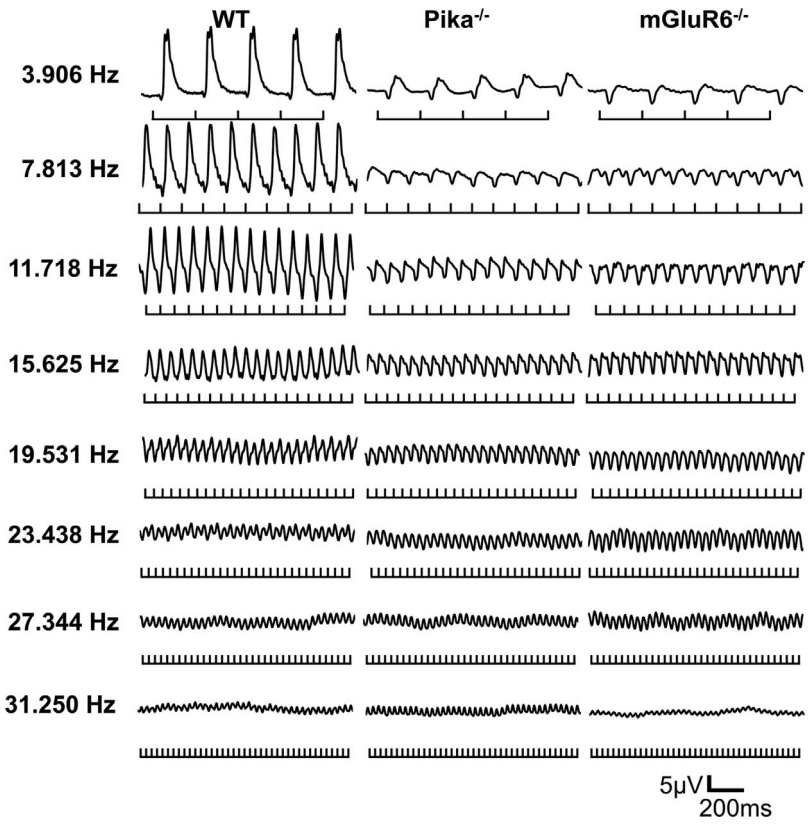

FIGURE 1. Representative flicker ERGs elicited by stroboflash stimuli from a WT mouse (*left*), Pika$^{-/-}$ mouse (*middle*), and mGluR6$^{-/-}$ mouse (*right*). The rates of temporal frequency stimulations ranged from 3.906 to 31.250 Hz. The stimulus traces are shown in *black* beneath each ERG waveform.

with past ERG recordings of Pika$^{-/-}$ and mGluR6$^{-/-}$ mice.[14,16,18]

Representative flicker ERGs recorded from the three types of mice at frequencies ranging from 3.906 to 31.250 Hz are shown in Figure 1. The ERGs comprised several components and shapes at lower intensities but became simpler and sinusoidally shaped at higher stimulation frequencies. There were distinct differences between the waveforms of the WT and those of the other mice. The amplitudes of the WT mice were large at low temporal frequencies but reduced at frequencies greater than 23.438 Hz. The flicker amplitudes did not much differ among the three types of mice at higher frequencies; but, at lower frequencies (3.906–11.718 Hz) the ERG flicker amplitudes of the Pika$^{-/-}$ mice were slightly different from those of the mGluR6$^{-/-}$ mice.

Harmonic Analyses

In Figure 2, the amplitude (A) and phase (B) of the fundamental components of the flicker responses are shown for the three types of mice. In the WT mice, the amplitude of the fundamental components was largest at 5.859 Hz and then monotonically decreased with increasing stimulus frequency. In the Pika$^{-/-}$ and mGluR6$^{-/-}$ mice, the amplitude of the fundamental component was slightly larger, between 3.906 Hz and 13.672 Hz, and then gradually decreased monotonically as the stimulus frequency increased. The amplitudes of the fundamental components of the three types of mice were approximately the same for temporal frequencies greater than 21.484 Hz and approached the noise level at frequencies greater than 29.297 Hz. The amplitudes of the fundamental components for Pika$^{-/-}$ and mGluR6$^{-/-}$ mice were significantly smaller than those for WT mice for frequencies between 3.906 and 17.578 Hz. However, there were no significant differences in the amplitudes of the fundamental components for Pika$^{-/-}$ and mGluR6$^{-/-}$ mice at any frequency.

The phases of the fundamental components for the mGluR6$^{-/-}$ and WT mice in response to 3.906 Hz stimuli differed by nearly 150°, and the phase for the Pika$^{-/-}$ mice was between those of the WT and mGluR6$^{-/-}$ mice. There were significant differences in the phases for the WT and Pika$^{-/-}$ mice at frequencies between 3.906 and 11.718 Hz and for the Pika$^{-/-}$ and mGluR6$^{-/-}$ mice at frequencies between 3.906 and 9.765 Hz. However, there were no significant differences in phase between the WT and Pika$^{-/-}$ mice at frequencies greater than 13.672 Hz, and the Pika$^{-/-}$ and mGluR6$^{-/-}$ mice at frequencies greater than 11.718 Hz.

Supplementary Figure S2 shows the amplitudes of the fundamental and harmonic components (2F–5F) of the three types of mice at 5.859, 11.718, 17.578, and 23.438 Hz. As with the response at the fundamental component, the amplitude of the harmonics decreased with increasing stimulus frequency.

Vector Model Analyses

Using the data shown in Figure 2, we calculated the vectors of the mean amplitudes and phases of the fundamental components at each frequency. The calculated vectors for the three types of mice for temporal frequencies of 5.859, 11.718, 17.578, and 23.438 Hz are shown in Figure 3. The vectors of the WT mice are shown in black, those for the Pika$^{-/-}$ mice in red, and those for the mGluR6$^{-/-}$ mice by blue dotted lines. We assumed that the ON pathway of the WT mice was normal and that the mGluR6$^{-/-}$ mice completely lacked contributions from

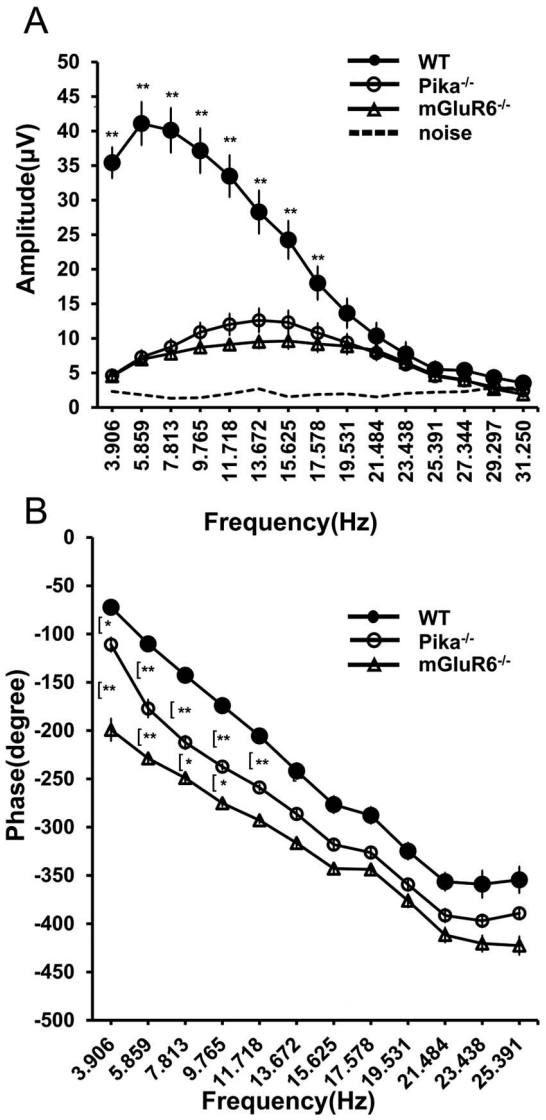

FIGURE 2. The mean amplitudes of the fundamental component (**A**) and the mean phase of fundamental component (**B**) of the flicker ERGs of WT (•), Pika$^{-/-}$ (○), and mGluR6$^{-/-}$ (△) mice are shown. *Error bars* represent the SEMs. Three times the average noise amplitude is shown as a *broken line* in (**A**). (**A**) The amplitudes of the WT mouse were significantly larger than those of the Pika$^{-/-}$ mouse between 3.906 and 17.578 Hz. There were no significant differences in the amplitudes of Pika$^{-/-}$ and mGluR6$^{-/-}$ at all frequencies. (**B**) There were significant differences in the phases of the WT and Pika$^{-/-}$ mice at frequencies between 3.906 and 11.718 Hz, and the Pika$^{-/-}$ and mGluR6$^{-/-}$ mice at frequencies between 3.906 and 9.765 Hz. $^{*}P < 0.05$, $^{**}P < 0.01$ (Tukey-Kramer test).

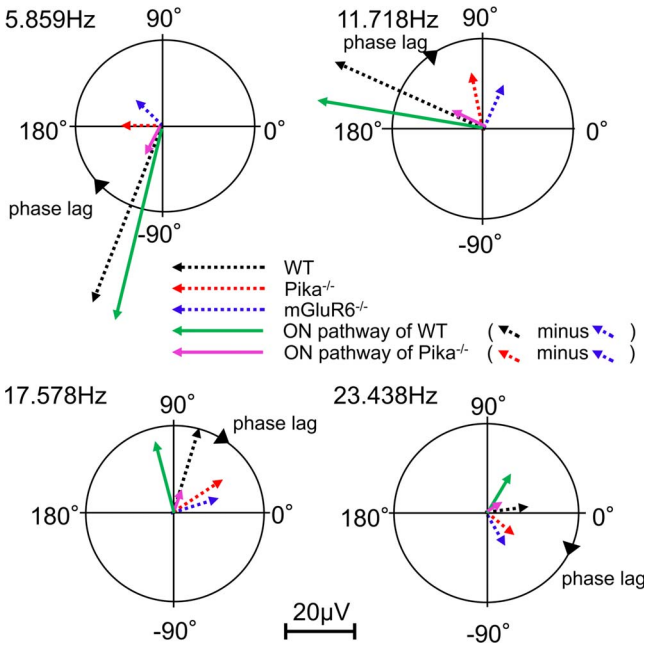

FIGURE 3. Representative vectors obtained from the amplitudes and phases of fundamental components of the flicker ERGs of the WT, Pika$^{-/-}$, and mGluR6$^{-/-}$ mice at 5.859 Hz (*top left*), 11.718 Hz (*top right*), 17.578 Hz (*bottom left*), and 23.438 Hz (*bottom right*). The length and direction of the *arrows* indicate the mean amplitudes and phases, respectively. The vectors of the WT (*black dotted arrow*), Pika$^{-/-}$ (*red dotted arrow*), and mGluR6$^{-/-}$ (*blue dotted arrow*) are shown. To estimate the contribution of the ON pathway of Pika$^{-/-}$ mice (*pink solid arrow*), the vector of the mGluR6$^{-/-}$ mice (*blue dotted arrow*) was subtracted from that of the Pika$^{-/-}$ mice (*red dotted arrow*). To estimate the contribution of the ON pathway of WT mice (*green solid arrow*), the vector of the mGluR6$^{-/-}$ mice (*blue dotted arrow*) was subtracted from that of the WT mice (*black dotted arrow*).

the ON pathway. To estimate the contribution of the ON pathway of Pika$^{-/-}$ mice, we subtracted the vectors of the mGluR6$^{-/-}$ mice from those of the Pika$^{-/-}$ mice and examined the residual ON pathway response of the Pika$^{-/-}$ mice. We also subtracted the vectors of the mGluR6$^{-/-}$ mice from those of the WT mice to evaluate the normal ON pathway. The obtained vectors for the ON pathway of Pika$^{-/-}$ and WT mice are shown in pink and green solid arrows, respectively. The differences of phase between Pika$^{-/-}$ and mGluR6$^{-/-}$ mice caused by ON pathway functioning in Pika$^{-/-}$ mice and the reduction of the phase lag between the two types of mice at higher frequencies corresponded to a decrease of the ON pathway in Pika$^{-/-}$ mice. The lengths of the vectors for the Pika$^{-/-}$ were much shorter than those for the WT mice, but the ON pathway was detected at frequencies of 5.859 and 11.718Hz. We plot the amplitudes of the calculated vectors for each frequency in Figure 4. The amplitude of the ON pathway in WT mice was largest (44.85 μV) at a frequency of 5.859 Hz, and it steadily decreased as the stimulus frequency increased. The amplitudes of the ON pathway in the Pika$^{-/-}$ mice were 5 to 7 μV at frequencies between 3.906 and 15.625 Hz, or 12% to 25% of those of the WT mice. The amplitudes of the ON pathway Pika$^{-/-}$ mice fell below 3 μV at the higher frequencies of 19.531 to 31.250 Hz, which, as shown in Figure 2A, is the noise level.

Direct Waveform Subtraction

To determine whether vector model analysis is a reasonable method for investigating the ON pathway, we subtracted the waveforms of the mGluR6$^{-/-}$ mice from those of the WT and Pika$^{-/-}$ mice directly (Supplementary Fig. S3A). The subtracted waveforms (ON pathway) of the WT mice differed from those of the Pika$^{-/-}$ mice. The amplitudes of the ON pathway for WT and Pika$^{-/-}$ mice were largest at the lowest frequency and decreased as the stimulus frequency increased (Supplementary Fig. S3B). The amplitudes of the ON pathway response in the WT mice were larger than those in the Pika$^{-/-}$ mice at frequencies between 3.906 and 15.625 Hz. The amplitudes of response in the ON pathway in the Pika$^{-/-}$ mice were 20% to 30% of those in the WT mice at frequencies between 3.906 and 11.718 Hz.

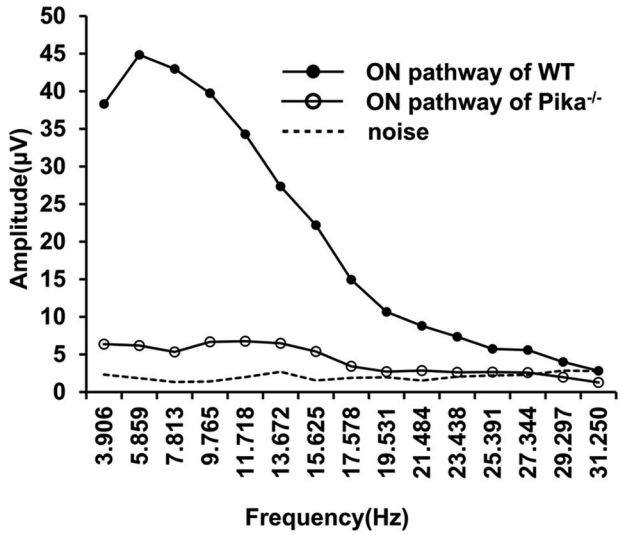

FIGURE 4. The averaged amplitudes of the ON bipolar component of WT (•) and Pika$^{-/-}$ (○) mice as functions of temporal frequency. Amplitudes were obtained from the vectors that were calculated from the averages of the ON pathway of WT and Pika$^{-/-}$ mice. Three times the average noise amplitude is shown as a *broken line*.

DISCUSSION

We evaluated ERG flicker responses in the ON pathway of Pika$^{-/-}$ mice using vector model analyses and identified how they varied with the temporal frequency of the stimulus.[8,13] Our results showed that the amplitudes and phases of the fundamental responses for Pika$^{-/-}$ mice were between those for mGluR6$^{-/-}$ and WT mice. In addition, the residual responses of the ON pathway of Pika$^{-/-}$ mice were 12% to 25% of those of the normal ON pathway at lower frequencies. At frequencies greater than 19.531 Hz, the estimated ON pathway response of Pika$^{-/-}$ mice was nearly at the noise level. Vector model analyses enabled us to estimate the ON pathway of Pika$^{-/-}$ mice over a wide range of stimulation frequencies.

In an earlier study, Krishna et al.[4] reported several features of the temporal responses of mice using vector model analyses. They noted that the temporal frequency range for mice was quite limited and that the flicker responses approached the noise level at frequencies of approximately 30 Hz. In addition, they showed that the mouse temporal response function was not as complicated as those of primates, which comprises summations and subtractions of the ON and OFF pathways. They concluded that these differences indicate that the mouse cone ERG responses are integrated in a more linear fashion than the primate responses.

Our results are in good agreement with their findings in that the amplitudes of the fundamental components for WT, mGluR6$^{-/-}$, and Pika$^{-/-}$ mice become smaller and are approximately equal at frequencies higher than 23.438 Hz and the responses approach the noise level at a frequency of 29.297 Hz. In addition, our results show that the mouse cone ERGs respond in a more linear manner than do the previously reported primate ERGs.[8] We suggest that this linear temporal response summation in mice might reflect differences in the relative contributions of the ON and OFF pathways to the cone ERGs. The mouse OFF pathway makes only a small contribution to the ERG[14,23]; however, the cause of the limited temporal frequency range of the mouse response was not determined.

We determined the contribution of the ON pathway to the flicker ERGs of WT and Pika$^{-/-}$ mice by subtracting the waveforms of the mGluR6$^{-/-}$ mice from those of the WT and Pika$^{-/-}$ mice, as shown in Supplementary Figure S3. Our results show that the contribution of the ON pathway measured by direct subtraction has similar properties to measurements made using vector model analysis. According to both methods, the residual responses of the ON pathway of Pika$^{-/-}$ mice are approximately 20% of those of the normal ON pathway at lower frequencies, and the contribution is severely reduced at frequencies higher than 19.531 Hz in both WT and Pika$^{-/-}$ mice. These results suggest that vector model analysis is appropriate for investigation of the mouse ON pathway. In addition, we found that vector model analysis provides additional information regarding the contribution of the ON pathway to the flicker ERG. The corresponding vectors comprise the phase and amplitude (length), and the summation and cancelation of these vectors are severely affected by phase lag. Kondo and Sieving[8] showed that differences in phase lag in the ON and OFF pathways at different frequencies contribute to the characteristic changes in amplitude ERG responses to flicker. In the present study, we unraveled the differences of phase lag among three types of mice, which contributed greatly to flicker ERG amplitudes. Thus, we conclude that vector model analysis is more informative than direct subtraction of the waveforms in the analysis of the temporal properties of flicker ERGs.

There were limitations in our study. First, the stimulus frequencies had to be rounded off to the third decimal place owing to equipment limitations. Consequently, the stimuli were not precise multiples of the sampling rate and the resulting phase information might not have been completely accurate.

Second, we used stroboscopic flashes instead of the sine wave stimuli used in earlier studies.[4,6,7] In clinical situations, rapid flicker ERGs are commonly elicited using stroboscopic flashing at approximately 30 Hz, although sinusoidal and square-wave stimuli have been used in some situations. For harmonic analysis, sine wave stimuli are probably preferable. Kondo and Sieving[13] compared the results of vector analysis obtained from primate flicker ERGs elicited by several types of stimuli, including sine waves and pulses. They showed that vector model analysis of ERGs using several types of stimulus revealed similar features, although there were fewer phase differences between ON and OFF pathways and a greater contribution from higher harmonic components when strobe flash stimuli were used. Our results indicate that the phase differences between WT and mGluR6$^{-/-}$ mice elicited by strobe flashes are smaller than those in previous reports on WT and nob mice recorded using sinusoidal stimuli.[4] In addition, for the WT mice, the contributions of higher harmonic components elicited by stroboscopic flashes were larger than those previously reported; these larger components might have affected the results of our vector analysis.[4] However, most of the features of our data were substantially similar to previously obtained WT mouse data; therefore, we believe that our methods are appropriate to detect ON pathway activities in Pika$^{-/-}$ mice.

Third, the specifications of our notch filter might have affected the FFT analysis of the ERGs. Our filter attenuated the 60-Hz frequency noise, which might have affected the higher harmonic components. Because we used fundamental components within 32 Hz of the flicker ERGs in our vector analysis, the effect of the notch filter on our results might be limited.

In conclusion, our results show the utility of vector model analysis in mice with defects in the photoreceptor-to–bipolar cell communication.

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