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Reliability and Significance of Measurements of a-Wave Latency in Rats

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Purpose: To determine whether measurements of the a-wave latency of the electroretinogram (ERG) can be made as reliably as that of the implicit time (IT) in rats. In addition, to determine the relationship between the potential level selected for the latency and the baseline potential level.

Methods: ERGs, elicited by different stimulus intensities, were recorded from Long-Evans rats. The a-wave latency was determined by measuring the time between the stimulus onset and the beginning of the negative-going a-wave, and the IT was measured as the time between the stimulus onset and the peak of the a-wave. To test the reliability of the measurements of the latency, the a-wave latency and the IT were measured by three independent observers for the same 15 ERGs.

Results: The mean a-wave latency was approximately 14 milliseconds, and the mean a-wave implicit time was approximately 36 milliseconds. The mean of the a-wave latency and the IT, as measured by the three observers, were within 1 millisecond of each other. The coefficient of variation was as good for the latency as for the IT of the a-wave. The potential level selected for the latency was lower than the mean baseline potential level by 1 to 2 standard deviations.

Conclusions: Selection of the a-wave latencies can be made as reliably as that for the IT. Because the a-wave latency is not affected by the activity of the second order neurons, the latency is a better measure than the IT of the time course of the a-wave. **Jpn J Ophthalmol 2002;46:419–425**

Key Words: Electroretinogram, implicit time of a-wave, latency of a-wave, rats, reliability

Introduction

The amplitudes of the a- and b-waves, and occasionally the c-waves, of the electroretinogram (ERG) are used to assess the physiological condition of the retina. Because the contributions of the different retinal cells to the a-, b-, and c-waves are fairly well established, changes in the amplitudes of these waves provide information on the physiological condition of the corresponding cells. However, only an estimate of the real amplitudes of these waves can be made because of the confounding effects of the a-, b-, and c-waves.

To examine the time course of the ERGs, the implicit time of the a- and b-waves is used almost universally. The implicit time (IT) is defined as the time

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between the onset of the stimulus and the peak of the response. The confounding effects of the a- and b-waves also apply to the IT, eg, the IT of the a-wave depends not only on photoreceptor activity but also on the activity of the bipolar cells.[1] It is not known with certainty what the IT represents and, thus, its physiological significance is not known.

In neurophysiological studies, the latency of a response is most commonly used to assess the timing properties of the system, eg, the compound nerve action potentials.[2] The latency of a response is defined as the time from stimulus onset to the beginning, as opposed to the peak, of the response. Thus for the a-wave of the ERG, the latency is the time from the stimulus onset to the beginning of the negative-going a-wave.

In early ERG studies, the latency of the a-wave was used to assess the time course of the ERG.[3] The reason why the IT was adopted to characterize the time course has not been stated; however, it is generally believed that a more reliable measurement of the IT can be made because the peak of a response is easier to identify than the slower potential changes at the beginning of a response. In addition, before the advent of digital computers with fast sampling rates, it was difficult, if not impossible, to measure the short latencies with any degree of accuracy with the equipment available then.

The purpose of this study was to determine whether reliable subjective measurements of the a-wave latencies can be made by different observers. We have defined reliability as the ability to obtain the same value of a response by different observers. We also determined the potential level (μV) of the latency values selected in relation to the baseline potential level. We shall show that the latency of the a-wave can be measured as reliably as the IT to determine the time course of the a-wave. The value selected as the a-wave latency in our experiment was a potential level that was lower than the pre-stimulus mean baseline potential level by 1 to 2 standard deviations (SDs).

Materials and Methods

Animals

The experiments were conducted on young adult Long-Evans rats weighing approximately 200–300 g. Immunohistochemical methods have shown that the cones make up only 0.85% of the photoreceptors in the rat retina.[4] The rats were housed in standard rat cages with lighting on a 12-h:12-h dark:light cycle. The ambient illumination of the room was 0.1 to 0.2 log cd/m^2, and the top of the cage racks shielded the rats from direct illumination. All animals used were treated in accordance with the ARVO Resolution on the Use of Animals in Research, and the experimental protocol was approved by the University of Miami Animal Care and Use Committee.

Recording the ERGs

The animals were anesthetized with a mixture of ketamine:xylazine:urethane (11 mg/kg:14 mg/kg:500 mg/kg body weight), and the pupils were dilated with 0.5% phenylephrine HCl and 0.5% tropicamide. The ERGs were recorded with a wick-Ag:AgCl electrode placed on the cornea with the reference electrode, and a 25-gauge hypodermic needle, placed subcutaneously on the head. The animal was grounded by another needle placed subcutaneously in the neck region.

The responses were fed to a Tektronix A39 preamplifier with the half-amplitude bandpass set at 0.1 Hz to 10 kHz. The output from the preamplifier was displayed on an oscilloscope and was also fed to a Biopac MP100 Acknowledge Data Acquisition program (Goleta, CA, USA). A sampling rate of 1000 samples/s and an analysis time of 2.0 seconds were selected.

Stimulus

The light for the stimulus was obtained from a 15-V 10A quartz-iodide lamp bulb. The filament of the bulb was focused in the plane of a Uniblitz shutter (Vincent Associates, Rochester, NY, USA), and by another lens onto the tip of a fiber optic bundle. The other end of the fiber optic bundle was brought into a Faraday cage and placed 1–2 mm from the cornea of the animal.

The maximum luminance of the stimulus was 2.53 log cds/m^2, and neutral density (ND) filters were used to reduce the full intensity stimulus. The stimulus intensities were increased in 1.0 log unit steps from threshold to the maximum available. The stimulus intensities are designated by the ND filter used to attenuate the full intensity stimulus. A pulse generator (S44; Grass Instruments, Quincy, MA, USA) drove the Uniblitz shutter and controlled the stimulus duration at 1 second. Two responses were averaged at the lower stimulus intensities (ND = 6.0 to 4.0) and only one response was recorded at the higher stimulus intensities (ND = 3.0 to 0). With each increase in stimulus intensity, the recovery period was increased stepwise from 1.5 minutes at the lowest stimulus intensity to 8 minutes at the highest stimulus intensities.

Procedures

After the animal was set up, the eye was dark-adapted for 30 minutes. ERGs were then recorded beginning with a stimulus intensity that was determined from earlier experiments to be close to the b-wave threshold (ND = 6.0). The stimulus intensity was increased in 1.0 log unit steps to the full intensity stimulus (ND = 0).

The amplitude of the a-wave was measured from the baseline to the trough of the a-wave, and the b-wave was measured from the baseline or trough of the a-wave to the peak of the b-wave.

Because we were interested only in the a-wave latency, only the ERGs with an a-wave that were elicited by the higher stimulus intensities were analyzed.

Measurement of a-Wave Latency and IT

The latency of the a-wave was measured from stimulus onset, #1, to the beginning of the negative-going a-wave, #2 (Figure 1). The IT of the a-wave was measured from stimulus onset, #1, to the peak of the a-wave, #3. The IT of the a-wave is also the latency of the b-wave. The IT of the b-wave was measured from stimulus onset, #1, to the peak of the b-wave, #4.

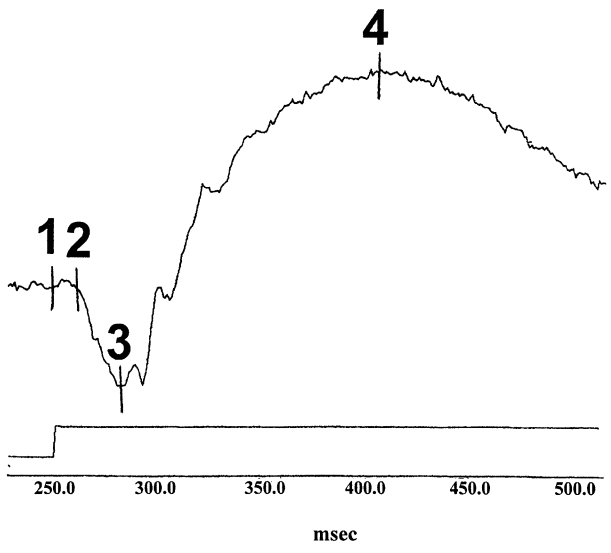

Figure 1. Electroretinogram (ERG) elicited by the full-intensity stimulus from the dark-adapted eye. #1: stimulus onset, #2: beginning of the a-wave, #3: peak of the a-wave, and #4: peak of the b-wave. a-wave latency: time between #1 and #2, a-wave implicit time (IT): time between #1 and #3, and b-wave IT: time between #1 and #4.

The Biopac MP100 Acknowledge Data Acquisition program provided a digital readout of the relative potential level (μV) and time (milliseconds) at any point along the response selected by a cursor. Using the program, we were able also to place two cursors on the response so that the time and potential level at each cursor, and the time (delta time, milliseconds) and the difference in the potential levels (delta amplitude, μV) between the two cursors could be read off.

To determine the a-wave latency, the first cursor was placed at the stimulus onset (#1 in Figure 1), and the second cursor was stepped along the baseline of the response in 1-millisecond steps (1000 samples/s) until a downward movement of the cursor was noted. The movement of the cursor was continued for several more steps to show that the potential continued to decrease. The cursor was then stepped back until the beginning of the response was reached. Then, the time between the two cursors was read in milliseconds and recorded as the a-wave latency. The cursor was then moved until the peak of the a-wave was reached, and the time between the onset of the stimulus and this point was read and recorded as the IT of the a-wave. The b-wave IT was measured in the same way.

Because it was possible to read the digital potential level (μV) at each point, we were also able to follow the changes in the potential level as the cursor was stepped along the response. By following these potential changes, a point could be found where a decrease in the potential level continued to decrease in subsequent steps. Then, the first point was selected and recorded as the a-wave latency. This is the procedure used in the early experiments and it required about 10 to 20 seconds to set the cursor at the beginning of the response. Later, with experience, the cursor was placed at the beginning of the response by "pointing" at the latency point and clicking the mouse. This took about 5 to 10 seconds to complete.

Statistical Analysis

Student unpaired *t*-tests were used to determine the significance of the differences. The P value $< .05$ was selected as statistically significant.

Results

ERGs

The ERGs elicited by the four highest intensities (ND = 3.0, 2.0, 1.0, and 0) that evoked an a-wave are shown in Figure 2A. At ND = 3.0, there was a slowly decaying negative a-wave with a latency of approximately 37 milliseconds. Increasing the stimulus inten-

Jpn J Ophthalmol
Vol 46: 419–425, 2002

sity to ND = 2.0 led to a larger amplitude and faster decaying a-wave. The latency and IT of the a-wave were also shorter with the increase in intensity. With the full intensity stimulus (ND = 0), there was a rapidly decaying a-wave of large amplitude and a short latency of 13 milliseconds and an IT of 37 milliseconds.

Effect of Stimulus Intensity on the a-Wave Latency

From ERGs such as those shown in Figure 2B, the a-wave latency was measured for 15 ERGs elicited by ND = 2.0, 1.0 and 0. As expected, there was a decrease in the latency with increasing intensity (Figure 2B), and the slope of the best-fit line was 7.95 ms/log unit.

Reliability of a-Wave Latency and IT Measurements

Because it has been suggested that measurements of the a-wave latency were not reliable and that more reliable measurements can be made of the IT, the latency and IT of the a-wave were measured by three observers for the same 15 ERGs elicited by the full-intensity stimulus. The first observer (D.I.H.) was an experienced neurophysiologist, the second (H.Q.) was familiar with ERGs as a clinician but had not performed ERG experiments, and the third (I.P.) was a bioengineering graduate student whose first experience with ERGs was when he made these measurements.

The mean ± SD of the a-wave latency and IT determined by the three observers for the 15 ERGs are shown in Table 1. Although the SD was larger for the inexperienced observer, the difference in the a-wave latency determined by each of the three observers was within 1 millisecond. The mean IT recorded by the three observers were also within 1 millisecond, and there was good agreement in the SD of the three individuals (Table 1).

We also calculated the coefficients of variation (SD/mean) for the measurements made by the

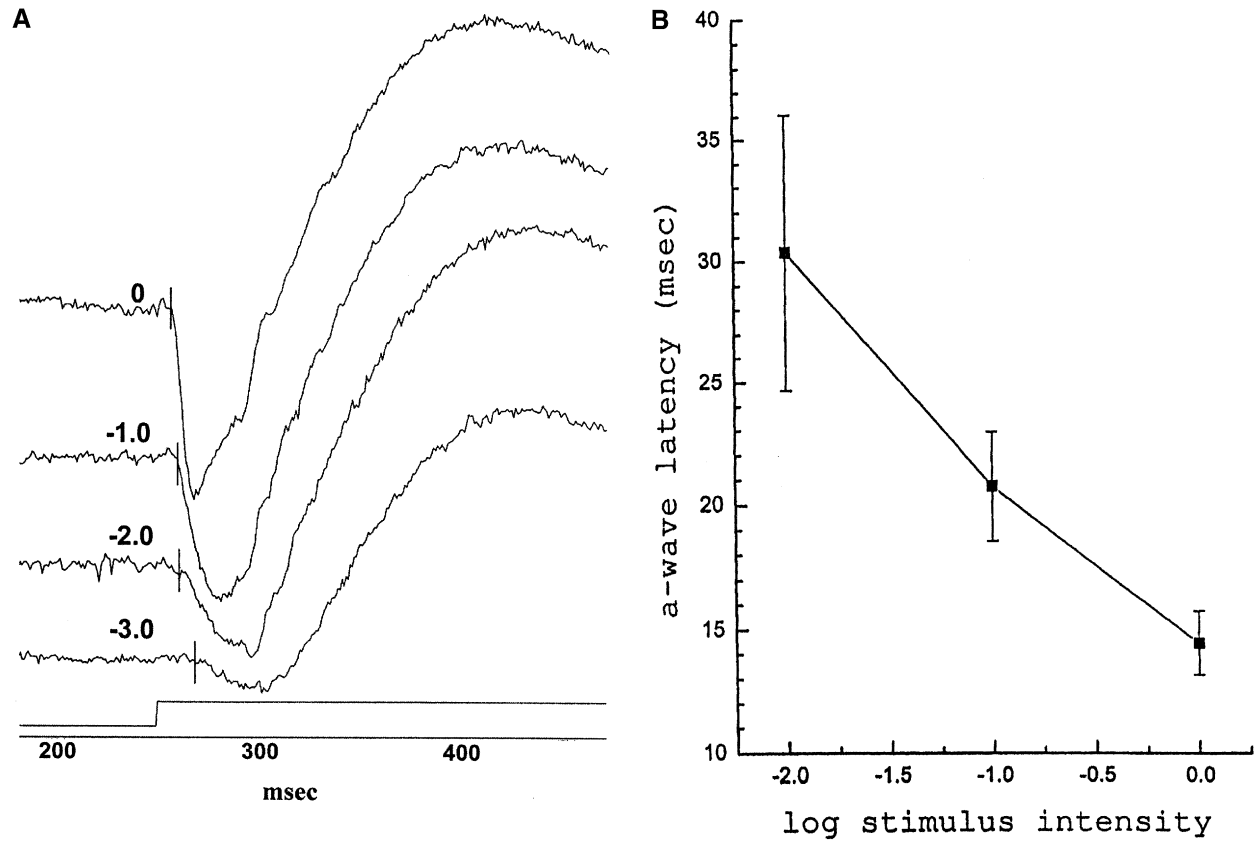

Figure 2. (**A**) Electroretinograms (ERGs) elicited by increasing stimulus intensities recorded from a dark-adapted Long-Evans rat. The number above each ERG represents the neutral density (ND) filter used to reduce the full intensity stimulus. At ND = 0, luminance = 2.53 log cds/m^2. (**B**) Effect of stimulus intensity on the a-wave latency. The mean ± SD for 15 ERGs are shown.

three observers. The coefficients of variation ranged from 9.0% to 14.7% for the a-wave latency and from 11.1% to 11.9% for the IT. Although the coefficient of variation of the latency was higher for the inexperienced observer, the coefficients for the other two observers were slightly better for the latency than for the IT.

What Does the Latency Value Measure?

The question arose as to what the potential value (μV) selected as the latency represented in reference to the baseline potential level. To answer this question, the mean ± standard deviation of the baseline potential level was determined for the 100 milliseconds just prior to stimulus onset. To do this, the first cursor was set at the stimulus onset, and the second cursor was moved 100 steps (100 milliseconds) back from the stimulus onset. We had the computer calculate the mean ± SD of these 100 potential values. This was then the mean (± SD) potential level of the baseline 100 milliseconds prior to stimulus onset, and this mean baseline potential level was determined in this way for each of the 15 ERGs.

The changes in the potential level during the early phase of 2 ERGs are plotted in Figure 3, with the ERG in Figure 3A having a noisier baseline. The onset of the stimulus is marked by the arrow (on), and the mean potential level 100 milliseconds prior to stimulus onset is marked by an "X." The mean minus 1 SD (mean − 1 SD) and the mean minus 2 SD (mean − 2 SD) potential levels are also shown. The latency values selected by D.I.H. are shown by the points labeled "Lat" in the figures. In both Figures 3A and 3B, the latency was between 1 and 2 SD from the mean baseline potential level.

In a similar way, the latency values selected by D.I.H. and I.P. were compared to the mean baseline potential. For D.I.H., 53.3% (8/15) of the latencies lay between the mean and − 1 SD and 66.7% (10/15) between the mean and − 2 SD. For I.P., the comparable values were 33.3% and 53.3%, respectively. There was one latency selected by D.I.H. and two latencies selected by I.P. that were lower than 3 SD from the mean baseline potential level.

Effect of Light-adaptation

After the dark-adapted responses were recorded, a weak adapting light was turned on, and after 2 minutes of adaptation to the steady light, responses were recorded with increasing stimulus intensities. The intensity of the adapting light was increased by 1.0 log unit, and after 2 minutes of adaptation to this level, ERGs were recorded. The results from one of the eyes are shown in Figure 4 for the ERGs that were elicited by the full intensity stimulus. The ERGs elicited in the dark, and with three levels of background illumination differing by 1.0 log unit, are shown.

With increasing background illumination, there was an increase in the a-wave latency and the IT. There was also a decrease in the amplitude and a slowing of the decay time of the a-wave.

Discussion

Reliability of a-wave Latency Measurements

The results have shown that the mean a-wave latency selected by an ERG-experienced and an ERG-inexperienced observer differed by less than 1 millisecond. This small variation was also found for the IT measurements. Based on our findings, we can conclude that the measurements of the latency were as reliable as those of the IT.

The coefficients of variation for the a-wave latency and for the IT were also used to determine the reliability of the measurements by the three observers. Although the coefficient was higher for the inexperienced observer, the coefficients for the other two observers were slightly lower for the latency than for the IT. Thus, we conclude from these results that measurements of the a-wave latency are as reliable as those made of the IT.

Table 1. Mean ± SD and Coefficients of Variation of the a-Wave Latency and Implicit Time Determined by Three Observers

	a-Wave Latency		a-Wave Implicit Time	
Observer	Mean ± SD (ms)	CV (%)	Mean ± SD (ms)	CV (%)
I.P.	13.9 ± 2.05	14.7	36.2 ± 4.30	11.9
H.Q.	14.7 ± 1.49	10.1	36.0 ± 4.05	11.2
D.I.H.	14.5 ± 1.30	9.0	37.1 ± 4.13	11.1

CV: coefficient of variation (SD/mean %).

Jpn J Ophthalmol
Vol 46: 419–425, 2002

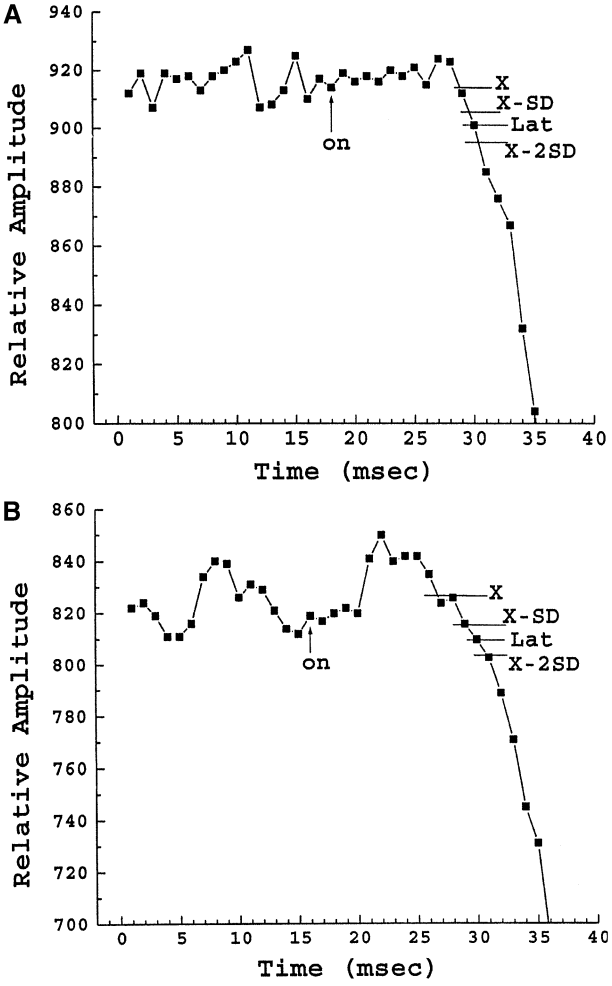

Figure 3. Plots of the early parts of two electroretinograms (ERGs) are shown. The digital potential level is plotted on the ordinate and time on the abscissa. Stimulus onset is marked by an arrow (on). The mean potential level 100 milliseconds before stimulus onset is marked by an "X," and the mean minus 1 SD (X − SD) and the mean minus 2 SD (X − 2 SD) are also marked. "Lat" in these figures indicates the point of the latency selected by one observer (see text). The time between any two points is equal to 1 millisecond.

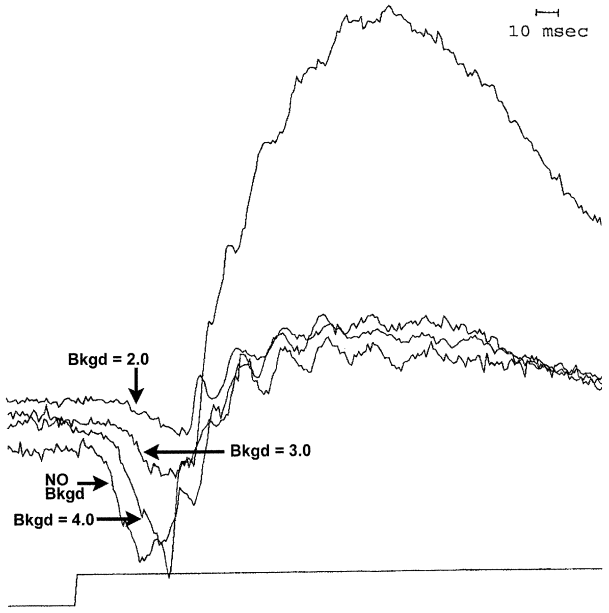

Figure 4. Effect of adapting background (bkgd) on the electroretinogram (ERG). The ERGs elicited by full intensity stimulus with no background and with three increasing background intensities in 1.0 log unit steps are shown. There is an increase in the a-wave latency and a decrease in the a-wave amplitude.

There are, however, two caveats. First, the ERGs must be collected with a sampling rate of at least 1000 samples/s to allow a resolution of at least 1 millisecond. And second, a computer data acquisition program with a readout of the digital potential level and the time at each point along the response should be used. For ease of analysis, the program should also be able to calculate the mean and SD between any two points selected by the cursors.

An analysis of the potential level selected as the a-wave latency showed that the point was lower than the mean baseline potential level by 1 to 2 SD. There was, however, some variation in the point selected by the two observers for some of the values greater than 3 SD from the mean baseline potential level. This variation in the latencies was due to several factors. First, the baseline potential level selected for the analysis was the 100 milliseconds before the stimulus onset. The 10 to 12 milliseconds after the stimulus onset and just prior to the beginning of the response were not considered in the calculations. In the subjective placement of the latency, on the other hand, the baseline values 10 to 12 milliseconds before the beginning of the response were considered as the cursor was moved along the baseline to determine the latency (see Figure 3). Thus, changes in the baseline during this period can alter the selection of the latency value and these values were not included in the calculation of the baseline.

Second, the change in the potential level is very rapid with the full-intensity stimulus. Thus, a movement of one step (1 millisecond) can move the potential level from a value that was within 1 SD to one that was 2 SD of the baseline potential level (Figure 3). A higher sampling rate would be needed to overcome this problem.

The a-Wave Latency, the IT, and Curve-fitted a-Wave

The time course of the a-wave has been of interest to visual neurophysiologists from the beginning of the studies of ERGs. Very early investigators compared the latency of the a- and b-waves to the neural discharges in the optic nerve and lateral geniculate nucleus to try to determine the cellular origin of the different components of the ERG.[3] Later, the isolated rod photo responses were fitted to exponential equations,[5] and a similar analysis was performed for single rod photoresponses[6] in order to characterize the time course of the phototransduction process. More recently, the a-wave has been fitted to an equation developed to describe the phototransduction process of rods.[7] This method consisted of fitting a family of Gaussian curves to the a-wave by first fitting the leading edge of the a-wave "by eye" to the photoreceptor model of phototransduction. Except for the studies of the recordings from single rods, the contribution of the bipolar cells to the a-waves had to be either blocked or mathematically subtracted out of the response.

These different methods give different information on the phototransduction process. The question then arises: Which of these methods should be used to characterize the time course of the ERGs in animal experiments and in patients? In the following paper, we shall show that the a-wave latency is determined solely by photoreceptor activity.[8] Thus, we recommend that the a-wave latency be used because, unlike the IT and the curve-fitting methods, changes in the a-wave latency can be attributed to alterations of photoreceptor activity. In addition, there is a difference in the time required to make the measurements. It required about 5 to 10 seconds to measure the a-wave latency, and, although the time to curve-fit the a-wave has not been reported, it certainly cannot be calculated within 5–10 seconds. Thus, in making measurements on a large number of patients, as was done for the data presented in the third paper of this series,[9] the ease and time required to make the measurements strongly favor measurement of the a-wave latency.

Factors Affecting the Timing of the ERG

The results showed that both the a-wave latency and IT decreased as the stimulus intensity increased. The curve was steep with a slope of about 8 ms/log unit, and thus, care must be taken in adjusting the animal's eye and the stimulus so that the same intensity is presented for each experiment.

The effect of stimulus intensity on the latency was also demonstrated by placing different levels of background light. As shown, the latency increased as the level of adapting light increased.

Summary and Conclusions

The results have demonstrated that the a-wave latency can be measured with very small inter-subject variation. The coefficients of variation for the a-wave latency were comparable to those for the IT measurements, and we thus conclude that measurement of the a-wave latency can be made as reliably as measurement of the IT. The potential value selected as the latency was 1 to 2 SD lower than the mean pre-stimulus baseline potential level. Because of the ease in making a-wave latency measurements and the reliability of the measurements, we recommend that the a-wave latency be used to measure the time course of the a-wave.

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