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The *a*-Wave of the Human Electroretinogram Recorded with a Minimally Invasive Technique

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A minimally invasive technique is described for recording the *a*-wave of the human ERG and extracting the parameters of transduction in the rod and cone photoreceptors. A corneal DTL fibre electrode is used, but the pupil is not dilated and the cornea is not anaesthetized. Although the amplitude of the signal collected by the DTL electrode varies from session to session, this is not a problem, as the photoreceptor fractional circulating current is obtained by normalization of the response family. A method is described for varying the effective flash intensity over a wide range, by controlling the duration of the xenon flash. In order to fit the kinetics of the responses, an analytical equation is derived for the convolution of the previous "delayed gaussian" expression with the cell's capacitive time constant. This equation provides a good description of both the rod and the cone response families. For rods, the capacitive time constant was found to be $\tau_{rod} \approx 1$ msec as reported previously, but for the cones a considerably longer time constant of $\tau_{cone} \approx 4$–5 msec was needed. For rods, the amplification constant ($A_{rod} \approx 5$ sec^{-2}) was close to previous estimates, but for cones the sensitivity (expressed in terms of corneal illuminance) was higher than in previous work. Calculation of the amplification constant of transduction within the cones requires knowledge of their light collection properties, and the absence of hard information makes this estimate somewhat speculative. However, when account is taken of the larger diameter of the inner segments of cones in the peripheral retina, then our estimated amplification constant for the cones ($A_{cone} \approx 3$–7 sec^{-2}) is of a similar order of magnitude to that obtained for the rods. © 1997 Elsevier Science Ltd

Electroretinogram *a*-Wave Phototransduction Photoreceptors Flash stimulus

INTRODUCTION

In recent years there has been a resurgence of interest in the *a*-wave of the electroretinogram (ERG), as a tool for determining the parameters of transduction in photoreceptors in the living human eye (Hood & Birch, 1994, 1995, 1996; Breton *et al.*, 1994; Jacobson *et al.*, 1994, 1995; Fulton & Hansen, 1996; Cideciyan & Jacobson, 1996), as well as in experimental animals (Chen *et al.*, 1995; Goto *et al.*, 1996; Robson & Frishman, 1996; Lyubarsky & Pugh, 1996). These approaches are based on two findings. Firstly, the *a*-wave has been shown to provide an accurate measure of the time course of the total circulating current in the massed photoreceptors of the eye (Hood & Birch, 1990, 1993a; Breton *et al.*, 1994). Secondly, molecular modelling of the transduction cascade has generated a relatively simple equation (the "delayed gaussian") that describes the complete family of responses to flashes of different intensity (Lamb & Pugh, 1992; Pugh & Lamb, 1993). Combining this knowledge, it is straightforward to design simple experiments that allow extraction of the "amplification constant" A of transduction in the rod and cone photoreceptors.

In the present work our aims have been two-fold. Firstly, we wished to develop an experimental technique that is as non-invasive as possible, and that can therefore readily be used on volunteers in the laboratory, without clinical supervision. Secondly, we wished to develop a means of fitting the experimental curves, taking account of the photoreceptor's membrane time constant in addition to its amplification constant.

A ganzfeld stimulator was constructed, based on a modified integrating sphere, and using a xenon flash unit that could provide flashes of variable duration. A CCD camera monitored the pupil diameter under IR illumination, so that the quantity of light entering the eye could be calculated. The *a*-wave was recorded with a DTL fibre electrode (Dawson *et al.*, 1979) placed on the cornea, without the use of any drugs. An equation was derived for the convolution of the simple delayed gaussian with an exponential time constant, and that expression is shown to provide a good fit to the experimental responses. The parameters of transduction in the rod and cone photo-

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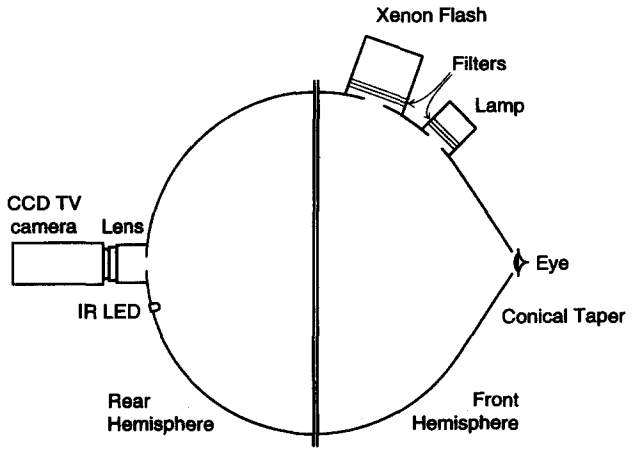

FIGURE 1. Schematic diagram of the ganzfeld, viewed from the side. The rear hemisphere was fixed, while the front one was hinged, to permit easy access to the interior. The front "hemisphere" was modified to a conical shape (angle approx. 135 deg), tapering to a small monocular viewing port. When the left eye was tested, the observer faced slightly to the right of the axis, and fixated a red LED at a location selected for viewing comfort. The flash gun was mounted behind a rectangular aperture above the viewing port, at an elevation of 70 deg, and was electrically shielded by a metallic cover to minimize pick-up of a transient. An incandescent lamp was also mounted above the viewing port, at an elevation of 45 deg, in order to provide a steady background. A CCD TV camera fitted with a 70 mm lens (Canon, 35–70 mm zoom) was located behind an aperture on the horizontal axis, diametrically opposite the viewing port.

receptors are extracted, and the significance of these values is discussed.

METHODS

Procedure and subjects

The corneal ERG was recorded, using a DTL fibre electrode (Dawson *et al.*, 1979) from six adult observers, in response to ganzfeld exposure of one eye to very brief flashes of light. Apart from slight myopia in two subjects, the observers had normal vision. No drugs were used: the pupil was not dilated, and the cornea was not anaesthetized. Ethical approval was obtained from the Cambridge Human Biology Ethics Committee. Informed consent was obtained from each subject, following detailed explanation of the procedures and risks.

Electrical recording

Electrical recordings were obtained using a Cambridge Research Systems optically-isolated pre-amplifier (ISO-1/D) and analogue board (AS-1), connected to an i486 computer running custom software. The DTL fibre electrode was placed loosely across the cornea, resting at the lower fornix, and was taped in place on both the nasal and temporal sides. The indifferent electrode was located on the temple on the same side, with the ground electrode on the forehead; both were disposable ECG electrodes. Signals were DC-coupled and low-pass filtered at 1 kHz (4-pole Bessel), and were sampled at 5 kHz with a resolution of 12 bits (approx. 1 μV at the input).

Ganzfeld

Ganzfeld illumination was delivered to one eye (Fig. 1) using an integrating sphere approximately 450 mm in diameter, painted white internally (Kodak, 6080 White Reflectance Coating). One of the aluminium hemispheres was modified, so as to taper conically to an aperture 35 mm in diameter, which served as a monocular viewing port. Diametrically opposite, an infra-red-sensitive CCD camera viewed the eye through a 10 mm aperture, and provided a means of measuring the pupil size and checking the position of the DTL fibre. With a lens of 70 mm focal length, the iris (approx. 11 mm) subtended a diameter approximately 30% of the height of the TV screen (Fig. 2). A fixation light was provided by one of several red LEDs, at a position selected for viewing comfort.

Illumination

The flash was supplied by a photographic flash gun (Mecablitz 60CT4, with N23 power pack; Metz) mounted behind a rectangular aperture, 50×80 mm, positioned above the observer at an elevation of about 70 deg. The light passed through a heat filter, a UV filter, a selectable colour filter, and a prismatic diffuser, before entering the sphere. Background light was provided by a 6 V, 20 W incandescent lamp run from a regulated DC supply. The colour filters for the flash and background were either "blue" (450 nm peak, Lee 195) or "red" (610 nm long-pass); when necessary, a neutral-density filter was also used. Infra-red illumination (usually at

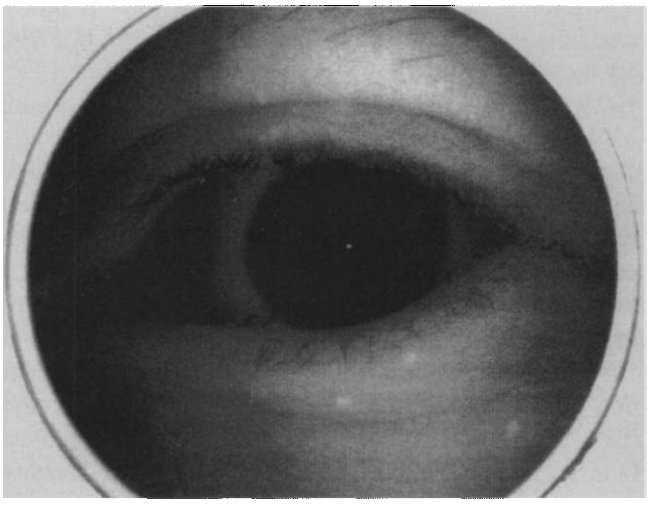

FIGURE 2. View of the eye during a recording session, digitized from a single frame of video. The diameter of the monocular port was approx. 35 mm, and its TV image slightly exceeded the vertical extent of the screen. For this subject, the diameter of the dark-adapted pupil was 6.5 mm. The DTL fibre electrode may be seen coiling loosely across the cornea at the lower fornix.

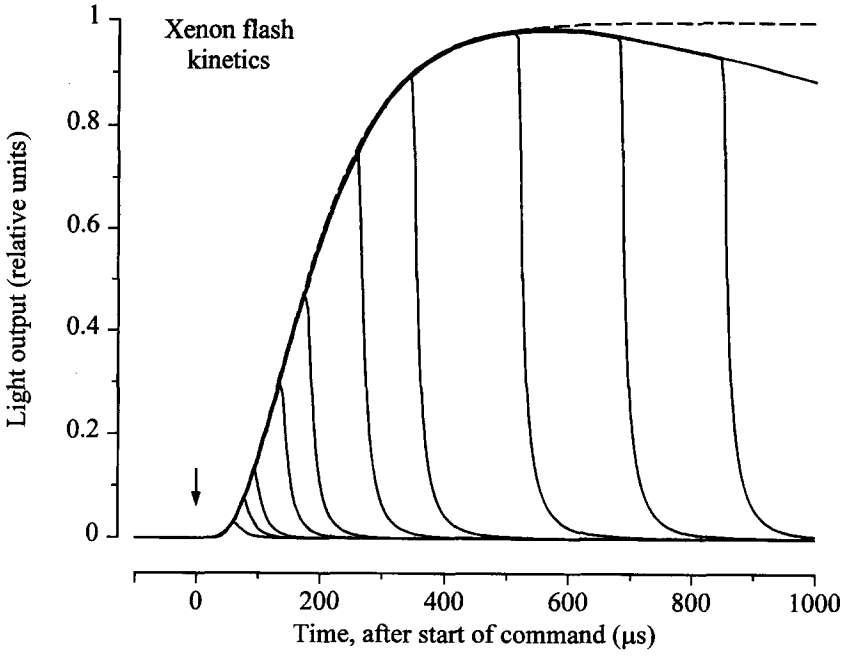

FIGURE 3. Time course of the flash output. Superimposed records of photodiode current, in relative units, recorded in response to command pulses ranging in duration from 50 μsec to greater than 1 msec. The S-shaped rising phase of light output at early times was well described by the broken line, given by the integrand of Eq. (1). That expression is the form expected if the arc current rises with a time constant τ_{rise}, and if the light output varies as the fourth power of the current.

930 nm, occasionally 880 nm) was provided by one of two GaAlAs LEDs positioned beneath the TV camera aperture. Intensities were measured with a calibrated photometer (IL1700, International Light) fitted with scotopic and photopic filters. The time course of the flash output was monitored by a reverse-biased photodiode.

The inter-flash interval was made long enough to allow full recovery of pupil diameter and response amplitude. It ranged from as short as 2 sec, for dim red flashes on a saturating background, to as long as 45 sec – 2 min following bright blue flashes (1000 cd m^{-2} sec).

Flash control and calibration

The effective intensity of the flash was varied by controlling its duration. The start of a command pulse from the computer triggered ignition of the flash, and the end of the same pulse triggered quench of the flash. Because of the presence of an inductor in the high-voltage supply, the arc current (and light output) built up gradually with time, as shown in Fig. 3. The broken curve gives the expected kinetics of light output; see below. Because of the S-shaped rise, the integrated light output was a non-linear function of flash duration, and could be controlled over a 10,000-fold range by varying the command pulse duration from about 20 to 400 μsec, as illustrated in Fig. 4. Although even higher light output could be achieved by increasing the duration to 1 msec or longer, the duration was restricted to a maximum of 400 μsec when recording the *a*-wave, since the rod's capacitive time constant is of the order of 1 msec (see Results). The curves in Fig. 4 were derived by integrating the rising phase fitted in Fig. 3, using the equation:

$$Q = \int_0^{\Delta t} I_{max}\left(1 - e^{-t/\tau_{rise}}\right)^4 dt \qquad (1)$$

where Q is the quantity of light delivered by the flash (i.e., the integrated flash intensity), and Δt is the flash duration. The term inside the integral in Eq. (1) represents the time course of the flash, as indicated by the dashed trace in Fig. 3. For the curves in Fig. 4, the time constant of the rise (τ_{rise}) was 72 μsec, while the maximum flash intensity (I_{max}) was 6×10^6 scotopic and 1.2×10^6 photopic cd m^{-2} for the blue and red flashes, respectively. Repeated measurements of individual flashes showed that the integrated output was reproducible to within ±1%.

Control of the flash duration has several advantages. Firstly, there is no need physically to change any neutral density filters during an intensity series. Secondly, a photopic match can be achieved at all intensities using a single pair of colour filters: a blue filter and a photopically matched red filter (i.e., a red filter with a suitable neutral filter). Thirdly, the flash duration will be briefer than in conventional experiments using neutral filters, since in those experiments the full flash duration is always used. Fourthly, it is possible to achieve a short inter-flash interval because the flash capacitor is only partly discharged. With the flash unit used here, an inter-flash interval as short as 250 msec was obtainable, even with the brightest flash used (1000 scotopic cd m^{-2} sec). Finally, there is less difficulty in shielding stray light

from the flash, because virtually no neutral filtering is used.

Separation of rod and cone signals

Rod and cone *a*-waves were separated using the approach of Hood & Birch (1993a) and Cideciyan & Jacobson (1996). Under fully dark-adapted conditions, signals of predominantly rod origin were obtained using the unattenuated blue filter (*B*), and the cone contribution was estimated using a photopically matched red filter (R_{Ph}). As proposed by Cideciyan & Jacobson (1996), the residual rod component elicited by the red filter was estimated using an attenuated blue filter, B_{Sc}, scotopically matched to the red filter. The dark-adapted rod-isolated signal was then obtained as $B-(R_{Ph}-B_{Sc})$. Cone-isolated *a*-waves were obtained by presenting red flashes during exposure to a steady blue background (48 scotopic cd m^{-2}) that saturated the rods. Cone isolation was confirmed by presenting blue flashes, and finding that the form of the family of responses was essentially the same.

Conversion of troland units to isomerizations

The retinal illuminance was determined as the product of the ganzfeld illuminance and the pupil area (in mm^2), and then the number of isomerizations per photoreceptor per flash was calculated using a conversion factor, *K*. For the rod system, this conversion is straightforward, and Breton *et al.* (1994) have estimated the factor to be K_{rod} = 8.6 photoisomerizations per rod, per scotopic td sec. For the cone system the conversion is more problematic, because of uncertainty about the effective collecting area of peripheral cones, and so, in accordance with previous work, our values are expressed first as a sensitivity, in terms of incident retinal illuminance. We consider the conversion factor further in the Discussion.

Curve fitting

The predicted form of the *a*-wave kinetics is developed subsequently, in Eq. (5) in the Results section. One methodological pitfall in the numerical evaluation of this equation is as follows. At low intensities the factor c^2 becomes very large, so that the term in [] in Eq. (5*a*) represents the difference between two numbers very close to unity. Although MAPLE (Waterloo Maple Software) will evaluate the equation accurately, a FORTRAN, MATLAB or similar program based on the form in Eq. (5) will fail under these conditions. An alternative approach is to evaluate the equation in this region using the expression for $e^{c^2}\mathrm{erf}(c)$ given in Section 7.1.6 of Abramowitz & Stegun (1964). For relatively high intensities (e.g. $c^2 < 25$), Eq. (5) may be evaluated using MATLAB (The Math Works, Inc.), with the following code:

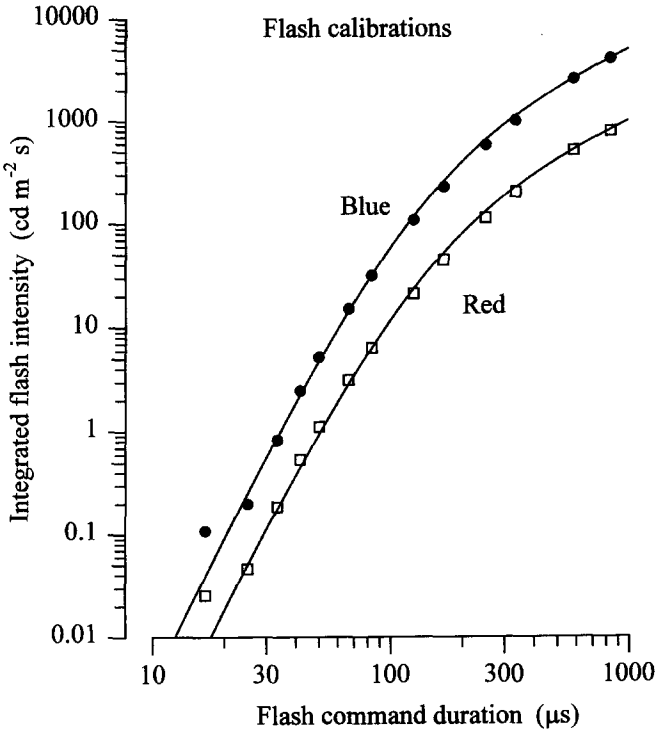

FIGURE 4. Flash intensity calibrations. Integrated flash intensity for flashes of different duration, with the standard blue and red filters. The measurements were obtained using the IL1700 photometer in its integrating mode, with the detector reverse-biased and with calibrated scotopic and photopic filters. The ordinate values are in scotopic units for the blue filter and in photopic units for the red filter. The curves plot Eq. (1) with τ_{rise} = 72 μsec for both curves, and with $I_{max} = 6\times10^6$ scotopic and 1.2×10^6 photopic cd m^{-2}, respectively. Using these values, a flash command duration of 333 μsec gave integrated intensities of about 1000 scotopic and 200 photopic cd m^{-2} sec at the viewing aperture. The photopically matched red filter used in the rod experiments was attenuated by about 0.3 log units from the full red filter illustrated.

where the variable names should be self-explanatory, and where t and F may be vectors. Copies of a complete MATLAB analysis program and example data are available on the World Wide Web at http://www.physiol.cam.ac.uk/staff/lamb/awave/.

RESULTS

Rod and cone response families

Families of rod-isolated and cone-isolated responses are illustrated as the solid traces in Fig. 5 and Fig. 6, respectively, for one observer (NPS). In each figure, six flash durations were used, and the traces plot averages determined from five to twenty responses. For the rod system, the conversion to photoisomerizations is unequivocal, and so the numbers near each trace give the

```
c2 = 1/(2*Phi*A*T^2); c = sqrt(c2);
F = (1+ sqrt(pi)*c*exp(c2) * (erf(c)-erf(c*(1-Phi*A*T*t)))).* exp(-t/T);
F = F.* (t>0) + (t<=0);
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photoisomerizations per rod, Φ. For the cone system, this conversion is less certain, and so the retinal illuminance (in photopic td sec) is given. The experimentally recorded response families have the characteristic form illustrated previously for rods by Hood & Birch (1990, 1994); Cideciyan & Jacobson (1993, 1996) and Breton *et al.* (1994), and for cones by Hood & Birch (1993b, 1995) and Cideciyan & Jacobson (1996). We conclude that our minimally invasive method is capable of resolving *a*-wave responses with quality comparable with that achieved in other studies employing contact lens electrodes, corneal anaesthesia, and dilation of the pupil.

Previous analysis of *a*-wave responses, using an ensemble of "delayed gaussian" theoretical curves, led to quite poor fits of the responses to extremely bright flashes. This occurred because all the short delay steps, including the membrane capacitive time constant, were lumped into a pure delay term in the delayed gaussian formulation. Hence, as the intensity is increased, the slope of the predicted rising phase of the electrical response increases indefinitely. To overcome this limitation, two recent papers have taken explicit account of the capacitive time constant, by numerical convolution (in cones: Hood & Birch, 1995; and in both rods and cones: Cideciyan & Jacobson, 1996). Here we describe an analytical approach that yields a manageable expression which fits the experimental responses up to very high intensities.

Predicted kinetics of the response

Lamb & Pugh (1992) analysed the molecular steps underlying phototransduction and derived a theoretical expression for the time course of the circulating current. In response to a brief flash of intensity Φ (isomerizations per photoreceptor) they predicted that the circulating current should decay as a gaussian function of time:

$$G(t) = \exp(-\Phi \tfrac{1}{2} A t^2), \qquad t \geqslant 0. \tag{2}$$

Here A is the amplification constant (in sec^{-2}), and Eq. (2) provides a single-parameter description of a whole family of responses at different intensities, Φ. For simplicity, a short delay time t_d has been omitted; thus, t in Eq. (2) represents $(t-t_d)$ in the earlier formulation.

That analysis did not take explicit account of the cell's capacitive time constant, τ, which was simply approximated as a pure delay and lumped into the total delay, t_d. However, with bright flashes this approximation is inappropriate, and the expression in Eq. (2) should instead be convolved with an exponential decay $E(t)$ representing the impulse response of a cell with a

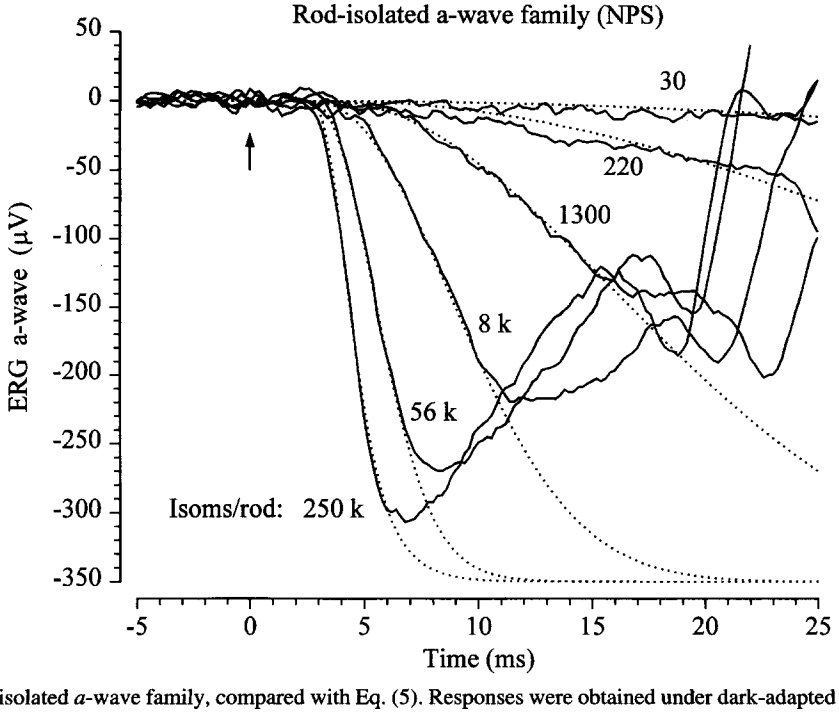

FIGURE 5. Rod-isolated *a*-wave family, compared with Eq. (5). Responses were obtained under dark-adapted conditions, using blue flashes, and with the double subtraction technique described in the Methods; pupil diameter 6.0 mm. Flash command durations were 16, 33, 50, 83, 166 and 333 μsec, and the integrated intensities of the blue flashes (shown in Fig. 4) were: 0.12, 0.89, 5.3, 33, 230 and 1030 scotopic cd m^{-2} sec; the numbers near each trace indicate the estimated numbers of photoisomerizations per rod (Φ). Each response was averaged from: 10 presentations for the full blue, 10 for the photopically matched red, and 10 for the scotopically matched blue, with the exception that only five presentations were used for the two most intense blue flashes. The inter-flash intervals ranged from 8 to 50 sec (blue), and from 5 to 20 sec (red). The curves plot the predictions of Eq. (5), using the measured intensities and pupil diameter, and with the following parameters: a maximal rod signal of $a_{rod} = -350\ \mu V$, a membrane time constant of $\tau_{rod} = 1.1$ msec, and a pure delay of $t_{d,\ rod} = 2.3$ msec. The fitted amplification constant for the ensemble was $A_{rod} = 4.5\ \text{sec}^{-2}$, with an intensity conversion factor of $K_{rod} = 8.6$ isomerizations per scotopic td sec. For the theory curves, the origin of time in Eq. (5) was set to the mid-point of the flash command pulse.

capacitive time constant τ:

$$E(t) = e^{-t/\tau}/\tau, \quad t \geqslant 0 \tag{3}$$

(Penn & Hagins, 1972; Hood & Birch, 1993a, 1995; Cideciyan & Jacobson, 1996).

To perform the appropriate convolution, it is necessary to take account of the initial conditions, i.e., for $t < 0$, $G(t) = 1$ in Eq. (2), and $E(t) = 0$ in Eq. (3). With those conditions the fractional circulating current $F(t)$ may be written as

$$F(t) = 1 - [1 - G(t)] * E(t) \tag{4}$$

where the * represents convolution. Evaluation of the convolution integral, using MAPLE (Waterloo Maple Software), gives $F(t)$ as:

$$F(t) = \left\{1 + \sqrt{\pi}ce^{c^2}[\mathrm{erf}(c) - (\mathrm{erf}(c[1 - \Phi A\tau t])]\right\}e^{-t/\tau}, \quad t \geqslant 0 \tag{5a}$$

where

$$c^2 = 1/(2\Phi A\tau^2). \tag{5b}$$

Eq. (5) thus describes the family of normalized circulating current responses using two main parameters: the amplification constant A and the capacitive time constant τ. For low intensities (small Φ), Eq. (5) is found to be indistinguishable from Eq. (2), with an added delay of τ, while for very high intensities Eq. (5) approaches an exponential decay, $e^{-t/\tau}$. Two further parameters are also needed: the maximal response amplitude, a_{max}, which is simply a scaling constant, and the residual delay, t_d, which comprises several short delays within the transduction cascade (Lamb & Pugh, 1992), as well as any electrical filtering delay.

Fit of the predicted kinetics, and parameters obtained

The fit of Eq. (5) to the experimental results is shown by the dotted traces in Fig. 5 and Fig. 6. In each figure, the family of responses was obtained using a single set of parameters and substitution of the measured flash intensities (ensemble fitting). Clearly, the theoretical traces provide a reasonable description of the rising phase of the experimental responses. The deviation between theory and experiment that occurs at later times arises from intrusion by the *b*-wave, which is generated by cells proximal to the photoreceptors. We tested six observers, and in each case the quality of fit of the rod-isolated responses was similar to that in Fig. 5. Four of these subjects were also tested under cone-isolating conditions, and in each case the quality of fit was similar to that in Fig. 6. Since the main aim of this work was to test the applicability of the minimally invasive approach, we did not attempt to examine a larger group of subjects. The values of the parameters in Eq. (5) extracted from fitting the *a*-wave responses of these observers will now be considered separately for rods and cones, and compared with other values in the literature (Table 1).

Rods. For the rod-isolated family in Fig. 5, the fitted amplification constant was $A = 4.5$ sec^{-2}, and the mean for our observers was 4.8 ± 1.7 sec^{-2} (SD, $n = 6$). Even at the highest intensity of 250,000 photoisomerizations per rod, the theoretical trace provides a reasonable description of the early rise. This fit is comparable to that obtained recently by Cideciyan & Jacobson (1996), with allowance for the membrane capacitive time constant, but is in contrast to the poorer fit at early times obtained previously when the capacitive time constant was ignored (e.g., Breton *et al.*, 1994). In order to obtain a good fit to the bright flash responses in Fig. 5, it was necessary to adopt a rod time constant of $\tau_{rod} = 1.1$ msec. For the six subjects, the mean value fitted was $\tau_{rod} = 1.2 \pm 0.2$ msec (SD, $n = 6$); see Table 1.

Cones. For cone-isolated responses to red flashes, the contribution of the blue-sensitive cones can safely be ignored, and only the contributions of the green- and red-sensitive cones need be considered. In fitting our recordings, we began with the assumption (Hood & Birch, 1995; Cideciyan & Jacobson, 1996) that the red cones dominate the response, so that the green cones may be ignored. However, we found, consistently across our four observers, that the families of responses could be fitted better by assuming roughly comparable contributions to the saturating response from the two cone classes; i.e., roughly similar saturating levels, of $a_{green} \approx a_{red}$. Although the fit was improved by using a non-zero value of a_{green}, the quality of fitting was not especially sensitive to the ratio a_{green}/a_{red}, and so we chose $a_{green}/a_{red} = 1$.

Inclusion of a contribution from green cones has two effects: it causes a change in the spacing between the theory traces (i.e., a change in the shape of the response–intensity relation), and it necessitates an increase in the fitted value of the amplification constant, A_{cone}. Thus, if the red cones are assumed to contribute only half the total of the cone response to intense flashes (and because the green cones absorb considerably less of the red light) then, in order to fit the dim-flash responses, it turns out to be necessary to use a cone amplification constant two to three times as large as when only a single class is considered.

We have assumed that the other parameters of transduction (A_{cone}, τ_{cone} and $t_{d,\ cone}$) are identical for the two classes of cone. The factors converting incident intensity to numbers of photons will be denoted K_{red} and K_{green} (in photoisomerizations per cone per photopic td sec). The ratio of these parameters is determined by the spectral sensitivity of the cones, in conjunction with the spectral composition of the stimulus. For the red filter used in these experiments, with 50% transmission at 610 nm, integration of the spectral templates for the human red and green mechanisms (Lamb, 1995) gives a ratio of $K_{green}/K_{red} \approx 1/6$, comparable to the ratio of approx. 1/7 determined by Hood & Birch (1995) with their red filter.

In the absence of a contribution from green cones, the best fit to the data in Fig. 6 was obtained with K_{red} $A_{cone} = 580$ sec^{-2} per td sec. However, an improved fit (illustrated in Fig. 6) was obtained using equal green and red cone contributions ($a_{green} = a_{red}$), for which the fitted amplification constant increased to K_{red} A_{cone}

TABLE 1. Transduction parameters determined from the *a*-waves of normal subjects

Rods		$-a_{rod}$ (μV)	A_{rod} (sec^{-2})	τ_{rod} (msec)	$t_{d,\ rod}$ (msec)	*n*
Breton *et al.* (1994)		463 ± 81	6.9 ± 1.1	0	2.6 ± 0.7	6
Hood & Birch (1994)		195 ± 39	4.0 ± 2.0	0	2.6 ± 0.5	15
Cideciyan & Jacobson (1996)		456 ± 54	3.9 ± 1.6	0.5	(2.6)	14
This study		292 ± 55	4.8 ± 1.7	1.2 ± 0.2	2.3 ± 0.2	6
Cones		$-a_{cone}$ (μV)	$K_{red}\ A_{cone}$ (sec^{-2} per td sec)	τ_{cone} (msec)	$t_{d,\ cone}$ (msec)	*n*
Hood & Birch (1995)		42 ± 11	178 ± 46	1.8	1.7	6
Cideciyan & Jacobson (1996)		83 ± 8	309 ± 117	2.0	(2.0)	16
	$a_{green} = 0$		606 ± 364			
This study		91 ± 41		4.4 ± 0.9	1.4 ± 0.2	4
	$a_{green} = a_{red}$		1440 ± 820			

Values given as "±" are mean ± SD, with *n* given in the final column. For the rods, values of A_{rod} were obtained from the previous studies using K_{rod} = 8.6 photoisomerizations per rod per scotopic td sec. For the cones, values of $K_{red}\ A_{cone}$ were obtained from the previous studies as 2*S* in the terminology of Hood & Birch (1995), and as σ_{ph} in the terminology of Cideciyan & Jacobson (1996). For these cone sensitivities, the corrections for Stiles–Crawford effect made by the previous authors (0.2 log units) have been moved to the calculation of retinal illuminance, in order to make their results with dilated pupils comparable with those presented here with small aperture natural pupils, for which no correction is necessary; see text. In the column for delay ($t_{d,\ rod}$, $t_{d,\ cone}$), values from Cideciyan & Jacobson (1996) given in parentheses are approximate values, since they used a delay plus a series of short time constants, rather than a pure delay.

= 1340 sec^{-2} per td sec. In both cases we used a total cone *a*-wave amplitude ($a_{cone} = a_{red} + a_{green}$) of −110 μV, a time constant of τ_{cone} = 4.4 msec and a delay of $t_{d,\ cone}$ = 1.5 msec. For the four observers tested under cone-isolating conditions, the mean sensitivity obtained was $K_{red}\ A_{cone}$ = 606 ± 364 sec^{-2} per td sec (no green cone contribution) or 1440 ± 820 sec^{-2} per td sec (equal green and red cone contributions); mean ± SD, *n* = 4.

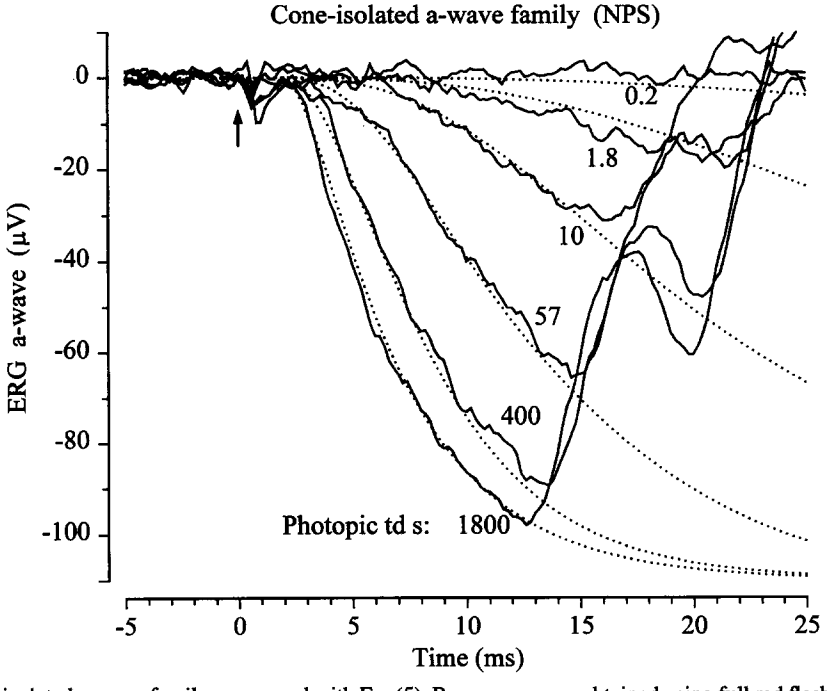

FIGURE 6. Cone-isolated *a*-wave family, compared with Eq. (5). Responses were obtained using full red flashes in the presence of a steady blue background (48 scotopic cd m^{-2}) that saturated the rods; pupil diameter 3.3 mm. Flash durations were as listed in Fig. 5, and the integrated flash intensities (in photopic cd m^{-2} sec) were 0.024, 0.18, 1.1, 6.6, 46 and 206 scotopic cd m^{-2} sec; the numbers near each trace indicate the retinal illuminance in photopic td sec. Each response is averaged from 20 presentations, and the inter-flash intervals ranged from 2 to 20 sec. The curves plot the predictions of Eq. (5), using the measured intensities and pupil diameter, and identical red- and green-cone parameters, of τ_{cone} = 4.4 msec, $t_{d,\ cone}$ = 1.5 msec, and maximal green- and red-cone signals of $a_{green} = a_{red} = -55$ μV. The ratio of conversion factors used for the green- and red-cones was K_{green}/K_{red} = 1/6 for the red light. The fitted sensitivity for the ensemble was $K_{red}\ A_{cone}$ = 1340 sec^{-2} per td sec.

The mean value of cone membrane time constant was $\tau_{cone} = 4.4 \pm 0.9$ msec, considerably larger than the values of 1.8 and 2 msec reported recently by Hood & Birch (1995) and Cideciyan & Jacobson (1996). We were not able to obtain an adequate fit to our data with a time constant as short as 2 msec.

DISCUSSION

A minimally invasive and simple procedure

The procedure described here is simple and minimally invasive, in that no drugs are used, and the DTL fibre electrode is comfortable and causes negligible abrasion of the cornea. Nevertheless, the results recorded are of comparable quality to those obtained using a contact lens electrode with corneal anaesthesia and dilation of the pupil. The method is therefore especially appropriate in a research laboratory, where clinical supervision may not be readily available.

Two potential shortcomings should be mentioned. Firstly, the maximum amplitude of the *a*-wave, a_{max}, was found to vary from session to session, presumably as a result of variability in the position or extent of contact between the DTL electrode and the cornea in different sessions. However, this variation was relatively unimportant, since what is needed in the fitting is the normalized response, $F(t) = a(t)/a_{max}$, and in practice a_{max} was found to be quite stable within a given recording session. Secondly, in the absence of pupil dilation, care must be taken to measure the size of the pupil, to ensure that it has returned to normal before a subsequent flash is delivered. Following intense flashes (1000 scotopic cd m^{-2} sec) it was necessary to wait for 45 sec – 2 min to obtain complete recovery of pupil diameter.

With a natural pupil, some experiments (such as those requiring double flashes, e.g. Lyubarsky & Pugh, 1996; Pepperberg *et al.*, 1996) would be complicated, though not impossible. Using a computerized video frame-grabber, together with an algorithm for rapidly estimating the pupil diameter (Daugman, 1993), it would, in principle, be possible to determine the pupil size immediately prior to delivery of the flash, and to look-up the flash duration needed to deliver the desired quantity of light.

Rod parameters

In dark-adapted rods we obtained a mean amplification constant of $A_{rod} = 4.8$ sec^{-2}, within the range of 3.9–6.9 sec^{-2} obtained in previous studies (Table 1). For the saturating rod response amplitude, we obtained a mean of $a_{rod} = -292$ μV. However, the precise value obtained here depends somewhat on the way the fitting is performed, and there is some leeway in the amount by which the saturating level a_{rod} can be set to exceed the largest response. In general, we found that satisfactory fits could be obtained by setting a_{rod} about 5–15% higher than the peak of the response obtained with a flash of 1000 scotopic cd m^{-2} sec.

The mean value that we found for the capacitive time constant, of $\tau_{rod} = 1.2$ msec, is very similar to the value of 1 msec reported by Penn & Hagins (1972) in rat rods, but is somewhat larger than the value of 0.5 msec obtained by Cideciyan & Jacobson (1996). Part of the reason for this difference may relate to their assumption of the existence of three additional time constants (τ_{sc}), each of 0.85 msec in rods; it is possible that one of those time constants instead represented the membrane capacitive constant.

Cone parameters

Our fitted cone parameters differ from those of the other two studies, in showing a higher sensitivity and a longer time constant. The sensitivity that we obtain, of $K_{red} A_{cone} = 606$ sec^{-2} per td sec (when the green cones are ignored), is approximately double the value of 309 sec^{-2} per td sec obtained from Cideciyan & Jacobson (1996), and is more than three times the value of 178 sec^{-2} per td sec obtained from Hood & Birch (1995). We have referenced both these latter values to illumination through a small pupil, by incorporating the authors' correction for the Stiles–Crawford effect of the dilated pupil into the calculation of retinal illuminance; see notes to Table 1. This correction is needed, but in the present context it is more appropriate to apply it to the calculation of illuminance, than to just the final estimate of photoisomerizations.

A possible reason for part of the difference in sensitivity is our use of a blue background, in contrast to the use of a white background in the other two studies. Our background delivered a retinal illuminance of approximately 420 scotopic trolands (and 42 photopic trolands), which was enough to saturate the rods, yet which is likely to have caused negligible desensitization of the cones. In the previous studies the white background delivered about 800 (Hood & Birch, 1995) or 1500 (Cideciyan & Jacobson, 1996) photopic trolands, and is likely to have caused desensitization of the cones of 0.1–0.2 log units; i.e. a factor of 1.25- to 1.6-fold (Hood & Birch, 1993a).

It is possible that a similar explanation may, in part, underlie the longer cone time constant that we find, of approx. 4.4 msec compared with previous values of 1.8 and 2.0 msec. In turtle cones it has been reported (Baylor *et al.*, 1974, p. 717) that hyperpolarization of the cell activates a conductance in the inner segment, and it seems possible that a similar phenomenon occurs in human cones during light adaptation. Since a cell's membrane time constant is the product of its capacitance and its resistance ($\tau = RC$), the existence of such a "shunt" conductance in the light-adapted state would decrease the time constant measured in response to a bright flash. Hence the larger time constant observed in our study may be representative of dark-adapted cones, whereas the smaller value in the other studies may be indicative of light adaptation.

The four-fold larger capacitive time constant of the cones (approx. 4.4 msec) compared with the rods (approx. 1.2 msec) may simply reflect the different topology of the outer segment membrane. In a cone

most (if not all) of the outer segment membrane is patent to the extracellular space (e.g. Steinberg *et al.*, 1980), rather than being closed-off in the form of discs, and so the cell's capacitance is larger than for a rod. During a bright flash, when the outer segment channels close and the outer segment conductance declines to zero, the cell's overall resistance will be determined by the inner segment (and not by the area of outer segment membrane). In this case, one would expect the membrane time constant to be roughly proportional to the surface area of outer segment plasma membrane. Hence, our observation of a larger time constant in the cones seems broadly consistent with the known anatomy.

Cone amplification constant

In order to determine the amplification constant A_{cone} of the cones, it is necessary to establish the value of the parameter K_{red} that characterizes light collection by the red-sensitive cones. If the cone sensitivity $K_{red}\,A_{cone}$ is not spatially homogeneous across the retina then, as argued by Hood *et al.* (1993), the experimentally measured sensitivity is likely to be set by the most sensitive cones, provided that that pool of cells contributes at least 20% of the total cone *a*-wave.

From the assumptions of Schnapf *et al.* (1990) for the dimensions and light-funnelling properties of *foveal* cones, Hood & Birch (1995) have estimated that $K_{red} = 20$ isomerizations per red-sensitive cone, per photopic td sec, for red light. However, the important factor that is not known with any degree of certainty is the extent of light-funnelling in cones in the *peripheral* retina. The inner segment diameter of cones in the mid-periphery is very large, with values of 6.0–7.5 μm reported by Polyak (1941) at an eccentricity of 30–50 deg. Even after allowance for the shorter length of outer segments at these eccentricities (approx. 15 μm, compared with up to 50 μm in the fovea), the effective collecting area for peripheral cones is likely to be a factor of approximately 10 times greater than for foveal cones. Hence we suggest that, for red-sensitive cones in the mid-periphery stimulated by red light, the appropriate conversion factor would be roughly $K_{red} = 200$ isomerizations per red-sensitive cone, per photopic td sec. However, we stress that considerable uncertainty surrounds the estimation of this value.

Further uncertainty surrounds the appropriate experimental value to adopt for the cone sensitivity $K_{red}\,A_{cone}$. On the assumption that the red-sensitive cones dominate the response (i.e., that $a_{green} = 0$), we found that our responses were best fitted with $K_{red}\,A_{cone} \approx 600$ sec^{-2} per td sec. However, it seems very unlikely that this assumption can be valid, since with very bright flashes the green-sensitive cones will also be stimulated, so that the saturating cone response will necessarily be the sum of $a_{green} + a_{red}$. Therefore, as the next step, we made the assumption that the green- and red-sensitive cones contribute equally to the saturating response, and we then found that the best fit to the cone families was obtained with a sensitivity of about 2.4-times greater, or $K_{red}\,A_{cone} \approx 1400$ sec^{-2} per td sec (see Table 1). Clearly, the assumption of negligible green cone contribution ($a_{green} = 0$) represents a lower limit, and it seems likely that the assumption of equal green and red cone contributions ($a_{green} = a_{red}$) represents an upper limit. Hence from the respective estimates for $K_{red}\,A_{cone}$, together with the approximate value for K_{red} in the previous paragraph, we obtain the amplification constant of the cones as $A_{cone} \approx 3$–7 sec^{-2}. Although there is considerable uncertainty in this estimate, it is interesting that the magnitude is similar to the rod amplification constant, as was suggested might be the case by Pugh & Lamb (1993).

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