Human cone photoreceptor responses measured by the electroretinogram *a*-wave during and after exposure to intense illumination

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1. We recorded the *a*-wave of the electroretinogram from human subjects with normal vision, using a corneal fibre electrode and ganzfeld stimulation under photopic conditions, so as to extract the parameters of cone phototransduction. The amplitude of bright flash responses provided a measure of the massed circulating current of the cones, while the amplitude of dim flash responses provided a measure of the product of the fraction of cone photopigment present, and the amplification constant of transduction within the cones.

2. In the presence of steady background illumination, the cone circulating current declined to half at 3000 photopic trolands, and to a quarter at 20 000 photopic trolands.

3. At very early times after the delivery of a near-total bleach, we could not determine the level of circulating current as our bright flashes did not appear to saturate the *a*-wave (presumably because so little pigment was present). However, by 20–30 s after a total bleach, the cone circulating current had returned to its dark-adapted level.

4. Following smaller bleaches (when *ca* 50 % of the pigment remained present) the bright flashes were able to saturate the *a*-wave even at very early times. Within 3 s of extinction of the illumination, the cone circulating current had returned to its dark-adapted level.

5. This is at least a factor of 300 times faster than the period of *ca* 15 min required for full recovery of rods exposed to the same level of bleach, and indicates a major difference between rods and cones in the way that they cope with the photoproducts of bleaching.

6. Despite the very rapid recovery of circulating current after bleaches, the recovery of dim-flash sensitivity was much slower, with a time constant of *ca* 1·5 min after a near-total bleach. This time course is very similar to previous measurements of the regeneration of cone photopigment, and it seems highly probable that the reduction in dim-flash sensitivity results from pigment depletion.

The sensitivity of the overall human visual system during light adaptation and dark adaptation has been studied exhaustively over many decades, and the electroretinogram (ERG) has long been used as a method for extracting information about photoreceptor responses. In the past few years there has been a resurgence of interest in the ERG, because of the development of a molecular model of phototransduction (Lamb & Pugh, 1992), which permits ready interpretation of the *a*-wave of the ERG in terms of photoreceptor responses (Breton *et al.* 1994). The application of the ERG to the study of photoreceptor responses has been reviewed by Hood & Birch (1996), and its application to the study of the responses of subsequent retinal cells has been reviewed by Robson & Frishman (1999). Human cone responses have recently been investigated using techniques of this kind by Hood & Birch (1993, 1995), Cideciyan & Jacobson (1996), Hood *et al.* (1996), Smith & Lamb (1997) and Cideciyan *et al.* (1998).

For human rod photoreceptors, Thomas & Lamb (1999) monitored light adaptation and dark adaptation *in vivo*, by measuring the *a*-wave of the ERG under rod-isolating conditions. From bright flash responses they obtained a measure of the massed circulating current of the rods, while from comparison of responses to dim and bright test flashes they extracted the apparent amplification constant of transduction within the rods. Adaptation to steady background lights suppressed the rod circulating current according to a Michaelis (or Naka-Rushton) relation, with half-suppression occurring at a steady intensity of around

70 scotopic trolands (Td), yet the amplification constant of transduction was found to be virtually unaffected. Following an almost total bleach of rhodopsin, the circulating current was completely suppressed for the first 5 min, with half-recovery taking around 15 min and full recovery around 30 min. During this period the amplification constant *appeared* to be greatly reduced initially, and to recover with a time constant of about 7 min. However, further analysis indicated that the true amplification constant did *not* in fact change, and that the apparent decrease resulted from the reduced quantal catch caused by pigment bleaching.

Our aim in the present study has been to extend that investigation to human cones, so as to provide a description of the suppression of circulating current by steady illumination, and to measure the kinetics of recovery of circulating current and responsiveness following bleaching exposures. Our principal finding is that, following extinction of a steady light that bleaches around 50 % of the photopigment, the recovery of circulating current in human cones *in vivo* occurs at least 300 times faster than found previously in rods. A preliminary report of some of our results has been presented (Mahroo *et al.* 1999).

METHODS

ERG recording, illumination and light calibration

The methods used for recording the ERG, for presenting stimuli, and for calculating bleaching levels, were very similar to those described by Smith & Lamb (1997) and Thomas & Lamb (1999). In brief, a conductive thread electrode (DTL, UniMed Electrode Supplies, Farnham, Surrey), placed in the lower fornix, was used to record the corneal ERG from four adult male subjects (the authors) with normal vision apart from minor errors of refraction. Light stimuli were delivered in a ganzfeld apparatus, and were viewed by the subject through a small monocular port. In experiments where pupil dilation was required, two drops of 1 % tropicamide were applied. Ethical approval was obtained from the Cambridge Human Biology Research Ethics Committee, and informed written consent was obtained from each subject following detailed explanation of the procedures and risks.

The test stimuli and adapting lights were designed to probe the cone system; thus the test flashes from the xenon flash gun were red, and were presented in the presence of a dim blue background that almost saturated the rods while minimally stimulating the cones. Bleaching exposures came from an incandescent source, and were either yellow (when delivered in a mini-ganzfeld, Thomas & Lamb, 1999) or white (in the main ganzfeld). All light stimuli passed through a heat filter, short-wavelength filter, and prismatic diffuser. The coloured filters were: 'red' = 610 nm long-pass, and 'yellow' = 475 nm long-pass (GG475, Schott, Mainz, Germany).

Light calibrations were performed using an IL-1700 photometer (International Light, Newburyport, MA, USA) with photopic (Y) and scotopic (revised Z-CIE) filters. Throughout this paper light measurements will be given in photopic units unless otherwise specified; i.e. corneal luminances will be in photopic cd m^{-2}, and retinal illuminances in photopic trolands (Td), where x Td is the retinal illuminance produced by a corneal luminance of 1 cd m^{-2} viewed through a pupil area of x mm^2.

Estimation of pigment bleaching levels

The equation for the fraction B of pigment bleached, at the end of an exposure of duration t, at a retinal illuminance I, has been given by eqn (2a) of Hollins & Alpern (1973), or eqn (4) of Thomas & Lamb (1999), as:

$$B = \frac{I}{I+I_{\mathrm{P}}}\left(1-\exp\left(-\left(1+\frac{I}{I_{\mathrm{P}}}\right)\frac{t}{\tau_{\mathrm{P}}}\right)\right), \quad (1)$$

where I_{P} is the steady retinal illuminance that bleaches half the pigment, and τ_{P} is the time constant of pigment regeneration. For cones, Hollins & Alpern (1973) measured $I_{\mathrm{P}} = 30\,000$ Td, and $\tau_{\mathrm{P}} = 105$ s, following a total bleach. In earlier work, Rushton & Henry (1968) reported $I_{\mathrm{P}} = 20\,000$ Td, while more recently Coile & Baker (1992, Fig. 3) have reported $\tau_{\mathrm{P}} \approx 90$ s at age 30 years (see later).

Equation (1) expresses the well-known feature that the steady level of bleaching is reached more rapidly as the intensity increases. Although the time constant of equilibration is near τ_{P} (*ca* 100 s) for very dim exposures, it should shorten to $1/(n+1)$ of this value when the intensity rises to n times I_{P}. Hence, for high intensities (as in the mini-ganzfeld) or long exposures, it is adequate to use the steady-state form:

$$B = I/(I+I_{\mathrm{P}}). \quad (2)$$

Two difficulties with these calculations should be mentioned. Firstly, the use of a single (photopic) light measurement cannot correctly calculate the bleach for both the red-sensitive and green-sensitive (L- and M-) cones, although the approach should be reasonably accurate in the case of white or yellow light, as used here for bleaching. Secondly, when the pupil is dilated, calculations employing raw troland values are bound to overestimate the absorption of light by the cones, because of the Stiles & Crawford (1933) directional effect. Thus much of the light that enters the pupil near its rim will be ineffective in exciting (or bleaching) the cones, yet will be included in the troland calculation.

The measurements of Stiles (1939) and Nordby & Sharpe (1988) have shown that the cone directional sensitivity follows a Gaussian profile, given by the expression $S = 10^{-pr^2}$, where S is the sensitivity for a ray entering the pupil at radius r (mm) relative to that for a ray incident centrally, and p (mm^{-2}) is the index of directional sensitivity. Integration of this expression out to very large radii gives a limiting value for the effective pupil area of $\pi/(p \ln 10)$. Substitution of Stiles' (1939) measured value of $p = 0{\cdot}064$ mm^{-2} for the cones gives an effective pupil area of 21 mm^2, in reasonable agreement with the value of 24·5 mm^2 estimated by Le Grand (1968; Table 14). Although variation in directional sensitivity has been reported at different retinal locations (e.g. Burns *et al.* 1997), this has mainly been studied in the central retina, and we are not aware of any reason to doubt the validity of Stiles' value as being applicable to our ERG measurements. Hence, when the pupil is dilated, the effective retinal illuminance of the cone system may be calculated by taking the pupil area to be about 20 mm^2, and we shall specify the retinal illuminance calculated this way in units of 'effective trolands'. On the other hand, for a constricted pupil (e.g. a natural pupil in a bright background) or for the rods, the correction is so small that it is appropriate to use the true pupil area.

Ganzfeld delivery of steady illumination, and pupil dilation

During the course of this study we used two configurations for background delivery and pupil dilation: the illumination was delivered either in a 'mini-ganzfeld' or in the main ganzfeld, and the pupil was either dilated or natural, as follows.

(1) Mini-ganzfeld, with dilated pupil. In one set of experiments (Figs 3 and 4) we delivered bleaching exposures using the same mini-ganzfeld as Thomas & Lamb (1999), and a dilated pupil. This had the advantage of providing an almost total bleach (corneal luminance *ca* $1{\cdot}6 \times 10^5$ cd m^{-2}; effective photopic retinal illuminance *ca* 3×10^6 Td), but had several disadvantages. Firstly, since this intensity of illumination through the dilated pupil was mildly uncomfortable, the subject tended to close his eye involuntarily, and unfortunately the operator could not gauge the extent to which this had occurred. If eyelid closure did occur, then one of its major effects is likely to have been to shield the peripheral retina, and thereby cause a spatially non-uniform bleach. Secondly, there was a problem that related to the large size of the bleach (rather than to the method *per se*): at the very earliest times after the bleach, when very little pigment was present, our brightest flashes were no longer sufficiently bright to saturate the response (see below). Thirdly, at the end of the bleaching exposure the subject was required to move from the mini-ganzfeld to the main ganzfeld before recording could resume, and there was inevitably a short delay (*ca* 5 s) in repositioning the subject's eye accurately. Although this delay had been of little consequence in the earlier rod study, it represented a potentially significant 'dead time' in studying the faster recovery of the cones.

(2) Main ganzfeld, with natural pupil. In order both to enable the state of the eyelids to be observed, and to permit repeated and reproducible timing of flash delivery at the extinction of the background, we presented the bleaching exposures using the main ganzfeld in another set of experiments (Figs 5 and 6). However, the maximum intensity available in the main ganzfeld was significantly lower than in the mini-ganzfeld (*ca* 6500 photopic cd m^{-2}), so that a total bleach could not be achieved. In preliminary trials with the pupil dilated, we found that the subject experienced a marked sensation of glare during bright exposures, which made it difficult for him to keep his eyelids fully open, and furthermore that during the exposures the ERG was accompanied by a high level of noise (>20 μV peak-to-peak), which presumably originated from involuntary activity of the eyelid muscles.

We therefore used the natural pupil, and found that the sensation of glare was much less pronounced and that the level of electrical noise during light exposures was dramatically reduced; this was the only way that we were successfully able to record ERG signals during bright backgrounds. A further advantage of using a natural pupil was that we could be more confident of the light absorption in the cones, because the correction for the Stiles-Crawford directional effect was negligible. However, the two disadvantages were, firstly, that we needed to continuously measure the pupil diameter (via the TV camera and monitor) and, secondly, that the maximum level of bleaching was not very high. With the maximum intensity (of 6200–6900 cd m^{-2} on different days), the diameter of the natural pupil was typically 2 mm, giving a retinal illuminance of around 20 000 Td, which appeared sufficient to bleach ~50% of the pigment.

Trace rejection

In attempting to specify which traces to reject from subsequent analysis, we experienced difficulty in dealing with those responses that appeared to contain 'noise' due to muscle activity. Thus it was not easy to determine whether a deflection represented signal or noise, and in particular we were not able to devise criteria for automated rejection. On the other hand, it was usually very obvious when a blink occurred, because of the occurrence of a large and abrupt deflection. The criteria that we set for manual rejection were as follows. Individual traces were rejected if, within a window from −10 to +15 ms relative to the flash, any of the following were present: (a) 'flats' on the trace, caused by reaching the limits of the recording range, (b) a blink artefact or other sudden large transient, (c) noise of greater than 20 μV peak-to-peak (presumably due to muscle activity or mains pick-up), or (d) continuous drift exceeding a slope of 20 μV in 25 ms (which typically occurred following a blink). Finally, we set our criteria marginally tighter in the period of 5 ms immediately preceding the flash, since this interval was used as the reference (or zero) level. In good experiments the proportion of traces rejected on these criteria did not exceed 10%, but in some experiments the rejection rate reached 20%.

RESULTS

Dark-adapted family of cone-isolated responses

Figure 1 shows a family of cone-isolated responses, obtained for one subject at a range of flash intensities. This family closely resembles those obtained in previous studies (Hood & Birch, 1993, 1995; Smith & Lamb, 1997), and comparable results were obtained from the other subjects. The responses have been fitted, as an ensemble, using the theoretical model for cone activation given by Smith & Lamb (1997), using the parameters listed in the legend to Fig. 1; that equation embodies the molecular description given previously by Lamb & Pugh (1992), modified to allow for the cells' membrane capacitive time constant. Furthermore, allowance is also made for the existence of two classes of cone (red- and green-, or L- and M-cones), with similar properties apart from their spectral sensitivities. However, as we discuss below, it is likely that the 'cone *a*-wave' illustrated in Fig. 1 includes some component of post-receptoral origin, and we therefore interpret the parameters of the fit with caution.

In the experiment of Fig. 1, we deliberately kept the background as dim as possible, in order to minimize adaptation of the cones. On its own, a blue background of this intensity (280 scotopic Td) would not have been quite sufficient to saturate the rods, but the experiment was performed before the rods had fully dark-adapted after previous illumination. Since the illustrated responses exhibit no sign of the late rise that is characteristic of a component of rod intrusion, we conclude that they originate in the cone system, though we cannot exclude some component of post-receptoral cone system activity.

Responses during steady illumination

The effect of a range of steady adapting intensities was studied in three subjects. The steady level of circulating current remaining was examined by delivering intense probe flashes, beginning at least 15 s after the background had been turned on. In an attempt to ensure that these probe flashes really were saturating, even in the presence of the brightest background, we made them as bright as possible (800 μs duration, delivering 550 cd m^{-2} s), corresponding to the brightest flash in Fig. 1. The retinal illuminance of the intense flashes varied with background intensity (because

of the induced change in pupil diameter) from 4200 Td s with the dimmest background down to 1400 Td s with the brightest. However, as may be seen by inspection of Fig. 1, a reduction in flash intensity by about this factor, from the brightest to the second brightest level, caused negligible change in response amplitude under dark-adapted conditions.

Figure 2*A* illustrates averaged responses to the intense flashes, obtained from one subject, in the presence of steady illumination ranging from a dim blue background almost sufficient to saturate the rods (the two largest responses) up to the most intense white background that we could deliver in the main ganzfeld (6700 cd m^{-2}, smallest response). Although the responses obtained in the presence of the white backgrounds exhibit a reasonably flat plateau after about 6 ms, the two responses obtained on the dim blue background (obtained before and after the other traces) instead continued to rise beyond 6 ms.

This behaviour strongly suggests the intrusion either of rod signals, or of post-receptoral signals from the cone system, under dim adaptational levels. In an attempt to minimize such intrusion, we chose to measure the responses at a relatively early time of 6 ms (Fig. 2*A*, dashed vertical line). This time was chosen as being close to the plateau level for the brighter backgrounds, but before the most obvious later ramping with the dim backgrounds. As a partial test of the adequacy of this approach, we re-analysed the results in Fig. 2 of Thomas & Lamb (1999), where rod-isolated families were measured at a range of background intensities. For the intense red flash used to derive the cone subtraction, we found that the response was unaltered up until 6 ms after the flash, at all background intensities up to the highest tested intensity of 960 scotopic Td, but that a slower component intruded at later times on the dimmer backgrounds. We interpret this to indicate that up until about 6 ms the response is of cone origin, rather than rod origin. However, we cannot exclude the possibility of intrusion by post-receptoral cone signals.

The measured amplitudes from Fig. 2*A* are plotted in Fig. 2*B* for this subject (●) and for two further subjects

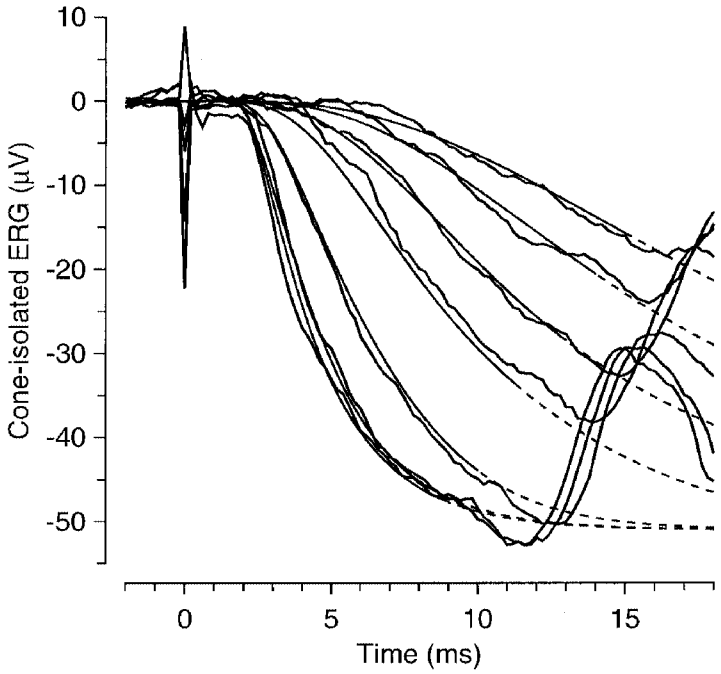

Figure 1. Cone-isolated family of *a*-wave responses

Cone-isolated family of responses to brief red flashes, presented on a blue background. The red flashes ranged in duration from 50 to 800 μs, and delivered 5, 10, 22, 54, 270, 1500 and 3700 photopic Td s. The traces plot the average of 18 to 42 flash presentations; the inter-flash interval was 0·5 s for the dimmer flashes, and 1·5 and 3 s, respectively, for the two brightest intensities. The blue background delivered 45 scotopic (and 4·2 photopic) cd m^{-2} through a natural pupil that averaged 2·8 mm in diameter, giving 280 scotopic (and 26 photopic) Td. The fitted curves plot the predictions of the ensemble fit of eqn (5) of Smith & Lamb (1997), where the following symbols are defined (see their Fig. 6). Total cone *a*-wave amplitude, $a_{cone} = a_{red} + a_{green} = -51$ μV; membrane capacitive time constant, $\tau_{cone} = 2{\cdot}4$ ms; delay time, $t_d = 1{\cdot}2$ ms; the fitting yielded a red-cone sensitivity of $K_{red}\,A_{cone} = 2100$ s^{-3} Td^{-1}. We assumed that the red- and green-cones (or L- and M-cones) contributed equally to the maximal response (i.e. that $a_{red} = a_{green}$), and that their sensitivities were determined by the measured spectral absorbance functions; for the red stimulus ($\lambda > 610$ nm), the calculated sensitivity ratio is $K_{green}/K_{red} = 1/6$. The theory traces are shown as continuous over the time window where the ensemble fitting was performed, and they are continued interrupted thereafter. See text for a discussion of the possibility of post-receptoral signal intrusion. Subject: T.D.L.

(□, ◇). For each subject the maximum response obtained with an intense flash declined with increasing background intensity, and the form of the relation appeared to be closely similar for the three individuals. The common curve drawn near all three sets of points is given by the equation

$$\frac{a_{\max}(I)}{a_{\max}(\mathrm{DA})} = \frac{1 + a_{\infty}(I/I_{½})^{n}}{1 + (I/I_{½})^{n}}, \quad (3)$$

with $a_{\infty} = 0{\cdot}15$, $I_{½} = 2400$ photopic Td, and $n = 0{\cdot}8$. Here $a_{\max}(I)$ is the maximal response obtained in the presence of a background of intensity I, and $a_{\max}(\mathrm{DA})$ is the corresponding dark-adapted value, while a_{∞} is the residual fraction of $a_{\max}$ at infinite background, n is an exponent, and $I_{½}$ is the background intensity that reduces the fractional current midway from unity to a_{∞}. We introduced a finite value of a_{∞} because of our expectation that the cone current should not be completely suppressed even by extremely bright steady illumination; thus, once an intensity is reached where substantial bleaching of pigment occurs, further increases in intensity will not greatly increase the rate of photoisomerization, because of the reduced pigment level. As we show subsequently in Figs 5

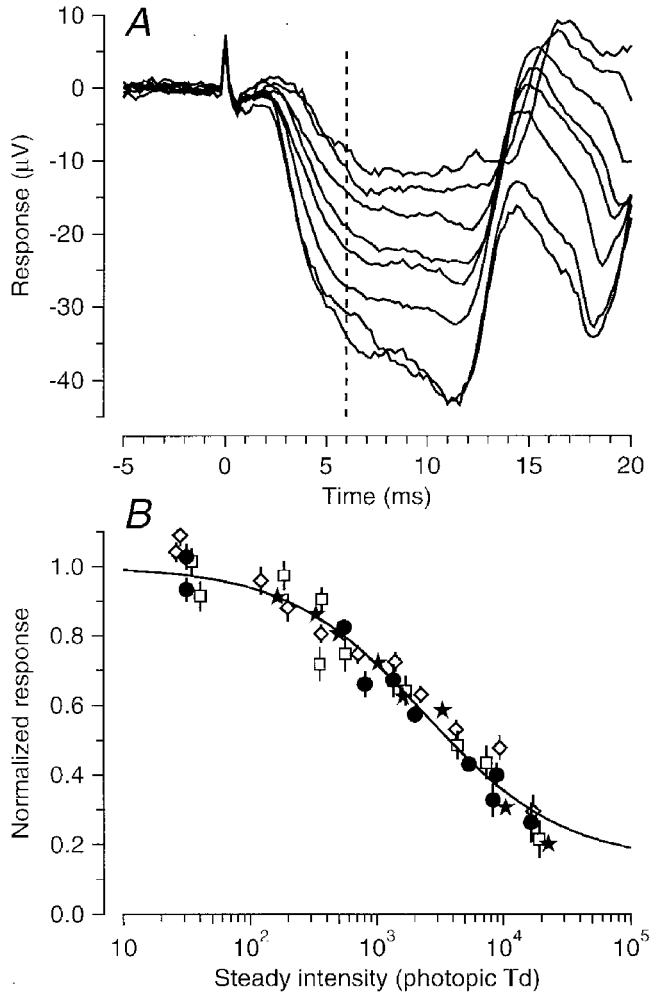

Figure 2. Suppression of cone circulating current by steady illumination

A, averaged responses to intense red flashes (800 μs duration) delivering 550 cd m^{-2} s, on different backgrounds. Largest two traces: on dim blue background, 4·1 photopic and 45 scotopic cd m^{-2} (31 photopic and 350 scotopic Td). Other traces: white backgrounds delivering 77, 300, 590, 1300, 2700 or 6700 cd m^{-2}. The diameter of the natural pupil varied from 3·1 mm to 1·8 mm; therefore the intense flashes delivered from 4200 Td s (on dim blue background) to 1800 Td s (on brightest background). Responses are averaged from 10 to 40 flash presentations; subject: A.A.V.P. *B*, normalized response amplitude, $a_{\max}(I)/a_{\max}(\mathrm{DA})$ (where $a_{\max}(I)$ is the maximal response obtained in the presence of a background of intensity I, and $a_{\max}(\mathrm{DA})$ is the corresponding dark-adapted value), measured 6 ms after the intense flash, for the subject in *A* (●, A.A.V.P.) and two other subjects (□, C.F.; ◇, T.D.L.); these measurements have been normalized using $a_{\max}(\mathrm{DA})$ for the three subjects equal to −33, −34 and −36 μV, respectively. Error bars are ± S.D. The stars (★) plot the measurements of Schnapf *et al.* (1990) Fig. 7*B*, of the steady response after 2 s of illumination, for monkey cones recorded with a suction pipette; their intensities have been converted using the factor 1 Td = 100 photons μm^{-2} s^{-1} (see text). The curve plots eqn (3) with $a_{\infty} = 0{\cdot}15$, $I_{½} = 2400$ Td, and $n = 0{\cdot}8$.

and 6, the brightest intensities in Fig. 2*B* correspond approximately to a bleach of 50% of the pigment.

For comparison, we have plotted as stars (★; Fig. 2) measurements from single primate cones made using the suction pipette method by Schnapf *et al.* (1990), which we shall consider in the Discussion.

Recovery of cones from bleaches

Recovery from a total bleach: mini-ganzfeld, dilated pupil

The recovery of cone responsiveness following delivery of a total bleach in the mini-ganzfeld is shown in Figs 3 and 4, for probe flashes that were dim (Fig. 3) or bright (Fig. 4). The bleaching intensity was $1{\cdot}6 \times 10^5$ cd m^{-2} through the dilated pupil, and lasted for 30 s; eqn (2) indicates that the effective retinal illuminance of *ca* 3×10^6 Td should have bleached about 99% of the cone pigment. Immediately upon cessation of the bleach, the subject rapidly moved from the mini-ganzfeld to the main ganzfeld, and the operator initiated a series of interleaved dim and bright test flashes (see legend to Fig. 3). The entire procedure was repeated with a second bleach after an interval of 9 min; the upper panels in Figs 3 and 4 illustrate traces obtained following the first bleach, while the lower panels show the recovery after both bleaches.

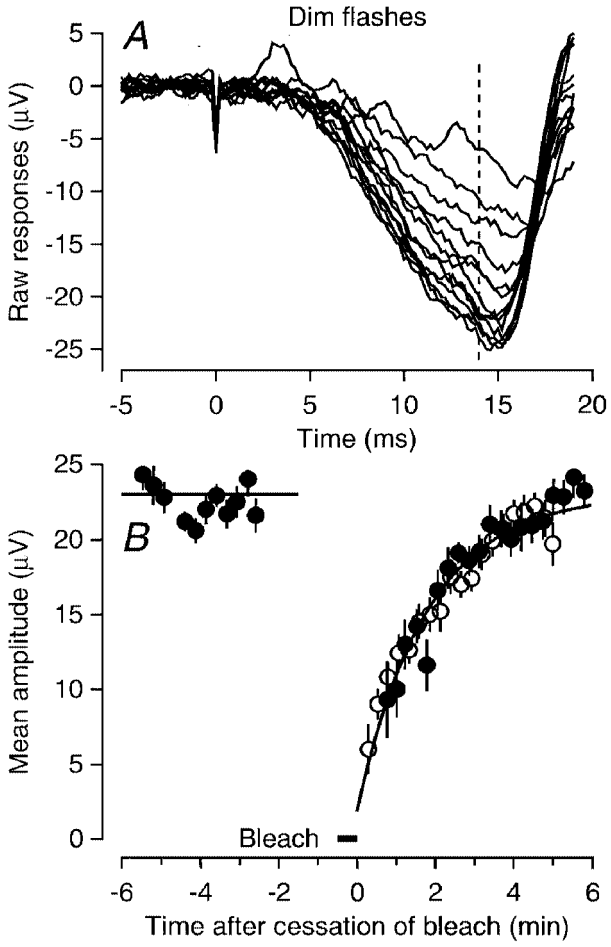

Figure 3. Recovery of cone responses to dim flashes following a total bleach

A, selected averaged responses to groups of dim test flashes presented at a range of times relative to delivery of a total bleach in the mini-ganzfeld. The test flashes were 50 μs in duration, and each delivered 0·92 photopic cd m^{-2} s to the dilated pupil, corresponding to about 16 effective photopic Td s. The flashes were presented in groups of 10 at intervals of 0·5 s, followed by two bright flashes at intervals of 1·5 s (see Fig. 4). Each trace is averaged from 13 to 20 responses. The mean time at which these selected traces were obtained (from top downwards) was: 0·3, 0·8, 1·3, 1·9, 2·4, 2·9, 3·5, 4·0 and 4·5 min after extinction of the bleach, and in addition four dark-adapted traces (the largest four) were obtained between 3·3 and 2·1 min before the start of the bleach. The bleach delivered $1{\cdot}6 \times 10^5$ photopic cd m^{-2} s for 30 s in the mini-ganzfeld. The dilated pupil behaved effectively as an area of about 20 mm^2 for the cones (see Methods), so that the effective retinal luminance was *ca* 3×10^6 photopic Td, which would be expected to bleach around 99% of the cone pigment; eqn (2). Prior to and after the bleaches there was a steady blue background of 32 scotopic cd m^{-2} in the recording ganzfeld. *B*, dim flash response amplitudes, measured at 14 ms (dotted vertical line in *A*), both before and after the first bleach (●), and after a second bleach delivered 9 min later (○). Error bars are ± S.D. The curve plots eqn (4) with $a_{\text{final}} = 23$ μV, $B = 0{\cdot}92$, and $\tau = 1{\cdot}8$ min. Subject: T.D.L.

Selected averaged responses to dim probe flashes (0·9 cd m^{-2} s, 16 effective Td s) are illustrated in Fig. 3*A*, for the first bleaching exposure, while Fig. 3*B* plots the recovery of all the dim flash response amplitudes (measured 14 ms after the test flash) following both the first exposure (●) and the second exposure (○). Clearly, the cone sensitivity to dim flashes was dramatically reduced following a total bleach, but recovered fully within 6 min. The curve drawn in Fig. 3*B* plots an exponential recovery of $a(t)$ towards a final level a_{final}, according to:

$$a(t)/a_{final} = 1 - B\exp(-t/\tau_P), \quad (4)$$

as would be expected to occur with first-order regeneration of photopigment and a linear dependence of dim-flash response on pigment level. The parameters used for this curve were a time constant of $\tau_P = 1{\cdot}8$ min (108 s) and a bleaching level of $B = 0{\cdot}92$. If a total bleach had been achieved, then the value of B expected in fitting eqn (4) would have been unity, but a better fit was obtained with $B = 0{\cdot}92$, suggesting that the level of bleaching obtained in the mini-ganzfeld was not total, possibly because of partial closure of the eyelids.

Two limitations of these experiments should be mentioned. Firstly, as for the case of the bright-flash experiments, it is entirely plausible that the *a*-wave signals might have included some component of post-receptoral response, as has been reported by Bush & Sieving (1994), e.g. from hyperpolarizing bipolar cells. One way to test this hypothesis in experimental animals would be by the judicious use of

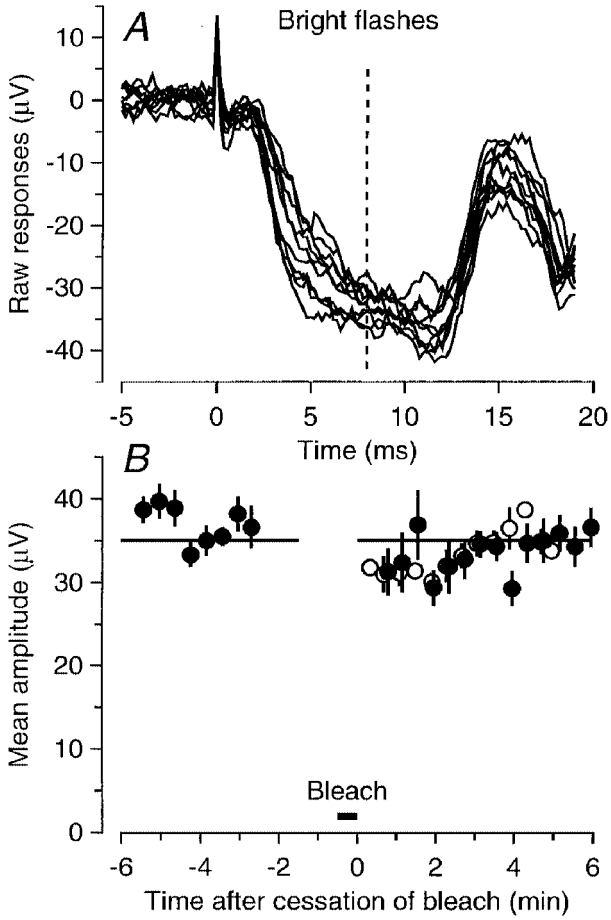

Figure 4. Recovery of cone responses to intense flashes following a total bleach

A, selected averaged responses to intense flashes, obtained after the same bleach (in the mini-ganzfeld) as illustrated for dim flashes in Fig. 3*A*. The test flashes were 400 μs in duration, and delivered 201 photopic cd m^{-2} s to a dilated pupil, corresponding to about 4000 effective photopic Td s. The flashes were presented in pairs, at 1·5 s intervals, following each group of 10 dim flashes (see Fig. 3). Each trace is averaged from two pairs of such responses. The mean times at which the selected traces were obtained were: 0·3, 0·5, 1·0, 1·5, 2·1, 2·6, 4·7 and 5·0 min after extinction of the bleach; in addition two dark-adapted traces were obtained 3·9 and 3·6 min before the start of the bleach. *B*, averaged bright flash response amplitudes, measured at 8 ms (dashed vertical line in *A*), both before and after the first bleach (●), and after the second bleach (○). To reduce the noise, the measurements in this panel were averaged from three pairs of bright test flashes (so the timing differs from the traces in *A*). Error bars are ± S.D. The horizontal line indicates the behaviour expected if the recovery of circulating current occurred very rapidly. Subject: T.D.L.

pharmacological agents (see Robson & Frishman, 1999). In the present experiments, though, we need to assume that any such intrusion of post-receptoral signals is linearly related to the underlying cone response. Secondly, one needs to be aware that the test flashes were not strictly 'dim', in the sense that the responses were probably out of the linear range. Ideally, it would have been preferable for the dark-adapted responses to have been smaller, but in that case the early post-bleach responses would have been submerged in noise, and hence unmeasurable. As a compromise, we selected a test-flash intensity that yielded a response no greater than that of the third trace in Fig. 1. The effect of any non-linearity will have been to compress the larger responses, so that the time constant of recovery will have been underestimated.

For another subject tested following twelve full bleaches on eight separate days, satisfactory fits to the dim-flash recovery were obtained using B in the range 0·9–1·0, with the fitted time constant τ_P ranging from 1·1 to 2·0 min. We suspect that these apparent variations in τ_P for results from the same subject arose from errors in fitting, due to noise in the measurements, rather than from systematic changes. The overall mean for this subject (O.A.R.M.) was $\tau_P = 1{\cdot}42 \pm 0{\cdot}29$ min (S.D., $n = 12$). In order to check whether the delivery of bright flashes influenced the recovery of the response to dim flashes, we compared experiments in which dim flashes were given alone with experiments in which they were interleaved with bright flashes. For the four bleaches monitored with dim flashes alone, the mean was $\tau_P = 1{\cdot}28 \pm 0{\cdot}13$ min (S.D., $n = 4$), while in the remaining eight bleaches with interleaved flashes, the mean was $\tau_P = 1{\cdot}50 \pm 0{\cdot}32$ min (S.D., $n = 8$). The small difference is not significant, indicating that the delivery of the bright flashes had little effect on the measured time constant of dim-flash recovery. For a third subject (C.F.), a value of $\tau_P = 1{\cdot}5$ min was obtained for each of two full bleaches.

Our results are in close agreement with the reflection densitometry measurements made by Coile & Baker (1992), which showed that the time constant of cone pigment regeneration increased with age, with a slope of 0·21 min per decade. For our subjects, the ages and time constants were: O.A.R.M., 21 years, 1·4 min; C.F., 30 years, 1·5 min; T.D.L., 50 years, 1·8 min. These values fall almost exactly on the correlation line plotted by Coile & Baker (1992) in their Fig. 3. We conclude that, for subjects aged about 30 years, the time constant of recovery of the dim-flash response is close to $\tau_P = 1{\cdot}5$ min (90 s), and that this value coincides very closely with the time constant of pigment regeneration determined by densitometry.

Figure 4 shows corresponding results for bright flashes (201 cd m^{-2} s, 4000 effective Td s), following the same pair of total bleaches as in Fig. 3. The traces in Fig. 4*A* are selected averaged responses obtained following extinction of the first bleaching exposure, while the symbols in Fig. 4*B* plot all the measured bright flash amplitudes (at 8 ms) for both the first exposure (●) and the second exposure (○). Clearly there is little change in the response amplitude to bright flashes following a total bleach.

However, inspection of the traces in Fig. 4*A* suggests that at early post-bleach times the onset phase of the response rose marginally more slowly, as would be expected if the effective flash intensity had been reduced by pigment depletion. Thus if the traces in Fig. 4*A* are compared with those of the family in Fig. 1, then the range of traces in Fig. 4*A* corresponds approximately to a transition from trace 5 to trace 6 in Fig. 1, as if the 400 μs flashes used in Fig. 4*A* had initially appeared about 5-fold dimmer at early post-bleach times. This is broadly as expected at 0·3 min after the bleach (the mean time at which the responses used to generate the first trace were obtained), if pigment regeneration occurs with a time constant of 1·5 min, as approximately 20 % of the pigment would then have been regenerated. Because we were already working at almost the maximum flash intensity available, we simply could not expect to saturate the response at earlier times than this, and accordingly we have not presented bright-flash measurements at times earlier than 15–20 s after a full bleach. However, in a subsequent section we present results for bright flashes delivered at much earlier times after a partial bleach.

The magnitude of bleaching induced by the bright flashes (4000 effective Td s) can be estimated using eqn (1) as approximately 4000 Td s/($I_P \tau_P$), where Hollins & Alpern (1973) have estimated the product $I_P \tau_P$ as *ca* 3×10^6 Td s. Hence each bright flash should have bleached only a little over 0·1 % of the cone pigment. These bright flashes were delivered in pairs every 8 s, so that eqn (2) gives the steady-state bleaching level as *ca* (2 × 4000 Td s)/(8 s × 30 000 Td) = 3 %. Hence, upon extinction of a near-total bleach, the quantity of photopigment should recover from near zero initially towards a final level not of 100 %, but instead of around 97 %. Ideally one would prefer that the flashes elicited no bleaching themselves, but unfortunately they would not then have been sufficiently bright to saturate the response reliably. As mentioned above in relation to Fig. 3, the delivery of these intense flashes appeared to have little effect of the kinetics of recovery of the responses to dim flashes.

Interpretation of the post-bleach recovery with dim and bright flashes

We now consider the interpretation of the measured recovery of dim and bright flash responses. In their recent analysis of the Lamb & Pugh (1992) model of phototransduction, Thomas & Lamb (1999) showed that the amplitude (at any fixed time) of the response to a suitably dim flash should be proportional to the product of three factors: (i) the maximal response a_{max} obtained with a very bright flash; (ii) the

amplification constant A of phototransduction; and (iii) the fraction f of photopigment present. The first of these factors, the maximal response to a bright flash, provides a measure of the circulating current in the photoreceptors at the time of flash delivery.

From the bright-flash results of Fig. 4 (and comparable experiments on the other subjects), we conclude that at the cessation of a near-total bleach the cone photoreceptor circulating current recovers completely within 20–30 s; i.e. that factor (i), a_{max}, returns rapidly to its pre-bleach level. Accordingly, the time course of dim-flash recovery, of the kind illustrated in Fig. 3, should be proportional to the product of the remaining factors (ii) and (iii): the amplification constant A, and the fraction of pigment present f. Since our measurements of dim-flash sensitivity and the measurements of Coile & Baker (1992) for pigment regeneration both found recovery to proceed exponentially with a time constant averaging 90 s (following a full bleach, and for subjects aged 30 years), the most parsimonious explanation is that the amplification constant A of phototransduction is unaltered following a bleach, and that the reduced sensitivity in Fig. 3 results solely from reduced quantal catch, due to reduced pigment level, f.

One of the main difficulties in accurately measuring the recovery of the circulating current (as monitored by the maximal response) following a near-total bleach arises from pigment depletion. Thus if one wished to saturate the a-wave at a very early time, when only 1 % of the pigment was present (say, 1 s after extinction), then it would be necessary to increase the flash intensity 100-fold, in order to deliver the same number of photoisomerizations as when all the pigment was present. However, since our red test flashes were already unattenuated, and 400 μs in duration, we could only increase their intensity severalfold. A second difficulty was that we could only achieve the very highest bleaching levels using the mini-ganzfeld, which meant that the eye could not be observed during the bleach, and that at the end of the exposure there was a short delay in reliably repositioning the observer's eye in the recording ganzfeld.

Recovery from a partial bleach at faster time resolution: main ganzfeld, natural pupil

To examine the recovery at higher time resolution, but following smaller bleaches, we next delivered bleaching exposures in the main ganzfeld. The advantages of this procedure were firstly that test flashes could be delivered as soon as desired after a bleaching exposure (and at reproducible times after successive bleaches), secondly that we could be confident of the total retinal illuminance delivered, as the pupil and eyelids could be monitored continuously throughout the bleach, and thirdly that we could monitor the responses to bright flashes during the exposure. An inevitable consequence, though, was that we could only deliver a partial bleach. As explained previously, we employed a natural pupil which, in combination with the lower corneal illuminance that could be delivered in the main ganzfeld, limited the cone pigment bleaching level to around 50 %.

In order to obtain averaged responses, we delivered a given bleach repeatedly. Figure 5 shows the recovery obtained for one subject using bright probe flashes (550 cd m^{-2} s, *ca* 1700 Td s), for six bleaching cycles, each comprising a 30 s exposure at 6900 cd m^{-2} followed by 60 s of recovery. The measurements are plotted in raw form in Fig. 5*A*, and averaged over the six repetitions in Fig. 5*B*. The probe flashes were delivered at intervals of 3 s, and the incandescent lamp was switched manually after each tenth or twentieth flash; hence the first flash after time zero occurred close to 2·5 s after extinction of the bleaching light. By this time, it appears that the cone circulating current had returned completely to its pre-bleach level; i.e. that full recovery of circulating current occurred in less than 3 s.

To estimate the size of the bleach in these experiments, it is possible to adopt either a theoretical or an experimental approach. Application of eqn (1), with an exposure of 6900 cd m^{-2} through a 2·0 mm diameter pupil ($I = 22\,000$ Td) for a duration of $t = 30$ s, yields an estimated bleach level of $B = 18$ % using $I_P = 30\,000$ Td (Hollins & Alpern, 1973), or $B = 26$ % using $I_P = 20\,000$ Td (Rushton & Henry, 1968), with the value of $\tau_P = 90$ s from Coile & Baker (1992) and the present study, for full bleaches. However, these calculations are likely to provide underestimates for several reasons. First, the values of $I_P = 20\,000$–$30\,000$ Td were measured in the fovea or parafovea, whereas our a-wave responses are summed over the entire retina, and it is possible that the value of I_P appropriate to our experiments is smaller, because of the larger cross-sectional area of cone inner segments in the periphery. Secondly, the magnitude of the regeneration time constant, τ_P, for this relatively short partial bleach may be considerably shorter than 90 s, as we show in Fig. 6.

Our experimental approach to estimating the size of the bleach was to monitor the recovery with dim flashes and to make the assumption that any reduction in response represented a reduction in pigment level (see above). One such experiment from another subject is shown in Fig. 6*A*, which plots the averaged amplitude of responses to dim flashes (*ca* 5 Td s) delivered in groups of ten at 0·5 s intervals. As in Fig. 5, we further averaged by repeating the bleach six times, with each cycle comprising 32 s of intense illumination at 6200 cd m^{-2} followed by 3 min of recovery in darkness. Examination of Fig. 6*A* shows that, at the extinction of the intense background, the amplitude of the response to a dim flash jumped immediately from near zero to around 50 % of its pre-bleach level, and then recovered fully over the next 3 min. The absence of response during the background occurred because the circulating current had been reduced to a low level, and the sudden jump is what would be expected in the case of a rapid recovery of

the circulating current in conjunction with an initial pigment level of about 50%. The curve plots an exponential recovery of the form given previously in eqn (4), with $B = 0{\cdot}5$ and $\tau_P = 0{\cdot}7$ min (42 s). By way of comparison, Fig. 6*B* plots the recovery of the same subject tested with bright flashes following an identical bleach (note the faster time scale). Clearly, the post-bleach recovery for the dim flash response is very much slower than that for the bright flash response. For the two subjects tested in Figs 5 and 6, the recovery for dim flashes could be described by an exponential with $B \approx 50\%$ and $\tau_P \approx 0{\cdot}7$–$0{\cdot}8$ min (40–50 s); this time constant appears substantially faster than that of *ca* 90 s obtained after a near-total bleach.

If we assume that the experimental results in Fig. 6*A* provide a better estimate of bleaching level than the theoretical calculations above using others' values for τ_P and I_P, then inspection of the fitted trace in Fig. 6*A* indicates that the 30 s of illumination should have caused a final bleaching level of around 50%, and that the 60 s period of recovery should have left about 10% of the pigment unregenerated. However, the exact level of bleaching in these panels is in no way critical – the importance of Figs 5 and 6*B* is to show that after a substantial bleach the circulating current recovers very rapidly.

Finally, it is worth noting that the intensities used in Figs 5 and 6 correspond to the highest intensities plotted in Fig. 2*B*; i.e. to the brightest white light that we could deliver in the main ganzfeld. Therefore the points on the right of Fig. 2*B*, obtained during steady illumination, are analogous to the final points obtained during the exposures in Figs 5*B* and 6*B*. Both correspond to a circulating current of about 20% of the dark-adapted level.

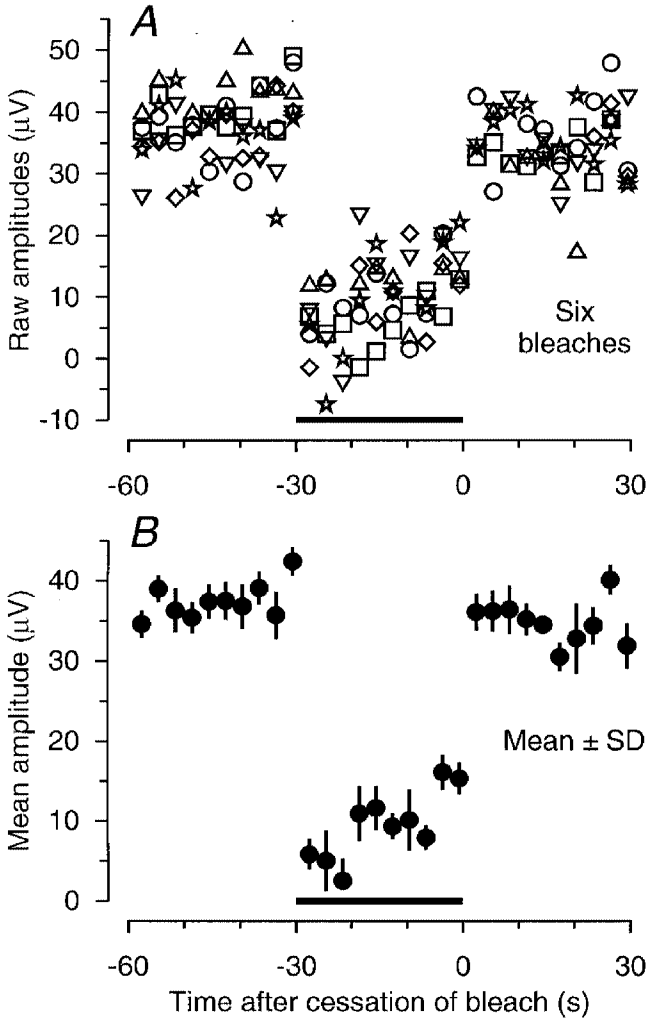

Figure 5. Recovery of cone responses to intense flashes following a bleach in the main ganzfeld

A, recovery of the response to intense flashes following six repetitions of an exposure in the main ganzfeld estimated to bleach about 50% of the pigment. Red flashes of 800 μs duration delivered 550 photopic cd m^{-2} s to the natural pupil 2·0 mm in diameter, giving 1700 photopic Td s; the flashes were given repetitively at intervals of 3 s. The white background of 6900 cd m^{-2} was switched on manually for 30 s (i.e. for 10 flash cycles), and then off for 60 s. The six repetitions of the procedure are indicated by different symbols. *B*, mean ± S.D. from *A*. Subject: C.F.

DISCUSSION

We have probed the activity of cone photoreceptors in the human eye using the *a*-wave of the ERG under photopic conditions. The amplitude of the response to a bright flash provides a measure of the circulating current in the cone photoreceptors.

Our principal finding is that, upon extinction of steady illumination sufficiently intense to bleach about 50% of the photopigment, the cone circulating current in the human eye recovers completely in less than 3 s. This time course is around 300 times faster than in rods, for which complete recovery of the circulating current takes about 15 min following a bleach of 50% (Thomas & Lamb, 1999). Following a total bleach, the cone circulating current recovers within 20–30 s (the earliest time at which we could saturate the response), compared with about 25 min in rods. The contrasting time course is indicative of a major difference between rods and cones in the handling of the photoproducts of pigment bleaching. The rapid recovery of the cones suggests either that the photoproducts are removed extremely rapidly from the cone outer segment following a bleach, or that the cone circulating current is essentially immune to their presence. If, as in rods, an 'equivalent background light' (Stiles & Crawford, 1932) is generated by the presence of photoproducts, then our results show that it has a negligible effect on the circulating current, indicating that its equivalent intensity must drop to below 10–100 Td

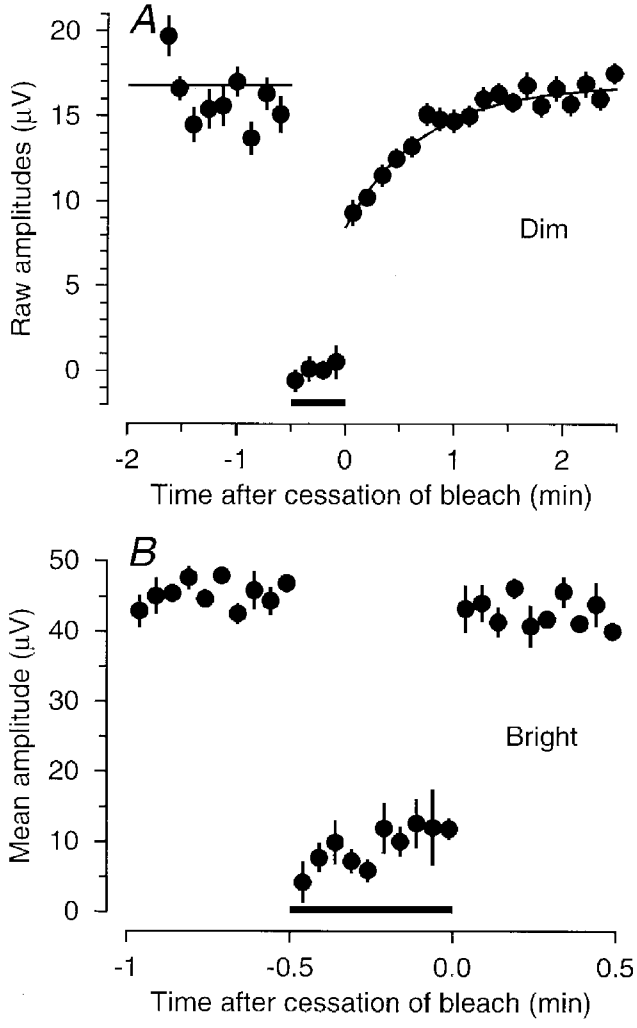

Figure 6. Recovery of cone responses to dim and bright flashes following bleaching in the main ganzfeld

A, time course of recovery of dim flash response, following a bleach delivered in the main ganzfeld. Test flashes 60 μs in duration, delivering 1·7 cd m^{-2} s (5·7 photopic Td), were given in groups of 10 at 0·5 s intervals every 8 s (permitting a few seconds for blinking). The white background of 6200 cd m^{-2} was switched on manually for 32 s, and then off for 184 s, for a total of six cycles. Response amplitudes were measured 15 ms after the flash. The curve plots eqn (4) with $a_{\text{final}} = 16{\cdot}8\ \mu$V, $B = 0{\cdot}5$, and $\tau_{\text{P}} = 0{\cdot}7$ min; this fit is consistent with a bleach of about 50%. *B*, recovery of the same subject's bright flash responses, following a 30 s bleach at the same intensity, determined with the protocol in Fig. 5. Natural pupil; 2·0 mm diameter during background exposure. Subject: T.D.L.

(see Fig. 2) within a couple of seconds. Whatever the molecular basis, the important consequence for the visual system is that, almost immediately after the extinction of an intense steady light, the cones are again generating a large circulating current that can be modulated by illumination.

Very recently, Pianta & Kalloniatis (2000) have investigated the 'equivalent background' underlying psychophysical dark adaptation of the human cone visual system. They find that following a 90 % bleach the equivalent background decays as two components, with time constants of *ca* 19 and 51 s, from initial levels of *ca* 1000 and 150 Td, respectively. If their experiments and ours are comparable, then it would suggest that the larger equivalent background that they observe at early post-bleach times has little effect on the cone steady current.

For the future, it will be interesting to improve the time resolution of the method, by using an electronically controlled shutter for the background, and brighter probe flashes. In recordings from monkey cones, using an extracellular electrode inserted into the eye, Valeton & van Norren (1983) have shown that recovery from a background of 10 000 Td occurs within about 100 ms (their Fig. 2). This time course is not greatly different from the recovery from a 'just saturating' flash under dark-adapted conditions, as measured in isolated monkey cones (Schnapf *et al.* 1990) or with the human ERG (Hood *et al.* 1996; Cideciyan *et al.* 1998).

The responses to dim flashes provide different information. From a theoretical analysis, it is predicted that the amplitude of the response to a dim flash should be proportional to the product of three factors: (i) the magnitude of the circulating current, (ii) the amplification constant of phototransduction, and (iii) the fraction of visual pigment present. The first of these factors has been measured above, and recovers extremely rapidly after a bleach. Hence, on the assumption that the amplification constant is unchanged, the amplitude of the dim flash response provides a measure of the fraction of pigment present.

Our dim flash recordings have shown that cone sensitivity recovers with a time constant of *ca* 90 s following a near-total bleach, and it is satisfying that this is indistinguishable from the value of the regression line for a subject aged 30 years in Fig. 3 of Coile & Baker (1992) who used reflection densitometry. Interestingly, this recovery is only a factor of about 4-fold faster than in rods, where the time constant of pigment regeneration has been reported as *ca* 7 min (Rushton, 1965; Alpern, 1971). Following partial bleaches, we found a shorter time constant for recovery of sensitivity, of *ca* 40–50 s after a bleach of around 50 %. The finding of a faster time constant following a partial bleach indicates that the regeneration of cone pigment may not follow first-order kinetics, as reported by Smith *et al.* (1983), and suggests that perhaps a rate limit is involved, similar to the rate limit in the removal of rod photoproducts that was reported by Lamb (1981). Although this suggestion is at variance with the exponential recovery assumed in eqn (4), we regard that form simply as an approximation. Thus if recovery after large bleaches initially proceeds roughly linearly with time, but with some dispersion between different photoreceptors in the actual rate, then the average time course is likely to approximate an exponential; however, with smaller bleaches the apparent time constant would be shorter.

We would point out one considerable shortcoming of *a*-wave measurements, in comparison with direct recordings of photocurrent from individual cones (Schnapf *et al.* 1990). The *a*-wave measurements presented here can only probe the rising phase of the photoreceptor response, prior to intrusion by the *b*-wave, whereas suction pipette measurements monitor the entire time course of the response. Accordingly, our measurements do not provide information about the time-to-peak of the cone response, or about possible changes in sensitivity that might result from changes in the time-to-peak. In their recordings from monkey cones, Schnapf *et al.* (1990) found that the dim-flash responses were invariant in shape. In order to obtain information from the ERG about the photoreceptor response at later times, it is possible to use a paired-flash technique (Pepperberg *et al.* 1997). This approach is time-consuming, but we have recently adopted it in preliminary experiments to extract the full time course of the cone response (Friedburg & Lamb, 2000).

Upon exposure to increasing intensities of steady illumination, the maximal response declined, as might be expected. At first, though, we were surprised to find that the maximal cone response decreased so substantially at steady intensities that were not particularly high; thus the maximal response was reduced to 25–30 % at around 20 000 Td (the highest intensity that we could deliver). It is well known in the psychophysical literature that the photopic visual system avoids saturation even for extremely high intensities of steady illumination (though transient saturation does occur), and previous extracellular measurements of cone activity in monkey eyes have shown that at high steady intensities, the steady level of response suppression stabilizes at not more than about 50 % of the dark-adapted maximal response (Boynton & Whitten, 1970; Valeton & van Norren, 1983). Comparable findings of a steady response of no more than 50 % of the total range have been made in cones recorded intracellularly in the turtle eye-cup (Burkhardt, 1994) and extracellularly in the frog retina (Donner *et al.* 1998). In view of these previous findings, is our measurement of 80 % suppression reliable?

To investigate this, we compared our results with recordings of circulating current from isolated monkey cone photoreceptors reported by Schnapf *et al.* (1990) in their Fig. 7*B*. Their measurements are re-plotted as the stars (★) in Fig. 2*B*, after horizontal scaling along the intensity axis.

The magnitude of horizontal scaling that we employed, to scale their intensity units of photons μm^{-2} s^{-1} to our units of Td, corresponds to a conversion factor of 1 Td = 100 photons μm^{-2} s^{-1}. For comparison, Schnapf *et al.* (1990, p. 684) had estimated the conversion factor to be 1 Td = 39 photons μm^{-2} s^{-1}, for transverse incidence with unpolarized light of 560 nm. However, they explicitly commented that the magnitude of this conversion factor depended heavily on the extent of light collection by the cone inner segment, which they estimated as a factor of 2. If instead, the large cone inner segments of the peripheral retina funnelled light into the outer segments with a collection factor of 5 (which seems not unreasonably large), then their calculated troland conversion factor would immediately increase to the value that we have used.

Hence we feel that there is no reason to doubt the general form for the suppression of cone circulating current implied by our results in Fig. 2, which indicate about 75% suppression at the highest steady intensity that we could deliver. The form of expression that we have adopted in eqn (3) to describe these results complies with the equations used by Boynton & Whitten (1970) and Valeton & van Norren (1983) in describing the response *versus* intensity relation, in that it employs an exponent of less than unity: we used $n = 0{\cdot}8$, whereas they used $n \approx 0{\cdot}74$. The main difference is that our equation predicts that *ca* 85% of the cone current will be suppressed in the presence of extremely intense steady illumination, whereas their results had suggested that *ca* 50% was suppressed.

In addition to the closely similar form of the circulating current *versus* intensity relation, there are three further similarities between our results and those of Schnapf *et al.* (1990) on monkey cones. Firstly, their results (Fig. 12) and ours (Figs 5*B* and 6*B*) show partial recovery of circulating current during a period of tens of seconds of bleaching exposure. Secondly, their results show a rapid return of circulating current at the cessation of a non-total bleach (this result was not discussed in their paper, but is apparent at the end of the upper trace in Fig. 12). Thirdly, they showed that the reduction in sensitivity during bleaching was accounted for solely by reduced quantal catch. Thus the findings of the two studies, employing very different experimental techniques, are in close agreement. The present results in the intact eye, where pigment regeneration occurs, confirm the earlier observations in isolated cones, and show that the recovery of cone sensitivity parallels the regeneration of photopigment.

Overall, our results indicate that cone photoreceptors in the living human eye are extremely well adapted to function in the presence of intense illumination. Even when half their pigment is bleached, they are able to maintain a respectable circulating current, and when the steady light eliciting this bleach is extinguished, they are able to recover their full circulating current within seconds.

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