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LOCAL EFFECTS OF BLEACHING IN RETINAL RODS OF THE TOAD

BY D. A. BAYLOR* AND T. D. LAMB

*From the Physiological Laboratory, Downing Street, Cambridge, CB2 3EG, and the *Department of Neurobiology, Stanford University School of Medicine, Stanford, CA 94305, U.S.A.*

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SUMMARY

1. Suction electrode recordings were used to study the recovery of responsiveness in single toad rods after bleaching a small fraction (less than 5%) of the rhodopsin.

2. After a spatially uniform bleach that initially abolished the dark current over the entire length of the outer segment, the more proximal regions recovered faster than the more distal regions. For a time the most basal region was almost fully recovered while the tip remained fully saturated.

3. Such a gradient of responsiveness did not occur during uniform steady *background* illumination of dark-adapted cells.

4. The entire outer segment recovered uniformly after a longitudinally graded bleach that simulated the pattern produced by self-screening in the intact eye.

5. The recovery of the distal end of the outer segment was not affected by a bleach at the proximal end. This suggests that the differences in recovery rate reflect intrinsic local properties of the outer segment rather than longitudinal diffusion of a substance from the inner segment.

6. For at least the first 3 min after bleaching with a narrow transverse slit the reduction of responsiveness remained most pronounced in the bleached region, suggesting that this effect of bleaching does not spread extensively.

7. The increased noise induced by bleaching is shown to originate locally in the bleached region of outer segment.

8. When the tip was locally saturated after a bleach or during steady light, the current recorded from the tip was predominantly capacitive, resulting from intracellular voltage change. This indicates that when the dark current is abolished the outer segment plasma membrane has negligible leakage conductance.

INTRODUCTION

Electrical recordings from vertebrate rods have shown that a bright 'bleaching' light causes a profound and long lasting reduction in responsiveness (see for example Dowling & Ripps, 1972; Penn & Hagins, 1972; Grabowski & Pak, 1975; Ernst, Kemp & Lake, 1978; Pepperberg, Brown, Lurie & Dowling, 1978; Donner, & Hemilä, 1979). With a few exceptions (Jagger, 1979; Hemilä & Reuter, 1981) the previous studies have not been designed to examine the contribution of different regions of the outer segment to the bleaching effect.

We have used the suction electrode technique (Baylor, Lamb & Yau, 1979*a*) with localized bleaching and testing lights to examine regional behaviour in the outer segment and to look for evidence of spread of bleaching effects. We also studied the residual membrane conductance and capacitance of a locally bleached region of outer segment. Preliminary reports of some of this work have been published previously (Baylor & Lamb, 1980, 1981).

METHODS

The methods were as described by Baylor, Lamb & Yau (1979*a*, *b*) and Lamb, McNaughton & Yau (1981). When it was necessary to draw the cell rapidly into and out of the pipette several times it was convenient to use rods which had become detached from the retina but which still retained intact inner and outer segments (as in Baylor *et al.* 1979*a*, Fig. 6). Experiments were performed using either bicarbonate-buffered Ringer (Cervetto, Pasino & Torre, 1977; see Lamb *et al.* 1981) or HEPES-buffered Ringer (see Baylor *et al.* 1979*a*); the bleaching behaviour was similar in both solutions.

Calibration of bleaches

The monochromatic 500 nm bleaching light was applied at right angles to the axis of the outer segment. Calibration of bleaches was performed in two ways. The unattenuated flux at 500 nm was measured after the experiment and converted to isomerizations using eqn. (14) of Baylor *et al.* (1979*b*). As the light was polarized, and about 40 μm of outer segment was effectively within the pipette, the collecting area A_c was typically about 30 μm^2. The number of isomerizations was then converted to a percentage bleach using the figure of $2{\cdot}7 \times 10^9$ rhodopsin molecules in a toad rod 60 μm long (Baylor, Matthews & Yau, 1980). As a check the dim flash response was measured so that the single quantum response could be resolved directly, or obtained from the mean and variance of the response amplitude. Then by simple extrapolation to allow for the change in neutral density filters and in the duration of the stimulus, the number of bleaching isomerizations was obtained.

Graded bleaches

In the intact eye self-screening by the rhodopsin causes the intensity of axial light to fall exponentially with distance along the outer segment. In toad rods Harosi (1975) measured the decline to be 0·016 log units μm^{-1} with 500 nm light, or one decade per 62·5 μm, corresponding to an exponential length constant of about 27 μm. One way to stimulate such a distribution with transverse illumination is to use a graded filter or 'wedge' with this density gradient. As an alternative we made a discrete filter which increased in density increments of 0·2 log units from 0 to 0·6 log units at intervals of about 12 μm referred to the retina (about 600 μm intervals before demagnification). This gave a reasonable approximation to the required gradient over a distance of at least 40 μm, which was about the length of outer segment normally sucked in. The results of these experiments are described on p. 60.

Drift compensation

Because of the long time course of the recordings slow DC drift in the reference electrode potential often gave a sloping base line. This drift could be monitored by observing the peaks of saturating flash responses, as these correspond to absolute zero of membrane current (Baylor *et al.* 1979*a*; and see p. 54). Provided that the drift was linear with time and less than 0·005 pA sec^{-1} it was subtracted from some records using a ramp generator circuit or the computer; such compensation is indicated in Figure legends.

Integration of capacitive currents

Capacitive currents in the bleached tip (Figs. 12–14) were integrated by the computer. Raw responses were low-pass filtered at 20 Hz and digitized at 10 msec intervals, and the time average was computed, usually over at least forty responses. The base line was determined as the mean level over the 1 sec interval before the flash and was subtracted from all points. The integral was

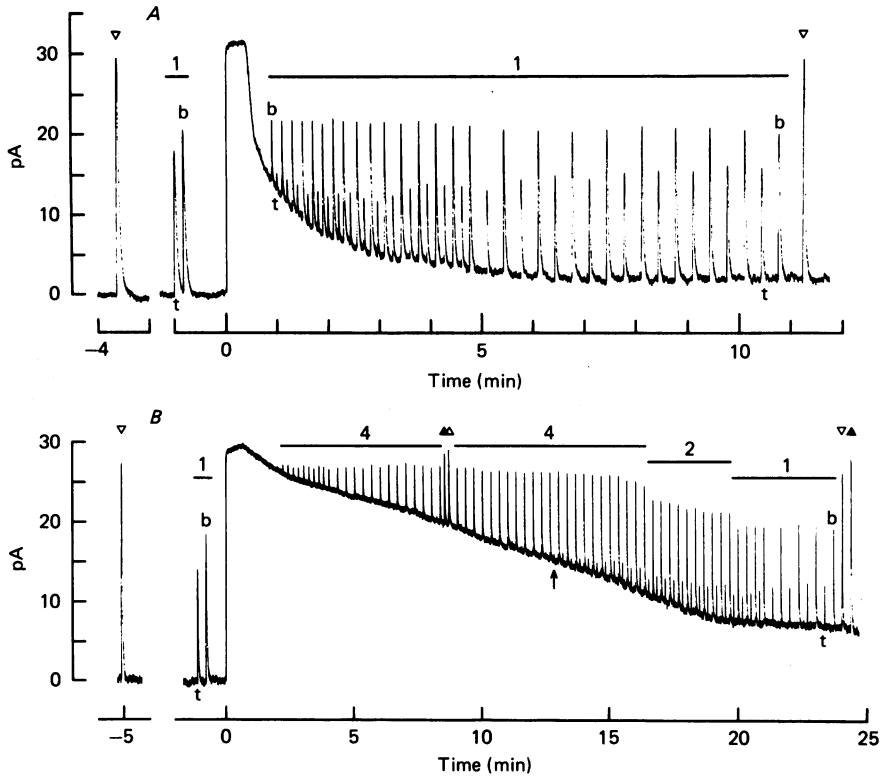

Fig. 1. Recovery of responsiveness to bright tip and base test flashes following two uniform bleaches; *A*, about 0·8 %; *B*, about 3·3 %. In each case t and b indicate flashes at tip and base respectively (25 μm diam.) and triangles indicate bright diffuse test flashes. Bars above each trace show the periods during which local tip and base flashes were alternated; numbers (1, 2 and 4) give flash strength relative to 42·5 photons μm^{-2} (about 740 isomerizations) per flash. Relative to this same intensity the diffuse test flashes had strengths of: ▽, 1; ▲, 4; Δ, 8. Arrow in *B* indicates first detectable response to a tip flash. Bleach in *A*, $1{\cdot}1 \times 10^5$ photons μm^{-2} sec^{-1} for 10 sec, isomerizing approximately 2×10^7 rhodopsins; *B*, $4{\cdot}7 \times 10^5$ photons μm^{-2} sec^{-1} for 10 sec, bleaching approximately $8{\cdot}2 \times 10^7$ rhodopsins. Slope compensation in both cases about 0·002 pA sec^{-1}. Bicarbonate/CO_2 Ringer: temperature 22·2 °C.

then computed by the trapezoid rule (i.e. on the assumption that the signal changed linearly in time between sampling points).

In most experiments this integral showed a small net charge movement over the cycle, as if a leakage conductance were in parallel with the capacitance. In these cases eqn. (4), with a finite time constant τ, was integrated numerically, again assuming that the signal changed linearly between sampling points. The means of estimating the appropriate time constant is given in the Results.

RESULTS

Slower recovery of distal regions after uniform bleaches

This section describes the spatial and temporal recovery of the outer segment following a uniform light incident transverse to the axis, and bleaching each element of outer length to the same fractional extent. The results confirm and extend our

earlier report (Baylor & Lamb, 1980) that after such a bleach the tip of the cell recovers more slowly than the base.

Fig. 1 shows a rod's responses to bright test flashes presented on either the base (b) or tip (t) half of the outer segment. In *A* both stimuli gave responses of around 20 pA in the fully dark-adapted state prior to bleaching, while the same intensity presented diffusely (▽) gave a maximal response, indicating that the local responses were of saturating intensity. At time zero an intense uniform light presented for 10 sec (and estimated to bleach about 1 % of the pigment) held the outer segment current fully saturated for about 30 sec, after which slow recovery of the dark current proceeded.

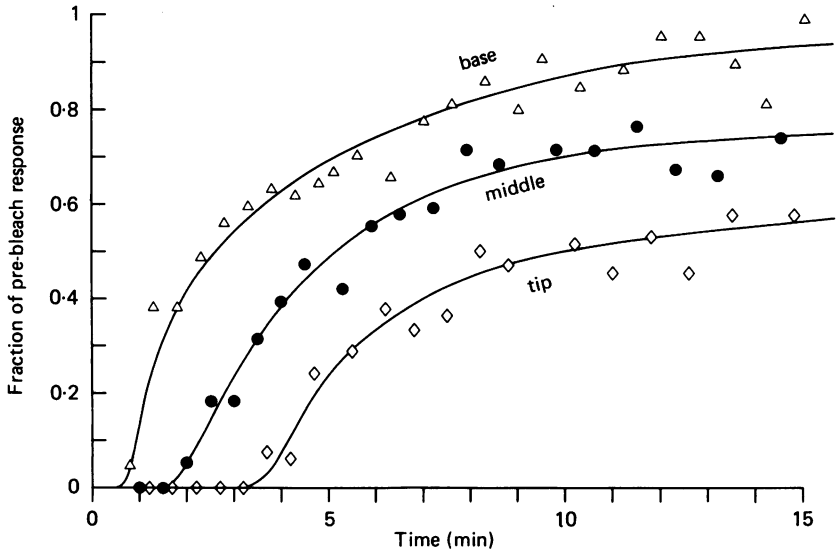

Fig. 2. Time course of fractional recovery of response at three test positions on the outer segment after a uniform bleach of about 2·5 %. Test stimuli were transverse slits 7 μm wide, spaced at 14 μm intervals, and are from three of the five positions shown in the inset of Fig. 3. For details of flash intensities and response amplitudes see legend of Fig. 3.

Beginning about 1 min after the bleach, test flashes of the same intensity as previously were presented alternately at the base and tip. Although the initial response elicited by flashes at the base was moderately large (about 8 pA) the first response from a flash at the tip was very small (about 1 pA); subsequent tip responses grew successively in size until reaching about 14 pA after 10 min of dark adaptation.

A more pronounced example of the different recovery rates is illustrated in Fig. 1 *B*, which is from the same cell, but with a bleach four times more intense. This record was obtained soon after that in Fig. 1 *A*, so that the tip and base responses before bleaching were similar to the responses at the end of record *A*; the time base is slowed by a factor of 2. Beginning about 2 mins after the bleach test flashes were again given alternately on the base and tip, at an intensity four times higher than previously (indicated by 4 above the bar). Despite the fact that flashes at the base elicited responses which slowly increased in amplitude to a level of about 10 pA after 10 mins,

no response whatsoever was obtained from the tip until about 13 mins after the bleach (arrow in Fig. 1*B*). Thereafter the tip responses slowly increased in amplitude until after 23 min a 5 pA response could be obtained with the original flash intensity.

This experiment demonstrates that after a uniform bleach the tip recovers more slowly than the base and that the reduction in tip responsiveness involves a reduction in the maximal amplitude of the local response. We show on p. 55 that this reduction in maximal response results from a local decrease in dark current.

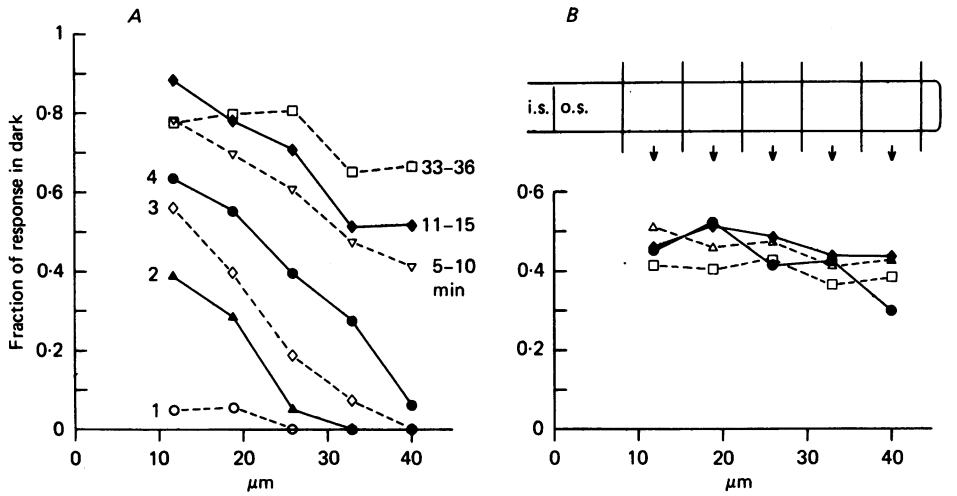

Fig. 3. Normalized profiles of responsiveness following a uniform bleach (*A*), and during uniform steady backgrounds (*B*). Same cell as Fig. 2; positions of test slit (7 μm wide) are shown in inset. *A*, profiles obtained at the indicated times after the same bleach (2·5 %) as Fig. 2. Bleaching light of 2×10^6 photons μm^{-2} sec^{-1} for 1 sec was estimated to isomerize $6{\cdot}2 \times 10^7$ rhodopsins. Test flash intensity 40·4 photons μm^{-2}, estimated to cause about 200 isomerizations per flash. Prior to the bleach the test flashes gave responses of 8·4, 9·1, 7·6, 8·0 and 6·6 pA respectively at the indicated positions (from left), and these values have been used to normalize all amplitudes in this and the previous Figure. The same intensity presented diffusely (and estimated to cause 1300 isomerizations) gave a saturating response of 20 pA. *B*, profiles obtained with uniform steady backgrounds prior to bleach in *A*. ●, dimmest background, 2·2 photons μm^{-2} sec^{-1}; test flash intensity as in *A*. For ◆, the background and test intensities were increased by 0·6 log units, and for △ and □ they were increased in further 0·6 log unit steps. Steady response amplitudes: ●, 6·7 pA; ◆, 9·6 pA; △, 12·5 pA; □, 14·4 pA. Bicarbonate/CO_2 Ringer.

Time course. In another cell narrow transverse slits were flashed at a series of positions along the outer segment, and Fig. 2 plots the time course of recovery at three of these positions; response amplitudes have been normalized with respect to their pre-bleach values. At the most basal position (△) recovery began less than 1 min after the bleach and reached 50 % after 2·5 min. At the middle (●) and tip (◇) recovery was significantly slower, with initial delays of about 1·7 and 3·5 min.

Profile of responsiveness. Using the same data from which Fig. 2 was drawn, the spatial profile of recovery is plotted in Fig. 3*A* at a series of fixed times for the five positions tested. The times after the bleach (in min) are shown against each set of

points, and the test positions are indicated by the inset in *B*; the responses have again been normalized.

At times up to at least 15 min after the bleach there is a distinct gradient of responsiveness along the outer segment. For example at 4 min (●) recovery at the most basal region, about 12 μm from the junction with the inner segment, was more than 60 % complete, while at the most distal point it was less than 10 %. At later times a given degree of recovery had been attained further outward along the cell, indicating that the return of responsiveness spread slowly from base to tip.

These and other experiments showed that with bleaches of more than 1–2 % the tip region of the cell did not fully recover over a period of an hour or so. This effect is presumably related to the absence of the pigment epithelium from the isolated retina preparation, as full recovery would be expected in the intact eye. With extracellular voltage measurements Donner & Hemilä (1979) have observed a similar permanent reduction of response in the isolated frog retina.

Although the possibility cannot be ruled out that part of the failure to recover in Figs. 1 and 2 may have resulted from 'deterioration' of the cell, we think that this is unlikely. The effect was seen reproducibly in many cells and appeared as a progression from the effect with small bleaches. On the other hand while deterioration of a cell's response was quite often observed, this generally occurred gradually over a period of an hour or more, and was usually accompanied by visible physical deterioration of the outer segment.

Comparison with the effects of steady backgrounds

To determine whether a spatial gradient of responsiveness also occurs during uniform steady background illumination, the cell was tested with the same bright local flashes superimposed on backgrounds of several intensities. Fig. 3*B*, which plots the response amplitudes normalized to the values in darkness, shows that the responsiveness was nearly uniform along the outer segment. This is despite the fact that at the highest intensity (□) the cell's mean steady response was about 70 % maximal, similar to that at 1–2 min in Fig. 3*A*.

Longitudinal density of dark current during recovery

Failure of the tip to respond to flashes after bleaching might result from disappearance of the dark current at the tip or from a failure of the distal disks to modulate the dark current. In each of several experiments of the kind illustrated in Fig. 1 a diffuse saturating flash applied during recovery from a bleach took the current to the same absolute level as did the bleaching light itself. Because a saturating light applied to a dark-adapted cell causes complete suppression of the inward dark current (Penn & Hagins, 1972; Baylor *et al.* 1979*a*), this result indicates that the reduction in saturating response amplitude after bleaching is due to suppression of the dark current rather than to failure of the disks to modulate the current.

To estimate the density of dark current as a function of position along the outer segment, the outer segment was drawn in and out to a series of positions while diffuse saturating flashes were given (see Baylor *et al.* 1979*a*, Fig. 6). Fig. 4 confirms that in the dark-adapted state (○, ●) the photocurrent rises approximately linearly with the length of outer segment drawn in, so that the density of dark current, given by

the slope of the line, was constant. This indicates that in the dark-adapted state each element of length of outer segment membrane contributes an equal component of dark current.

The remaining sets of symbols in Fig. 4 were obtained at the indicated times (approximately 5, 12 and 35 min) after a uniform bleach of about 15%. It is striking that for the points at 5 min no response at all could be obtained until the cell was drawn in to $d = 55$ μm, showing that only the most basal region responded, the dark

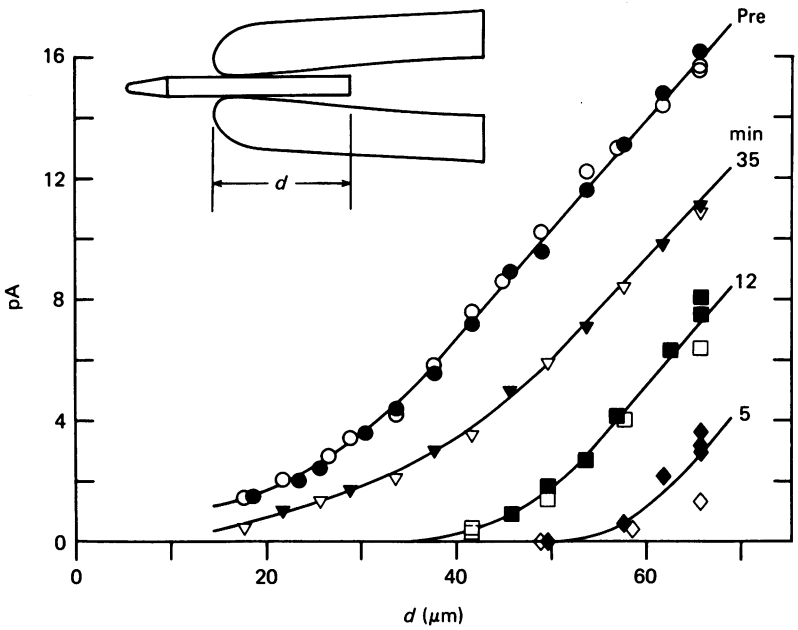

Fig. 4. Dependence of saturating response amplitude on length of outer segment drawn into the pipette. Symbols plot the peak response to a uniform saturating flash against the distance, d, from the tip of the pipette to the distal tip of the outer segment (see inset). Note that the length of outer segment effectively being recorded from is about $d-20$ μm, as the narrowest point of the pipette constriction corresponds to $d = 20$ μm (see Baylor *et al.* 1979*a*, Fig. 6). Open symbols were obtained first, as the cell was pushed out of the pipette, and filled symbols were obtained as it was subsequently drawn back in. Circles (○, ●) were obtained in the fully dark-adapted state, other symbols at approximately the indicated times after the bleach of about 15% (2×10^6 photons $\mu m^{-2} sec^{-1}$ for 5 sec, estimated to isomerize 9×10^7 rhodospins).

current being absent elsewhere. At later times the response began to appear when a lesser length had been drawn in, indicating that dark current had begun to flow in regions progressively further from the base. The curves drawn through the points all appear to have approximately the same slope in the region of $d = 60$ μm, when most of the outer segment was drawn in. This indicates that the current density in the responsive basal regions was roughly the same as in the dark-adapted state, while in the more distal regions no membrane current flowed. The conclusion is that after a uniform bleach recovery of the dark current proceeds longitudinally from the base to the tip of the outer segment.

Spatially restricted bleaches

Independence of effects of spatially separated bleaches

To test whether different parts of the outer segment recover independently or whether they interact after a bleach, the tip and base were bleached either alone or together, when the cell was either fully or only half drawn in.

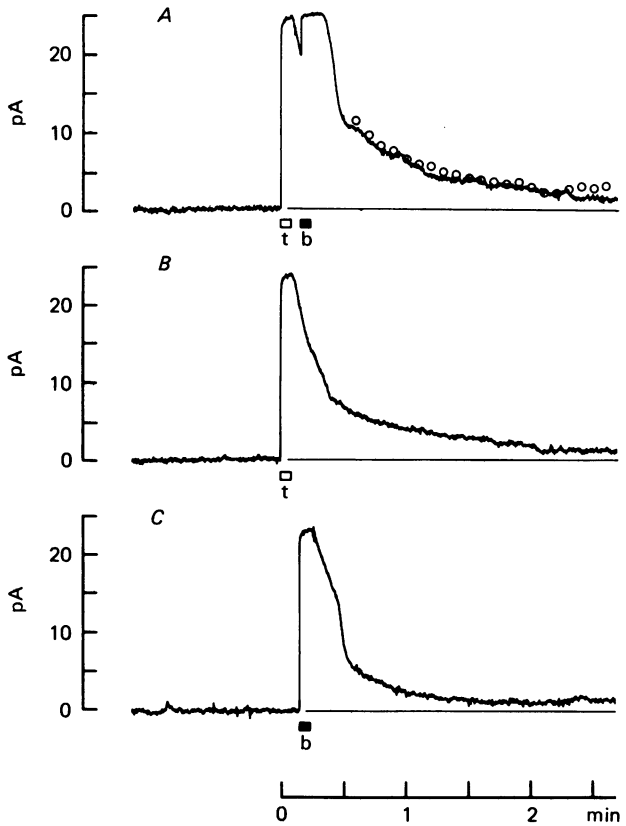

Fig. 5. Tip and base bleaches with outer segment fully drawn in. In *A* tip (t) and base (b) bleaches were delivered in succession; in *B* and *C* the same bleaches were delivered separately; circles in *A* are the sum of tails in *B* and *C*. Each bleach delivered 6×10^4 photons μm^{-2} sec^{-1} for 5 sec, estimated to bleach approximately 0·5 % locally. Records separated by 7 min each. Drift was less than 0·0005 pA sec^{-1} over 20 min, and has not been compensated, Bicarbonate Ringer, 23·0°C.

Outer segment fully drawn in. Fig. 5 shows the effect of tip and base bleaches (*ca.* 0·5 % locally) delivered one after the other (*A*) or separately (*B* and *C*) with an outer segment fully drawn in. In *C* the basal bleach has been slightly displaced in time to coincide with the basal bleach in *A*. Following an initial period of fairly rapid recovery each record exhibits a slowly recovering component at times of 30 sec or more after the bleach. The amplitudes of the separate components in *B* and *C* have been added to give the circles which provide a good fit to the recovery after the dual bleach in

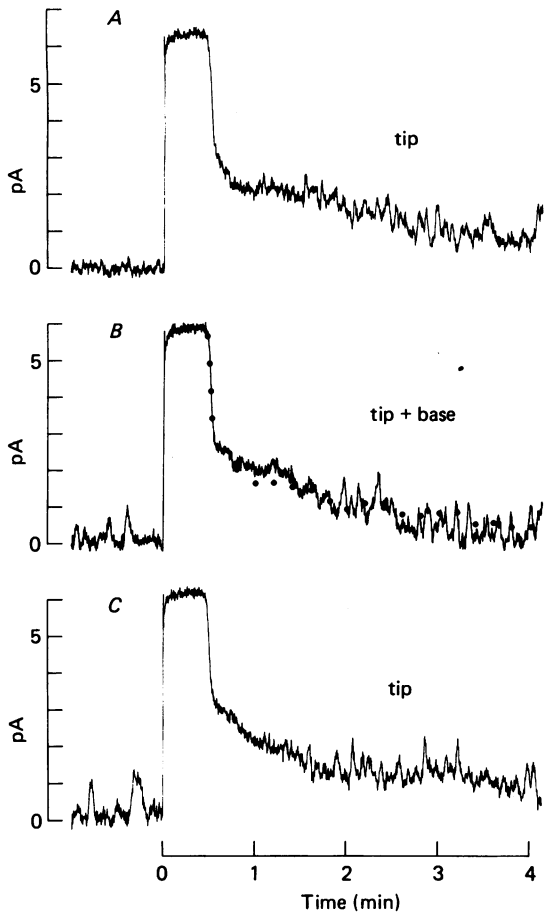

Fig. 6. Lack of effect of a basal bleach on recovery of the tip, with only the tip of the outer segment in the suction electrode. *A*, tip half of the outer segment bleached at time $t = 0$ with a 1 sec pulse of 500 nm light estimated to bleach 0·1 % locally. *B*, same tip bleach followed by a 0·5 % local bleach on the base half. *C*, tip bleach again as in *A*. Filled circles in *B* plot the average recovery in traces *A* and *C*. Saturating response amplitude with entire outer segment in electrode 19 pA, temperature 21·8°C, HEPES Ringer. Intervals between bleaches in *A*, *B*, and *C* approximately 6 min.

A. Thus, when the two separate bleaches were in the recorded region, the effect of the dual bleach was simply the sum of the effects of the separate bleaches, indicating that during recovery the separate regions acted independently of each other.

Outer segment drawn half-way in. When instead the outer segment was drawn half-way in, only the tip bleach was on the recorded region. An example of the behaviour is shown in Fig. 6. In traces *A* and *C* only the tip half of the cell was bleached, while in trace *B* the tip bleach was followed rapidly by a 4-fold stronger bleach on the basal half of the outer segment. The filled circles in *B* plot the average recovery in traces *A* and *C* and give a close fit to the response. Thus, addition of a basal bleach, although more intense than the tip bleach, caused no apparent

alteration in the recovery from the tip bleach. This again indicates that recovery of the tip proceeded independently of recovery in the base. Similar results were obtained from four other cells, with bleaches up to 2 %. Larger bleaches were not used because it was necessary to allow the cell to recover fully between bleaches.

In two cells addition of the base bleach caused a slight increase in the apparent rate of recovery at the tip, perhaps because additional hyperpolarization increased the driving force on the dark current at the tip. Consistent with this, the late recovery from a basal bleach alone showed a small underswing in these cells (see for example Fig. 10*B*).

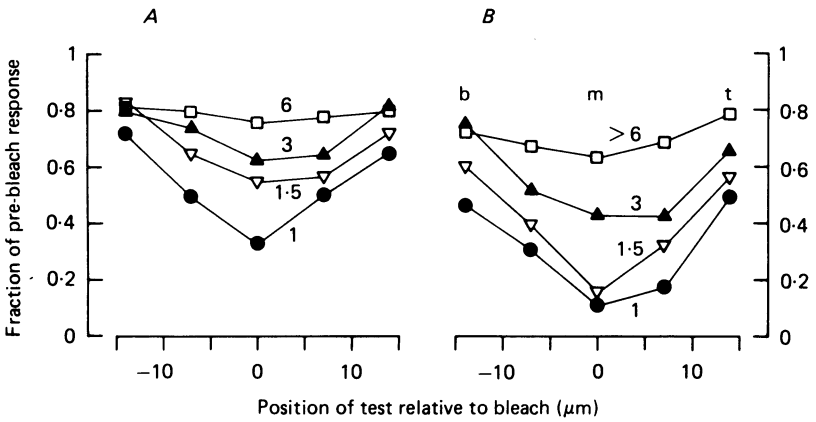

Fig. 7. Recovery of responsiveness following two local bleaches. Abscissa indicates position of 7 μm wide test slit relative to position of 7 μm wide bleaching slit placed near middle of outer segment (positive displacement is distal). Ordinate gives response as a fraction of the pre-bleach response at corresponding positions; pre-bleach amplitudes were: 7·5 pA, 9·1 pA, 9·1 pA, 9·0 pA and 7·3pA, from left to right; test flashes delivered 37 photons μm^{-2}, estimated to cause about 180 isomerizations. Bleaches delivered $8{\cdot}8 \times 10^5$ photons μm^{-2} sec^{-1}; *A*: 2 sec bleach, estimated to isomerize $8{\cdot}7 \times 10^6$ rhodopsins, or about 2·7 % locally; *B*: 5 sec bleach, presented 11 min after bleach in *A*, and estimated to isomerize $2{\cdot}2 \times 10^7$ rhodopsins, or about 6·8 % locally. Test flashes were given at 6 sec intervals and points were obtained from left to right (basal to distal); numbers near curves indicate time (in min) after bleach. Upper two sets of points in *A* and *B* are averages of 2–5 complete scans from left to right. The letters *b*, *m*, *t* indicate base, middle and tip positions used in Fig. 8.

Localization of effects of a spatially restricted bleach

The reduction in responsiveness induced by bleaching a narrow region near the middle of the outer segment is illustrated in Fig. 7 for bleaches of two strengths. The flash response, as a fraction of its pre-bleach value, is plotted against test position at a series of times after the bleach. In both cases the response at early times was less at the bleaching position than elsewhere: in *B* the initial reduction was to one tenth at 0 μm separation but only to about one half at 14 μm separation.

At successively later times all regions proceeded to recover and the 'notch' at the bleaching position became less apparent. The persistence of the notch up to at least 3 min after the bleaches shows that the reduction in responsiveness retained a

degree of spatial restriction over this period; the bleaching effect did not spread so widely as to cause uniform behaviour over the entire outer segment. Indeed, considering the finite widths of the bleaching and test flashes as well as light scatter, the profiles in Fig. 7 seem consistent with minimal longitudinal spread of the influence that reduces the dark current.

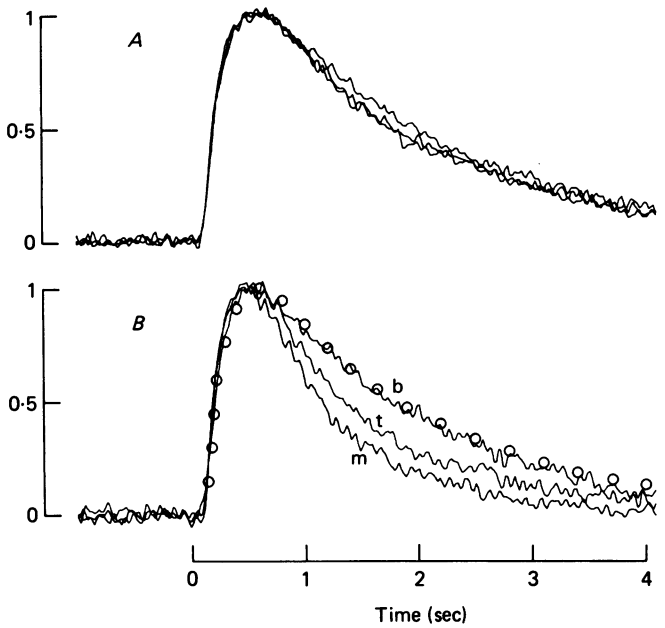

Fig. 8. Form of response to local flashes: *A*, in dark-adapted state; *B*, after bleach at middle of cell. Test flash positions at base, middle and tip (*b*, *m*, *t*) are indicated in Fig. 7. All responses have been normalized to unit height; test flash intensity constant, and same as in Fig. 7. Absolute response amplitudes at *b*, *m*, *t* respectively: *A*, 7·5 pA, 9·1 pA, 7·3 pA (four responses each); *B*, 5·4 pA, 5·8 pA, 5·6 pA (five responses each). Circles in *B* show average of responses in *A*.

Fig. 8 illustrates that the bleach in Fig. 7*B* caused a long-lasting change in response kinetics within the bleached region. Prior to the bleach (*A*) the shape of the normalized response to bright test flashes was independent of the test position, but after substantial recovery (*B*), at times greater than 6 min, the falling phases of the test responses differed significantly. The flash response at the position of the bleach (m) showed a faster falling phase than did responses from either the base or tip, even though the amplitudes at all three positions were about 75 % recovered (corresponding to symbols □ in Fig. 7*B*). At the most basal test position (b) the shape of the normalized response in Fig. 8*B* was indistinguishable from the pre-bleach responses (○), while at the two more distal positions the response had an accelerated recovery. Although the amplitude of the local dark current had nearly recovered, the kinetics of the processes that modulate the dark current were still affected.

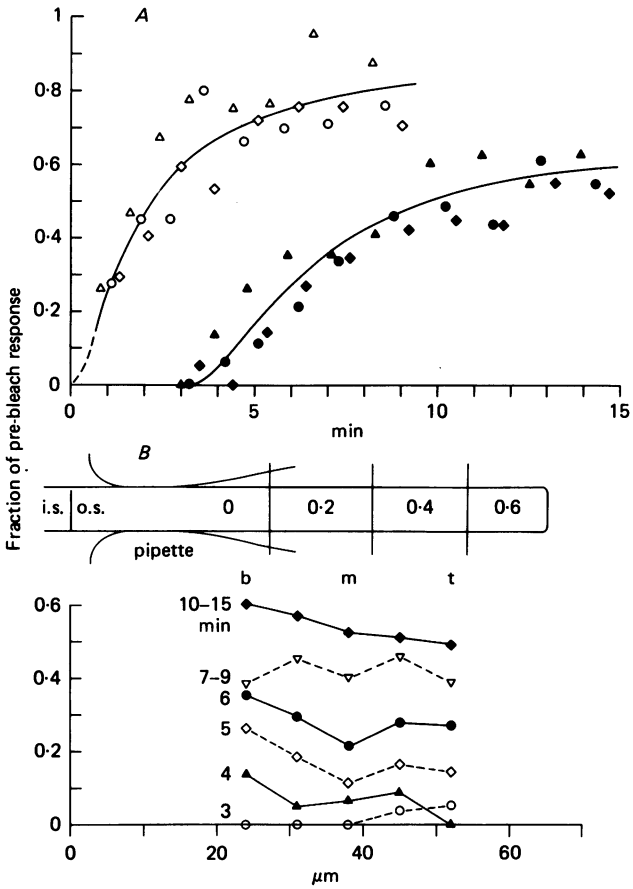

Fig. 9. Recovery of responsiveness following spatially graded bleaches. *A*, recovery as a function of time following two bleaches; open symbols: after 1·6 sec exposure estimated to bleach about 2·2 % at the basal end and progressively less more distally; filled symbols: after 6·4 sec exposure. Test position: △, ▲, base; ○, ●, middle; ◇, ◆, tip (see *B*). *B*, recovery as a function of test position following the more intense bleach in *A*. Prior to the two bleaches the test flashes gave responses of 8·8, 8·2, 8·0, 7·9 and 7·8 pA at the indicated positions (from left), and these values have been used to normalize all amplitudes in both *A* and *B*. Test stimuli 7 μm wide delivered 37 photons μm^{-2}, estimated to cause about 180 isomerizations per flash. Inset shows positions of density changes in bleaching filter (0, 0·2, 0·4, 0·6 log units).

Spatially graded bleaches

On p. 52 it was shown that for a uniform bleach recovery is slower in the more distal regions of outer segment. *In vivo*, however, light enters the outer segment axially and is attenuated exponentially with distance as it is absorbed, producing a graded bleaching pattern. To reproduce the physiological situation with transverse incidence requires an exponential wedge of appropriate density gradient (0·016 μm^{-1} for 500 nm light). As an approximation we used a discrete filter having 0·2 log unit density steps at intervals of approximately 12 μm referred to the retina (see Methods

and inset of Fig. 9). Results obtained with such spatially graded bleaches showed nearly synchronous recovery of the different regions, as illustrated in Fig. 9 which presents an experiment analogous to those in Figs. 2 and 3.

In Fig. 9 the recovery of the response to a bright narrow slit is plotted as a function of time following two spatially graded bleaches. For the filled symbols the bleach was four times greater than for the open symbols, and in both cases the three different symbols represent different test positions: △, ○, ◇ for base, middle and tip respectively, as in Fig. 2.

In contrast to the behaviour in Fig. 2 the time course of recovery does not appear to depend on test flash position, at either bleaching strength. Recovery at all positions following the smaller graded bleach (open symbols) is roughly comparable to the recovery of the base after the uniform bleach in Fig. 2, while recovery at all positions with the larger graded bleach (filled symbols) is roughly comparable to the recovery of the tip in Fig. 2. Hence the 4-fold increase in graded bleaching strength in Fig. 9 has much the same effect as movement of the test position 28 μm more distally in Fig. 2 with a uniform bleach. The results of Fig. 2 and 9 are therefore reasonably consistent internally, as the 28 μm displacement in the one corresponds to a density change of about 0·5 log unit, or 3-fold in the other.

The results from the larger graded bleach are replotted as a function of test flash position in Fig. 9*B*. This shows that at all times after the bleach the recovery is more spatially uniform than after the uniform bleach in Fig. 3*A*.

These results suggest that *in vivo*, with axial passage of 500 nm light, small bleaches such as these would be followed by roughly synchronous recovery of dark current in different regions of outer segment. Since the pigment absorption varies with wave-length, the pattern of recovery after axial bleaching lights at other wave-lengths would be expected to differ. For wave-lengths beyond about 580 nm, for example, weak axial bleaching lights should give nearly uniform bleaching patterns, with relatively slower recovery at the tip.

Spatial origin of the noise resulting from bleaching

It has recently been shown that following a small bleach the rod membrane current becomes noisy (Lamb, 1980). If the current noise arose from a generalized metabolic effect or if it reflected intracellular voltage noise arising elsewhere in the cell, then its apparent source would be distributed along the whole outer segment (e.g. Baylor *et al.* 1980). If instead the noise were closely linked to rhodopsin bleaching in the disks, then its source might be as spatially localized as the spread of excitation (Lamb *et al.* 1981). To investigate this we measured the noise and the saturating response with different lengths of outer segment drawn in (Baylor *et al.* 1980) following localized bleaching of either the base or the tip.

Fig. 10 shows the response to a base bleach when the region of outer segment sucked in was: *A*, the entire length, and *B*, only the tip. In *C* the noise variance from traces *A* and *B* is plotted, except during the steeply sloping period immediately after the bleach and at times when test flashes were delivered. A substantial noise increase is apparent from the whole cell (●), whereas no significant change can be seen from the tip (○). This shows that following a bleach at the base the noise originated in the base.

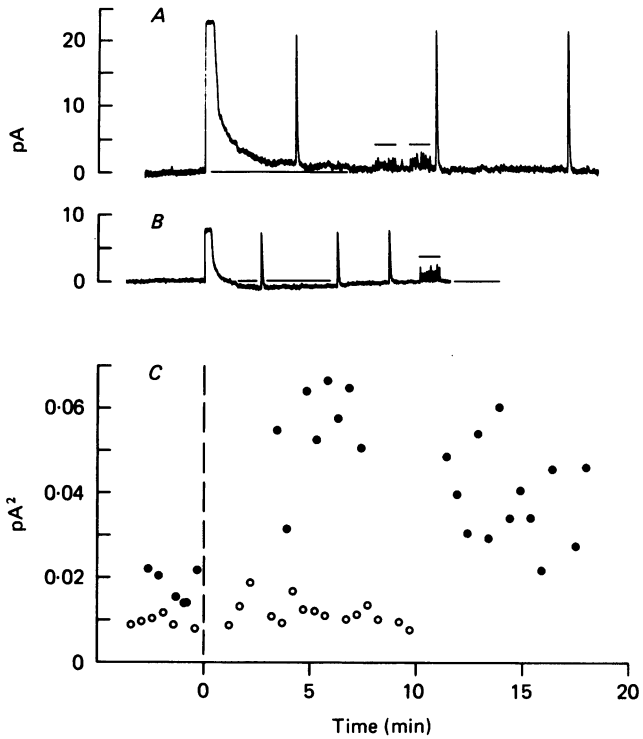

Fig. 10. Responses (*A* and *B*) and noise variance (*C*) following base bleaches. *A*, and ● in *C*: outer segment fully drawn in; *B*, and ○ in *C*: only tip drawn in. Bleach of approximately 1·4 % to the basal half was given at time zero in the two cases. Variance was measured over 25·6 sec records (256 points at 100 msec intervals) with the signal filtered from DC to 2 Hz; least-squares linear trend removal was used to eliminate DC drift from individual records. The three large responses in *A* and *B* resulted from bright flashes, and the bars indicate presentation of dim test flashes; variance could not be determined at these times. Bicarbonate Ringer; 23·7 °C; traces *A* and *B* corrected for drift of 0·002 and 0·001 pA sec^{-1}.

This phenomenon is shown in another way in Fig. 11*A*. The same cell was drawn in and out to several positions following two further base bleaches, and the symbols plot the noise variance as a function of saturating response in the two cases (○, ●). The interrupted horizontal line plots the expected thermal noise variance in the measured sealing resistance of the pipette, while the sloping continuous line is drawn by eye near the points in the right half of the Figure. This shows that in the bleached basal region of outer segment the noise variance rose approximately linearly with saturating response, a measure of length effectively drawn in; it also indicates that little noise was recorded from the tip. Now a linear variation of noise with saturating response is as expected for a uniformly distributed source of uncorrelated noise along the outer segment (Baylor *et al.* 1980). This experiment shows that only the bleached base exhibits substantially increased noise.

In another cell the tip was bleached, and the variation of noise as a function of saturating response is shown in Fig. 11*B*. In contrast to Fig. 11*A* the steeply sloping

region in this experiment corresponds to small saturating currents, representing the tip half of the cell, and shows that with a tip bleach the noise arises predominantly from the tip. We conclude that following a localized bleach the noise increase originates in the bleached region.

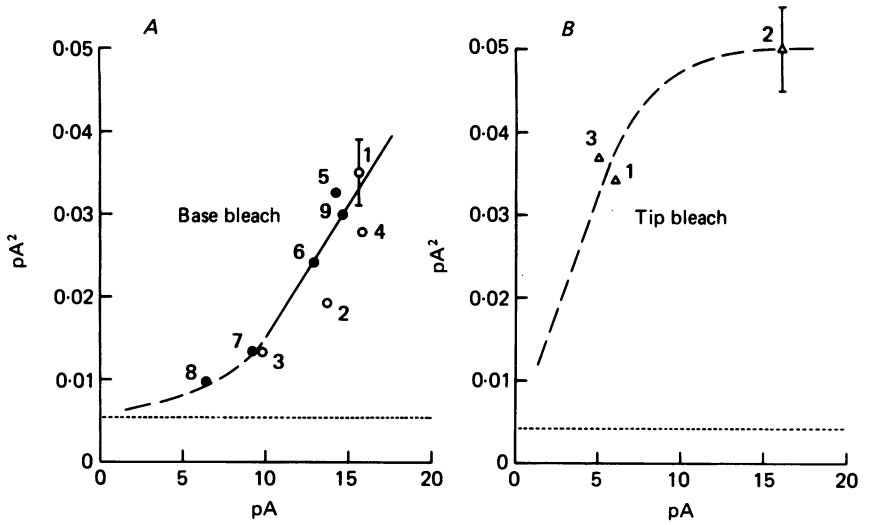

Fig. 11. Noise variance as a function of saturating response when outer segment was drawn in and out to various positions. *A*, following two further basal bleaches of 1·4 % in same cell as Fig. 10. Saturating response and noise had declined somewhat, as the records were obtained about 1½ hr after the first bleach in Fig. 10. *B*, following a tip bleach of approximately 1 % in another cell. Variance measurements from records 100 sec long (*A*) and 200 sec long (*B*) taken about 5–20 min after the bleaches, in the sequence indicated by the numbers near the symbols. Representative error bars show ±1 S.D. estimated from record length and signal band width. Dotted lines give expected thermal noise in measured seal resistance (*A*, 6 MΩ; *B*, 7·7 MΩ) over the band width used, DC-2 Hz. Curves drawn by eye according to form expected if the noise arose principally in the bleached region.

Capacitive currents in the bleached tip

A consequence of the elimination of dark current in a bleached region of outer segment is that the surface membrane should act as a capacitance in parallel with any remaining leakage conductance. In this section we examine the current in the bleached tip induced by intracellular voltage changes generated in the unbleached basal region.

In Fig. 12 the responses in the left column were obtained in the fully dark-adapted state, and those in the right column after bleaching the tip of the outer segment. All test stimuli were bright flashes illuminating only the basal part of the outer segment. In the upper row, *A*, the outer segment was fully within the pipette, and basal stimuli gave normal responses. In the second row, *B*, the pipette was drawn back so that only the tip of the outer segment remained inside, and in this case rapid transient responses were obtained.

The right-hand trace of this pair (*B*), recorded from the bleached tip, represents

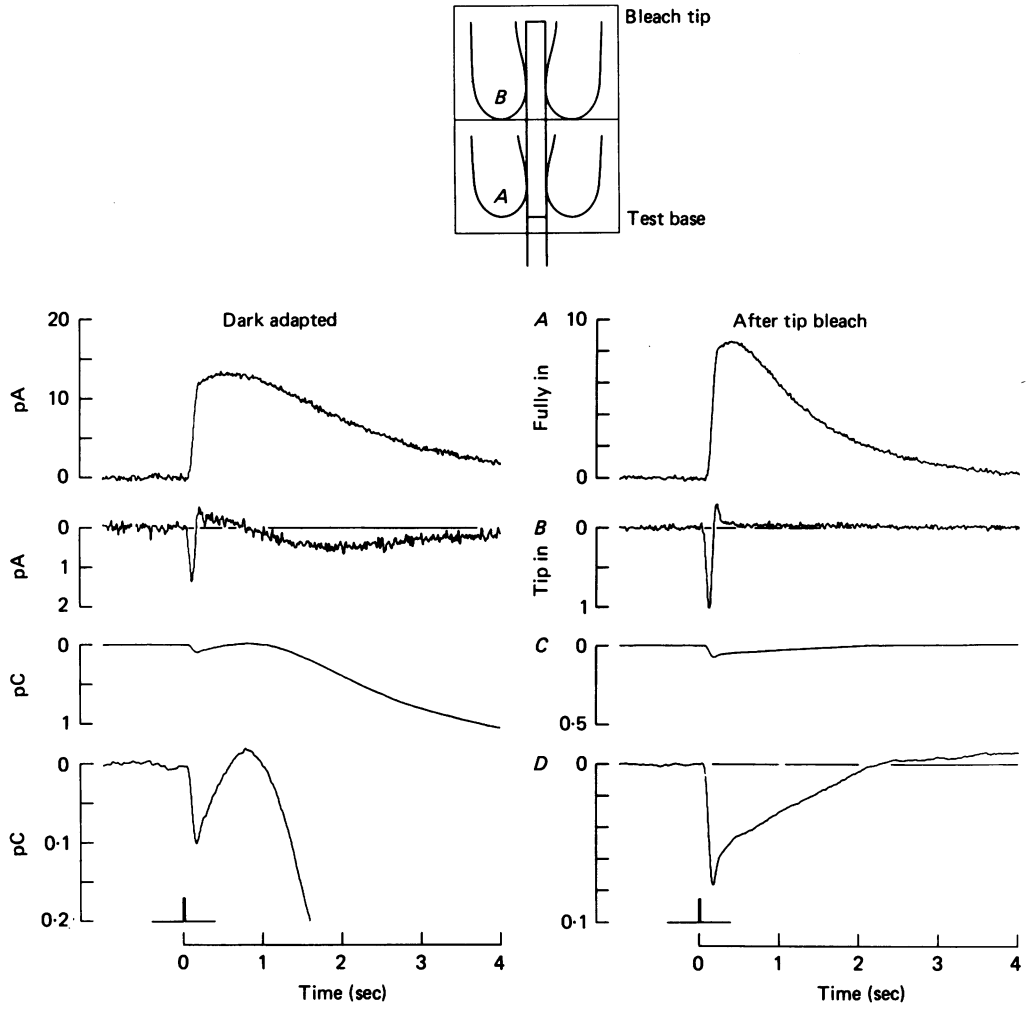

Fig. 12. Charge movement across bleached and unbleached membrane. Responses on left were obtained from dark-adapted cell; those on right following bleaching of the tip. All stimuli were basal flashes delivering 38 photons μm^{-2}, and were estimated to isomerize 950 rhodopsins per flash. In row *A* the outer segment was fully drawn into the pipette; in row *B* only the tip was drawn in, but the pipette was moved up so that the outer segment was in the same position relative to the light stimulus (see inset). Rows *C* and *D* show time integral of row *B* at two vertical gains; for this computation the zero level in *B* was taken as the average over the period of 1 sec immediately preceding the flash (see Methods). Traces were averaged from the following numbers of sweeps: left, *A*, 2; *B*, 10; right, *A*, 20; *B*, 216. The 216 responses were obtained in groups of about 60 at 6 sec intervals following four succesive bleaches. Each bleach delivered $9{\cdot}0 \times 10^5$ photons μm^{-2} sec^{-1} for 1 sec to the most distal 35 μm of outer segment, and was estimated to isomerize about $2{\cdot}2 \times 10^7$ rhodopsins, or about 1·5% of the local concentration of rhodopsin. Ordinate scales on left and right (before and after the bleach) differ by a factor of 2. Drift was less than 0·0002 pA sec^{-1}.

the local membrane current generated by the intracellular voltage change. If this current were purely capacitive it would be proportional to the time derivative of the intracellular voltage, and its time integral would have the form of the voltage response. This integral has been computed in *C* and *D* at two vertical gains, and has roughly the form which would be expected for the voltage response, with a rapid rise, a distinct 'nose' and subsequent recovery to near the initial level. This recovery takes about 2·5 sec, similar to that of the photocurrent in the base induced by the same stimulus (*A*), and the two curves bear the qualitative relationship expected between voltage and current in this preparation (Nunn, Matthews & Baylor, 1980). The extent of final recovery of the integrated curve is important: a return to the original level indicates a total charge movement of zero, as expected for a pure capacitance, while any residual conductance would cause a net charge movement over the cycle.

The trace in *B* was averaged from more than 200 responses, given as groups of about sixty at 6 sec intervals following four successive tip bleaches of about 1·5%. This procedure was employed to ensure that the tip remained unresponsive during the tests, and control flashes presented to the tip showed that this was so. At later times, some 10 min after the bleaches, gradual recovery of the tip began to occur.

It is possible to estimate the membrane capacitance and to put an upper limit on the residual conductance from these measurements by comparing the charge movement (curves *C* and *D*) with the expected intracellular voltage response. We estimate the peak voltage response to have been of the order of 15 mV, from both the form of the response and the fact that only half of the outer segment was responsive, while Fig. 12*D* (right) shows the peak charge movement to have been 0·08 pC. From the equation for the charge on a capacitor

$$\Delta q = \int_o^t i \, dt = C\Delta V, \tag{1}$$

where the symbols have their usual electrical meaning, the capacitance of the tip membrane would have been $C = 0{\cdot}08$ pC/15 mV = 5 pF. The exact length of outer segment effectively within the pipette is difficult to estimate because of the distributed nature of the pipette constriction. The total length of the outer segment was about 70 μm, and in the fully and partly drawn in configurations we estimate that about 50 and 20 μm respectively were effectively inside the pipette. The value of 20 μm together with the outer segment diameter of 6 μm gives a plasma membrane surface area of about 400 μm^2, so that the specific membrane capacitance would be 1·3 μF cm^{-2}. This value relies heavily on the estimates of intracellular voltage change and membrane area and can only be considered approximate.

The net charge movement after 4 sec in Fig. 12*D* (right) is less than 0·01 pC. If a residual conductance had remained, then the hyperpolarization of the membrane would have been expected to lead to a *negative* net charge movement, and the small positive value is probably the result of very slight drift. On the assumption that the net charge movement is not larger than −0·01 pC and that the time integral of the voltage response was of the order of 10 mV sec then the residual conductance can be found from

$$\Delta q = \int_o^t i \, dt = g \int_o^t V \, dt \tag{2}$$

to be $g < 0{\cdot}01$ pC/10 mV sec or $g < 1$ pS. This indicates that the leakage resistance is greater than 10^{12} Ω, and that the specific resistance is greater than 4 MΩ cm². This extremely high specific resistance suggests that no ionic channels of any kind remain open in the bleached state.

The form of responses obtained in dark-adapted conditions is different (Fig. 12, left). The transient current from the tip induced by a basal flash (*B*) has a large negative component at times around 2 sec. Analysis of this trace is complicated by the fact that light scattered from the base inevitably stimulated the tip and directly caused an outward (positive) component of recorded current. This would be expected to have had a time to peak of less than 1 sec and it presumably accounts for the form of the late inward current component being different from the form of the presumed voltage response. The time integral of this curve is shown in Fig. 12*C* and *D* (left), and there is clearly a net inward charge movement of at least 1 pC, two orders of magnitude greater than obtained when the tip had been bleached. Allowing for the direct light response, the net charge movement induced by the intracelullar voltage change may have been greater than 2 pC.

Leaky cells

The net charge movement in the bleached tip was close to zero in Fig. 12*D* but this was not so in all cells. Fig. 13 shows a more typical example. The current transient in *A* has been integrated in *B* and shows a net charge movement of about −0·12 pC. In the 2 sec following the initial peak there is a charge movement of about −0·08 pC, indicating an average current during this time of roughly −0·04 pA or about 0·5 % of the normal saturating current from the tip half of the cell. Despite the small size of this current its effect on the integral is substantial. Assuming that this current represents a leakage component induced by the intracellular voltage change, and that the leakage resistance is constant, the total current i would be

$$i = C\,\mathrm{d}V/\mathrm{d}t + V/R. \tag{3}$$

Writing $q = CV$ for the charge on the capacitive element and $\tau = RC$ for the membrane time constant in the bleached state, this equation may be rewritten as

$$i = \mathrm{d}q/\mathrm{d}t + q/\tau. \tag{4}$$

Equation (4) may be integrated to give the charge on the capacitor, and hence the voltage, if the time constant τ is known. The appropriate time constant was estimated by requiring firstly that with the brightest flashes the voltage response should show a horizontal plateau after the initial peak, and secondly that the net charge movement on the capacitive element be zero over the cycle, as the voltage returns to its original value. A first estimate for τ was obtained by extrapolating the linearly rising region in Fig. 13*B* back to zero charge, and taking the magnitude of the time intercept. For Fig. 13 a value of $\tau = 1$ sec satisfied these conditions, and the time course of the charge q on the capacitive element calculated from eqn. (4) is shown as the continuous curve in Fig. 13*C*. This has the form expected for the intracellular voltage response to a bright flash.

We also performed this experiment with a flash intensity four times lower, and the dashed curve in Fig. 13C plots the calculated charge movement for the same time

constant. This curve again has qualitatively the expected form, with a later peak and earlier return from the plateau level, providing further support for the value of $\tau = 1$ sec in this cell. In three other cells time constants of 1, 1·5 and 2 sec were estimated to best describe the capacitive transient data.

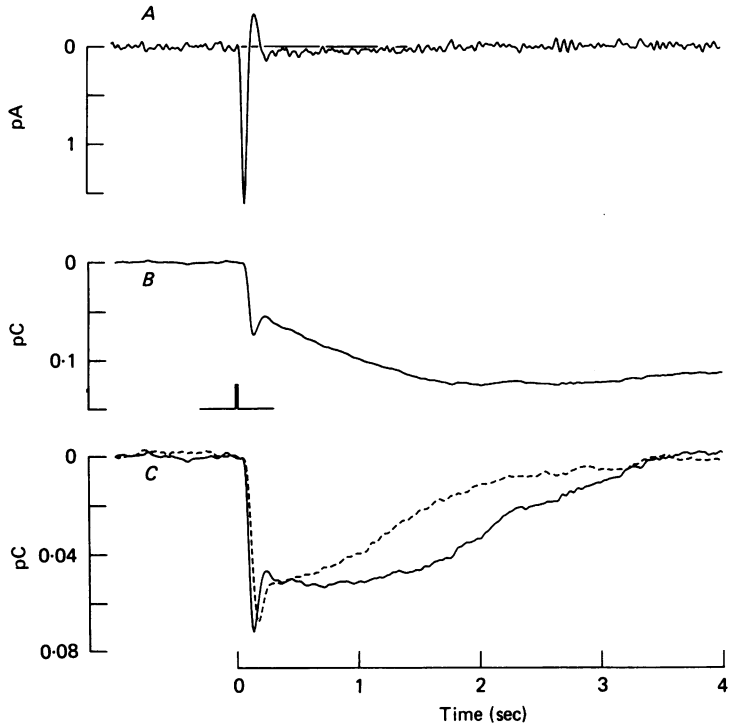

Fig. 13. Charge movement in 'leaky' case, with only the tip of the cell drawn into the pipette. The tip was bleached and flashes were delivered at the base. *A*, transient current response to bright flashes. *B*, time integral of *A*. *C*, continuous: 'leaky' integral of *A*, with $\tau = 1$ sec. Flashes delivered 570 photons μm^{-2}, estimated to cause about $1{\cdot}4 \times 10^4$ isomerizations; average of 101 responses; traces corrected for measured drift of $-0{\cdot}002$ pA sec^{-1}. *C*, dashed curve: as continuous curve, except flash intensity reduced by a factor of 4; average of 141 responses. HEPES Ringer; saturating response: fully drawn in, 16 pA; with only tip in, 6 pA.

The membrane capacitance and resistance can again be calculated from the estimated peak voltage change, but another method when the time constant has been found explicitly is to assume a specific membrane capacitance of 1 μF cm^{-2}. Then, from $\tau = 1$ sec, the specific membrane resistance would be 1 MΩ cm^2. Although this is a large value it may nevertheless represent a lower limit for the true resistivity, as it is possible that some membrane damage occurred in drawing the cell into the pipette.

Capacitive current during steady illumination

If the light-sensitive channels were closed by bright steady illumination rather than a bleach, one would expect qualitatively similar capacitive transient currents.

However for technical reasons the experiment is more difficult. To obtain a reliable average transient it is necessary to present a large number of test flashes and the bright steady light must be applied for a considerable time, causing a certain degree of bleaching. A more serious problem is that scatter from the very bright light at the tip causes a considerable direct response in the base, so that superimposed basal

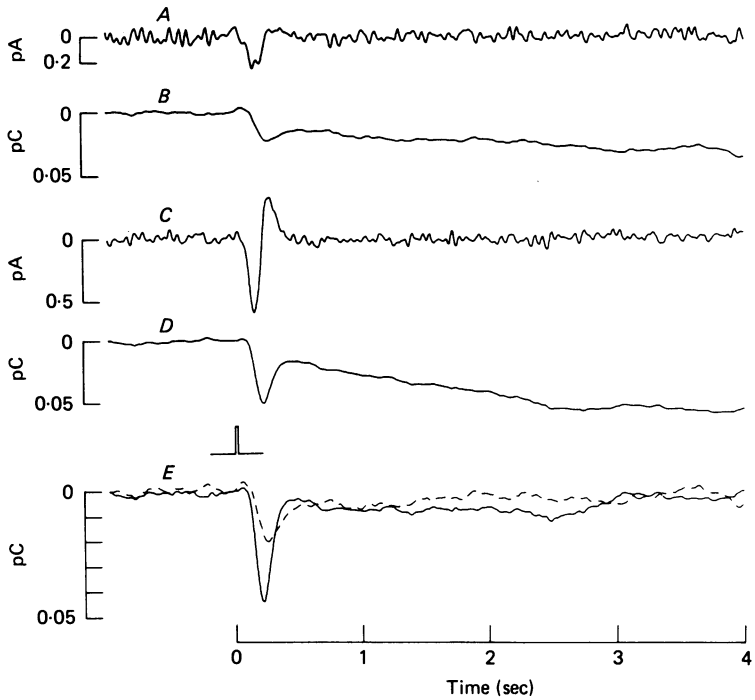

Fig. 14. Charge movement with steady light on tip of outer segment. *A*, average response to base flashes delivering approximately 300 isomerizations; sixty trials. *B*, time integral of *A*. *C*, average response to base flashes about four times brighter, delivering approximately 1200 isomerizations per flash; ninety trials. *D*, time integral of *C*. *E*, 'leaky' integrals of traces *A*, (interrupted curve) and *C* (continuous curve), with $\tau = 500$ msec. Steady light on tip delivered 70 photons μm^{-2} sec^{-1} (approximately 1000 isomerizations sec^{-1}); initially the tip current was completely saturated (5·5 pA), but after a few minutes about 25 % had recovered, as saturating responses of approximately 1·4 pA were obtained. The steady light was on for a total of 25 min, and was calculated to bleach less than 0·2 % of the rhodopsin in the tip. Traces *A* and *C* were filtered DC to 20 Hz, and have been corrected for the measured drift of 0·005 pA sec^{-1}. Bicarbonate Ringer, 20·8 °C

flashes can elicit only small currents and therefore small voltage changes. If, instead the background light is too dim and the dark current in the tip is not completely suppressed, scattered light from the basal test flash may cause a direct response from the tip in addition to the capacitive transient.

Fig. 14 shows an experiment in which a compromise was achieved. The steady light on the tip, although bright enough to suppress its dark current initially, was not bright enough to maintain the suppression. After a minute or so about 25 % of the current had recovered, as saturating responses of 1·4 pA could be obtained, compared with 5·5 pA before the light was applied. Scattered light to the base was not a limitation,

as good transients were obtained (e.g. Fig. 14*C*), nor was bleaching significant, as the 25 min of illumination was calculated to bleach less than 0·2 % of the rhodopsin in the tip.

Traces *A* and *C* in Fig. 14 show the average transients obtained with flashes delivering about 300 and 1200 isomerizations to the base; traces *B* and *D* are the time integrals of *A* and *C* respectively. These integrals show sloping regions at times from about 0·5 to 3 sec after the stimuli, suggesting the existence of a leakage component. In *E* the 'leaky' integral has been calculated from traces *A* and *C*, with $\tau = 500$ msec. These curves show no significant net charge movement and are qualitatively of the form expected for the intracellular voltage change. This suggests that 500 msec is a reasonable lower limit for the value of τ in this experiment. As this value was obtained with the tip current reduced only to 25 %, one would expect to measure a considerably longer time constant with total saturation, if the effects of light scatter could be avoided.

DISCUSSION

After the outer segment's dark current had been eliminated by a uniform bleach, recovery began at the base, and the front of dark current advanced towards the tip. An obvious explanation for this pattern of recovery would involve the longitudinal diffusion of a substance inside the cell. Supposing the rate of recovery to be limited by the local internal concentration of a substance synthesized in the inner segment, a bleach at the base should reduce the supply of material to the tip and slow its recovery. The finding that recovery of the tip proceeded independently of bleaching at the base seems to rule out this explanation and suggests instead the importance of intrinsic differences in the transduction machinery at different longitudinal positions. It is well known that the distal disks in a rod are older than their counterparts at the base (Young, 1976); perhaps associated with this is the finding of a longitudinal gradient of rhodopsin phosphorylation in disks along the outer segment (Shichi & Williams, 1979). It seems attractive to suppose that the gradient of bleaching recovery rates observed here, as well as the previously observed gradient in the speed of the linear response (Baylor *et al.* 1979*a*), may be connected in some way with the age of the disks.

Following a localized bleach the dark current was reduced most strongly near the bleaching location, and the 'notch' in the dark current profile lasted for at least 3 min. This indicates that the bleaching effect is confined to a region near the bleached disks, any spread of the effect being quite limited. Supporting evidence for the spatial restriction of the bleaching effect is provided by the existence following a uniform bleach of a steep longitudinal gradient of dark current. In both experiments, extensive spread would abolish gradients and produce a uniform dark current. Restricted spread was also found for the current noise induced by bleaching, to the extent that the fluctuations arose in the half of outer segment that had been exposed to the light.

Our experiments do not provide a quantitative measure of the degree of spread, although when allowance is made for the finite width of the bleaching and test stimuli and for light scatter, it would seem likely that the effects are highly localized. In

particular the results are probably consistent with the hypothesis that the primary effects of bleaching are restricted to the disks containing isomerized rhodopsins, any spread resulting from longitudinal diffusion of the internal transmitter. Such longitudinal spread has recently been measured by Lamb *et al.* (1981) to have a length constant of about 6 μm in the steady state. We cannot exclude the existence of widespread effects due to general metabolic changes (such as reduction in ATP levels), but these may be of minor importance with the small bleaches (less than 5%) used here.

The rods in these experiments were detached from the pigment epithelium and there was probably little if any regeneration of the bleached rhodopsin (see Grabowski & Pak, 1975). In the presence of pigment regeneration in the intact eye the kinetics of recovery and the spatial patterns of the bleaching effects might differ from those observed here.

It is well established that bleaching reduces both the amplitude of the saturating response and the sensitivity to dim flashes (see references in the Introduction). This paper is concerned mainly with recovery of the local saturating response. We have estimated this parameter from the amplitude of the response to a bright local flash; but, because of possible complications (light scatter to adjacent regions, failure to cause complete local saturation), we have referred to the measured large-signal parameter as 'responsiveness'.

When the light-sensitive conductance at the tip of the outer segment was blocked by a bleach or by a bright steady light, the plasma membrane behaved as an almost pure capacitance. The estimated membrane time constant was at least 1 sec, so that if the specific capacitance were 1 μF cm^{-2} as in other cells (Cole, 1940), the specific resistance must have at least 10^6 Ω cm^2. This is about three orders of magnitude higher than the specific resistance of resting nerve membrane.

The ionic channels of axonal and post-synaptic membranes have open state conductances of 1–30 pS, so that the density of such channels would have to be less than one per 100 μm^2. As the surface area of a toad rod outer segment is of the order of 1000 μm^2, there could be no more than a few such channels open when the dark current is suppressed. This calculation does not exclude the possible existence of voltage-sensitive channels that conduct in the dark-adapted state and close when the cell hyperpolarizes. Such channels could only be partially closed, however, because the steady hyperpolarization generated by bleaching half the outer segment should be 10 mV or less. This would be inconsistent with a large population of the channels. The implication is that the plasma membrane of the outer segment contains few if any ion channels (pores or carriers) other than those that admit the dark current.

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