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RESPONSE PROPERTIES OF CONES FROM THE RETINA OF THE TIGER SALAMANDER

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SUMMARY

1. Spectral sensitivity measurements using the suction electrode technique reveal three types of cone in the retina of the tiger salamander, showing maximum sensitivity at wavelengths 610 nm (red-sensitive cone), 444 nm (blue-sensitive cone) and below 400 nm (UV-sensitive cone).

2. The absolute sensitivities of red- and blue-sensitive cones to flashes of optimal wavelength are 0·022 and 0·33 pA photon^{-1} μm^2 respectively.

3. The time-to-peak of the dim flash response and the recovery of membrane current after a flash of any intensity are fastest in red-sensitive and slowest in blue-sensitive cones.

4. In blue- and UV-sensitive cones the flash response peaks progressively earlier as the flash strength is increased, as in rods. In red-sensitive cones, however, bright flash responses take longer to peak than dim flash responses.

5. In all three cone types, voltage clamping at -40 mV reduces the time-to-peak of the response to a bright flash, showing that the rising phase of the bright flash response is normally limited by the time constant of the cell. Under voltage clamp, all cones show a decrease in time-to-peak with increasing flash intensity.

6. Voltage clamping red-sensitive cones reveals two components of the rising phase of the response to a bright flash. Most of the current is rapidly suppressed by a bright flash, and represents the closure of light-sensitive channels. The residual current decays with a mean time constant of 20 ms, and is probably attributable to the decline of electrogenic Na^+–Ca^{2+}, K^+ exchange. The amplitude of this exchange current suggests that the proportion of the dark current carried by calcium ions is greater in red-sensitive cones than in rods of the same species.

7. In UV-sensitive cones, a prominent oscillation of light-sensitive current is observed during the recovery from flashes of intermediate intensity. A similar, but slower and less prominent oscillation is usually seen in blue-sensitive cones.

8. When a red-sensitive cone is voltage clamped an oscillation similar to those in the other two cone types is revealed. An underswing of up to 2 pA is also observed after recovery from intermediate or bright flashes in the majority of red-sensitive cones, and voltage clamping increases the amplitude of this underswing. These observations demonstrate that the oscillations and underswing are not caused by

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changes in membrane potential and must therefore reflect oscillations in the cytosolic concentration of cyclic GMP.

9. Rapid solution change experiments indicate that the selectivity of the light-sensitive channel for monovalent cations is different in the rod and the red-sensitive cone.

10. About 60% of the membrane current remains after replacement of external sodium ions with an impermeant cation, and this residual current is abolished by removal of calcium ions.

INTRODUCTION

Rods from the larval form of the tiger salamander have been extensively studied, partly because they are large, robust, and operate normally when isolated from the rest of the retina. The cones of this species share these advantageous features, and in this study we extend many of the experiments performed on rods to salamander cones. We wished in particular to examine calcium regulation in cones and the ionic selectivity of the cone light-sensitive channel. As a first step we sought to classify the cones by spectral sensitivity, since cones of different spectral sensitivity may also differ in their other properties.

The absorption spectrum of cones from the salamander has been studied by microspectrophotometry, and was found to be identical to that of cones from the tadpole, peaking at 620 nm (Liebman & Entine, 1968; Liebman, 1972). An action spectrum has also been obtained, using the technique of intracellular recording, and was broadly consistent with Liebman & Entine's absorption spectrum (Attwell, Werblin & Wilson, 1982). The suction electrode technique (Baylor, Lamb & Yau, 1979) allows a much fuller study of spectral sensitivity in the cones of this species since flash responses can be obtained from a single cone for several hours. We have used this technique to obtain full spectral sensitivity functions (within the wavelength range 400–770 nm) of twenty salamander cones. These experiments revealed three types of single cone in the salamander: a red-sensitive cone and two less common types showing maximum sensitivities to blue and UV light.

Each type of cone gives flash responses which are characteristically different in form, and the responses of all three are different from those previously obtained in rods. Results from voltage-clamped cones suggest, though, that the phototransduction mechanism is qualitatively similar in all of these photoreceptors, and that many of the differences arise from the more rapid response time course in cones. The much higher rate of calcium turnover in cones is one important factor in the more rapid time course of the cone response. A second quantitative difference between rods and cones is in the proportion of the light-sensitive current carried by calcium, which appears to be about twice as great in the red-sensitive cone as in the rod, probably because of a difference in the ionic selectivity of the light-sensitive channel. Preliminary reports of some of these experiments have appeared (Craig & Perry, 1988; McNaughton & Perry, 1989).

METHODS

Solutions

Cells were bathed in Ringer solution of composition 110 mM-NaCl, 2·5 mM-KCl, 1·0 mM-$CaCl_2$, 1·6 mM-$MgCl_2$, 10 mM-HEPES, neutralized to pH 7·6 with tetramethylammonium hydroxide

(TMA-OH); 3 mM-glucose was included where stated. Whole-cell pipettes were filled with a pseudo-intracellular solution which contained 110 mM-potassium aspartate, 3 mM-$MgCl_2$, 1 mM-Na_2ATP, 1 mM-Na_2GTP, 10 mM-PIPES (piperazine-*N*-*N'*-bis(2-ethanesulphonic acid)), neutralized to pH 7·2 using KOH. The liquid junction potential between this solution and Ringer solution is −14 mV (Lagnado & McNaughton, 1988); all the membrane potentials which we quote, whether applied under voltage clamp or measured under current clamp, have been corrected appropriately. In solution change experiments the cone outer segment was first exposed briefly to a solution from which KCl had been omitted (the pre-test solution, of composition 112·5 mM-NaCl, 1·0 mM-$CaCl_2$, 1·6 mM-$MgCl_2$, 10 mM-HEPES, neutralized to pH 7·6 with TMA-OH). To obtain a series of test solutions for the investigation of ionic selectivity of the light-sensitive current, the 112·5 mM-NaCl in the pre-test solution was replaced with 112·5 mM of LiCl, KCl, RbCl, CsCl, choline chloride or tetraethylammonium (TEA) chloride. Zero-calcium (0 Ca^{2+}) test solutions were made by omitting calcium and adding 2 mM-ethyleneglycol-bis-(β-aminoethylether)*N*,*N'*-tetraacetic acid (EGTA), and 0 Ca^{2+}, 0 Mg^{2+} solutions were made by omitting calcium and magnesium and adding 2 mM-ethylenediaminetetraacetic acid (EDTA). Experiments were also performed with either 100, 200 or 500 μM-3-isobutyl-1-methylxanthine (IBMX) in both the pre-test and test solutions. All experiments were conducted at room temperature (18–22 °C).

Isolation procedure and suction electrode recording

Tiger salamanders (*Ambystoma tigrinum*, Lowrance Waterdog Farms, Tulsa, OK, USA) were dark adapted for at least an hour before experiments. The methods for preparing electrodes, for isolating photoreceptors and for viewing them under infra-red illumination were similar to those described by Hodgkin, McNaughton & Nunn (1985). To facilitate the use of high-power objectives with the inverted microscope the floor of the recording chamber consisted of a cover-slip which had been coated with tri-*n*-butyl chlorosilane to prevent cells from adhering to it.

Cones were drawn into a suction pipette of internal diameter 5–8 μm, either with the outer segment in the pipette (for experiments on spectral sensitivity or for voltage-clamp experiments) or with the inner segment in the pipette (for experiments requiring rapid changes in the solution bathing the outer segment). The apparatus for recording the membrane current was as described by Hodgkin, McNaughton, Nunn & Yau (1984). The suction pipette technique was used to examine the full spectral sensitivity of twenty cones between the wavelengths 400 and 770 nm. In a further forty cones the spectral sensitivity was briefly checked near the peak wavelength and flash responses over a range of intensities were recorded using the optimal stimulating wavelength, or white light in the case of UV-sensitive cones, to give a response family (see Fig. 3). In seventy more cones the spectral sensitivity near the peak wavelength was briefly checked in order to establish the cone type prior to solution change experiments (see below).

Outer segment membrane current was recorded on a Racal 7DS FM tape-recorder with bandwidth 0–312 Hz. For later analysis signals were digitized off-line with a PDP 11-73 computer and a CED 502 laboratory interface. Sampling frequency was 400 Hz for experiments involving flash responses and 200 Hz for solution-change experiments; signals were low-pass filtered to half the sampling frequency before digitizing. Signals were further smoothed by convolution with a Gaussian filter of standard deviation 5 ms.

Optical stimuli

The system for optical stimulation was similar to that of Baylor & Hodgkin (1973). Monochromatic stimuli were produced using nineteen interference filters, which were calibrated for peak wavelength transmitted and for half-bandwidth (*ca* 20 nm) using a Varian DMS 90 UV/Visible spectrophotometer. Each filter was carefully checked for transmission at wavelengths other than the peak; no such transmission was detected. A constant duration of test flash between 10 and 40 ms was set for each cell, depending on its absolute sensitivity and on the relative spectral output of the light source.

Variation in the intensity of the light source was achieved by attenuating the beam with neutral density filters. These filters were not quite ideal (i.e. wavelength independent) in their behaviour, and in addition the output of the quartz–halogen light source varied with wavelength. A calibrated photometer (United Detector Technology, 111A) was therefore used to measure the power of the stimulating light source at the bath with each combination of filters (neutral density and monochromatic) in position. The intensity of light (photons μm^{-2} s^{-1}) at the floor of the bath was calculated in each case.

Determination of absolute and spectral sensitivities

In all cones tested the form of the flash response was found to be independent of the stimulating wavelength (the principle of univariance, Naka & Rushton, 1966), and therefore the spectral sensitivity of each cone type was determined by the methods of Baylor, Nunn & Schnapf (1984, 1987; see also Baylor & Hodgkin, 1973). The reference wavelengths were 400, 455 and 600 nm for the UV-, blue- and red-sensitive cones respectively.

For dim flashes, the amplitude of the response was found to be proportional to the flash strength. The sensitivity of a cone to flashes of optimal wavelength, S_F (in pA photon^{-1} μm^2), is therefore defined by eqn (1):

$$S_F = \frac{R}{F}, \tag{1}$$

where R (in pA) is the peak amplitude of the response to a dim flash of strength F (in photons μm^{-2}, cf. Baylor & Hodgkin, 1973). R is obtained by multiplying the response amplitude recorded by the suction electrode by 1·8 to correct for current lost through the seal (see Results and Fig. 5).

The theoretical amplitude of the response to a single photoisomerization, S_Φ (in pA photoisom.$^{-1}$) is given by

$$S_\Phi = \frac{S_F}{A}, \tag{2}$$

where A is the effective collecting area. Values for A were calculated from the measured mean volumes, assuming an optical density at the optimal wavelength of 0·012 μm^{-1} (Harosi, 1975) and a quantum efficiency of 0·67 (Dartnall, 1972). Finally, this value S_Φ can be divided by the total dark current to give S_Φ^f (in photoisom.$^{-1}$), i.e. the fraction of dark current suppressed by a single photoisomerization.

Recording under whole-cell voltage clamp

Bright flash responses or complete response families were obtained under voltage clamp in ten cones. Voltage clamping was achieved by a method similar to that applied to red-sensitive cones by Cobbs, Barkdoll & Pugh (1985), but without cyclic GMP in the pipette. Whole-cell pipettes were pulled in two stages on a BB-CH puller (Mechanex, Geneva) to give bubble numbers in methanol of 5·4–5·6. Recordings were made with a patch clamp amplifier (Model 8900, Dagan Instruments, Minneapolis, MN, USA) with a 10 GΩ feedback resistor in the head stage.

Before voltage clamping, the cone was held with the outer segment in a suction pipette. The spectral sensitivity of the cone near the peak wavelength was checked and a response family obtained as above. A whole-cell pipette was then applied to the inner segment of the cone (see inset to Fig. 5). After sealing, a command potential of −40 mV was applied, and the whole-cell configuration was obtained spontaneously or by a brief burst of suction. Provided the seal was good, the suction electrode recorded a current similar to that observed before sealing. In one red-sensitive cone, the whole-cell pipette was carefully withdrawn after recording responses under voltage clamp. The membrane resealed and a further response family was obtained.

Rapid solution changes

The perfusion system described by Hodgkin *et al.* (1985, 1987) was used for rapidly changing the solution bathing the cone outer segment. The cone was held with its inner segment in a right-angle bend suction electrode, so that only its outer segment was exposed to the bathing solution. A few seconds before exposure to a test solution, the cone was stepped from Ringer solution to the pre-test solution, in which the 2·5 mM-KCl in Ringer solution had been replaced by NaCl. All records shown represent the further rapid step between this pre-test solution and the test solution. The junction currents indicated that the solution change was complete within 50 ms (or considerably less in some preparations); these currents have been subtracted from all traces shown, as described by Hodgkin *et al.* (1985, 1987).

RESULTS

Spectral sensitivities

Three cone types were identified, with spectral sensitivities as illustrated in Fig. 1*A*. The wavelengths of peak sensitivity (λ_{max}) were at 610 nm (red-sensitive cone),

444 nm (blue-sensitive cone) and below 400 nm (UV-sensitive cone). The sensitivity of the UV-sensitive cone showed no sign of peaking as the wavelength approached 400 nm, suggesting that the peak sensitivity of this cone occurs well within the UV range. The part of the curve shown for the UV-sensitive cone has therefore been

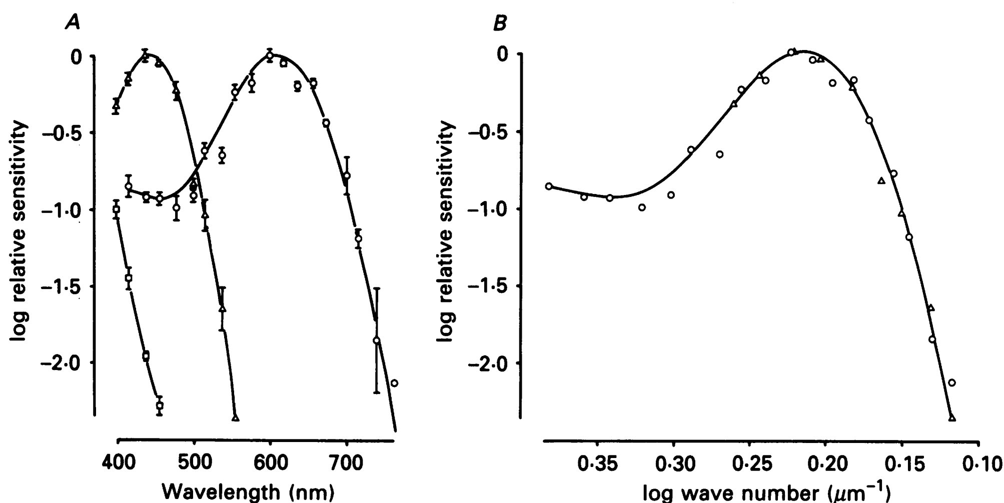

Fig. 1. Spectral sensitivities of three types of cone identified in the tiger salamander. *A*, action spectra of red-sensitive cones (○, $n = 12$), blue-sensitive cones (△, $n = 5$) and UV-sensitive cones (□, $n = 3$). Points from UV-sensitive cones do not include peak wavelength and were therefore aligned arbitrarily on sensitivity axis. Bars represent standard errors. Smooth curves drawn according to eqn (6) of Baylor *et al.* (1987) with $\lambda_{max} = 610$ nm for red cones and 444 nm for blue cones; curve through points for UV-sensitive cone drawn by eye. *B*, action spectra of red- and blue-sensitive cones plotted as a function of log wave number (symbols as in *A*). Blue-sensitive cone spectrum shifted 0·138 log units along the abscissa to superimpose with red-sensitive cone spectrum. Curve drawn according to eqn (6) of Baylor *et al.* (1987) with $\lambda_{max} = 610$ nm.

arbitrarily aligned on the vertical axis, since the sensitivity at the peak wavelength could not be obtained with our existing optical system.

Of the 131 cones from which we have recorded, 106 were red-sensitive (81%), thirteen were blue-sensitive (10%) and twelve were UV-sensitive (9%). All three cone types generated similar dark currents (Table 1) and usually gave consistent flash responses for several hours, suggesting that they are all equally able to survive the preparative procedure. We conclude therefore that red-sensitive cones are by far the most abundant in the retina of the tiger salamander. The only apparent difference in the morphology of cones of different spectral sensitivity is that the outer segments of UV-sensitive cones are significantly shorter (Table 1).

Figure 1*B* shows that when the spectra of red- and blue-sensitive cones are plotted as functions of log wave number, the spectrum of the blue-sensitive cone can be made to superimpose exactly on that of the red-sensitive cone simply by shifting it 0·138 log units along the abscissa. This observation is consistent with those made on the action spectra of macaque cones (Baylor *et al.* 1987; see also Mansfield, 1985), and confirms that this transformation provides a good means of predicting the entire

spectral sensitivity function of a photoreceptor from its λ_{max}. The continuous curves in Fig. 1*A* and *B* have been drawn according to eqn (6) of Baylor *et al.* (1987), using the constants derived by these authors to fit the spectral sensitivity of macaque cones. There are no free parameters in this function, apart from λ_{max}, and the fact that the function also provides a good description of the spectral sensitivity of cones from the salamander suggests that it may be widely applicable to spectral sensitivities from many species.

TABLE 1. Summary of properties of salamander cones

	Red-sensitive (λ_{max} = 610 nm)	Blue-sensitive (λ_{max} = 444 nm)	UV-sensitive (λ_{max} < 400 nm)
Outer segment dimensions			
Length (μm)	9·0 ± 0·4	7·9 ± 0·6	5·5 ± 0·5
Volume (μm³)	79 ± 7	80 ± 6	61 ± 12
Collecting area (μm²)	0·73	0·74	0·56
Sample size	7	5	3
Absolute sensitivity			
S_F (pA photon^{-1} μm^2)	0·022 ± 0·003	0·33 ± 0·13	
S_Φ (pA photoisom.$^{-1}$)	0·030 ± 0·004	0·45 ± 0·18	
S_Φ^f (× 10^{-4} photoisom.$^{-1}$)	7·6 ± 0·8	179 ± 71	
Sample size	38	9	
Form of response			
Measured dark current* (pA)	20 ± 1	18 ± 2	18 ± 1
Dim flash time-to-peak (ms)	131 ± 4	518 ± 75	209 ± 21
$t_{\frac{1}{2}}$† (ms)	220 ± 16	1120 ± 180	349 ± 49
Period of oscillation (ms)	105‡	278 ± 30	138 ± 6
Sample size	40	11	9

Standard errors given where appropriate. Symbols S_F, S_Φ and S_Φ^f are the flash sensitivity, the photon sensitivity and the fractional photon sensitivity (corrected by a factor of 1·8 to allow for incomplete collection of current by the suction pipette – see eqn (1) in Methods).
* The measured value represents about 56% of total dark current; see p. 571 and Fig. 5.
† Time taken for recovery of half of the current suppressed by a half-saturating flash.
‡ Period measured in the voltage-clamped cone shown in Fig. 5.

Comparison with absorption spectra

The continuous line in Fig. 2*A* shows the absorption spectrum of the tadpole cone (Liebman & Entine, 1968); the absorption spectrum from the tiger salamander cone is identical (Liebman, 1972). This absorption spectrum does not provide a good fit to the action spectrum of red-sensitive cones (○); the chief discrepancy is below about 550 nm, where the absorption spectrum is up to 0·5 log units greater than the action spectrum of the red-sensitive cone.

Ebrey & Honig (1977) have derived nomograms which predict the complete absorption spectra given the λ_{max}, and Fig. 2*B* shows their predicted absorption spectra for vitamin A_2-based pigments of λ_{max} = 444 and 610 nm (continuous lines), together with the observed action spectra of the red-sensitive cones (○) and blue-sensitive cones (△). Once again, the main discrepancies are on the short wavelength side of the peak of each spectrum.

A weakness of microspectrophotometry is that light which is scattered by the

outer segment is measured as having been absorbed. Since the degree of light scattering often increases with frequency, this is probably the main reason for the overestimation of the relative absorption at short wavelengths.

Absolute sensitivities

When each photoreceptor is stimulated at its optimal wavelength, the flash sensitivities (S_F) of red- and blue-sensitive cones are 0·022 and 0·33 pA photon^{-1} μm^2 respectively (Table 1), while the flash sensitivity of a salamander rod is about 5 pA photon^{-1} μm^2 (from Fig. 3 of Baylor & Nunn, 1986). The rods of this species are therefore about 230 times more sensitive than red-sensitive cones but only about 15 times more sensitive than blue-sensitive cones.

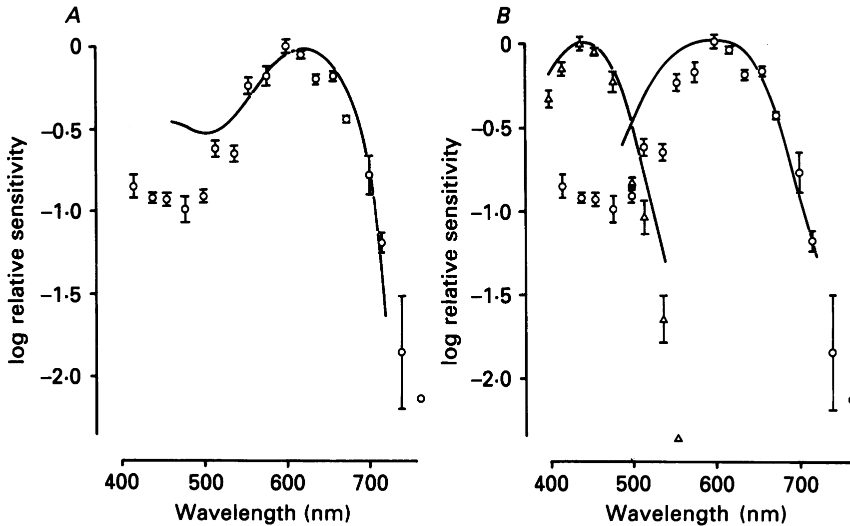

Fig. 2. Comparison of action spectra (from Fig. 1*A*) and various absorption spectra. *A*, action spectrum from red-sensitive cone (○) with absorption spectrum of single cone from tadpole (Liebman & Entine, 1968; continuous line); *B*, action spectra from red-sensitive (○) and blue-sensitive (△) cones with predicted A_2-pigment nomogram (continuous line) of Ebrey & Honig (1977). Bars represent standard errors.

One major reason for the lower sensitivity of cones is that the outer segment volume, and therefore the effective collecting area, is about 27 times smaller than that of the rod. Another factor is the gain of the phototransduction pathway, which can be expressed independently of collecting area by estimating the current suppressed by each photoisomerization (S_Φ, see Methods). In salamander rods S_Φ is about 0·2 pA per photoisomerization (Nakatani & Yau, 1989). As Table 1 shows, in the red-sensitive cone S_Φ is 0·03 pA per photoisomerization, comparable to the values obtained in red-sensitive cones by other authors (Schnapf & McBurney, 1980; Nakatani & Yau, 1989) but considerably lower than in the rod. The transduction gain of the blue-sensitive cone, however, is of the same order of magnitude as the rod (0·45 pA per photoisomerization). The difference in sensitivity between the blue-sensitive cone and the rod can therefore be explained entirely by the smaller size of the blue-sensitive cone's outer segment.

The gain of the transduction pathway can also be expressed as the fraction of the dark current shut off by a single photoisomerization, S^f_Φ, thereby removing errors which may be introduced by variations in the proportion of membrane current collected by the suction electrode. For thirty-eight red-sensitive cones the mean

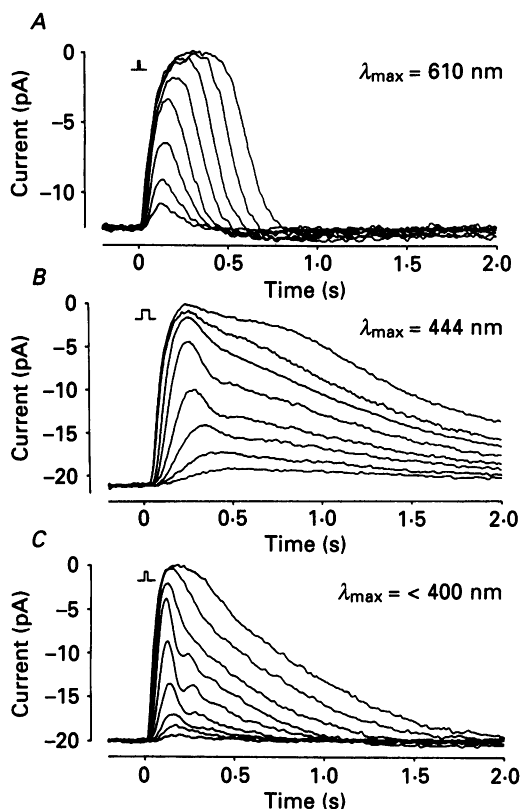

Fig. 3. Response families from red-, blue- and UV-sensitive cones. *A*, red-sensitive cone stimulated with 10 ms flashes at $\lambda = 600$ nm. Log_{10} of flash strength in photons μm^{-2}: 3·08, 3·40, 3·74, 4·10, 4·46, 4·79, 5·13, 5·46. *B*, blue-sensitive cone stimulated with 40 ms flashes at $\lambda = 455$ nm. Log_{10} of flash strength in photons μm^{-2}: 2·26, 2·63, 3·02, 3·41, 3·77, 4·14, 4·79, 5·09. *C*, UV-sensitive cone stimulated with white 20 ms flashes. Log_{10} of flash strength relative to brightest flash: −2·92, −2·58, −2·20, −1·80, −1·39, −1·02, −0·64, −0·30, 0. Each trace represents an average of five to ten flash responses. Traces not corrected for current lost through seal.

value of S^f_Φ was 8×10^{-4} per photoisomerization (see Table 1), comparable with the figures of 5×10^{-4} per photoisomerization obtained by Schnapf & McBurney (1980) and 3×10^{-4} per photoisomerization obtained by Nakatani & Yau (1989).

Form of flash response

As Fig. 3 illustrates, the response properties of the three cone types were so different that the spectral sensitivity of a cone could be predicted from the form of its flash responses. The time-to-peak of the dim flash response was slowest in blue-

sensitive cones and the fastest in red-sensitive cones (Table 1). The time taken for the recovery of half of the current suppressed by a half-saturating flash, $t_{\frac{1}{2}}$, is a useful index of the rate of recovery of the flash responses, and again blue-sensitive cones are slowest and red-sensitive cones are fastest (Table 1).

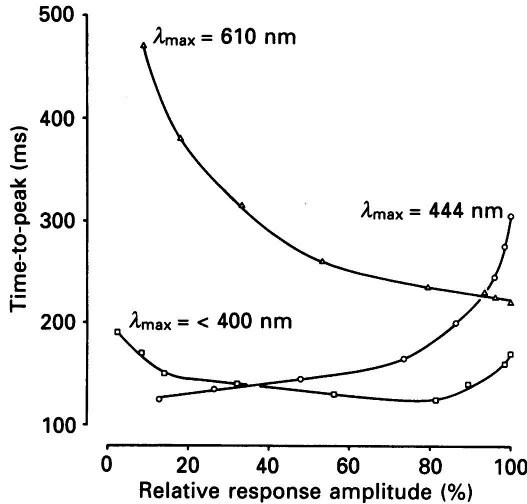

Fig. 4. Time-to-peak of flash response as a function of the proportion of current suppressed by the flash, plotted separately for each of the cones in Fig. 3. Curves drawn by eye.

Twenty-three out of forty red-sensitive cones (58 %) showed an underswing in the falling phase of the response to all but the dimmest flashes (Fig. 3*A*). This underswing is of particular interest because the responses of cones from the cynomolgus monkey (Baylor *et al.* 1987) or from man (Schnapf, Kraft & Baylor, 1987) show a similar but much larger underswing.

UV-sensitive cones consistently showed a damped oscillation during the recovery of light-sensitive current after flashes of intermediate intensity (see Fig. 3*C*). The period of oscillation during the recovery from a flash which suppressed 60 % of the membrane current was about 140 ms (see Table 1), equivalent to a frequency of 7·1 Hz. Blue-sensitive cones usually showed a less pronounced oscillation after flashes of intermediate intensity (see for example the fourth trace up in Fig. 3*B*), of frequency about 3·6 Hz.

There are two possible explanations for this oscillation in current: it could result from an oscillation in the number of open light-sensitive channels, caused by oscillations in the level of internal cyclic GMP, or alternatively it could reflect an oscillation in the driving force on the light-sensitive current due to an oscillation in membrane potential. In the latter case, the underswings and oscillations would be abolished by voltage clamping the cone (see below, p. 575).

Figure 4 shows the effect on the time-to-peak of increasing the flash intensity, for each of three cones shown in Fig. 3. Blue- and UV-sensitive cones are like the rod (Baylor *et al.* 1979), in that increasing the flash strength decreases the time-to-peak,

although in about half of these cones the time-to-peak begins to increase again as the responses approach saturation (e.g. the UV-sensitive cone in Fig. 4). Red-sensitive cones, however, are unlike rods in that the time-to-peak generally *increases* as the flashes are made brighter. In about 30% of red-sensitive cones, though, there is a

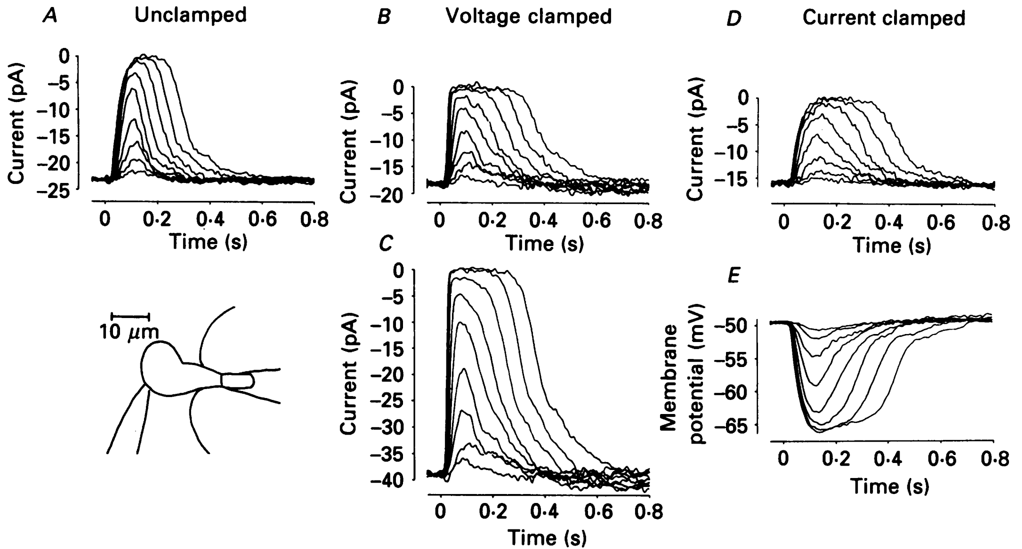

Fig. 5. Effect of voltage clamping on the form of the family of flash responses from a red-sensitive cone. Responses first obtained with the suction electrode alone (*A*). Cone membrane then voltage clamped via whole-cell electrode applied to inner segment membrane, holding potential −40 mV. Inset shows configuration of cone and electrodes, traced from photograph of preparation. Voltage-clamped responses recorded simultaneously by the suction electrode (*B*) and whole-cell electrode (*C*). Whole-cell trace offset by −45 pA to give a measure of the current across the outer segment membrane. Whole-cell electrode then clamped to pass zero current, and current responses recorded by suction electrode (*D*), with freely changing membrane potential simultaneously recorded by whole-cell electrode (*E*). Log_{10} of flash strengths in photons μm^{-2}: 2·28, 2·56, 2·88, 3·22, 3·58, 3·94, 4·27, 4·61. Dimmest flash omitted in panels *D* and *E*. Each trace is the average of four or five flash responses.

small initial decrease in time-to-peak for responses below one-half saturating (see for example the curve labelled 'unclamped' in Fig. 6). A possible explanation for the apparently anomalous behaviour of the red-sensitive cones is that the response time course is significantly distorted by the membrane time constant, in which case the anomaly should disappear under voltage clamp (see below).

Response characteristics under voltage clamp

Figure 5 shows the general characteristics of light responses in a red-sensitive cone under voltage clamp (Fig. 5*B*) and unclamped (Fig. 5*A* and *D*). A family of responses was first obtained with suction electrode alone (Fig. 5*A*). Flash responses were then recorded by the suction electrode in the same way whilst the cone membrane was voltage clamped via a whole-cell electrode applied to the inner segment (Fig. 5*B*). The total membrane current was recorded simultaneously by the whole-cell pipette

(Fig. 5*C*), and the form of these responses to light was, as expected, identical to those recorded by the suction electrode. The difference in amplitude is due to imperfect collection of current by the suction electrode; the current recorded by the suction electrode was 56 ± 9% (mean ± S.E.M.; six cones) of that recorded by the whole-cell electrode.

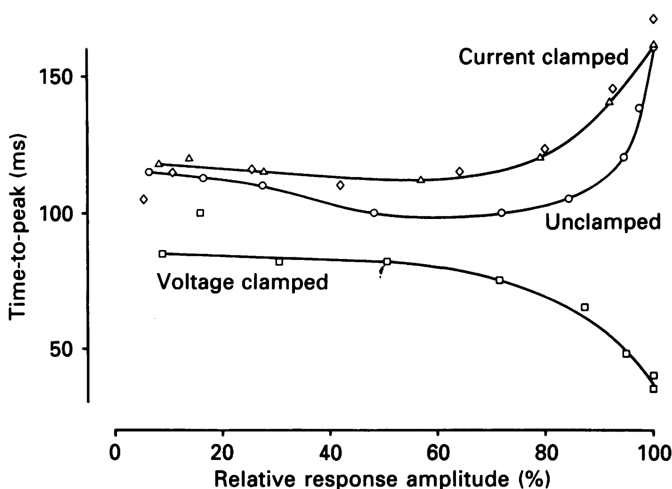

Fig. 6. Effect of voltage clamping on the time-to-peak of the flash responses of the cone shown in Fig. 5. ○ from Fig. 5*A*, □ from Fig. 5*C* and △ from Fig. 5*D*. Curves drawn by eye. ◇ near the curve labelled 'current clamped' were obtained by filtering the voltage-clamped responses with an exponential time constant of 27 ms (see Fig. 9).

The current passed by the whole-cell electrode was then clamped at zero, so that the membrane potential could again change freely, and a further response family was recorded by the suction electrode (Fig. 5*D*). The general form of the responses is similar to those in Fig. 5*A*, though the maximum amplitude is smaller and the response time course somewhat slowed; similar changes are often observed in the course of a long experiment even in an intact cell. The membrane potential was simultaneously recorded via the whole-cell electrode (Fig. 5*E*), and the response family recorded by this means is remarkably similar to that obtained from cones *in situ* in the eye-cup of the tiger salamander (Lasansky & Marchiafava, 1974), suggesting that the isolation procedure used in the present study does not change the response properties of cones.

Effect of membrane time constant on the flash response

Figure 6 shows the times-to-peak of the flash responses from panels *A*, *C* and *D* of Fig. 5, plotted against the proportion of membrane current suppressed by the flash. The shortening of the time-to-peak by voltage clamping is most marked for bright flash responses in red-sensitive cones (see Fig. 6 and Fig. 7*A*; Cobbs *et al.* 1985; Lamb, Matthews & Murphy, 1989) but it is observed in all three cone types. Furthermore, Fig. 6 shows that in red-sensitive cones the shortening is observed across all flash intensities. These findings show that the form of the rising phase of

the response of a cone to a saturating flash is normally affected substantially by the time constant of the outer segment membrane, and that at least in the red-sensitive cone the dim flash response is also significantly slowed by the membrane time constant.

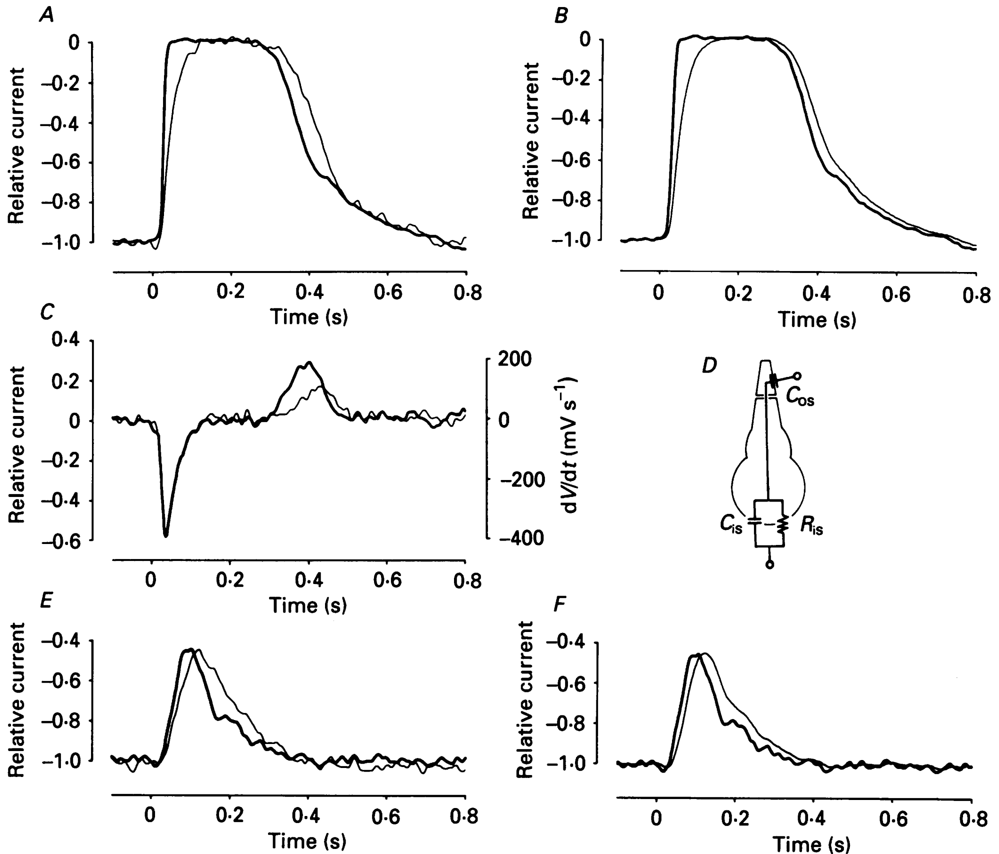

Fig. 7. Slowing of the response of a red-sensitive cone by the low-pass filtering properties of the cell membrane. Same cone as shown in Fig. 5. *A*, thick trace: bright flash response under voltage clamp, recorded by whole-cell electrode from red-sensitive cone under voltage clamp (from Fig. 5*B*); thin trace: response to same bright flash recorded by suction electrode under current clamp, i.e. with membrane potential free to change (from Fig. 5*D*). *B*, thick trace: voltage-clamped response as in *A*; thin trace: same response low-pass filtered with a single exponential time constant of 27 ms. *C*, thick trace: difference between currents shown in *A*, recorded under voltage clamp and under current clamp; thin trace: differential of membrane potential recorded while rod was under current clamp (see Fig. 5*E*); calculated membrane capacitance required to bring thick and thin traces into coincidence was 59 pF (see eqn (6)). *D*, equivalent circuit for cone membrane in light (see text). *E*, responses to half-saturating flash recorded by suction electrode under voltage clamp (thick trace) and under current clamp (thin trace), as in *A*. *F*, voltage-clamped response from *E* (thick trace), and filtered by 27 ms time constant (thin trace).

Figure 7*A* provides a direct comparison of the kinetics of the bright flash responses of a red-sensitive cone under voltage clamp (thick trace) and under current clamp (thin trace). From the voltage-clamped trace it can be seen that the light-sensitive

current is suppressed within 40 ms of the flash, and that the relaxation of membrane current in the unclamped cell after the bright flash is therefore determined by the time constant of the cell membrane. The entire rising phase of the bright flash response was well approximated by an exponential of time constant 27 ms in this cell, and low-pass filtering the voltage-clamped response with this time constant provides a good approximation to the bright flash response in the unclamped cell (Fig. 7*B*, thin trace).

A simple equivalent circuit for the cone membrane is shown in Fig. 7*D*. We assume as a first approximation that the outer segment membrane resistance R_{os} is large compared to the inner segment resistance R_{is}. This approximation is likely to be strictly true in the light, because the outer segment conductance in light is known to be at most a few picosiemens in salamander rods (Baylor & Lamb, 1982; Baylor & Nunn, 1986; Lagnado, Cervetto & McNaughton, 1988), but the circuit may also provide a good approximation in darkness if $R_{os} \gg R_{is}$ (as in the rod; see Baylor & Nunn, 1986). The membrane time constant τ_m is given by:

$$\tau_m = R_{is}(C_{os} + C_{is}), \tag{3}$$

where C_{os} and C_{is} are outer and inner segment membrane capacitance. The total outer segment membrane current, I_{os}, is given by

$$I_{os} = I_L + C_{os}\frac{dV}{dt}, \tag{4}$$

where I_L is the current through light-sensitive channels and V is the membrane potential. Under voltage clamp, therefore,

$$I_{os} = I_L, \tag{5}$$

while in the current-clamped cell

$$I_{os} - I_L = C_{os}\frac{dV}{dt}. \tag{6}$$

The difference between the voltage-clamped and current-clamped responses (equal to left-hand side of eqn (6) if I_L is voltage independent) is shown in Fig. 7*C* (thick trace), and the derivative of the membrane potential is superimposed (thin trace). The correspondence between the two traces is excellent during the rising phase of the flash response, when $I_L = 0$, and the value of outer segment membrane capacitance, C_{os}, required to bring the two traces into coincidence was 59 pF. During the recovery phase the correspondence is less good, in part because $I_L \neq 0$, although the main reason for the disagreement was a small change in the kinetics of the flash response between the two series of measurements.

The responses to a half-saturating flash under voltage and current clamp (thick and thin traces, respectively) are shown in Fig. 7*E*. Filtering the voltage-clamped record with the same time constant of 27 ms provides a good approximation to the current-clamped trace (Fig. 7*F*). The effect on the time to peak of low-pass filtering the voltage-clamped responses is shown in Fig. 6 (◇), and is clearly successful in reproducing the time-to-peak of the current-clamped responses across the whole range of intensities. A single time constant is therefore adequate in this cone to

describe the electrical behaviour of the cell membrane, in support of the idea that the light-sensitive conductance has little influence on the cone time constant.

Time constant of the cone membrane

The membrane time constant can be obtained from the time constant of relaxation of outer segment current in an unclamped cone after the light-sensitive current has

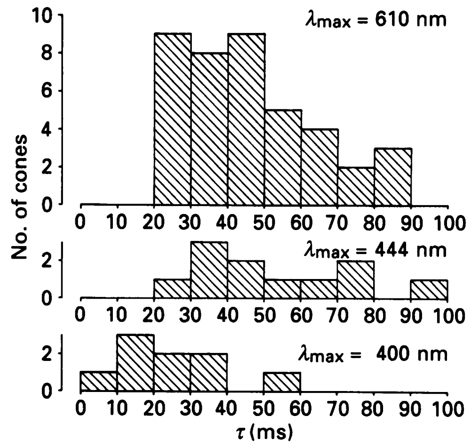

Fig. 8. Frequency distributions of electrical time constants of red-sensitive (λ_{max} = 610 nm), blue-sensitive (λ_{max} = 444 nm) and UV-sensitive cones ($\lambda_{max} < 400$ nm) in the light. Time constants were obtained from the final phase of relaxation of the outer segment current, measured by a suction pipette in unclamped cones, in response to a bright flash. Mean time constants were 46, 54 and 25 ms respectively; median time constants were 45, 45 and 25 ms respectively.

been suppressed by a bright flash. In all three classes of cone the distributions of the membrane time constants were markedly skewed (Fig. 8), probably reflecting a skewed distribution of membrane capacitances in the cones of this species (Barnes & Hille, 1989). The median values of the time constant were 45 ms in both red- and blue-sensitive cones, but only 25 ms in UV-sensitive cones, a difference which probably arises because the outer segments of UV-sensitive cones are relatively small (see Table 1) and therefore have a smaller membrane capacitance than those of red- and blue-sensitive cones.

The decay of current in unclamped cones after a bright flash was usually reasonably well approximated by an exponential function over most of its time course, but two types of deviation were commonly observed. Firstly, time-dependent conductances in the inner segment membrane cause a small depolarization after the initial peak in membrane potential (Baylor & Hodgkin, 1973; Attwell *et al.* 1982; Barnes & Hille, 1989; see Fig. 5*E*). The current flowing through the outer segment membrane capacitance consequently exhibits a small outward transient before relaxing to zero. Secondly, in a few red- and blue-sensitive cones there was a marked discontinuity in the rising phase of the response to a bright flash, and the last 35% of the current decayed along a slower exponential with a time constant of about 110 ms. The discontinuity occurred well after all light-sensitive channels had been

closed by the bright flash, and so cannot be attributed to an increase in the time constant caused by the closure of light-sensitive channels. Assuming that the outer segment capacitance is fixed, the change in time constant implies that in this population of cones hyperpolarization from the resting potential decreases the inner segment slope conductance. In support of this argument, the steady-state current–voltage relation of cones in the light has been shown to exhibit outward rectification (Barnes & Hille, 1989).

Oscillation and underswing under voltage clamp

Red-sensitive cones rarely show oscillations when the membrane potential is not controlled, but oscillations are observed at intermediate flash intensities when these cells are voltage clamped (see for example Fig. 7*E*). The frequency of this oscillation (*ca* 12 Hz in the cell of Fig. 7) is such that it would be largely filtered out in the unclamped cone by the time constant of the membrane ($\tau = 27$ ms, corresponding to a half-power frequency of 5·9 Hz). Figure 7*F* shows that the oscillations are indeed largely removed by filtering with a time constant of 27 ms. All three cone types, therefore, show an oscillation in light-sensitive current during the recovery from flashes of intermediate intensity. In the red-sensitive cone this oscillation can be observed under voltage clamp, and it therefore seems likely that the oscillatory mechanism present in all three cone types does not involve changes in membrane potential.

Figure 9*B* shows the underswing after a bright flash response, recorded from a red-sensitive cone with the membrane potential unclamped (thin trace) and clamped at −40 mV (thick trace). Voltage clamping increased the amplitude of the underswing in those cones which normally showed it (Fig. 9*B*), and revealed a similar underswing in those which did not (Fig. 5*B*, cf. Fig. 5*A*). We conclude that the underswing is not caused by changes in membrane potential.

The oscillations which can be observed in all three cone types, and the underswing in the red-sensitive cones, are therefore not generated by voltage-dependent processes, but are produced by an oscillation in the concentration of internal transmitter. A likely mechanism is described in the Discussion.

Na^+–Ca^{2+}, K^+ exchange current

Figure 9*A* shows that under voltage clamp the rising phase of the bright flash response divides into two components. About 95% of the current is entirely suppressed within the first 30–40 ms of the flash, but there is then a discontinuity in the rising phase, indicated by the arrow in Fig. 9*A*, and the last 5% shuts off more slowly. The separation of the rising phase into two components can probably be explained by analogy with similar observations in toad and salamander rods. The current entering the rod outer segment in the dark consists of two components: the majority (the light-sensitive current) enters through the light-sensitive channels, but a small proportion (the exchange current) enters via electrogenic Na^+–Ca^{2+}, K^+ exchange. Both components are suppressed after a bright flash, the light-sensitive current as a direct result of the fall in cyclic GMP within the outer segment, while the exchange current decays more slowly as a result of the fall in $[Ca^{2+}]_i$ after light-sensitive channels are closed. The exchange current therefore shows up as a separate

component in the response to a bright flash (Yau & Nakatani, 1985; Cervetto & McNaughton, 1986; Cobbs & Pugh, 1987; Hodgkin *et al.* 1987; Nakatani & Yau, 1988; Cervetto, Lagnado, Perry, Robinson & McNaughton, 1989). It seems likely that the slowly decaying component of the cone current has a similar origin to that

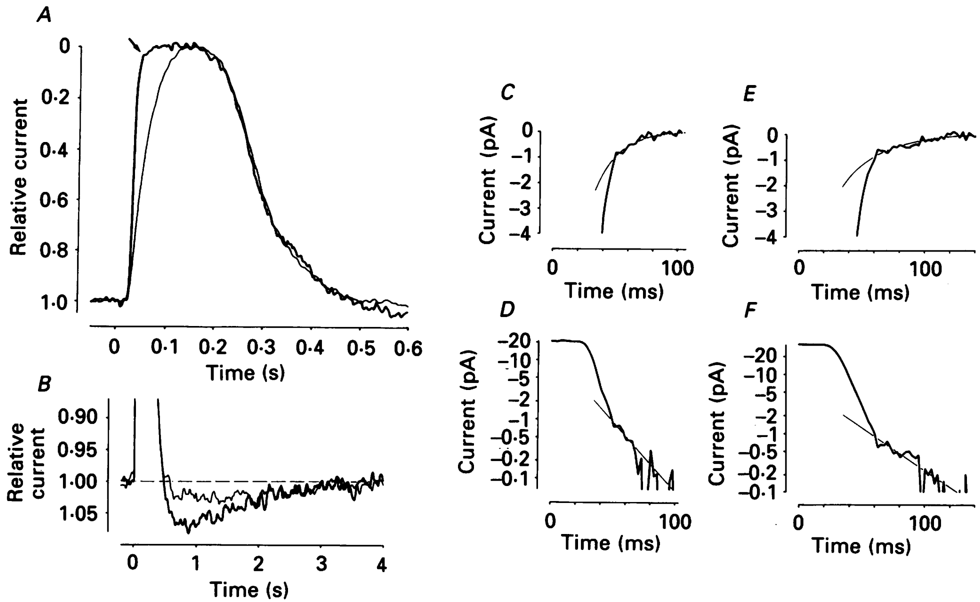

Fig. 9. Rising phase of the light response under voltage clamp, and effect of voltage clamping on the underswing in red cones. *A*, comparison of bright flash responses recorded by a suction electrode while the red-sensitive cone is voltage clamped at −40 mV via a whole-cell electrode (thick traces, average of 9), and after pulling away the whole-cell electrode (thin traces, average of 5). Traces normalized for light-sensitive current: uncorrected dark currents were 21·2 pA under voltage clamp and 23·5 pA after pulling away. Arrow indicates separation of rising phase into light-sensitive current and exchange current (see expanded traces in *C* and *D*). *B*, same responses on a slower time base, showing effect of voltage clamping on the underswing after the bright flash response. *C*, final portion of rising phase of voltage-clamped trace in *A*, magnified to show decay of exchange current. Thin curve is a single exponential function with τ_{exch} = 18 ms, fitted to the exchange current and extrapolated to the moment at which half of the light-sensitive current has been suppressed; magnitude of exchange current in dark obtained by this method was 2·3 pA, or 11 % of the dark current. *D*, log plot of data in *C*, showing the separation of the two phases. *E* and *F*, similar data to that shown in *C* and *D*, but from another cone. Light-sensitive current 32·5 pA; exchange current 2·1 pA, or 6·5 % of dark current; τ_{exch} = 30 ms.

in rods, particularly since the presence of an exchange mechanism has been demonstrated in the salamander cone outer segment (Nakatani & Yau, 1989), and we shall therefore refer to this component as the exchange current.

The exchange current is shown magnified in Fig. 9*C*, and the log plot in Fig. 9*D* shows the separation of the exchange current from the suppression of light-sensitive current. The decay of the current is consistent with a single exponential, as in the rod, but the time constant is much shorter; the thin line in Fig. 9*C* is an exponential of time constant of 18 ms. In all cones an exchange component could be distinguished

from the faster suppression of light-sensitive current after a bright flash, but the distinction is particularly clear in the cone shown in Fig. 9*E* and *F*, in which the decline of the exchange current was slower ($\tau = 30$ ms). In seven red-sensitive cones the mean time constant of decline was 21 ± 3 ms (mean $\pm$ S.E.M.).

What proportion of the dark current enters the cone outer segment via the exchange? In toad and salamander rods the initial amplitude of the exchange current is 4–7% of the total dark current at the point when all of the light-sensitive current has been suppressed (Cervetto & McNaughton, 1986; Cobbs & Pugh, 1987; Hodgkin *et al.* 1987) or about 9% if the exponential decay is extrapolated back to the moment when half of the light-sensitive current has been suppressed (Nakatani & Yau, 1988). The latter estimate is probably preferable since it allows for the small decay in exchange current during the suppression of the light-sensitive conductance. When this value is corrected for the hyperpolarization in response to light by allowing for the dependence of the exchange activity on membrane potential (Lagnado *et al.* 1988), an estimate of 6% is obtained for the proportion of the dark current carried by the exchange. As Fig. 7*C–E* shows, the same method can be applied to the red-sensitive cone of the salamander, although the much faster decline of the exchange current makes this estimate less accurate than in the rod. When half of the light-sensitive current has been suppressed the amplitude of the extrapolated exchange current was $3{\cdot}0 \pm 1{\cdot}0$ pA (mean $\pm$ S.E.M.; 7 cells), or 10·4% of the total dark current. No correction is needed for hyperpolarization in these voltage-clamped cones.

Since every calcium ion which enters the outer segment via the channel carries a charge of +2, but every calcium ion leaves in exchange for a charge of +1 (Yau & Nakatani, 1984; Hodgkin *et al.* 1987; Lagnado *et al.* 1988), these results show that the proportion of the total membrane current carried by calcium ions is about 12% in the rod and about 21% in the red-sensitive cone.

An exchange current component has also been observed on the rising phase of the bright flash response in one blue-sensitive and two UV-sensitive cones under voltage clamp. The time constant of decline of the exchange current in the UV-sensitive cones was similar to that of the red-sensitive cones, but the value of $\tau = 50$ ms observed in the blue-sensitive cone is considerably longer than was recorded in any red-sensitive cone. The difference may be related to the slower response time course in blue-sensitive cones (see Fig. 3 and Table 1).

Relaxation of light-sensitive current in the absence of external sodium

Hodgkin *et al.* (1985) demonstrated that when the sodium in the solution bathing the outer segment of a toad rod is rapidly replaced by another cation, the change in the membrane current consists of a rapid change (an increase or decrease in current with the time course of the solution change) followed by a slower exponential decline. The rapid change is mainly caused by the replacement of sodium as the chief current carrier through the light-sensitive channel, though there is also a small reduction in current owing to the inhibition of the electrogenic Na^+–Ca^{2+}, K^+ exchange. The slower decline in current results from an increase in $[Ca^{2+}]_i$ when the exchange has been inhibited. In the rod this decline is speeded by light adaptation and is slowed by inhibitors of the cyclic GMP phosphodiesterase (PDE), as expected if the rate of decline is determined by the activity of the PDE (Hodgkin *et al.* 1985).

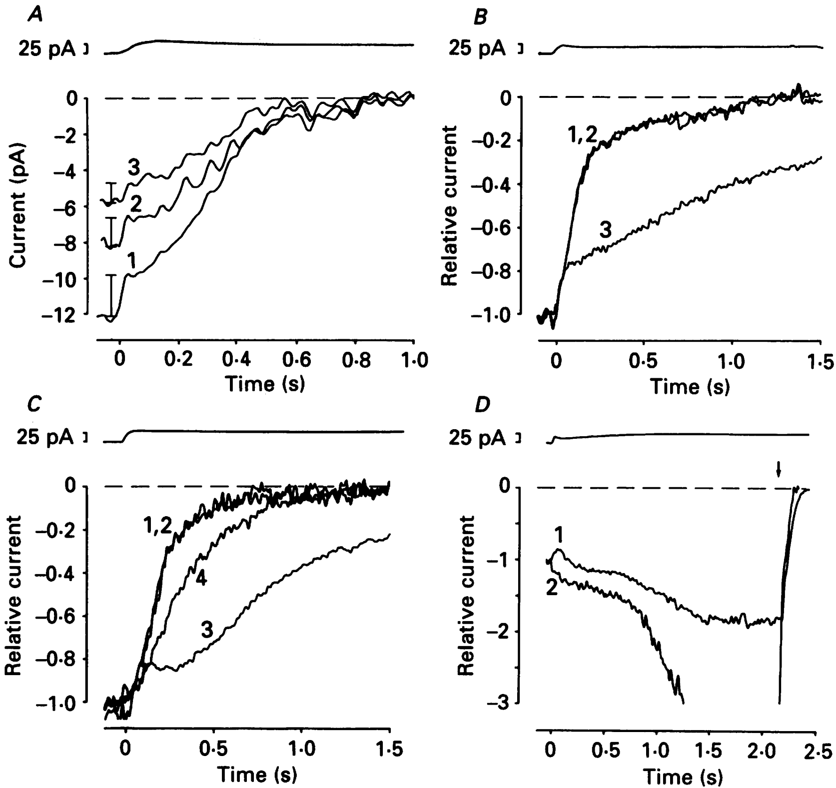

Fig. 10. Effect of rapid replacement of Na^+ in solution bathing outer segment by Li^+ on outer segment membrane current in red-sensitive cones under various conditions. *A*, cone transferred to a test solution in which all Na^+ had been replaced by Li^+: trace 1 in darkness, traces 2 and 3 in the presence of steady white lights sufficient to reduce the light-sensitive current to 0·68 and 0·48 of its dark value. Vertical bars indicate, for each trace, 19% of the current in the pre-test solution. *B*, effect of the PDE-inhibitor IBMX. All traces normalized for the light-sensitive current in the pre-test solution. Trace 1 obtained in absence of IBMX (light-sensitive current 14·0 pA), trace 2 in 10 μM-IBMX (15·8 pA) and trace 3 in 100 μM-IBMX (21·4 pA). *C*, effect of background light on relaxation of current, with and without IBMX. Traces 1 and 2 obtained in absence of IBMX: trace 1 in dark (light-sensitive current 20·2 pA) and trace 2 in steady white light (9·0 pA); trace 2 is the more noisy of the normalized traces. Traces 3 and 4 obtained in presence of 500 μM-IBMX: trace 3 in dark (33·6 pA) and trace 4 in steady white light (17·8 pA). *D*, effect of withdrawal of Ca^{2+}. Both traces obtained with 0 Ca^{2+} and 2 mM-EGTA in the test solution, with 112·5 mM-Li^+ (trace 1) or 112·5 mM-Na^+ (trace 2). Arrow indicates timing of bright flash of duration 10 ms; complete suppression of current confirms that current is light sensitive. Junction currents, indicating time course of solution changes, shown at top of each panel; all records have had junction currents subtracted. Traces in panel *A* represent single solution changes, whilst traces in panels *B–D* each represent averages of two solution changes.

Figure 10*A* (trace 1) shows a similar experiment in which the sodium bathing the outer segment of a red-sensitive cone was rapidly replaced by lithium. There was an initial rapid suppression of current, and the subsequent relaxation occurred about six times faster than in the rod. Traces 2 and 3 in Fig. 10*A* show the effect of performing the same solution change in the presence of steady background lights which

suppressed one-third and one-half of the dark current. In this cone, 19% of the current (shown by the vertical bars) was rapidly suppressed on stepping to lithium, a result which was independent of the level of light adaptation.

Red-sensitive cones are unlike rods in that the relaxation in current after removal of external sodium is not exponential. The speed of the delayed relaxation was not limited by the membrane time constant, which was determined from the time constant of current decay after a bright flash (see above) and was found to be considerably shorter than the relaxation of current after removal of Na^+. Two lines of evidence show that the delayed relaxation of current in the absence of external Na^+ is not limited by the rate of break-down of cyclic GMP by the PDE. Firstly, the rate of decline was unaffected by 10 μM-IBMX (Fig. 10*B*, trace 1 in absence of IBMX, trace 2 in 10 μM-IBMX, traces normalized to same level), although higher concentrations did slow the decline (trace 3), as in the rod. Secondly, increasing PDE activity with a steady background light did not affect the rate of decline (compare traces 1 and 2 in Fig. 10*C*, see also Fig. 10*A*). In the presence of 500 μM-IBMX, however, a background light did speed the rate of decline (compare traces 3 and 4 in Fig. 10*C*). Figure 10*D* (trace 1) shows that the delayed decline of current in the absence of $[Na^+]_o$ depended on an influx of calcium, as it was not observed in the absence of $[Ca^{2+}]_o$.

These experiments show that removal of external Na^+ suppresses the light-sensitive current in cones, and by analogy with the rod it seems reasonable to conclude that Na^+ removal causes an increase in $[Ca^{2+}]_i$, by inhibiting the Na^+–Ca^{2+}, K^+ exchange, and consequently inhibits the production of cyclic GMP by the guanylate cyclase (Lolley & Racz, 1982; Hodgkin *et al.* 1985; Koch & Stryer, 1988). Unlike in the rod, though, the delayed decline is not limited by the rate of break-down of cyclic GMP by the PDE, unless the activity of the PDE is reduced by inhibition with IBMX. Since the delayed decline is at least 6 times faster in the cone the turnover of cyclic GMP in darkness must be faster by at least a factor of six. One possible explanation for the lack of effect of IBMX and light adaptation on the rate of decline is that the entry of calcium through the light-sensitive channels may be rate limiting, which could also explain why the decline is not exponential.

Ionic selectivity of the light-sensitive channel of the red-sensitive cone

In toad rods the currents initially observed in Li^+, K^+, Rb^+ and Cs^+, relative to the total current supported by sodium, were 1·1, 0·8, 0·5 and 0·4 respectively (Hodgkin *et al.* 1985). The observation that the current initially increases on replacing sodium with lithium has been confirmed in salamander rods (Hodgkin *et al.* 1987).

In six red-sensitive cones in which a rapid and a slow phase of relaxation were visible after removal of external Na^+, the current initially supported by lithium was $0{\cdot}79 \pm 0{\cdot}01$ (mean ± S.E.M.) of the total current carried in sodium (see for example Fig. 10*A*). To obtain the effect on the light-sensitive current independent of the effect on the electrogenic Na^+–Ca^{2+}, K^+ current we need the fraction of membrane current carried by the exchange in Ringer solution; we estimate this to be approximately 10% (see p. 577). Allowing for the abolition of the exchange current in Li^+, therefore, the current carried through the light-sensitive channel by lithium, relative to that in sodium, is about 0·9 in the red-sensitive cone, compared to 1·2 in the rod. The lower

current seen in Li^+ cannot be attributed to a reduction in the component of the light-sensitive current carried by Ca^{2+}, because a similar ratio of the initial currents in the two solutions was observed in the absence of $[Ca^{2+}]_o$ (0·75, see Fig. 10*D*). The observation that the ratio of current carried by Na^+ to that carried by Li^+ is different

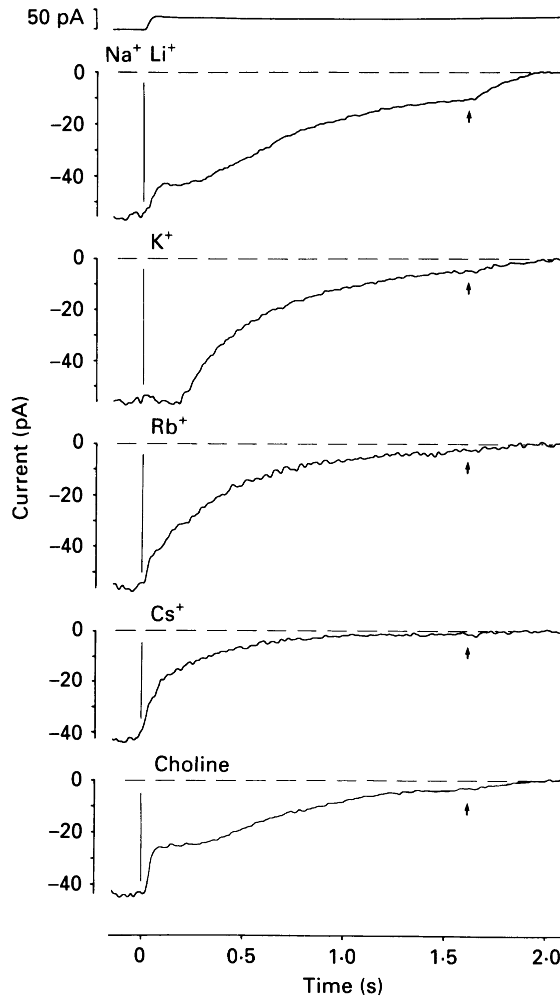

Fig. 11. Current recorded by suction electrode holding inner segment of red-sensitive cone during rapid replacement of Na^+ in solution bathing outer segment by other monovalent ions as indicated, with 500 μM-IBMX present throughout. Junction current during Li^+ substitution shown at top of figure; all records have had junction current subtracted. Vertical lines indicate start of solution change as indicated by junction current. Arrows indicate timing of bright flashes (10 ms duration); suppression of all remaining current confirms validity of subtraction. Each trace represents average obtained from two to four solution changes.

in rods and cones suggests that the light-sensitive channel itself is different in the two types of photoreceptor.

A clear separation between the instantaneous and delayed changes in current, as

shown in Fig. 10*A*, was only seen in the minority of experiments in which very rapid solution changes were achieved, and in the majority of experiments the rapidity of the delayed decline obscured the distinction between the two phases. In order to investigate the ionic series more fully the phosphodiesterase inhibitor IBMX was

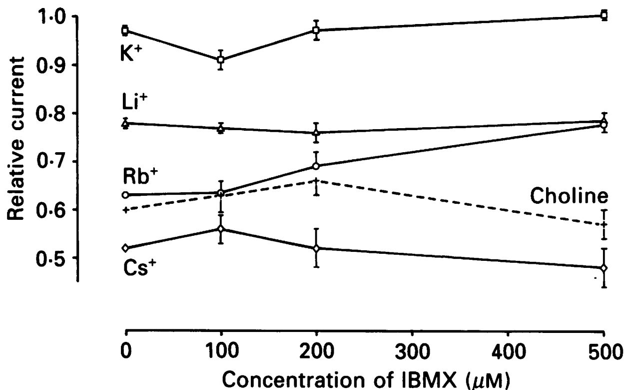

Fig. 12. Light-sensitive currents initially supported in the presence of each monovalent indicated (normal divalents in each case), relative to the total membrane current (i.e. light-sensitive and exchange current) in the Na^+-containing pre-stepping solution, with various concentrations of IBMX present throughout. Light-sensitive current supported by Na^+ is about 0·9 on this scale (see text). Bars show S.E.M. of at least four observations; points without error bars are from single observations.

used to slow the delayed decline and give a clear separation between the two phases (Fig. 11). In six cells in which 500 μM-IBMX was used, the initial currents, Li^+:(Na^+):K^+:Rb^+:Cs^+, were in the ratios 0·78:(1):1·00:0·78:0·48. Assuming that the proportion of the membrane current in sodium which is carried by the exchange current is 10·4 %, the ratios of currents carried through the light-sensitive channels, Li^+:(Na^+):K^+:Rb^+:Cs^+, are 0·87:(1):1·12:0·87:0·54.

A possible objection to the use of IBMX in experiments such as that shown in Fig. 11 is that the ionic selectivity may be affected by the presence of IBMX. This point is investigated in Fig. 12, in which the currents initially supported in the presence of alkali metal cations, relative to the total current in sodium, are plotted as a function of the IBMX concentration. If there is any effect of IBMX on the selectivity of the channel for monovalents, it is only small, and the selectivity sequence remains the same.

Replacing the sodium in the solution bathing a rod outer segment with the organic cation choline causes the current to fall to a level about 0·2 times that observed in sodium. This residual current must be carried mainly by calcium, since it is abolished when the calcium concentration is reduced to 1 μM (Hodgkin *et al.* 1985). The lowest panel in Fig. 11 shows a similar experiment on a red-sensitive cone. In fourteen observations on six cones the initial current supported in choline was $0·57 \pm 0·03$ (mean $\pm$ S.E.M.), equivalent to 0·64 times the current in sodium when allowance is made for the suppression of Na^+–Ca^{2+}, K^+ exchange.

Figure 13 shows that, as in the rod, the current in the presence of choline is almost entirely carried by Ca^{2+}, because if calcium is also removed then only a small residual current remains (trace 2). The cone light-sensitive channel is therefore much more permeable to calcium in the absence of external Na^+ than is the rod channel. As in

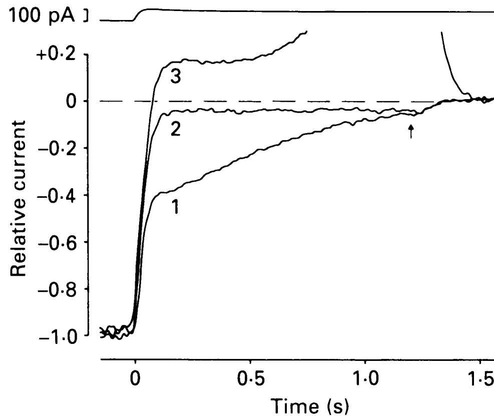

Fig. 13. Current recorded by suction electrode holding inner segment of red-sensitive cone during rapid replacement of Na^+ in solution bathing outer segment by choline in the presence of 500 μM-IBMX. Trace 1 was obtained with 1 mM-Ca^{2+} and 1·6 mM-Mg^{2+}, trace 2 with 0 Ca^{2+} and 1·6 mM-Mg^{2+}, and trace 3 with 0 Ca^{2+} and 0 Mg^{2+} in the test solution. Pre-test solution contained 1 mM-Ca^{2+} and 1·6 mM-Mg^{2+} in each case. Junction current for trace 1 shown at top of figure. Arrow indicates timing of bright flash (10 ms duration). Each trace is average obtained from two to three substitutions.

the rod, if both Ca^{2+} and Mg^{2+} are removed from the test solution, an outward current is observed (trace 3), which is probably carried mainly by K^+. This current increases after a delay, presumably because the calcium level in the outer segment falls as calcium escapes through the light-sensitive channel, and the level of cyclic GMP therefore rises (see Discussion). Similar results to these have also been obtained using TEA as the substituting cation. The observation that the current initially supported in choline is greater than that carried in caesium (Fig. 12) implies that caesium ions can exclude calcium from the channel to a greater extent than do choline ions.

DISCUSSION

In this study we have shown that the larval tiger salamander possesses three types of cone, maximally sensitive to red (λ = 610 nm), blue (444 nm) and UV (< 400 nm) light. Blue-sensitive cones were found to give slower flash responses than the other cones types and to be about fifteen times more sensitive than red-sensitive cones. When the cone membrane was voltage clamped at −40 mV, the time-to-peak of the bright flash response was considerably reduced in all cones. Oscillations in light-sensitive current can be seen during the recovery of all three types of cone from flashes of intermediate intensity, but in red-sensitive cones oscillations are only observed under voltage clamp. Sixty per cent of red-sensitive cones show a small underswing in their flash responses; this underswing is enhanced under voltage

clamp, which also reveals an underswing in the remaining 40%. Solution change experiments show that the ionic selectivity of the light-sensitive channel is different in the rod and the red-sensitive cone.

Spectral sensitivity

The 166 nm separation between the peak wavelengths of the red-sensitive cone (λ_{max} = 610 nm) and the blue-sensitive cone (λ_{max} = 444 nm) is surprisingly wide, but there does not appear to be a green-sensitive cone in the salamander retina to fill this gap. However, most salamander rods show peak sensitivity at 523 nm (Cornwall, MacNichol & Fein, 1984) and have a powerful mechanism of light-adaptation which may enable them to provide green sensitivity over most of the intensity range encountered by the salamander.

Many species have been credited with sensitivity to UV light (see Stark & Tan, 1982, for a review), and UV-sensitive photoreceptors have been identified by microspectrophotometry in several species of fish (see for example Bowmaker & Kunz, 1987). We report here the first electrophysiological recordings of UV-sensitive photoreceptors in a vertebrate, and confirm that absorption in the UV range is indeed physiologically relevant, rather than (for example) representing absorption by the 11-*cis* or all-*trans* isomers of free retinal.

Time-to-peak of the cone response

Figures 6 and 7 show that the time constant of the cone membrane significantly reduces the response speed of cones in the salamander, particularly to bright flashes. The measured outer segment capacitance of around 60 pF in the red-sensitive cone has a substantial contribution from the discs, as only around 3 pF can be attributed to the capacitance of the surface membrane, assuming that $C_m = 1\ \mu F\ cm^{-2}$. The capacitance of the rest of the cell is, from measured dimensions, about 30 pF, so it is clear that the time constant of the cell is significantly influenced by the capacitance of the disc membrane. The cone discs must be open to the external solution for a compelling reason, because the cone suffers a considerable loss of time resolution as a result of the consequent increase in cell membrane capacitance. One possibility is that the external solution must have direct access to the visual pigment in order for the chromophore to be regenerated sufficiently rapidly.

Fall of internal calcium after a bright flash

The decay of the Na^+–Ca^{2+}, K^+ exchange current in toad and salamander rods is approximately exponential, with a mean time constant of about 500 ms (Yau & Nakatani, 1985; Cervetto & McNaughton, 1986; Cobbs & Pugh, 1987; Hodgkin *et al.* 1987; Nakatani & Yau, 1988). Assuming that a bright flash gives a sustained hyperpolarization of about −20 mV, and correcting for the voltage dependence of the exchange (Lagnado *et al.* 1988) the time constant of turnover of free calcium in the dark is about 660 ms in darkness.

The decline of the exchange current after a bright flash in the voltage-clamped cone shows that, as in the rod, the internal calcium concentration falls in response to light. The mean time constant of this decline is about 20 ms in cones which have been clamped at −40 mV. The turnover of exchangeable calcium in the outer segment of

a red-sensitive cone is therefore about 30–40 times faster than in the rod. A comparison of the dimensions of the rod outer segment (Hodgkin *et al.* 1987) and those of the red-sensitive cone (Table 1) shows that the outer segment volume is about 26 times greater in the rod than in the red-sensitive cone (assuming that the proportion of the volume occupied by the discs is the same in each case). Since the time constant of turnover is directly proportional to volume (McNaughton, 1990, eqn (4)) the more rapid turnover of exchangeable calcium in the outer segment of the red-sensitive cone is principally due to its smaller volume, but differences in Na^{+}–Ca^{2+}, K^{+} exchange activity or calcium buffering may also exist.

Nakatani & Yau (1989) obtained a value of 100 ms for the time constant of decline of the exchange current in the red-sensitive cone, or about five times greater than that which we have measured. The most likely reason for the discrepancy with the present results is that these authors measured the exchange current in cones which had not been voltage clamped, and in which the decline was therefore limited by the time constant of the cone membrane. The time constant of 100 ms for the decline of the exchange current in their experiments was longer than our estimate of 45 ms for the membrane time constant of red-sensitive cones, but it was obtained under different conditions: all of the cones had been calcium-loaded and maintained in bright light for at least a second, and in some cases caesium was applied to the inner segments. It is possible that these treatments blocked some inner segment conductances and thereby increased the time constant. Recently Hestrin & Korenbrot (1990) have reported a value of around 100 ms in voltage-clamped cones, in agreement with the value of Nakatani & Yau and considerably slower than the values reported in the present study. A possible cause of the discrepancy is the inclusion of 50 μM of the calcium buffer EGTA in the perfusion solution used by Hestrin & Korenbrot; the increased calcium buffering power of the cytoplasm may have a significant effect on the turnover time constant of intracellular calcium (see McNaughton, 1990, eqn (4)). The perfusion solution used in the present study contained no added calcium buffer, and no relaxation time constant longer than 30 ms was observed in any of our red-sensitive cones.

Proportion of light-sensitive current carried by calcium in cones

The proportion of the light-sensitive current which is carried by calcium can be estimated from the extrapolated amplitude of the exchange current after a flash, giving values of about 12% in salamander rods and 21% in cones (see p. 577). The rapid decay of the exchange current limits the accuracy of this measurement in cones (cf. Fig. 9*C–F*), but some support for the idea that a larger fraction of the light-sensitive current is carried by calcium in cones than in rods is given by experiments in which the sodium bathing the outer segment is replaced by the impermeant cation choline (Figs 11–13). Around 20–25% of the normal light-sensitive current is carried by calcium in rods and 50–60% in cones.

Solution-change experiments indicate that the light-sensitive channel has a different structure in rods and in cones. The light-sensitive channel of the red-sensitive cone does not discriminate in favour of cations of small ionic radius to the extent that the rod channel does, implying that the field strength of the binding site is lower in the cone.

Negative feedback control of the light-sensitive current

The flash responses of cones from the cynomolgus monkey or man, measured by suction electrode at 37 °C, show a marked underswing (Baylor *et al.* 1987; Schnapf *et al.* 1987). Voltage clamping monkey cones would be a great help in elucidating the mechanism of the underswing, but may be difficult in these small cells. Salamander cones provide a useful model for studying the mechanism of the underswing, since the larger amphibian cones can be voltage clamped more easily than mammalian cones.

The underswing of the salamander red-sensitive cone response is increased under voltage clamp, showing that it arises from a change in the number of open light-sensitive channels and not from a change in membrane potential. Evidence has recently been accumulating for a delayed negative feedback system controlling the light-sensitive current in rods (Hodgkin *et al.* 1985; Hodgkin & Nunn, 1988; reviewed in McNaughton, 1990). In the dark, a proportion of the light-sensitive current is carried by calcium, and this calcium is pumped back out of the outer segment via Na^+–Ca^{2+}, K^+ exchange. The suppression of the light-sensitive conductance in response to light therefore results in a fall in free calcium (McNaughton, Cervetto & Nunn, 1986; Ratto, Payne, Owen & Tsien, 1988), which appears to disinhibit guanylate cyclase (Lolley & Racz, 1982; Koch & Stryer, 1988), leading to an increase in the level of cyclic GMP and a corresponding increase in light-sensitive current.

In the present study we provide three pieces of evidence supporting a similar scheme in the cone. Firstly, the amplitude of the Na^+–Ca^{2+}, K^+ exchange current after a bright flash and the size of the residual current when Na^+ is replaced by choline both suggest that a significant proportion of the light-sensitive current is normally carried by Ca^{2+} (Fig. 9). Secondly, the decline in the exchange current after a bright flash shows that the intracellular calcium level falls in response to light. Finally, the decline in the light-sensitive current when external sodium is removed (and Na^+–Ca^{2+}, K^+ exchange therefore inhibited) suggests that the level of cyclic GMP is reduced by the accumulation of calcium in the outer segment.

Oscillations in membrane current have been observed in salamander rods when the concentration of sodium ions bathing the outer segment is rapidly changed, and appear to be a product of the negative feedback system described above. The observation that oscillations are superimposed on the recovery of the flash response in physiological conditions constitutes a clear demonstration of the operation of the negative feedback system controlling the light-sensitive current in cones.

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