Journal of Physiology (1988), **403**, *pp.* 473–494
With 8 text-figures
Printed in Great Britain

EXTERNAL AND INTERNAL ACTIONS IN THE RESPONSE OF SALAMANDER RETINAL RODS TO ALTERED EXTERNAL CALCIUM CONCENTRATION

BY T. D. LAMB AND H. R. MATTHEWS

From the Physiological Laboratory, University of Cambridge, Downing Street, Cambridge CB2 3EG

(*Received 9 December 1987*)

SUMMARY

1. The membrane current was recorded from retinal rods isolated from *Ambystoma tigrinum* using the suction pipette and whole-cell patch pipette techniques, while the concentration of calcium bathing the outer segment was rapidly reduced.

2. The increase in outer segment current induced by lowered external calcium in darkness could be resolved into two components, one as rapid as the time course of the solution change (as judged by the junction current) and the other somewhat slower.

3. Introduction of the calcium buffer BAPTA (1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid) into the cell from a patch pipette led to a progressive slowing of the second component of current increase.

4. When several minutes had elapsed following rupture of the patch, to allow a substantial amount of BAPTA into the cell (*ca.* 10 mM in the patch pipette), the second component was slowed by a factor of about 20-fold, while the first component continued to have the same rapid time course as the solution change.

5. The rapid component is attributed to a direct effect of external calcium, Ca_o^{2+}, and the delayed component to an indirect effect mediated by a reduction in internal calcium, Ca_i^{2+}.

6. These results confirm that, in previous experiments in which BAPTA was introduced into photoreceptors, the internal calcium concentration was very significantly buffered.

7. When Ca_o^{2+} drops from 1 mM to $< 10^{-8}$ M, the rapid external component corresponds to an increase in circulating current of 3- to 4-fold, and the internal component corresponds to an increase of at least 4- to 5-fold. However, the total current at late times is limited by electrical factors, so that the size of the internal effect is bound to be considerably greater.

INTRODUCTION

It has been known for many years that the size of the circulating dark current in photoreceptors is strongly dependent on the concentration of external calcium, Ca_o^{2+}, bathing the outer segment. Elevated Ca_o^{2+} rapidly reduces the dark current (Yoshikami & Hagins, 1973), whereas lowered Ca_o^{2+} rapidly increases the dark

current (Hagins & Yoshikami, 1974). These results were taken as support for the calcium hypothesis of phototransduction (Hagins, 1972; Yoshikami & Hagins, 1973), but that hypothesis has now been discredited (for review see Pugh & Cobbs, 1986). Effects of the same form, but with poorer time resolution and based on intracellular voltage recordings, were reported by Brown & Pinto (1974), Lipton, Ostroy & Dowling (1977), Bertrand, Fuortes & Pochobradsky (1978) and Bastian & Fain (1979). Subsequently the effects of altered external calcium were investigated with improved time resolution by Yau, McNaughton & Hodgkin (1981), Hodgkin, McNaughton, Nunn & Yau (1984) and Hodgkin, McNaughton & Nunn (1985).

Nevertheless, the extent to which a change in external calcium concentration, Ca_o^{2+}, acts via a direct mechanism or via a change in cytoplasmic calcium concentration, Ca_i^{2+}, has not been clear. In earlier experiments (Yoshikami & Hagins, 1973; Hagins & Yoshikami, 1974; Yau *et al.* 1981; Hodgkin *et al.* 1984) it was not possible definitively to separate internal and external actions. More recently, a fast solution change technique has been applied (Hodgkin *et al.* 1985; Lamb, Matthews & Torre, 1985*b*), and this has revealed separate rapid and delayed components, which have been attributed to external and internal actions of calcium.

The aim of our study has been to investigate these two components, and in particular to demonstrate that the slightly delayed component is brought about as a result of changes in Ca_i^{2+}. Our approach has been to buffer the internal calcium concentration of the rod by introducing calcium chelator into the cytoplasm. A whole-cell patch pipette was used to incorporate the buffer BAPTA (1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tsien, 1980) into the photoreceptor using the simultaneous suction pipette and patch pipette method of Lamb, Matthews & Torre (1986).

The results distinguish a very rapid effect, attributed to a direct action of external calcium, and a delayed effect which is greatly slowed in the presence of BAPTA in the cytoplasm and which is attributed to an internal action.

Some of our early experiments were performed in collaboration with Dr V. Torre. A preliminary account of this work was presented to the Physiological Society (Lamb *et al.* 1985*b*).

METHODS

Simultaneous suction pipette and whole-cell patch pipette recordings were made from rod photoreceptors isolated from the retina of the tiger salamander, *Ambystoma tigrinum*, using the methods described by Lamb *et al.* (1986). As previously, the intracellular voltage of the rod was clamped at −40 mV during whole-cell recordings (after allowance for the 10 mV liquid junction potential). Rapid solution changes were accomplished with the θ-tube and stage-stepping method of Hodgkin *et al.* (1985), modified so that the hydraulic piston which translated the microscope stage was driven by a solenoid, and with the solution flow rate controlled by peristaltic perfusion pumps. From measurements of single frames from video recordings the velocity of movement of the stage was approximately 5 μm ms^{-1}, so that the boundary would have crossed the outer segment within 5 ms; this, however, takes no account of mixing and diffusional delays. In good experiments the junction current rise time (10–90%) was less than 30 ms.

In early experiments the responses were low-pass filtered from DC to 20 Hz with a 6-pole Bessel filter, and were sampled at 10 ms intervals. For later experiments (including Figs 2, 3, 4 and 7) the bandwidth was increased to DC to 40 Hz and the responses were sampled at 5 ms intervals.

Solutions

The salt composition of the normal Ringer, 0-Ca^{2+} Ringer and 50 μM-Ca^{2+} Ringer solutions is shown in Table 1 (in mM). Each solution was buffered to pH 7·7–7·8 with NaOH, and the normal Ringer solution also contained 10 mM-glucose. The free calcium concentration of the '0-Ca^{2+} Ringer solution' was probably around 10^{-9} M, but to be cautious we take it simply as $< 10^{-8}$ M.

The patch pipette filling solutions containing BAPTA were based on those used previously by Lamb *et al.* (1986). The concentration of BAPTA (BDH) was either 10 or 11 mM, with the free Ca^{2+} concentration buffered to either 0·5 or 1 μM, and with the free Mg^{2+} concentration buffered to 5 mM. The total K^+ concentration (after buffering to pH 7·4) was approximately 98 mM and the predominant anion was aspartate. On the day of an experiment 1 mM-Na_2ATP and 1 mM-Na_4GTP (Sigma) were added to the stock solution and the pH was adjusted back to 7·4 with KOH.

TABLE 1. Salt composition of bathing solution (in mM)

	NaCl	KCl	$CaCl_2$	$MgCl_2$	Na_4BAPTA	NaHEPES	EDTA
Normal Ringer solution	111	2·5	1	1·6	—	3	0·01
0-Ca^{2+} Ringer solution	103	2·5	—	1·6	2	3	—
50 μM-Ca^{2+} Ringer solution	111	2·5	0·05	1·6	—	3	0·01

We have no direct way of estimating the amount of BAPTA incorporated into a cell, but we suspect that the final cytoplasmic concentration may be substantially less than that in the patch pipette. By analysing experiments such as that illustrated in Fig. 6*A* and making certain assumptions, it is possible to show that the results are consistent with a cytoplasmic BAPTA concentration in the region of 0·5–5 mM; the wide range reflects uncertainty in the free concentration of calcium in the cytoplasm.

Series resistance of outer segment and patch pipette

In a later section (p. 492) we consider the limitation on the maximal circulating current caused by the total series resistance of the outer segment and the patch pipette. In most experiments the apparent access resistance of the patch pipette, estimated from the size of the initial transient elicited by a 2 mV 100 Hz square-wave voltage, was 10–30 MΩ with an average value around 20 MΩ; series resistance compensation was not employed. The series resistance of the outer segment may be estimated from the model of baffling caused by the stack of discs, put forward by Lamb, McNaughton & Yau (1981). In this model the only path available for axial conduction is the small gap at the circumference of the discs (and at any incisures), giving a series resistance R_s of

$$R_s = \frac{\rho l}{\pi d g},$$

where ρ is the resistivity of the cytoplasm, l is the length of outer segment under consideration, d is the diameter of the outer segment, and g is the width of the gap between the rim of the discs and the plasma membrane. In a salamander rod $d = 11$ μm, and the length of the whole outer segment is about 22 μm so that we can probably take $l = 10$ μm. For the patch pipette filling solution which we used, the resistivity should be approximately $\rho = 200$ Ω cm, and we shall take $g = 15$ nm from the estimate of Lamb *et al.* (1981). This gives an effective series resistance R_s of the outer segment of approximately 40 MΩ, and a total series resistance of around 60 MΩ after allowance for the patch pipette access resistance. For a clamping voltage of −40 mV and a reversal voltage around 0 to +10 mV, the driving potential would be about 45 mV so that the maximum current possible would be 45 mV/60 MΩ or about 750 pA, quite close to the maximum currents observed. It should be emphasized, however, that this value for the effective series resistance is only a rough estimate, and will depend critically on the size of the circumferential gap, g. Nevertheless, this calculation shows that the current recorded is likely to be limited by series resistance to a maximum of the order of 1 nA.

RESULTS

Incorporation of BAPTA and withdrawal of patch pipette

The sequence of events involved in incorporating the buffer BAPTA into the cell and then withdrawing the patch pipette is illustrated in Fig. 1. In this experiment the outer segment was drawn into the suction pipette, and the middle trace monitors the current recorded with this pipette, while the lower trace monitors the patch

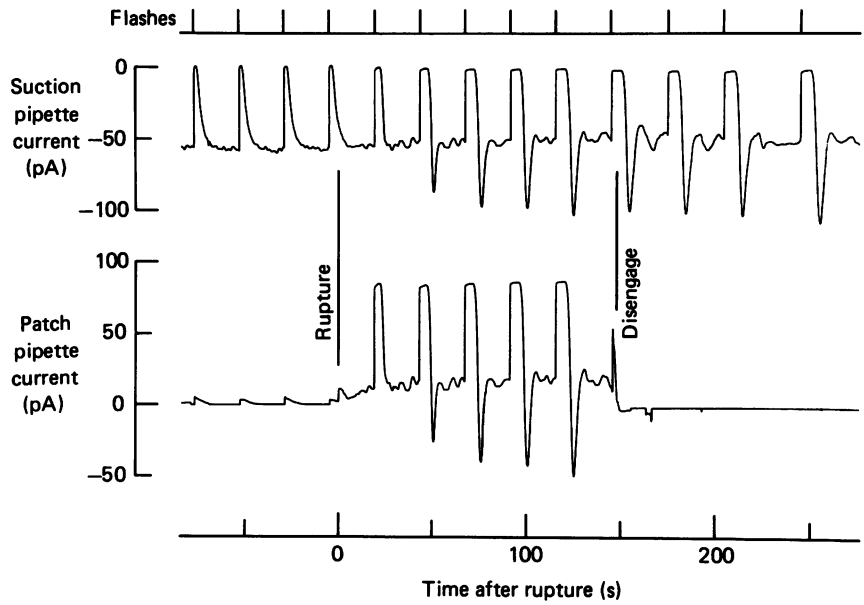

Fig. 1. Record illustrating incorporation of BAPTA into a rod via a whole-cell patch pipette on the inner segment, and subsequent disengagement of the patch pipette. Top trace, timing of delivery of bright flashes; middle trace, suction pipette current (outer segment drawn in); bottom trace, patch pipette current. The patch pipette contained 10 mM-BAPTA with 8 mM-total Ca^{2+}, giving a free Ca^{2+} concentration in the patch pipette of 0·56 μM. Immediately prior to the beginning of the illustrated traces the patch pipette was sealed against the inner segment membrane. At time zero the patch was ruptured with a gentle pulse of suction (access resistance approx. 15 MΩ), and at time 145 s the patch pipette was successfully disengaged. Small responses from the patch pipette prior to patch rupture resulted from leakage through the membrane patch. Very dim infra-red illumination was on until time 220 s.

pipette current measured simultaneously. Bright flashes (indicated at the top) elicited saturating responses of about 55 pA in the suction pipette recording. Just prior to the beginning of the illustrated traces the patch pipette was sealed against the membrane of the protruding inner segment and was voltage clamped to −40 mV. The small responses from the patch pipette during this period presumably resulted from leakage through the patch of inner segment membrane.

At time zero this patch was ruptured by a gentle pulse of suction, giving diffusional access from the patch pipette to the cell interior. From this time until the patch pipette was gently disengaged about 2·5 min later, the current recorded by the patch pipette had the same form as that recorded by the suction pipette, and the

characteristic changes in response waveform previously described by Lamb *et al.* (1986) were seen. In contrast to our earlier experiments the free calcium concentration in the patch pipette solution was here set to approximately 0·5 μM, rather than to an extremely low value. This minimized disturbances in the normal dark resting level of Ca_i^{2+}, and avoided the large increase in mean dark current seen upon incorporating BAPTA with no added calcium. Here the mean dark current was barely affected by incorporation of buffer.

By slowly withdrawing the patch pipette while attempting to hold the rod at a fixed position in the suction pipette, it was usually possible to disengage the patch pipette without damage to the cell (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). As shown in the lower trace in Fig. 1 disengagement happened to occur during the rising phase of a flash response near time 145 s, and the patch pipette current quickly disappeared. In this particular example the suction pipette current was very stable at the instant of disengagement, although in other cases a small step often occurred, perhaps as a result of movement of the cell within the suction pipette. Following disengagement of the patch pipette the photoresponses recorded with the suction pipette remained unchanged from their form immediately prior to removal. Although not shown in this figure such responses often remained little changed for an hour or more, and this we have previously interpreted to indicate that the BAPTA was 'trapped' in the cytoplasm, neither crossing the membrane nor being metabolized (Torre, Matthews & Lamb, 1986).

In the experiments reported subsequently in this paper BAPTA was incorporated into cells using the method described here but with the modification that, in order to superfuse the outer segment, the inner segment was drawn into the suction pipette (Yau *et al.* 1981) and the patch pipette was sealed onto the protruding outer segment. Figures 5 and 6 show responses to lowered external calcium while in the whole-cell mode, and Fig. 8 shows responses following disengagement.

Throughout this paper we use the term 'circulating current' to refer to the light-suppressible current recorded with the suction pipette, and its value in the dark steady state is termed the dark current. Strictly speaking, however, the suction pipette collects only a fixed fraction (*ca.* 60–80%) of the total current which circulates. Note also, that for the patch pipette, zero current corresponds to the dark resting state since this pipette clamps the intracellular voltage at its dark level.

0-Ca^{2+} perfusion of normal cells

Records of 0-Ca^{2+} perfusion of the rod outer segment in darkness have been presented by Hodgkin *et al.* (1984, Figs 9 and 19). Responses with improved time resolution are illustrated in Fig. 2, where 0-Ca^{2+} Ringer solution (containing 2 mM-BAPTA) was perfused onto the outer segment of a normal rod, both in darkness and in the presence of several intensities of background light. In order to avoid Na^+ loading of the cell the presentations of 0-Ca^{2+} Ringer solution were kept brief (approx. 1 s).

Figure 2*A* shows the 0-Ca^{2+} exposures on a moderate time base, together with the light responses on which they were presented. These light responses are shown magnified in Fig. 2*C*; the traces are noisy because the recording bandwidth was set to 40 Hz so as to minimize distortion of the rising phase of the response of 0-Ca^{2+}. In

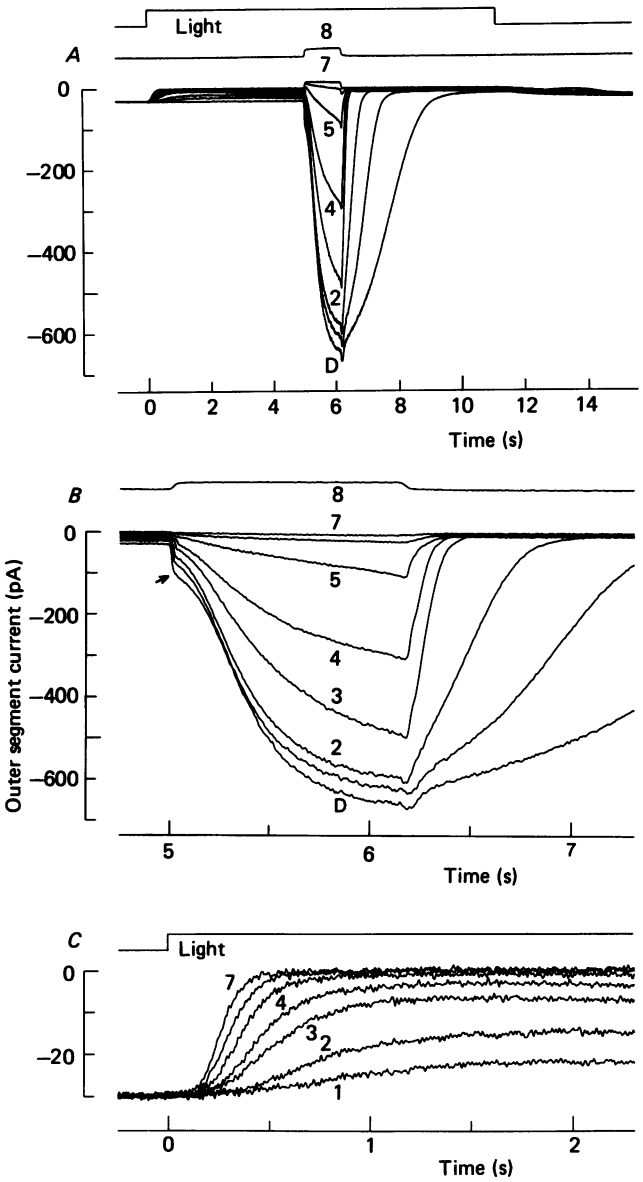

Fig. 2. Exposure of the outer segment of an intact rod to low external calcium concentration. Ordinate gives suction pipette current recorded with the inner segment drawn in, plotted inverted so as to give outer segment membrane current. *A*, raw responses to steady illumination of different intensities presented at time zero followed by brief exposures to 0-Ca^{2+} Ringer solution after 5 s. Trace 8 is the junction current obtained during bright steady illumination. *B*, light-sensitive currents in response to 0-Ca^{2+} exposures, obtained from the traces in *A* after subtraction of the junction current, and plotted on an expanded time base. The arrow indicates the knee of the rising phase for the trace in darkness (see text). *C*, rising phase of the light responses from *A*, magnified vertically 10×, and on the same time base as in *B*. Intensities of illumination (in isomerizations s^{-1}) were: *D* (dark), 0; 1, 44; 2, 170; 3, 650; 4, 1300; 5, 2600; 6, 5000; 7, $1{\cdot}05 \times 10^4$; 8, $2{\cdot}6 \times 10^5$. Recordings were filtered DC−40 Hz with a 6-pole Bessel filter, and were sampled at 5 ms intervals.

darkness, or in dim light, exposure to 0-Ca^{2+} rapidly increased the circulating current from 30 pA or less to around 600 pA, but with brighter lights the rate of increase was considerably reduced. In the presence of the brightest background a small change in current of inverted polarity was observed, and it is assumed that this residual response represents a junction current (Hodgkin *et al.* 1985) which must be subtracted to give the light-sensitive current.

After subtraction of this junction current the responses induced by 0-Ca^{2+} Ringer solution are replotted in Fig. 2*B* on an expanded time base. The trace in darkness shows a prominent 'knee' in the rising phase immediately after switching to 0-Ca^{2+} Ringer solution (see arrow in Fig. 2*B*). Such a knee was a reproducible feature observed in forty-seven of fifty-three cells in which rapid perfusion with 0-Ca^{2+} Ringer solution was examined. In the remaining six cells, from early experiments, the speed of solution change was apparently too slow (e.g. with a rise time of perhaps 100 ms in comparison with about 30 ms in Fig. 2); we presume that the absence of a knee in the records of Fig. 19 of Hodgkin *et al.* (1984) has a similar explanation. Recently a similar knee has been reported by Nakatani & Yau (1988) and Rispoli, Sather & Detwiler (1988). We usually attempted to set the perfusion at a rate sufficiently high to give an abrupt transition, but not so high as to damage the cell when it was later in the delicate whole-cell configuration; unfortunately this was not always successful.

In the traces in Fig. 2*B* for dim backgrounds the knee is still present, although smaller. It would appear that the reason for the reduced prominence of the knee in these traces in the light is that, as explained later (p. 489), the immediate effect of a 0-Ca^{2+} exposure is simply to scale-up whatever circulating current is already flowing.

Upon return to normal Ringer solution with 1 mM-Ca_o^{2+} there was, in darkness, a distinct delay in the turn-off of the current (Fig. 2*A*). This delay was comparable with that illustrated by Hodgkin *et al.* (1984, Fig. 19), and as in their results, the delay was reduced by light. Figure 2 shows that in the presence of illumination of increasing intensity the turn-off became progressively faster, and was in fact much faster than the original suppression of the dark current elicited by the onset of the same intensity. Consider, for example, the response labelled 4 with 2600 isomerizations s^{-1}. As may be seen from the expanded traces in Fig. 2*C* the dark current of 30 pA was suppressed by 80% in about 750 ms, whereas the current of about 300 pA in 0-Ca^{2+} was suppressed by a similar fraction within 150 ms of the return to normal Ringer solution. The lesser speed of suppression of dark current at the onset of illumination is not altogether surprising, as a chain of events intervenes between light absorption and hydrolysis of cyclic GMP.

Light-sensitive nature of the current increase

It might be argued that a substantial part of the change in current elicited by a 0-Ca^{2+} exposure could represent a change in the electrogenic Na^+–Ca^{2+} exchange current (Yau & Nakatani, 1984; Hodgkin *et al.* 1985). In order to exclude this possibility we examined whether the increased current was light suppressible.

Figure 3*B* illustrates the response to intense illumination presented at different times relative to the switch to 0-Ca^{2+} Ringer solution; the traces have been junction

compensated (Hodgkin *et al.* 1985). In the terminology of Hodgkin, McNaughton & Nunn (1987; see p. 351 and Fig. 1), their response L obtained during bright saturating light has been subtracted from each trace. Hence our trace *b*, in which an intense flash was presented just before the solution change, corresponds to their response DL−L. Under our conditions this trace represents the normal light-sensitive current plus the exchange current in 0-Ca^{2+} Ringer solution.

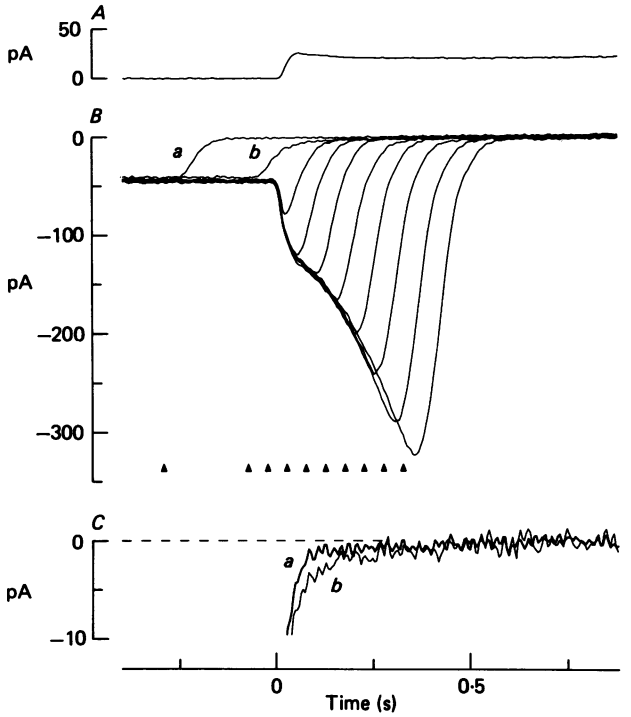

Fig. 3. Light sensitivity of the current induced by 0-Ca^{2+}, in a cell under control conditions. *A*, junction current. *B*, junction-compensated exposures to 0-Ca^{2+} Ringer solution, with bright flashes delivered at the times indicated by the arrow-heads. Trace *a* was obtained with the flash alone; trace *b* was obtained for presentation of the flash just prior to the stage movement, so that the light response and junction current were nearly synchronous. *C*, traces *a* and *b* from *B*, replotted at higher gain, and with trace *a* (heavy) shifted so that the flash timings coincide. The tail currents represent decay of electrogenic exchange in normal Ringer solution (*a*) and in 0-Ca^{2+} Ringer solution (*b*).

This trace has been replotted at a higher gain in Fig. 3*C* together with the flash response in normal Ringer solution at the same intensity (heavy trace), which shows the normal decay of the exchange current (Yau & Nakatani, 1985; Torre *et al.* 1986; Hodgkin *et al.* 1987). The exchange current in 0-Ca^{2+} Ringer solution appears increased in amplitude, perhaps by a factor of about 2. In investigating the saturating amplitude of the exchange current Hodgkin & Nunn (1987) found an approximate doubling in 1 μM-Ca_o^{2+}, so that our result seems consistent with theirs. However, in Fig. 3*B* the presence of noise makes detailed comparison of the traces difficult; in particular the final asymptotic level is rather uncertain. We conclude from this experiment that alteration in the size of the exchange current cannot

account for more than a small proportion of the increase of about 100 pA which occurs within 100 ms of switching to 0-Ca^{2+} Ringer solution.

Exposure to low-calcium solution containing no BAPTA

It might be thought that the presence of the knee in the response to lowered external calcium could arise somehow from the presence of BAPTA in the 0-Ca^{2+} Ringer solution, but this is not the case. Figure 4*A* shows the effect of rapidly switching to 50 μM-Ca^{2+} Ringer solution (containing no buffer) for periods of different duration. The form of the initial rise and knee is similar to that obtained in the same cell using 0-Ca^{2+} Ringer solution containing 2 mM-BAPTA (Fig. 4*B*). However, the amplitudes of both the early and later phases are smaller than with 0-Ca^{2+} Ringer solution. Upon return to normal Ringer solution following exposure to 50 μM-Ca^{2+}, rapid and delayed components are also apparent, similar to the behaviour reported by Hodgkin *et al.* (1985) upon raising Ca_o^{2+} from 1 to 10 mM.

For the shortest exposure to 50 μM-Ca^{2+} in Fig. 4*A* the rapid change at onset corresponded to a scaling up of the circulating current of approximately 2·0 times, when measured at the knee of the rising phase (arrow). Upon return to Ringer solution at the end of this short exposure the circulating current was rapidly scaled down by about the same ratio (2·0). Although it was not possible to make an entirely clear separation of the rapid and delayed components during the rising phase in normal cells, later experiments (e.g. Fig. 6) with calcium buffer in the cytoplasm suggested that the rapid change was about three-quarters complete at the time of the knee.

Similar behaviour in another cell is shown at higher magnification in Fig. 4*C*. In nine cells exposed to 50 μM-Ca^{2+} the amplitude of the current at the knee in the rising phase averaged 1·9 times the resting dark current (range 1·6–2·1); in five of these same cells exposed to 0-Ca^{2+} Ringer solution the amplitude at the knee averaged 2·8 times the dark current. In other experiments using 10 μM-free Ca^{2+} buffered with 2 mM-EDTA–Mg, the initial rise and knee were similar in form to that with 0-Ca^{2+} Ringer solution, but slightly smaller in amplitude. We have not, however, undertaken a systematic study of the effects of different external Ca^{2+} concentrations.

0-Ca^{2+} exposure during introduction of BAPTA into the cytoplasm

In order to separate external and internal actions of altered Ca_o^{2+}, the calcium buffer BAPTA was incorporated into the rod and 0-Ca^{2+} exposures were presented as the buffer diffused into the cell. One complication in performing this experiment, with the inner segment drawn into the suction pipette, is that once the membrane patch is ruptured to give the whole-cell configuration the response recorded with the suction pipette disappears, since the interior of the cell is voltage clamped (Bodoia & Detwiler, 1985; Lamb *et al.* 1986). This has the advantage that the quality of voltage clamping may be assessed from the suction pipette current; when the interior is adequately voltage clamped the suction pipette current simply gives a measure of the junction current, providing precise information about the timing of solution changes. A disadvantage of the configuration, however, is that the outer segment current can only be monitored with the whole-cell patch pipette, and we have

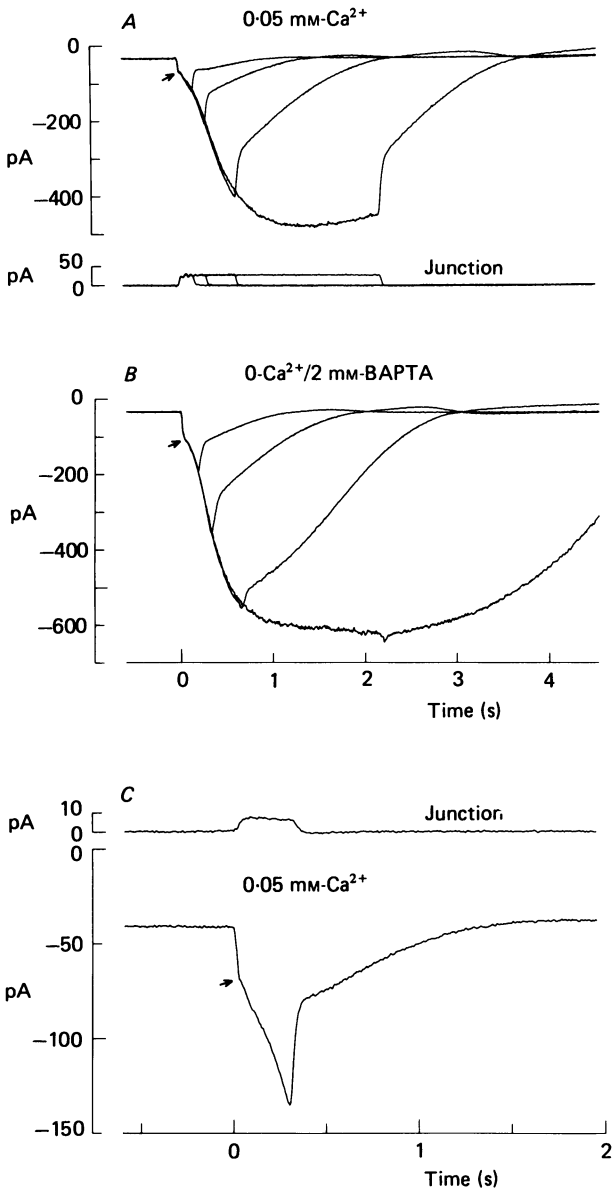

Fig. 4. Comparison of effects of brief exposures of the outer segment to Ringer solution containing nominally 50 μM-Ca^{2+} with no BAPTA (*A*), and 0-Ca^{2+} with 2 mM-BAPTA (*B*). The arrow in each panel indicates the knee of the rising phase of the current increase. The 50 μM-Ca^{2+} Ringer solution (and the normal Ringer solution) contained 10 μM-EDTA to chelate heavy metals. After allowance for the presumed presence of trace quantities of calcium in the water and chemicals, the free calcium concentrations were probably around 50 μM and 10^{-9} M respectively. The durations of the exposures, indicated by the junction currents, were approximately 0·2, 0·3, 0·6 and 2·2 s. The ringing in the junction current was atypical, and may have resulted from the presence of a small bubble which was seen above the θ perfusion tube. Resting dark current of cell in *A* and *B*, 35 pA. *C*, magnified view of a short-duration exposure to 50 μM-Ca^{2+} Ringer solution in another cell; resting dark current 41 pA.

generally found that this gives a noisier record than does the suction pipette over the low bandwidth of interest (Lamb *et al.* 1986).

Figure 5 shows the results of such an experiment. In order to avoid excessive influx of Na^+ the duration of each 0-Ca^{2+} exposure was again kept brief, and additionally a bright flash was delivered towards the end of each exposure (arrows) to shut off the

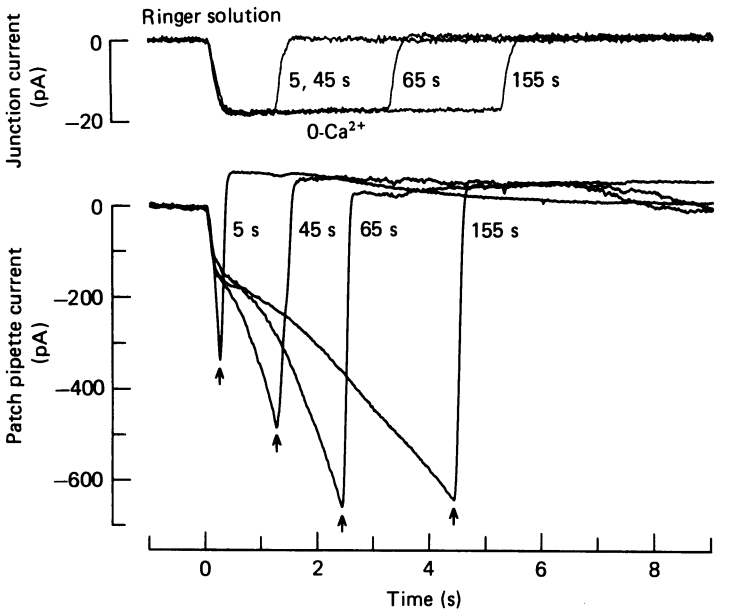

Fig. 5. Superimposed responses to lowered external calcium concentration during diffusion of BAPTA from the patch pipette into the cell; abscissa plots time after switching to 0-Ca^{2+} Ringer solution. Lower traces show responses to 0-Ca^{2+} exposures recorded with the patch pipette; the indicated times are from rupture of the outer segment membrane patch until onset of the 0-Ca^{2+} exposure. Since the patch pipette was voltage clamped at the dark resting level, zero current represents the dark resting state. Arrows show the time of presentation of bright flashes. Upper traces show the junction currents, recorded simultaneously using the suction pipette, indicating the timing of the solution changes. Note that the 0-Ca^{2+} exposure was increased in duration, and the timing of the bright flash presentation was delayed, in successive presentations. Patch pipette contained 11 mM-BAPTA; access resistance 12 MΩ.

light-sensitive current. The lower traces in Fig. 5 show the outer segment current recorded by the patch pipette, and the upper traces show the junction current measured simultaneously using the suction pipette, while the numbers against the traces indicate the time after rupture of the membrane patch at which each 0-Ca^{2+} exposure was presented.

With the earliest presentation (5 s after patch rupture) the outer segment current rose very rapidly, although a knee is not apparent because the solution change was not fast enough (10–90 % rise time *ca.* 150 ms). With successively later presentations, however, a knee is clearly apparent, and the slope of the subsequent rise in current becomes progressively reduced. This result is consistent with the notion that BAPTA slowly diffuses into the cell from the patch pipette, causing progressively slower changes in Ca_i^{2+} to be induced by changes in Ca_o^{2+}.

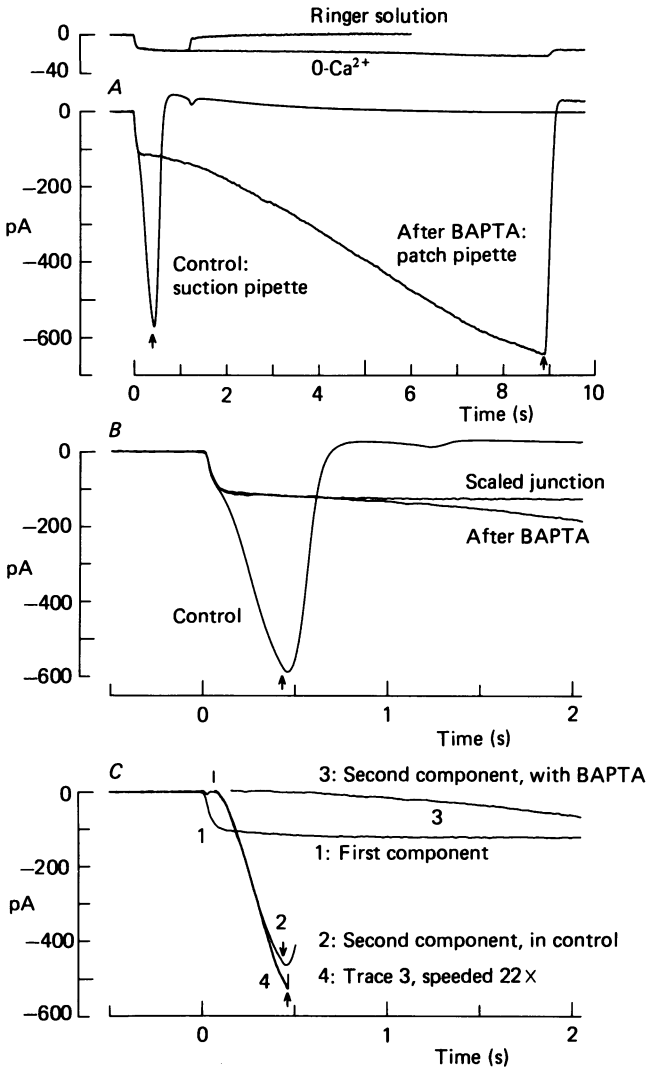

Fig. 6. Comparison of effects of 0-Ca^{2+} exposures in darkness, in control conditions and after allowing BAPTA to diffuse into the rod; patch pipette contained 11 mM-BAPTA. *A*, outer segment current in control conditions recorded with the suction pipette (average of six presentations), and approximately 3 min after patch rupture recorded with the patch pipette voltage clamped at −40 mV (average of four presentations between 150 and 250 s after rupture). The junction current has been inverted for ease of comparison. *B*, traces from *A* on an expanded time base; the junction current has been scaled vertically by 7·5×. *C*, separation of components. Trace 1 plots the scaled junction current from *B* (i.e. the first component). Trace 2 plots the control response from *B* minus the scaled junction current (i.e. the second component in control conditions). Trace 3 plots the response from *B* after BAPTA minus the scaled junction current (i.e. the second component with BAPTA incorporated). Trace 4 is trace 3 speeded in time by 22-fold, from a time origin indicated by the vertical mark. The junction current in *A* was measured with the suction pipette. Under control conditions (a 1·2 s exposure to 0-Ca^{2+}) it was measured separately during a bright step of light (which was turned off at time 6 s). During whole-cell voltage clamping (an exposure to 0-Ca^{2+} extending beyond the end of the record) the junction current was measured simultaneously, and exhibits a small error current at the time of the flash, corresponding to approx. 1 % of the patch pipette current response; this is due to imperfect voltage clamping, see text. Timing of bright flashes is indicated by the arrows.

Comparison of 0-Ca^{2+} exposures before and after incorporation of BAPTA

Responses to 0-Ca^{2+} exposures in another cell are compared in Fig. 6 under control conditions and about 3 min after rupture of the patch of outer segment membrane. In Fig. 6*A* a relatively slow time base is used, and the upper pair of traces plot the junction current to indicate the timing of the solution changes.

The lower pair of traces plot the outer segment current relative to its level in darkness, recorded with the suction pipette under control conditions (Control) and with the patch pipette in the whole-cell mode (After BAPTA). In each case the arrow indicates the timing of the bright flash used to suppress the light-sensitive current. As in the case of Fig. 2 a knee is apparent in the control response recorded with the suction pipette. The whole-cell trace, obtained after allowing several minutes for BAPTA to diffuse into the cell, shows a plateau phase rather more distinct than that in Fig. 5, followed by a second slower increase in current.

The rising phase of the current induced by exposure to 0-Ca^{2+} Ringer solution is examined on a faster time base in Fig. 6*B*, where the junction current has been scaled to fit the rising phase of the other two curves. In this cell it was not in fact necessary to scale the suction pipette current with respect to the patch pipette current, because the saturating response amplitudes happened fortuitously to match. The original suction pipette dark current was 35 pA and, although the patch pipette always measured a larger current than the suction pipette, by the time the 0-Ca^{2+} exposures were given with trapped BAPTA, the amplitude of the patch pipette photocurrent response had declined somewhat, to 35 pA also. The fact that the rising phases of the suction pipette current, of the patch pipette current, and of the scaled junction current do coincide would appear to indicate that the response to 0-Ca^{2+} Ringer solution, both under control conditions and with buffer incorporated, has an initial phase which is as rapid as the solution change. The secondary rise in current can be seen from Fig. 6*A* and *B* to be slightly delayed from the solution change under control conditions and to be very considerably delayed once buffer has been allowed to diffuse into the cell.

Separation of the two components

On the assumption that the control response, and the response with buffer incorporated, are each composed of a component having the time course of the solution change, then the time course of the remaining current may be investigated by subtracting the scaled junction current. The results of this subtraction are shown in Fig. 6*C*. The trace labelled 1 plots the scaled junction current from Fig. 6*B*, and represents the first component of current increase. Trace 2 plots the control suction pipette current minus the scaled junction current, and represents the second component under control conditions. Its onset is not apparent until about 70 ms after the solution change. The trace labelled 3 similarly plots the patch pipette current minus the scaled junction current, and represents the second component of current increase in the presence of buffer in the cytoplasm. After acceleration 22-fold in time this response has been replotted as trace 4, and is not dissimilar to trace 2.

Our interpretation of these results is as follows. Within the time resolution of our

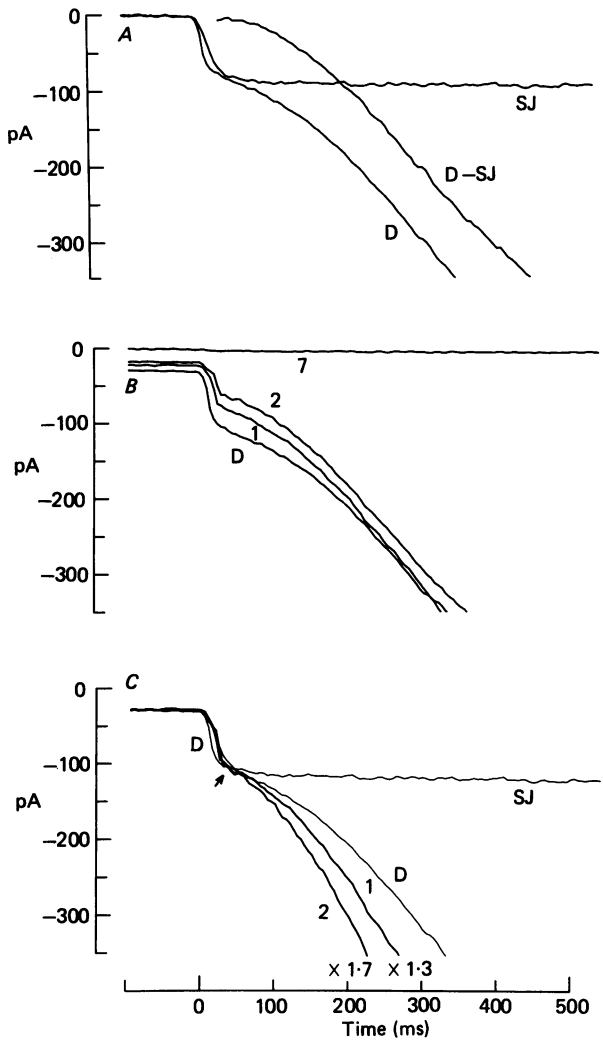

Fig. 7. Onset of response to 0-Ca^{2+} exposure in a cell under control conditions; junction-compensated records from Fig. 2*B* on a further-expanded time base. *A*, comparison of the response in darkness (D) with the scaled junction current (SJ, magnified −10·2× from trace 8 of Fig. 2*A*). As described in the text trace D leads trace SJ by about 10 ms. The difference between these traces (D−SJ) is plotted, and represents the second component of current increase; the initial transient resulting from the lead has been omitted for clarity. *B*, traces obtained in darkness (D), for the two dimmest backgrounds (1 and 2), and for a bright background (7) from Fig. 2*B*, showing reduced size of the rapid component. *C*, traces 1 and 2 from *B* have been scaled in amplitude (by 1·3× and 1·7× respectively). This provides approximate coincidence both of the initial steady level and of the amplitude at the knee. Traces D and SJ as in *A*. Responses have been filtered DC to 40 Hz and sampled at 5 ms intervals; light intensities are given in the legend to Fig. 2.

method the first component of current increase is as rapid as the solution change, and we presume that this rapid component represents a direct action of altered external Ca^{2+}. We interpret the effect of internal calcium buffer in slowing the second component to indicate that this delayed component represents an internal action of Ca^{2+}: lowered Ca_o^{2+} leads with a delay to a reduction of Ca_i^{2+} which causes the increased current.

As we have argued previously (Lamb *et al.* 1986), the presence of BAPTA ought not to alter the cytoplasmic calcium concentration in the steady state, but should simply slow down the rate at which any changes in Ca_i^{2+} take place. Therefore, the similarity of traces 2 and 4 in Fig. 6*C* may be taken to indicate that the amount of buffer which had diffused into the cell within 3 min was sufficient to cause an approximately 20-fold increase in the cell's calcium-buffering power over its normal level, at the dark resting Ca_i^{2+}. Qualitatively similar results to those of Figs 5 and 6 have been observed in twelve rods in which it was possible to perfuse 0-Ca^{2+} Ringer solution while recording with a whole-cell patch pipette containing BAPTA.

An attempt to separate the rapid and delayed components for a cell in which BAPTA had not been incorporated is shown in Fig. 7*A*; this is the same cell as in Fig. 2. Note that in comparison with Fig. 6*C* the time base is faster and the low-pass filtering frequency has been set to 40 Hz rather than 20 Hz. Figure 7*A* replots the trace in darkness from Fig. 2*B*, together with the junction current scaled in amplitude so as to correspond in size with the rapid component of change in dark current.

Somewhat surprisingly the rapid component appears to lead the junction current by about 10 ms. Since the two curves begin rising at the same instant, it is clear that the outer segment plasma membrane experiences the onset of altered calcium concentration at the same time as the junction current is recorded. Nevertheless, the rapid component of increase in dark current appears to be essentially complete in a shorter time than the junction current. This phenomenon was observed in each of thirteen cells, examined at a bandwidth of 40 Hz, in which the solution change was very fast. Hodgkin *et al.* (1984) have shown that the junction current and the change in ion concentration near the pipette need not have an identical time course. In our case it seems likely that the half-way transition point in the junction current would correspond roughly to equal mixing of the 0-Ca^{2+}/BAPTA solution with the normal Ringer solution, at which point the free Ca_o^{2+} would be 10^{-7} M. On this basis the slight lead would be explained if the rapid component were triggered by a relatively small drop in Ca_o^{2+}; i.e. if the concentration range for activation of this component were nearer to 1 mM than to 10^{-7} M. And indeed this appears to be the case according to the result presented in Fig. 4. Hence this lead seems in no way unphysical, although it slightly complicates the subtraction of scaled junction current required to separate the rapid and delayed components. We conclude that within the time resolution of the method, the onset of the first component is as rapid as the change in Ca_o^{2+}. From inspection of the earliest rising phase in Fig. 6, we think that the method would be capable of detecting a delay of greater than 10 ms.

Separation of the delayed component is shown in Fig. 7*A* by the trace D−SJ, which plots the difference between the dark current and the scaled junction current (except at the earliest times). Here it again appears, in a normal cell, that

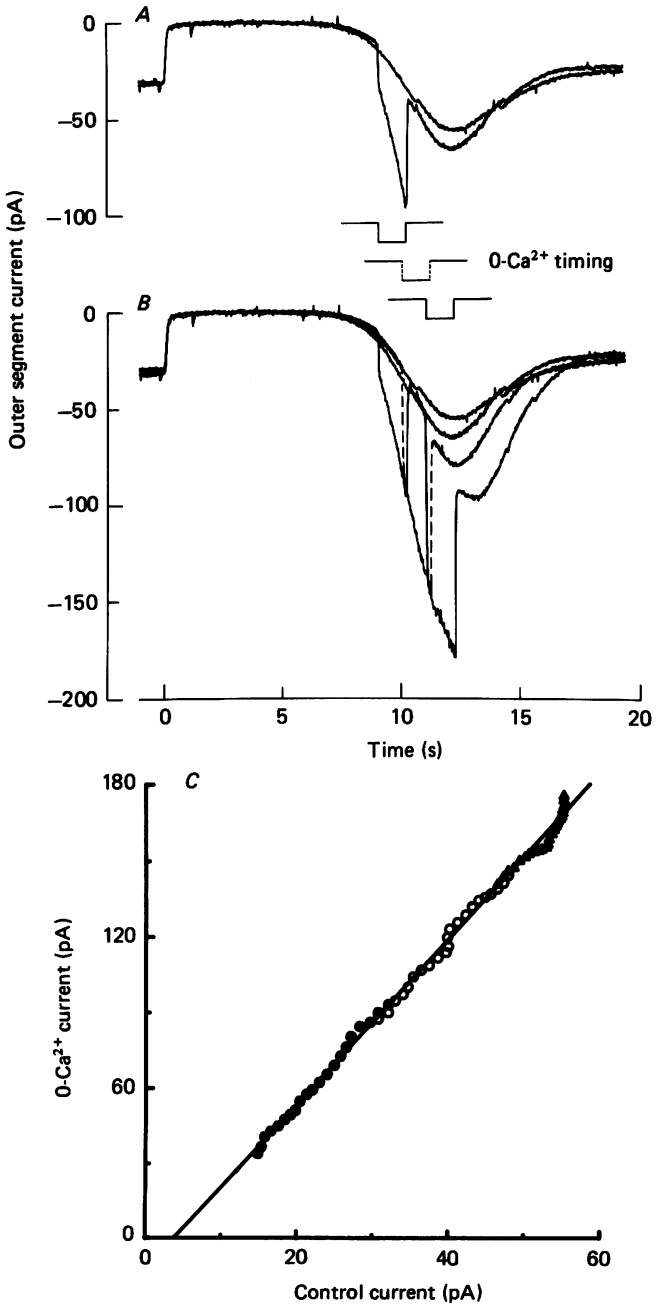

Fig. 8. Junction-corrected responses to 0-Ca^{2+} exposures during the overshoot, for a rod with BAPTA trapped in the cytoplasm. *A*, response to flash alone, superimposed with response to a single presentation of 0-Ca^{2+}. *B*, superimposed responses to three such exposures at different times during the overshoot; the rising and falling phases are shown interrupted for the middle exposure. *C*, current flowing during 0-Ca^{2+} exposure is plotted as a function of the current in normal Ringer solution for these three exposures (●, ○ and ▲ respectively). The slope of the regression line is 3·3; the non-zero intercept on the abscissa probably resulted from the fact that the early part of the control response was slightly larger than the subsequent responses. Patch pipette contained 11 mM-BAPTA, and the rod had been in the whole-cell configuration with access resistance approx. 20 MΩ for 2 min.

development of the second component does not begin appreciably until about 70 ms after the rapid component. In addition, as shown in Fig. 2 this component continues growing in amplitude for at least 1 s in 0-Ca^{2+} solution. It is perhaps worth mentioning that in cells which had been in the fast-flowing solution for some time, where the outer segment was bent, the onset of the second component often became delayed and the knee was more prominent; however, we usually tried to study cells which appeared as nearly normal as possible.

Scaling of the first component

So far we have examined the magnitude of the first component only for 0-Ca^{2+} exposures presented when the circulating current was at its normal dark resting level. In order to examine the first component more fully we presented the 0-Ca^{2+} exposure when the circulating current was altered. This was done in two ways: firstly during recovery of flash responses in a cell containing trapped BAPTA (Fig. 8), and secondly during steady illumination in a normal cell (Fig. 7*B* and *C*).

Figure 8 illustrates 0-Ca^{2+} exposures during the recovery phase of the flash response in a cell in which BAPTA had been trapped. For simplicity Fig. 8*A* shows the response in Ringer solution together with just a single 0-Ca^{2+} exposure lasting 1 s. During this exposure the circulating current was increased by approximately 3-fold above the level obtained in Ringer solution. Figure 8*B* shows superimposed traces from three such 0-Ca^{2+} exposures given at successive times during the overshoot. The aim of presenting several short exposures rather than a single long one was to attempt to avoid significantly altering the internal calcium concentration, so that predominantly the first component could be examined.

The currents obtained during the 1 s exposures in Fig. 8*B* have been plotted in Fig. 8*C* against the current obtained in Ringer solution. To a reasonable approximation these results are consistent with the idea that the initial effect of exposure to 0-Ca^{2+} solution is simply to scale up whatever current is circulating by a factor of around 3 times.

The experiment of Fig. 8 proved difficult to perform because cells with trapped BAPTA often failed to pass large currents, and behaved as if the inner segment was limiting the passage of current. As a result we were able to attempt the experiment on only three cells, each of which gave qualitatively similar results.

The second method for examining the effect of 0-Ca^{2+} exposure when the circulating current began not at its dark level is shown in Fig. 7*B*, redrawn on a faster time base from Fig. 2. In the presence of steady illumination, which reduced the circulating current, the size of the early change upon exposure to 0-Ca^{2+} Ringer solution was reduced in amplitude. In Fig. 7*C* the traces for the two dimmest backgrounds have been scaled-up by the amount required to bring the original circulating current in steady light back to the level in darkness, and it may be seen that the first components are then very similar. Hence the magnitude of the first component of current increase upon switching to 0-Ca^{2+} solution represents an approximately constant scaling of the initial circulating current. In this cell the first component corresponded to approximately a 4-fold increase in current, both in darkness and in dim light.

The main difference between the scaled traces in Fig. 7*C* and the trace in darkness

is that, rather than *leading* the junction current as the trace in darkness does, the traces in dim light rise with a time course quite similar to that of the junction current. We are not certain of the explanation for this shift in time but, extending our previous explanation for the lead of the dark trace, one possibility would be that during illumination the calcium sensitivity of the rapid external component is shifted to a lower concentration range.

DISCUSSION

The extent to which the effects of lowering the external calcium concentration bathing the rod outer segment are due to a direct action of Ca_o^{2+}, or are mediated by a change in cytoplasmic calcium concentration, has not been entirely clear. By examining the effects of reduced Ca_o^{2+} using rapid solution changes we have been able to separate external and internal actions, which manifest themselves as rapid and slightly delayed components of current increase (Lamb *et al.* 1985*b*). Similar components following rapid elevation of Ca_o^{2+} have been reported by Hodgkin *et al.* (1985), who also attributed them to external and internal effects. In our experiments, separation of the two components and more definitive assignment to direct and to internal actions has been greatly assisted by incorporation of the calcium buffer BAPTA into the cytoplasm, thereby slowing the onset of any changes in Ca_i^{2+} which would normally occur when Ca_o^{2+} was lowered.

Very recently Rispoli *et al.* (1988) have developed a method for investigating the role of external Ca^{2+} on the rod. Exposure of a rod outer segment, containing normal levels of ATP and GTP, to lowered Ca_o^{2+} gives results very similar to those presented here. In the absence of nucleotide triphosphates but with exogenous cyclic GMP in the outer segment, only a rapid component is observed. By disabling the biochemistry in this way these authors are able to study the two components separately.

External action

The first component of current increase has a time course at least as fast as that of the solution change, in as far as this is represented by the junction current. The rapid component, which we attribute to a direct action of Ca_o^{2+} on the light-sensitive channels, caused an increase in circulating current of approximately 3 to 4 times when Ca_o^{2+} dropped from 1 mM to $< 10^{-8}$ M.

Menini, Rispoli & Torre (1988) have reported apparent dissociation constants for external Ca^{2+} and Mg^{2+} of 50 μM and 8 mM respectively. In the experiment of Fig. 4 in which Ca_o^{2+} was dropped to 50 μM, with bathing solution containing no calcium buffer, the knee in the rising phase remained quite prominent, but the amplitude of the current at this point corresponded to a scaling-up of about 2·0 times the dark current. In nine cells the ratio averaged 1·9, which would appear broadly consistent with the findings of Menini *et al.* (1988).

In darkness the rapid component began rising at the same time as the junction current, but appeared to be complete more quickly, leading the junction current by about 10 ms (Fig. 7*A*). As argued on p. 487, a lead of this kind would be expected if the rapid external component were elicited by the earliest part of the drop in Ca_o^{2+}; for example, if the range of Ca^{2+} sensitivity of this mechanism were in the range

of 50 μM as reported by Menini *et al.* (1988). In the presence of dim illumination the lead became less prominent (Fig. 7*C*). A possible explanation would be that the Ca^{2+} sensitivity of the external action moved to a lower range during illumination, as has recently been suggested by L. Cervetto, A. Menini, G. Rispoli & V. Torre (personal communication from V. Torre). It must be emphasized, however, that these time shifts are near the resolution of the method, so that the interpretation is tentative. From comparison of the earliest signs of onset of the junction current and of the rapid component in Fig. 7, we think that the method would have resolved a time delay of 10 ms. In earlier experiments, however, a lower recording bandwidth of 20 Hz was used, and the time courses of the junction current and of the rapid component were then indistinguishable.

The magnitude of the external component ought to be comparable with the effects of lowered external Ca^{2+} concentration on excised patches of outer segment membrane exposed to cyclic GMP. Unfortunately such results do not appear to be available explicitly. Haynes, Kay & Yau (1986) and Zimmerman & Baylor (1986) have shown that, by removing all divalent cations (primarily Ca^{2+} and Mg^{2+}) from both sides of the membrane, the conductance is increased by a factor of perhaps two orders of magnitude. Similarly Yau, Haynes & Nakatani (1986) have altered the divalent concentration bathing the cytoplasmic surface. But we are not aware of results which would show the effect of altered Ca_o^{2+} alone, when Mg_o^{2+} and Mg_i^{2+} remained constant.

Internal action

The fact that the second component of current increase becomes progressively delayed as BAPTA diffuses into the cytoplasm is strong evidence for an action mediated by Ca_i^{2+}. Furthermore, the effect of BAPTA on the second component confirms our previous argument, used in rejecting the calcium hypothesis of phototransduction (Lamb *et al.* 1986), that incorporation of BAPTA very significantly increased the calcium-buffering power of the cytoplasm. From the results of Fig. 6*C* the calcium-buffering power appeared to increase by a factor of approximately 20, when the patch pipette contained 11 mM-BAPTA and when several minutes had been allowed for diffusion.

Recently Hodgkin *et al.* (1987) have shown that the recovery of dark current upon return to Ringer solution following exposure to elevated Ca_o^{2+} is delayed by an amount dependent on the size of the calcium load (their Fig. 7). It seems likely that their phenomenon is analogous to what we refer to as the second or delayed component, since both are likely to result from a change in cytoplasmic calcium concentration.

We presume that the internal action of Ca^{2+} is mediated by effects on the cyclic nucleotide cascade of transduction (reviewed by Stryer, 1986; Pugh & Cobbs, 1986). A considerable body of evidence indicates that lowered Ca_i^{2+} leads to an elevation of the cyclic GMP level in the outer segment, although it is not yet clear whether it is the synthesis by guanyl cyclase or the hydrolysis by the phosphodiesterase which is affected, or whether indeed both are affected by Ca_i^{2+} (see for example Hodgkin *et al.* 1985; Torre *et al.* 1986). It has become important to understand the role of cytoplasmic Ca^{2+} concentration, since it is now clear that Ca_i^{2+} declines during the

normal light response (Yau & Nakatani, 1985; Gold, 1986; McNaughton, Cervetto & Nunn, 1986; Ratto, Payne, Owen & Tsien, 1988).

The true magnitude of the second component in 0-Ca^{2+} Ringer solution in our experiments is difficult to gauge because the outer segment current (typically exceeding 600 pA) may well have been limited by purely electrical factors (see below). Since, overall, the current increased by a factor of 15–20 typically, while the first component represented an increase of about 3–4 times, then the second component must have corresponded to at least a 4- to 5-fold increase, but it seems certain that this is an underestimate. With 50 μM-Ca_o^{2+} the total current eventually increased by 10- to 15-fold, while the first component represented an approximate doubling, so that the second component then corresponded to a factor of about 5–8 times. Obviously, however, the second component in $< 10^{-8}$ M-Ca_o^{2+} is unlikely to have been smaller than that in 50 μM-Ca_o^{2+}, and we conclude that the current in 0-Ca^{2+} has been limited.

One of the factors which will limit the maximal current is the total series resistance of the outer segment and the patch pipette. As shown in the Methods (p. 475) the total resistance is likely to be about 60 MΩ, so that the current recorded would be limited by series resistance to a maximum of the order of 1 nA, and therefore the value above for the peak magnitude of the second component of current increase is bound to be an underestimate. In cells recorded with only a suction pipette (i.e. not under voltage clamp), the current is likely to limit at an even smaller value.

Other features of the response

As mentioned previously, a feature of the response which requires explanation is the apparent absence of a rapid component upon return from low-calcium solution to normal Ringer solution with 1 mM-Ca_o^{2+}. Sometimes there was even a hint of a component of inward current at this time (Fig. 2). The delayed recovery was more prominent with 0-Ca^{2+} Ringer solution than with 50 μM-Ca^{2+} Ringer solution, and was particularly noticeable after a 2 s exposure, which led to saturation of the current. As shown in Fig. 4*A*, following a brief exposure to 50 μM-Ca_o^{2+} there was a rapid component of decrease upon return to 1 mM-Ca^{2+} Ringer solution, representing approximately the inverse of the scaling up at the beginning (see also Fig. 4*C*, different cell). The slow component of recovery following 50 μM-Ca^{2+} became more prominent following longer exposures, but was never as pronounced as that following 0-Ca^{2+} Ringer solution; with 50 μM-Ca^{2+} a clear rapid component of recovery remained, although it was somewhat less prominent.

The absence of the rapid component upon return to normal Ringer solution in Figs 2*A* and 4*B* might therefore result in part from the fact that the current was limiting. However, another explanation would involve an increased Ca^{2+} permeability of the channel as a result of elevated cyclic GMP levels. On this idea the dissociation constant of the channel for Ca_o^{2+} might increase (from the 50 μM estimated by Menini *et al.* 1988, normally) to a considerably higher value with elevated cyclic GMP levels, as has been suggested by L. Cervetto, A. Menini, G. Rispoli & V. Torre (personal communication from V. Torre). The brief inward component of current at the end of the 0-Ca^{2+} exposure in darkness in Fig. 2 might therefore represent a calcium current.

A phenomenon which may be related concerns the kinetics of the response to 0-Ca^{2+} exposure in the presence of dim backgrounds. As shown in Fig. 2*B*, backgrounds 1 and 2 (which reduced the circulating current to approximately 0·77 and 0·59 of its dark level) had almost no effect on the rise of the second (delayed) component. Nevertheless they had major effects on the time course of the recovery of current upon return to normal Ringer solution. This observation is qualitatively consistent with previous findings indicating that the light sensitivity of the transduction mechanism is lower when Ca_i^{2+} drops and is higher when Ca_i^{2+} rises (Yau *et al.* 1981; Lamb, Matthews & Torre, 1985*a*; Torre *et al.* 1986; Hodgkin, McNaughton & Nunn, 1986).

We are most grateful to Dr V. Torre for helping with the early experiments, and to Professor A. L. Hodgkin for suggesting the experiments on light sensitivity and on short exposures. We wish to thank Professors D. A. Baylor, P. B. Detwiler and A. L. Hodgkin and Dr P. A. McNaughton for helpful discussions and for reading the manuscript. Supported by the MRC, the US NIH (EY-06154), the Wellcome Trust, NATO, and St John's College, Cambridge.

REFERENCES

BASTIAN, B. L. & FAIN, G. L. (1979). Light adaptation in toad rods: requirement for an internal messenger which is not calcium. *Journal of Physiology* **297**, 493–520.

BERTRAND, D., FUORTES, M. G. F. & POCHOBRADSKY, J. (1978). Actions of EGTA and high calcium on the cones in the turtle retina. *Journal of Physiology* **275**, 419–437.

BODOIA, R. D. & DETWILER, P. B. (1985). Patch-clamp recordings of the light-sensitive dark noise in retina rods from the lizard and frog. *Journal of Physiology* **367**, 183–216.

BROWN, J. E. & PINTO, L. H. (1974). Ionic mechanism for the photoreceptor potential of the retina of *Bufo marinus*. *Journal of Physiology* **236**, 575–591.

GOLD, G. H. (1986). Plasma membrane calcium fluxes in intact rods are inconsistent with the 'calcium hypothesis'. *Proceedings of the National Academy of Sciences of the U.S.A.* **83**, 1150–1154.

HAGINS, W. A. (1972). The visual process: Excitatory mechanisms in the primary receptor cells. *Annual Review of Biophysics and Bioengineering* **1**, 131–158.

HAGINS, W. A. & YOSHIKAMI, S. (1974). A role for Ca^{++} in excitation of retinal rods and cones. *Experimental Eye Research* **18**, 299–305.

HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

HAYNES, L. W., KAY, A. R. & YAU, K.-W. (1986). Single cGMP-activated channel activity in excised patches of rod outer segment membranes. *Nature* **321**, 66–70.

HODGKIN, A. L., MCNAUGHTON, P. A. & NUNN, B. J. (1985). The ionic selectivity and calcium dependence of the light-sensitive pathway in toad rods. *Journal of Physiology* **358**, 447–468.

HODGKIN, A. L., MCNAUGHTON, P. A. & NUNN, B. J. (1986). Effects of changing Ca before and after light flashes in salamander rods. *Journal of Physiology* **372**, 54*P*.

HODGKIN, A. L., MCNAUGHTON, P. A. & NUNN, B. J. (1987). Measurement of sodium–calcium exchange in salamander rods. *Journal of Physiology* **391**, 347–370.

HODGKIN, A. L., MCNAUGHTON, P. A., NUNN, B. J. & YAU, K.-W. (1984). Effects of ions on retinal rods from *Bufo marinus*. *Journal of Physiology* **350**, 649–680.

HODGKIN, A. L. & NUNN, B. J. (1987). The effect of ions on sodium–calcium exchange in salamander rods. *Journal of Physiology* **391**, 371–398.

LAMB, T. D., MCNAUGHTON, P. A. & YAU, K.-W. (1981). Longitudinal spread of activation and background desensitization in toad rod outer segments. *Journal of Physiology* **319**, 463–496.

LAMB, T. D., MATTHEWS, H. R. & TORRE, V. (1985*a*). Trapped calcium buffer alters the light-adaptation properties of rod photoreceptors from *Ambystoma tigrinum*. *Journal of Physiology* **365**, 37*P*.

LAMB, T. D., MATTHEWS, H. R. & TORRE, V. (1985*b*). Rapid and delayed components in the response of salamander retinal rods to reduced external calcium. *Journal of Physiology* **369**, 34*P*.

LAMB, T. D., MATTHEWS, H. R. & TORRE, V. (1986). Incorporation of calcium buffer into salamander retinal rods: a rejection of the calcium hypothesis of phototransduction. *Journal of Physiology* **372**, 315–349.

LIPTON, S., OSTROY, S. E. & DOWLING, J. E. (1977). Electrical and adaptive properties of rod photoreceptors. I. Effects of altered extracellular Ca^{++} levels. *Journal of General Physiology* **70**, 747–770.

MCNAUGHTON, P. A., CERVETTO, L. & NUNN, B. J. (1986). Measurement of the intracellular free calcium concentration in salamander rods. *Nature* **322**, 261–263.

MATTHEWS, H. R., TORRE, V. & LAMB, T. D. (1985). Effects on the photoresponse of calcium buffers and cyclic GMP incorporated into the cytoplasm of retinal rods. *Nature* **313**, 582–585.

MENINI, A., RISPOLI, G. & TORRE, V. (1988). The ionic selectivity of the light-sensitive current in isolated rods of the tiger salamander. *Journal of Physiology* **402**, 279–300.

NAKATANI, K. & YAU, K.-W. (1988). Calcium and magnesium fluxes across the plasma membrane of the toad rod outer segment. *Journal of Physiology* **395**, 695–729.

PUGH JR, E. N. & COBBS, W. H. (1986). Visual transduction in vertebrate rods and cones: a tale of two transmitters, calcium and cyclic GMP. *Vision Research* **26**, 1613–1643.

RATTO, G. M., PAYNE, R., OWEN, W. G. & TSIEN, R. Y. (1988). The concentration of cytosolic free Ca^{2+} in vertebrate rod outer segments measured with fura2. *Journal of Neuroscience* (in the Press).

RISPOLI, G., SATHER, W. A. & DETWILER, P. D. (1988). Effect of triphosphate nucleotides on the response of detached rod outer segments to low external calcium. *Biophysical Journal* **53**, 388a.

STRYER, L. (1986). Cyclic GMP cascade of vision. *Annual Review of Neuroscience* **9**, 87–119.

TORRE, V., MATTHEWS, H. R. & LAMB, T. D. (1986). Role of calcium in regulating the cyclic GMP cascade of phototransduction in retinal rods. *Proceedings of the National Academy of Sciences of the U.S.A.* **83**, 7109–7113.

TSIEN, R. Y. (1980). New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. *Biochemistry* **19**, 2396–2404.

YAU, K.-W., HAYNES, L. W. & NAKATANI, K. (1986). Roles of calcium and cyclic GMP in visual transduction. In *Membrane Control of Cellular Activity*, ed. LUTTGAU, H. C., pp. 343–366. Stuttgart: Gustav Fischer.

YAU, K.-W., MCNAUGHTON, P. A. & HODGKIN, A. L. (1981). Effect of ions on the light-sensitive current in retinal rods. *Nature* **292**, 502–505.

YAU, K.-W. & NAKATANI, K. (1984). Electrogenic Na–Ca exchange in retinal rod outer segment. *Nature* **311**, 661–663.

YAU, K.-W. & NAKATANI, K. (1985). Light-induced reduction of cytoplasmic free calcium in retinal rod outer segment. *Nature* **313**, 579–582.

YOSHIKAMI, S. & HAGINS, W. A. (1973). Control of the dark current in vertebrate rods and cones. In *Biochemistry and Physiology of Visual Pigments*, ed. LANGER, H., pp. 245–255. Berlin: Springer.

ZIMMERMAN, A. L. & BAYLOR, D. A. (1986). Cyclic GMP sensitive conductance of retinal rods consists of aqueous pores. *Nature* **321**, 70–72.