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THE EFFECTS OF PHOSPHODIESTERASE INHIBITORS AND LANTHANUM IONS ON THE LIGHT-SENSITIVE CURRENT OF TOAD RETINAL RODS

BY L. CERVETTO* AND P. A. MCNAUGHTON

*From the †Physiological Laboratory, Downing Street, Cambridge CB2 3EG and the *Istituto di Neurofisiologia del CNR, Via San Zeno 51, 56100 Pisa, Italy*

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SUMMARY

1. The light-sensitive current of isolated toad rods was recorded using the method of Yau, McNaughton & Hodgkin (1981) and the effects of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) and of La ions were examined.

2. IBMX caused an increase in the light-sensitive current and a prolongation of the time course of the response. A small inward current which may reflect the operation of an Na–Ca exchange pump was also increased in IBMX. With low doses of IBMX the time course of the dim flash response could be mapped onto that in Ringer solution by a linear transformation of the time scale.

3. Light adaptation had opposite effects to those of IBMX on the time course of the dim flash response, and a steady background light could exactly neutralize the effects of IBMX on the time course. Light adaptation had the additional effect of strongly reducing the amplitude of the dim flash response.

4. La ions caused a rapid inhibition ($t_{\frac{1}{2}} < 1$ s) followed by slow inhibition ($t_{\frac{1}{2}} \approx 30$ s) of the light-sensitive current. In low [Ca] the rapid inhibition became more prominent, perhaps because of a competition between La and Ca for a blocking site near the light-sensitive channel.

5. The time constants of the falling phases of responses to both bright and dim flashes were slowed by La. The dim flash response could be fitted by a model in which a single time constant in the chain underlying the flash response is slowed by La.

6. La reduced the rate of activation of light-sensitive current in response to a reduction of external [Ca]. A concentration of La sufficient to block the activation of current in low [Ca] did not prevent the activation of current in IBMX.

7. Light-sensitive currents carried by Mn, Ca or Mg in the absence of Na and in the presence of IBMX were inhibited by La. An outward current observed in the absence of permeable ions was inhibited by La.

8. The effects of La on the time course of the response and on the rate of activation of current when [Ca] is reduced are consistent with an inhibition of the Ca pump. La ions also have a high affinity for the light-sensitive channel and can block current carried by another ion.

† Experiments were carried out in Cambridge.

INTRODUCTION

Our knowledge of the internal events occurring in a photoreceptor following the absorption of a photon is still far from complete. An internal transmitter is clearly required to carry the signal from the disk membrane to the surface membrane where the suppression of light-sensitive current takes place, and both Ca ions (Yoshikami & Hagins, 1971; Hagins, 1972) and 3′,5′-cyclic guanosine monophosphate (GMP) (Hubbell & Bownds, 1979; Fesenko, Kolesnikov & Lyubarsky, 1985) have been proposed in this role.

One way of deciding between these and other possibilities is to examine the effects of inhibitors of the metabolism of putative internal transmitter substances. In the present study we have recorded the light-sensitive current from single toad rods using the methods developed by Baylor, Lamb & Yau (1979) and Hodgkin, McNaughton, Nunn & Yau (1984), and have investigated the effects of 3-isobutyl-1-methylxanthine (IBMX), which inhibits the phosphodiesterase (PDE) responsible for breaking down cyclic GMP (Beavo, Rogers, Crofford, Hardman, Sutherland & Newman, 1970) and of La ions, which inhibit both the Na–Ca exchange pump and the uncoupled, ATP-dependent Ca pump in the squid axon and other systems (van Breemen & de Weer, 1970; Baker & McNaughton, 1978). Some of the results of this work have been presented in brief (Cervetto & McNaughton, 1983*a*, *b*). Our conclusions concerning the antagonistic effects of light adaptation and IBMX are similar to those drawn from recordings of the intracellular potential of photoreceptors in the intact retina (Capovilla, Cervetto & Torre, 1982, 1983*b*).

METHODS

The preparation and recording system were essentially as described by Baylor *et al.* (1979) with the modifications for recording the light-sensitive current from isolated rods while perfusing the outer segment described by Hodgkin *et al.* (1984). The perfusion system described by Hodgkin *et al.* (1984) was modified by positioning the six-way taps closer to the preparation. This reduced the dead space and enabled multiple solution changes to be made in quick succession. The solution change rate was estimated from the time course of the junction current, and is given in the Figure legends where appropriate.

The use of La in the present experiments precludes the use of Ca chelating agents to buffer Ca since all such chelators have an extremely high affinity for La. Consequently the 'low-Ca' solution used in this work was usually 0·1 mM-Ca, except in the experiment of Fig. 10*C* where a Ca- and Mg-free solution without chelator was used. Recovery from the application of La was speeded by the addition of 100 μM-Mg EDTA to the Ringer solution, and the rod was always returned to normal Ringer solution before performing subsequent solution changes. Applying IBMX for long times sometimes had irreversible effects, in that the light-sensitive current on return to Ringer solution was small and the light response slow. In all of the experiments presented in this paper good recovery was observed on return to Ringer solution.

Healthy specimens of the toad *Bufo marinus* were pithed and red rods isolated by mechanical dissociation under infra-red illumination. The stimulus was unpolarized light of wave-length 500 nm, flash duration 20 ms. A collecting area of 20 μm^2 was assumed in calculating the number of photoisomerizations (Rh*) per flash.

RESULTS

Effects of PDE inhibitors applied in Ringer solution

Application of IBMX to the outer segment of a rod caused a rapid increase in light-sensitive current (Fig. 1*A*). The effect was specific to the outer segment since applying IBMX to the inner segment of the rod, with the outer segment shielded from the inhibitor by being inside the suction pipette, caused a much smaller and slower increase (Fig. 1*B*). It seems likely that even the small effect seen in Fig. 1*B* was due to diffusion of IBMX to the outer segment either within the cell or via the imperfect seal between the suction pipette and the cell membrane.

The fractional increase in current caused by various concentrations of IBMX is summarized in Table 1, column 1. A concentration of 20 μM caused a doubling of current, and the dependence of the current increase on concentration could be approximately fitted by a Michaelis relation with $K_M = 120$ μM and a maximum current increase of 9·3-fold.

Treatment with PDE inhibitors also had a strong effect in prolonging the time course of the response to a flash of light. Fig. 1*C* shows four flash responses taken during an exposure to 20 μM-IBMX. Trace 2, taken 10 s after application of IBMX, exhibits a substantial increase in current, but no change in the plateau duration and early falling phase of the response. In trace 3 the current increase had reached a maximum, but a substantial prolongation of the response occurred only after longer exposure (trace 4). This delay between the increase in current and the prolongation of the time course was seen in all rods, but the clear separation of the falling phase of the bright flash response into two phases, as seen in Fig. 1*C*, was not evident in all preparations (see for example Fig. 2).

A third effect of IBMX application was to increase the size of an early 'notch' in the plateau of the bright flash response (arrowed in Fig. 1*C*). This component could be due either to an inward current which is greatest immediately after the flash and declines during the plateau phase, or to an outward current slowly activated during the plateau. The first explanation is supported by the observation that the level of current late in the plateau remained constant when the notch increased in IBMX. It seems likely that this inward current reflects the operation of an electrogenic Na–Ca exchange in which 3 or more Na ions are exchanged for 1 Ca (Yau & Nakatani, 1984, 1985; Cook, Hodgkin, McNaughton & Nunn, 1984), which is supported by the disappearance of the notch in the absence of Na (see Fig. 10*B* below).

The characteristics of the notch in Ringer solution and the effects of IBMX and high [Ca] are summarized in Table 2. In Ringer solution the notch had an initial amplitude of about 1 pA below the level of current late in the plateau, and relaxed exponentially with a time constant of about 0·5 s; the total charge transferred was therefore about 0·5 pC. In IBMX the initial amplitude of the notch increased dramatically, to as much as 20 pA in some cells (see Table 2, column 3), though in high concentrations of IBMX the notch current always saturated at a steady level before declining along a sigmoidal time course.

Two further points concerning the effects of IBMX on the notch component of the light response are apparent from Table 2. First, the total charge transferred during the notch increased much more than the dark current; column 5 shows that the ratio

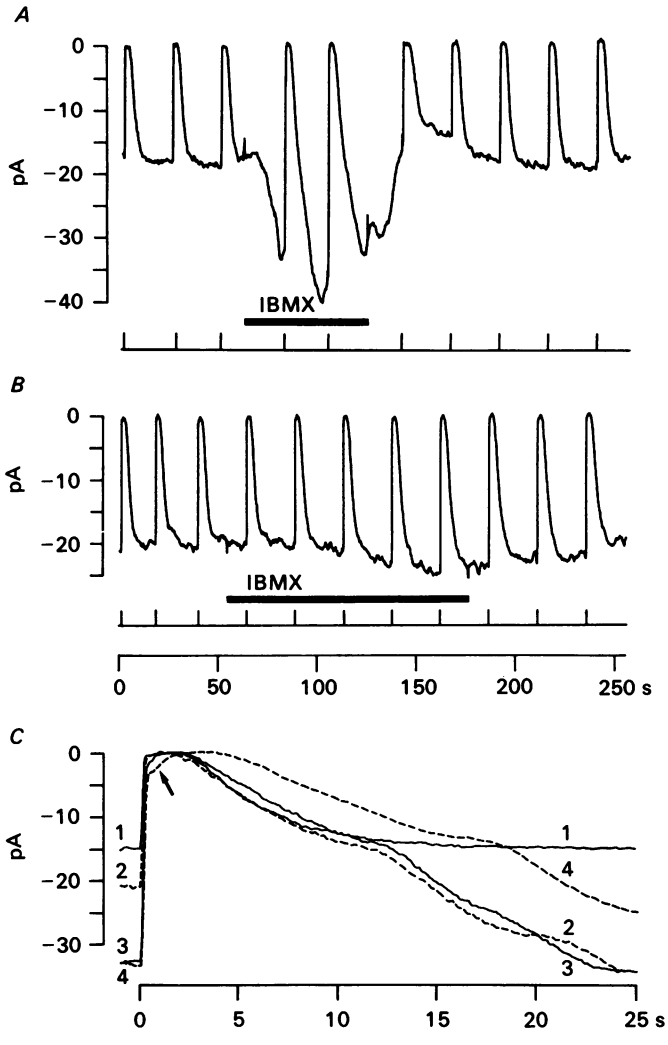

Fig. 1. The effects of 20 μM-IBMX applied to the outer segment (*A*) and to the inner segment (*B*) of the same rod. Duration of exposure to IBMX shown by the black bar. For trace *A* the inner segment was within the suction pipette; before recording trace *B* the rod was expelled and the outer segment drawn into the pipette. Current (ordinate) shown according to the convention that current flowing inwards across the outer segment membrane is negative. Flashes (2200 Rh*) given as shown by the flash monitor below each trace. Solution change complete in 18 s. *C* shows responses to bright flashes (2200 Rh*, given at time zero) during the application of 20 μM-IBMX; trace 1 in Ringer solution; traces 2–4 taken 10, 36 and 300 s respectively after exposure to IBMX. The arrow shows the 'notch' component referred to in the text. Solution change complete in 30 s.

of the charge to the dark current increased almost by a factor of 10 in 500 μM-IBMX. Elevating the Ca concentration also had the effect of increasing the charge transferred relative to the dark current (last row of Table 2). Secondly, the time constant of relaxation of the notch (or of its final phase if exhibiting saturation) was not significantly affected by IBMX in spite of the large change in amplitude (see column 4). The implications of these results are considered in the discussion (p. 107).

To check the specificity of the effects of IBMX the non-xanthine PDE inhibitors papaverine and *dl*-4-(3-butoxy-4-methoxybenzyl)-2-imidozolidinone (R01724; Roche Chemical Co.) were also tested. Both these compounds were less effective than IBMX and acted more slowly, probably because of a lower membrane permeability, but apart from this produced similar effects to those of IBMX.

TABLE 1. Effects of IBMX on dark current and dim flash response parameters

1 [IBMX] (μM)	2 Current	3 Sensitivity	4 Time-to-peak
5	1·36 (3)	1·8 (3)	2·5 (3)
20	2·1 (9)	0·92 (2)	3·1 (2)
50	3·6 (1)	—	—
500	7·7 (5)	—	—

Columns 2–4 give maximum light-sensitive current, dim flash sensitivity and time-to-peak of the dim flash response respectively, all quantities being expressed relative to their values in Ringer solution. Number of experiments given in parentheses. The dim flash sensitivity (column 3) has been normalized by the increase in light-sensitive current; when expressed in absolute terms an increase in sensitivity in IBMX was always observed.

TABLE 2. Effect of IBMX on the 'notch' component in the bright flash response

1 [IBMX] (μM)	2 Dark current (pA)	3 Notch amplitude (pA)	4 Time constant (s)	5 Charge (pC)	6 Charge / Dark current	7 *n*
0	18·2	0·78	0·43	0·47	0·026	8
5	29·1	2·79	0·50	1·52	0·052	3
20	34·7	3·36	0·47	2·01	0·058	6
500	108·2	13·5	0·68	23·9	0·22	5
20 + 5 mM-Ca	10·6	2·04	1·25	1·81	0·17	2

Column 1 gives the concentration of IBMX in μM, with 1 mM-Ca present in the Ringer solution except for the cells in the last row, where 5 mM-Ca was used. Column 2 gives dark current; column 3 gives the amplitude of the notch immediately after the rapid phase of current suppression by the bright flash; column 4 gives the time constant of relaxation of the notch (or of its final phase if exhibiting saturation); column 5 gives the integral of the charge flowing during the notch; column 6 gives the ratio of charge transferred during the notch to the dark current, i.e. the ratio of column 5 to column 2; column 7 gives the number of experiments.

Response time course in IBMX

With low doses of IBMX the effect on the dim flash response time course was equivalent to a change in scale along the time axis, as was shown by Capovilla *et al.* (1982, 1983*b*). This result is illustrated in Fig. 2*A*, in which the dim flash response in Ringer solution is a good fit to the responses to the same flash delivered in IBMX if the time scale is expanded by a factor of 1·54 (○, lower panel in Fig. 2*A*). The same horizontal scaling factor predicted a response too long to match the bright flash response (see Fig. 2*A*), but this failure might be explained by a non-linear effect of saturating flashes.

The sensitivity to dim flashes always increased in IBMX when expressed in absolute terms, but when scaled by the increase in light-sensitive current a small

decrease was sometimes observed (Table 1, column 3). With higher concentrations of IBMX the response became very long, and exhibited supralinear behaviour (Yau, McNaughton & Hodgkin, 1981; Capovilla *et al.* 1982, 1983*b*) in that doubling the flash strength produced more than a doubling of the response.

The behaviour of the dim flash response in IBMX can be explained by a multi-stage model of the type developed by Fuortes & Hodgkin (1964) and Baylor, Hodgkin & Lamb (1974) if the effect of IBMX is to increase the time constants of all the stages by an approximately equal amount. Light adaptation has a similar but opposite effect, in that the rate constant of more than one stage is increased (Baylor, Matthews & Yau, 1980), but light adaptation has in addition a strong effect in decreasing the gain of at least one stage (see below).

Interaction between light adaptation and PDE inhibition

If PDE inhibition has an effect opposite to that of background light, then it should be possible to neutralize a given dose of IBMX by an appropriate background light. Fig. 2 shows an experiment to test this point. In Fig. 2*A* the flash responses are shown in darkness and in the presence of two background light intensities, both in Ringer solution (panel 1) and in 20 μM-IBMX (panel 2). Qualitatively the effects of IBMX in increasing the dark current and in prolonging the time course of the flash response were neutralized by weak or moderate background lights. An exact match to the time course of the response could be obtained by choice of an appropriate background light, as shown in Fig. 2*B*, in which the dim flash responses have all been scaled to the same height to facilitate comparison of the time course. Panel 1 shows responses in darkness, panel 2 shows the effect of the brightest background light in IBMX, panel 3 shows that this light was sufficient to neutralize exactly the effects of IBMX on the time course. However, this level of light adaptation reduced both the flash sensitivity and the dark current well below their values in Ringer solution. For instance, the amplitude of the response labelled 'IBMX + bg' in Fig. 2*B* had to be increased by a factor of 6·5 to match the amplitude of the response to the same flash in Ringer solution. This fits with the idea that light adaptation has effects more complex than a simple activation of the PDE; while IBMX increases the time constants of the chain of events producing the light response, light adaptation both decreases the time constants in the chain and reduces the gain of at least one stage.

The results of Fig. 2 are presented in more detail in Fig. 3. Fig. 3*A* shows the effect of background light on the dark current; here 1·9 photons μm^{-2} s^{-1}, equivalent to about 38 Rh* s^{-1}, was sufficient to restore the dark current to its level in Ringer solution, while 3·3 photons μm^{-2} s^{-1}, equivalent to 66 Rh* s^{-1}, was required to restore the time-to-peak (Fig. 3*B*). The difference is due to the different effects of IBMX and light adaptation on the amplitude and time course of the light response, and provides further evidence that while IBMX acts in some respects in an opposite way to light adaptation it is not to be regarded as 'negative light'.

In Fig. 3*C* the effects of background lights on the flash sensitivities in Ringer solution and IBMX are compared. While the flash sensitivity expressed in absolute terms was increased in IBMX, the form of the curve relating flash sensitivity to background light level was little affected by the presence of the inhibitor. Another way of expressing this is to plot the desensitization parameter $T = (S_F^D/S_F - 1)$, where

S_F^D and S_F are the flash sensitivities in darkness and in the presence of a background light, respectively. The parameter T is linearly related to background light intensity if the flash sensitivity obeys the Weber law (McNaughton, Yau & Lamb, 1980; Lamb, McNaughton & Yau, 1981). Fig. 3*D* shows the dependence of T on light intensity; the straight line is the prediction of the Weber law with I_0, the background required to halve the flash sensitivity, equal to 0·65 photons μm^{-2} s^{-1}. The same dependence

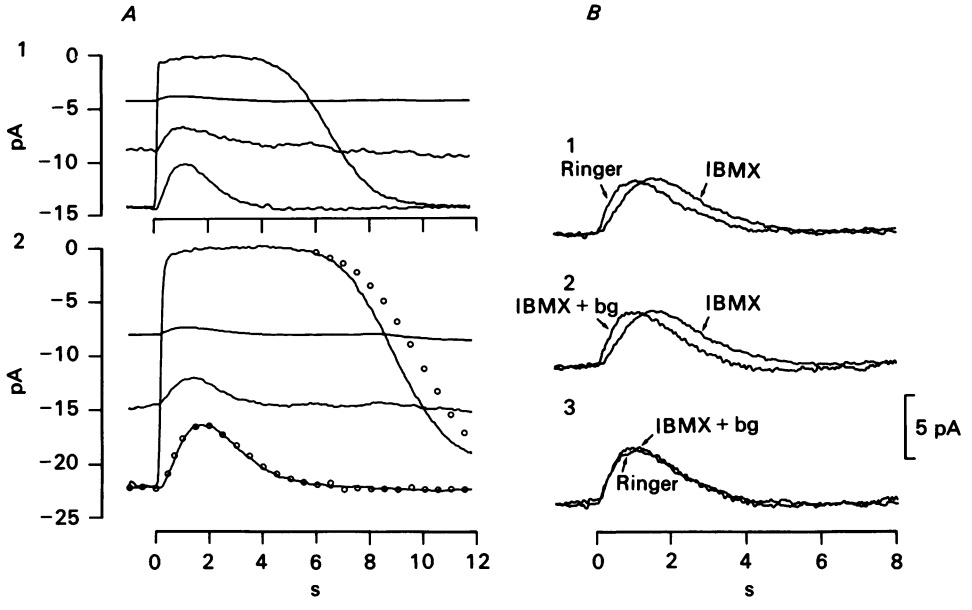

Fig. 2. Interaction between background light and IBMX. *A*, responses to bright flashes, given in darkness, and to dim flashes, given in darkness and in the presence of two intensities of background light. Top series of traces (1) taken in Ringer solution and bottom series (2) in the presence of 20 μM-IBMX. Bright flash strength 2200 Rh*. Dim flash strength 21 Rh*, except on the brighter background where the dim flash strength was increased by a factor of 4·7; the response shown has accordingly been scaled down by the same factor for comparison with the other responses. Background light intensity for the dim flash reponses: lowest trace, 0; middle trace, 22 Rh*/s; upper trace, 94 Rh*/s. The traces obtained in Ringer solution in darkness have been replotted in the lower panel (○) with the time scale expanded by a factor of 1·54. The ordinate has been magnified by a factor of 1·59 for the bright flash responses and 1·41 for the dim flash responses. *B*, dim flash responses scaled to the same height for comparison of time course. Panel 1: responses in Ringer solution and in IBMX, both in darkness. Panel 2: responses in IBMX, in darkness and in the presence of the brighter background (bg) shown in *A*. Panel 3: responses in Ringer solution, in darkness, and in IBMX, with the brighter background light. All traces are the average of five or six responses.

of T on background light, with the same value of the 'dark light', I_0, was observed in the presence of IBMX. This result is interesting in view of the possibility that the parameter T may be proportional to the concentration of a 'desensitizing substance' responsible for reducing the amplitude of the flash response in the presence of a background light (McNaughton *et al.* 1980; Lamb *et al.* 1981). If T indeed describes the concentration of a desensitizing substance responsible for controlling the gain of

the chain of events producing the flash response then it seems that the generation of this substance is independent of cyclic nucleotide metabolism in the cell. In terms of the multi-stage model proposed by Fuortes & Hodgkin (1964) and Baylor *et al.* (1974) we might suppose that T controls the over-all gain of the chain of events, and is little affected or unaffected by IBMX, while the activity of the PDE controls in some way the rate constants of the stages in the chain, affecting all of them in a roughly equal manner.

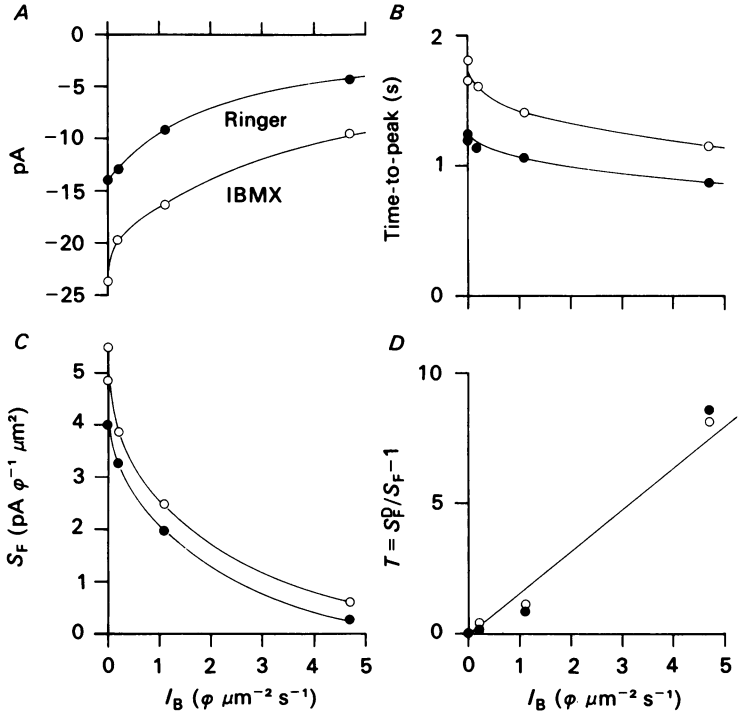

Fig. 3. Effects of background light on parameters of the flash response in Ringer solution (●) and in 20 μM-IBMX (○). Taken from the same experiment as shown in Fig. 2. Background light intensity, on the horizontal axis, can be converted into Rh*/s by assuming a collecting area of 20 μm^2. Parameters plotted are: A, dark current; B, time-to-peak of the dim flash response; C, flash sensitivity, S_F, expressed in absolute terms; D, the desensitization parameter $T = S_F^D/S_F - 1$ (see McNaughton *et al.* 1980).

Effects of La ions applied in Ringer solution

Fig. 4*A* shows that applying La ions to the rod outer segment caused a slow inhibition of the light-sensitive current. The effects were specific to the outer segment, since applying a larger dose to the inner segment (Fig. 4*B*) caused only a small and slow inhibition which can probably be attributed to the La leaking along the imperfect seal between the glass suction pipette and the rod membrane. Reversal of the inhibition on return to Ringer solution was slow, but could be speeded by the addition of small amounts of EDTA to the Ringer solution, as has been done in Fig. 4. La ions were very potent, with the dark current being about half-inhibited by 0·1 μM-La, though it was difficult to tell if this was steady state in view of the slowness of the inhibition. Concentrations above 2 μM produced a nearly complete inhibition,

although even with the highest concentration used (50 μM) a small dark current could still be detected. The rate of inhibition with a concentration of 2 μM or above showed little dependence on the concentration of La.

The early effects of La on the responses to bright and dim flashes are shown in Fig. 5. The plateau phase of the bright flash response was prolonged and the falling phase slowed (Fig. 5*A*). The dim flash response showed a small increase in peak height when the response was normalized to allow for the reduction in dark current, and the time-to-peak was increased (Fig. 5*B*, upper traces). A similar increase in sensitivity and prolongation of the time-to-peak has been observed in low Na (Yau *et al.* 1981), where the activity of Na–Ca exchange should be reduced.

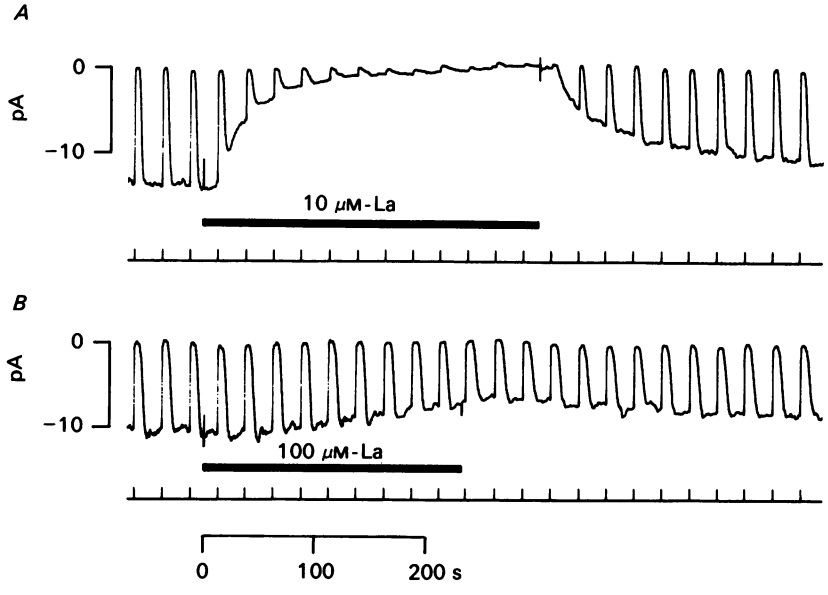

Fig. 4. Application of 10 μM-La to the outer segment (*A*) and 100 μM-La to the inner segment (*B*) of two different rods. Rods in Ringer solution before application of La; recovery takes place in Ringer solution to which 100 μM-Mg EDTA has been added. Solution change was complete in 15 s. Flashes (2200 Rh*) given as shown by the flash monitor.

The effects on the time course of the flash responses shown in Fig. 5 were observed consistently and appeared rapidly after the application of La. For example, in the experiment of Fig. 5*A* a similar prolongation of the falling phase was observed in a flash given 36 s after admission of La to the bath. The interpretation of such experiments is complicated, however, by a loss of flash sensitivity which appeared with long exposure to La concentrations of 10 μM or more. The effect of this loss of sensitivity was to reduce the amplitude of the dim flash response without a change in time course, and to reduce the plateau duration of the bright flash response while preserving the slowed time course of the falling phase. A possible interpretation of these results is that the early effects shown in Fig. 5 could be due to an external action of La (perhaps an inhibition of Ca pumping), while the later effects could be due to an entry of La into the cell and a direct interference with the light response. Other possibilities are not excluded, however.

The effects of La on the time course of the dim flash response differed from those of IBMX in that the response in La could not be mapped onto that in Ringer solution by a linear transformation of the time scale. Instead, a fair correspondence could be obtained by reducing one of the rate constants in a four-stage chain, as shown in Fig. 5*B*. In this reconstruction the chain has been assumed to have three equal rate constants followed by a smaller rate constant (last stage long model, Baylor *et al.* 1974). In Fig. 5*B* a reasonable fit to the response in La was obtained by reducing the final rate constant by a factor of 1·54. In the same experiment the time constant of the falling phase of the bright flash response was prolonged by the similar factor of 1·53.

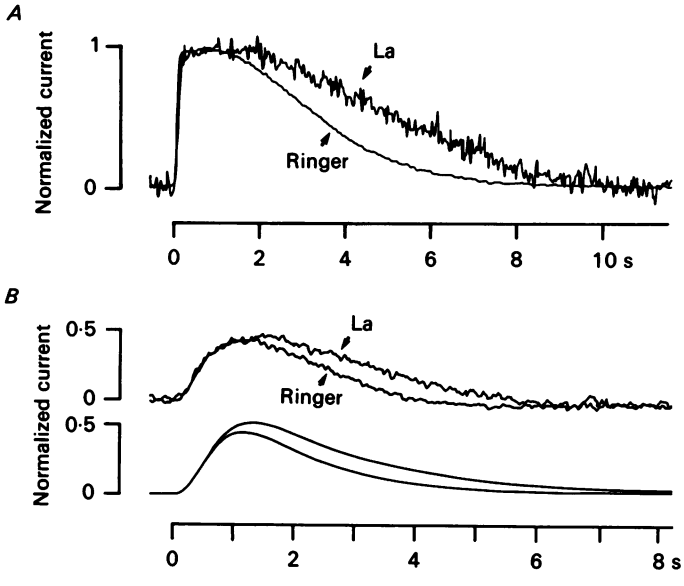

Fig. 5. Effect of La on flash responses. *A*, bright flash responses (2200 Rh*) in Ringer solution and after 110 s in 10 μM-La. Responses in La scaled up by a factor of 7·5. Traces are the average of three responses each. *B*, top: dim flash responses (21 Rh*) in Ringer solution and after 8·6 min in 0·1 μM-La (responses in La scaled up by a factor of 1·54); bottom: four-stage models of the flash response, in which three equal first-order stages, each with time constant 0·26 s, lead to production of a blocking substance, and a single stage of time constant 1·3 s (lower trace) and 2 s (upper trace) removes it.

Similar effects to those seen in La were produced by Co and by Mn ions, though both were considerably less potent. A concentration of 1 mM-Co inhibited the dark current to 0·57 in one experiment, prolonged the responses to both bright and dim flashes and increased the dim flash sensitivity when expressed as a fraction of the dark current. The same concentration of Mn ions had similar but weaker effects.

Effect of La on the rate of activation of current in low [Ca]

When the external Ca concentration is reduced the light-sensitive current activates at a rate substantially slower than the rate at which the solution can be changed, and the suppression of current on returning the Ca concentration to its original level

is also a slow process, although in some circumstances a fast component can precede the slow suppression (Yau *et al.* 1981; Hodgkin, McNaughton & Nunn, 1985). It is likely that the delayed activation of current when the cell is exposed to low Ca results from a fall in intracellular [Ca] (Hodgkin *et al.* 1985). In the present study we have observed a striking effect of La on the rate of current activation in response to lowering external [Ca], which can be explained if La inhibits the extrusion of Ca from the cell.

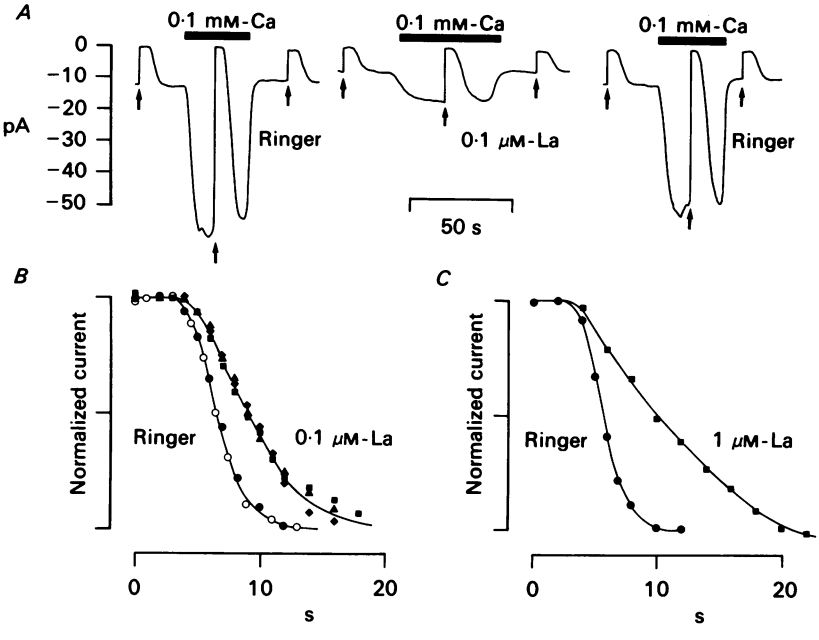

Fig. 6. Effect of La on the activation of current when [Ca] is lowered from 1 mM to 0·1 mM. *A*, representative exposures to 0·1 mM-Ca (duration shown by black bar) while the rod is otherwise in normal solution (left and right traces, before and after exposure to La) or has been maintained for 11 min in 0·1 μM-La (middle trace). Flashes (2200 Rh*) given at arrows. Solution change complete in 3 s. *B*, rate of activation of light-sensitive current in 0·1 mM-Ca without La (○, before La; ●, after La) and in 0·1 μM-La (three exposures to 0·1 mM-Ca, time after exposure to La being 160 s (◆), 370 s (■) and 650 s (▲)). Currents normalized to same height using factors of 1·0 (○), 2·24 (◆), 3·6 (■), 5·12 (▲) and 1·14 (●). *C*, as in *B*, in Ringer solution (●) and 20 s after exposure to 1 μM-La (■). Scaling factors 1·0 and 3·11; with longer exposure to La the response to low Ca concentration was completely abolished.

In Fig. 6*A* three sample records are shown in which Ca was changed from its normal level of 1 mM to 0·1 mM for a brief period. The middle record was taken with the rod maintained throughout in 0·1 μM-La, and the first and last records are in the absence of La. La had a strong effect in reducing the current attained in low Ca concentration, and it also reduced the rate of activation when the Ca concentration was lowered. This is shown in Fig. 6*B*, where the activation of current in 0·1 mM-Ca has been scaled by the maximum current attained. In three exposures carried out in the presence of 0·1 μM-La the rate of activation scaled in this way was similar on each occasion. The

rate of increase of normalized current was lower by a factor of 1·8 in the presence of 0·1 μM-La than in its absence.

Fig. 6*C* shows that a more pronounced inhibition of the activation rate was observed in 1 μM-La, with the rate of increase being slowed by a factor of 3·8. Higher concentrations of La completely inhibited the activation of current in low [Ca] (see below, Fig. 9). The action of La in slowing or preventing the current increase in low [Ca] came on immediately after the application of La, in contrast to the slow

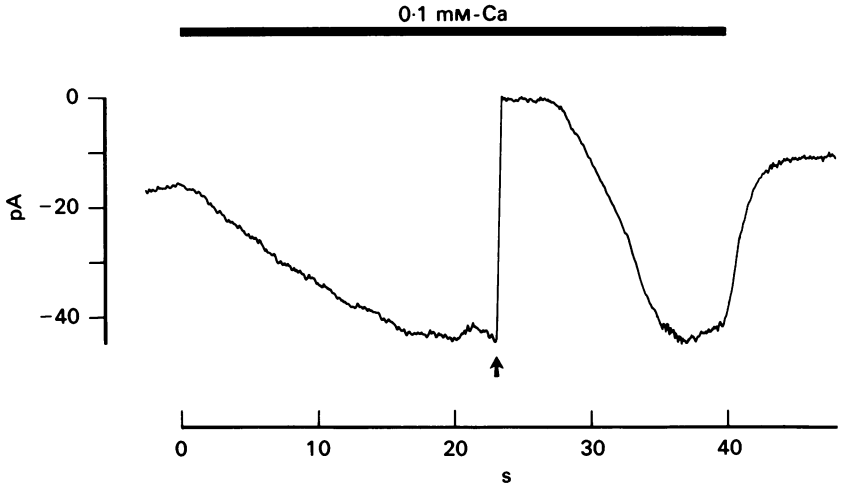

Fig. 7. Comparison between the activation of current in 0·1 mM-Ca (duration of application shown by dark bar) and the activation of current after a flash of light (2200 Rh*, given at arrow). La (1 μM) was present throughout. Solution change was complete in 3 s.

effect on the light-sensitive current, since in experiments in which La was applied at the same time as the Ca concentration was reduced the increase of current normally seen in low [Ca] was reduced or abolished. These results can be explained by assuming that Ca must be pumped from the cell, probably by a Na–Ca exchange mechanism, before current activation occurs, and that the effect of low concentrations of La is to reduce the rate of Ca efflux and thereby delay the activation of current.

Similar results to those shown in Fig. 6 were obtained in other experiments, though the potency of La was somewhat variable in different preparations. In one experiment the effect of 0·1 μM-La was to delay the onset of activation of current in 0·1 mM-Ca by 400 ms without affecting the rate of rise; in the same experiment, however, 0·5 μM-La slowed the rate of rise by a factor of 1·6 in addition to delaying the onset of activation. La ions also slowed the rate of suppression of current on return to 1 mM-Ca. This effect was not detected in the experiment of Fig. 6, probably because the rate of suppression of current was limited by the rate of change of solution. In another experiment with a faster flow 0·1 and 0·5 μM-La inhibited the rate of suppression of current by factors of 1·7 and 3·4 respectively.

One experiment was carried out to test whether the activation of current caused

by an elevation of [Na] was also affected by La. In this experiment the Na concentration was elevated from 0 to 110 mM in the presence of 1 mM-Ca. Concentrations of 0·5 and 1 μM-La were observed to inhibit the rate of activation of current on return to normal [Na] by factors of 1·5 and 1·8 respectively.

In a simple version of the Ca hypothesis the falling phase of the light response might be governed by the rate at which Ca released by light can be pumped from the cell, and it seemed worthwhile to test this idea by comparing the effects of La on the activation of current in low [Ca] and on the bright flash response. Fig. 7 shows that there was no straightforward correspondence between the activation of current in low [Ca] and the recovery from the flash response; a concentration of La sufficient to reduce substantially the rate of activation of current had a much weaker effect on the rate of recovery from a bright flash. This experiment supports others (Yau *et al.* 1981) showing that there is no simple relation between Ca extrusion from the cell and the recovery from a bright flash.

Even though the rate of Ca transport probably cannot be identified in any simple way as one of the rate constants of the flash response, it seems likely from the results of the preceding section that one rate constant in the chain of events underlying the flash response is affected by the concentration of internal Ca. One possible explanation is that the activity of the guanylate cyclase may be inhibited by a rise in internal Ca concentration (Hodgkin *et al.* 1985).

Rapid actions of La ions

When La ions were applied in Ringer solution the inhibition of light-sensitive current could usually be resolved into a rapid and slow phase, with the rapid phase seldom exceeding 20 % of the dark current, although in one rod an inhibition of 34 % was observed. (In salamander rods the rapid phase is usually larger: B. J. Nunn, personal communication.) Fig. 8 shows that the rapid inhibition became much more prominent in the presence of reduced [Ca]. If the application of La was kept brief enough then the rapid inhibition was rapidly reversed (Fig. 8*B*). The rapid inhibition was not detectably slower than the rate of change of solution, measured from the junction current during Na withdrawal, and we conclude that the half-time of the rapid action is less than 1 s (see Fig. 8). By contrast, the slow phase of inhibition was substantially slower than the solution change rate, with a mean half-time of 29 s (seven rods).

These experiments show that La ions interact rapidly and reversibly with the light-sensitive current. The simplest explanation is that La ions interact directly with the channel, perhaps by displacing Na ions from the site of entry. The two-phase inhibition of current shown in Fig. 8*A* is reminiscent of the effects of rapid elevations of external Ca concentration (Hodgkin *et al.* 1985) and this, together with the observation that the rapid phase of inhibition by La only becomes prominent in low Ca, may imply that La and Ca compete for a blocking site in or near the channel.

Interactions between the effects of La and IBMX

Fig. 9 shows that IBMX is able to produce an increase in light-sensitive current in the presence of a concentration of La sufficient to inhibit completely the increase in

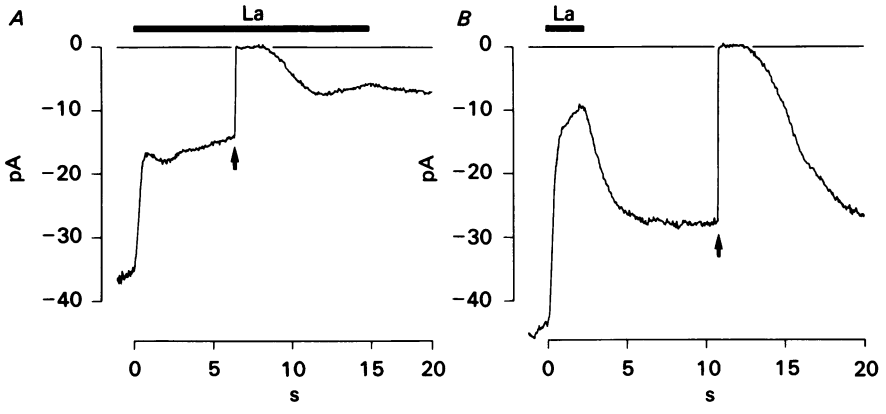

Fig. 8. Rapid suppression of current by La in the presence of 0·1 mM-Ca. Duration of exposure to La (5 μM) shown by dark bars. Flashes (2200 Rh*) given at arrows. Solution change complete in 1 s; no significant delay was detected between the solution change, as measured from the junction current during a change from Ringer to Na-free solution, and the suppression of current by La.

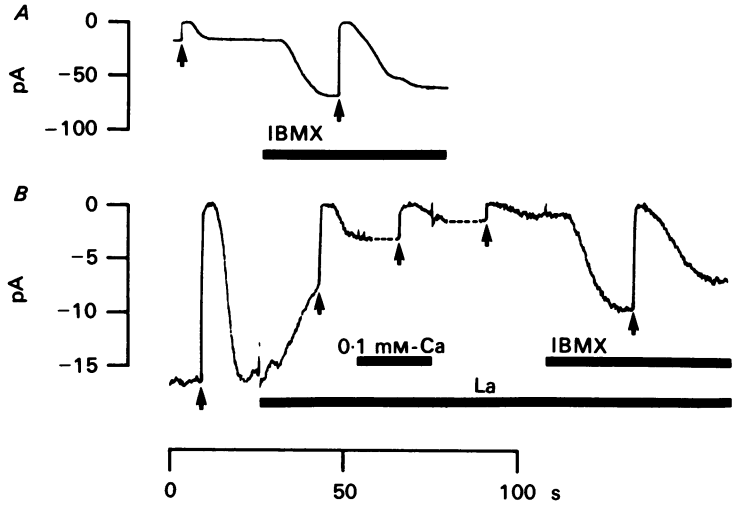

Fig. 9. Activation of current by low [Ca] and by IBMX in the presence of 5 μM-La. *A*, effect of IBMX applied in Ringer solution. *B*, effects of 0·1 mM-Ca and 50 μM-IBMX applied for short periods after exposing the rod to 5 μM-La. Flashes (2200 Rh*) given at arrows. Junction current caused by the reduction of [Ca] has been removed at the points indicated by the dashed lines. Solution change complete in 6 s.

current produced by low [Ca]. In Fig. 9*B* La (5 μM) was applied and the response to 0·1 mM-Ca was tested shortly afterwards; this concentration of La was sufficient to suppress the substantial activation of current which is normally observed in low [Ca]. As discussed above, this blocking of the effect of low [Ca] probably resulted from an inhibitory action of La on Ca transport. However, a subsequent application of IBMX produced a substantial increase in current, which although much less in

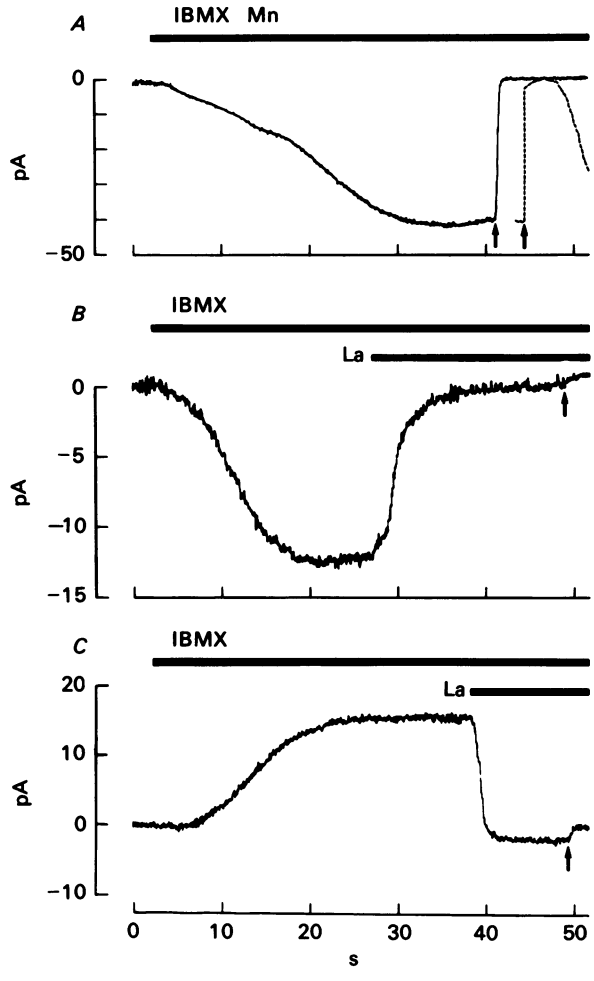

Fig. 10. Activation of currents by IBMX in the absence of Na, and the effects of La on these currents. In all traces Na was replaced by choline. Flashes (2200 Rh*) given at arrows. *A*, large currents observed in the presence of 1 mM-Mn. Solution contained 1 mM-Ca and 1·6 mM-Mg; 1 mM-Mn and 500 μM-IBMX added as shown by the dark bar. Note absence of early notch in the flash response in the absence of Na; a notch was clearly visible in the flash response in Ringer solution, which for comparison has been replotted as the dashed trace at the right, scaled to the same height as the flash response in Mn plus IBMX. The current observed in Mn plus IBMX was suppressed by 5 μM-La (not shown). Solution change complete in 4 s. *B*, inward current activated by 500 μM-IBMX in the presence of 1 mM-Ca and 1·6 mM-Mg, and the suppression of this current by 5 μM-La. A small residual inward current was observed in La, as shown by the small response to the flash delivered at the end of the trace. Solution change complete in 2·5 s. *C*, outward current activated by 500 μM-IBMX in the nominal absence of Ca and Mg (no chelating agents added), and its suppression by 5 μM-La. Note small inward current in the presence of La, as shown by flash response at end. Same rod as in *B*.

absolute terms than the increase observed in Ringer solution (Fig. 9*A*) is similar in magnitude and time course when compared with the reduced level of dark current. The most straightforward interpretation of this result is that the activation of current caused by IBMX is not due simply to a stimulation of Ca transport across the surface membrane.

Light-sensitive currents in the absence of Na

The light-sensitive current can be carried by ions other than Na, provided the light-sensitive channels can be maintained open in the absence of the Na gradient required to sustain the Na–Ca exchange (Hodgkin *et al.* 1985). This can be achieved either by bathing the rod in low [Ca] (Yau *et al.* 1981; Hodgkin *et al.* 1984) or by the application of PDE inhibitors (Torre, Pasino, Capovilla & Cervetto, 1981; Hodgkin *et al.* 1984). Capovilla, Caretta, Cervetto & Torre (1983*a*) have shown from recordings of membrane potential that a light response can be sustained by Ca, Mg or Mn in Na-free solutions containing IBMX. Fig. 10*A* shows a recording of the light-sensitive current with Mn and IBMX present in the Na-free solution; in the presence of Mn a particularly large current was observed, perhaps because Mn is more permeable than Ca or Mg. It is noteworthy that under these conditions the light response completely lacked the early notch seen in the presence of Na (compare with the dashed trace in Fig. 10*A* inset). Responses lacking the early notch were also seen when the current was carried by Ca or Mg, so its absence in Fig. 10*A* was not due to a direct effect of Mn. The sensitivity of the notch to Na withdrawal provides further evidence that this component of the light response results from the operation of an electrogenic Na–Ca exchange.

Currents in Na-free solution with IBMX were sensitive to the application of La. Fig. 10*B* shows that the inward current observed in the presence of Ca and Mg was strongly suppressed by La. Fig. 10*C* shows the outward current recorded when IBMX is applied in the nominal absence of Na, Ca and Mg. This current can also be recorded in the absence of IBMX when Ca and Mg are reduced to very low levels using chelating agents (Yau *et al.* 1981) and is probably carried by K ions leaving the cell (Capovilla *et al.* 1983*a*). The effect of La on the outward current was to cause a rapid inversion, with a small light response of normal polarity being recorded in La. A similar result was obtained in three other experiments.

A probable explanation of Fig. 10*C* is that La is able to carry current through the light-sensitive channel, although we cannot rule out the possibility that the inward current was carried through the proximal part of the outer segment by Na ions leaking from the pipette. If La can pass through the light-sensitive channel then it resembles Ca, which has the ability both to block the channel and to carry current in its own right (Hodgkin *et al.* 1985).

DISCUSSION

Light is known to stimulate the PDE which hydrolyses cyclic GMP to GMP in rod outer segments, and on a simple level one might expect that the PDE inhibitor IBMX would have opposite effects to those of light. This expectation is largely fulfilled, since IBMX increases the light-sensitive current and slows the time constants of the chain

of events underlying the flash response. Both effects are opposite to those of light, and both can be neutralized by an appropriate steady light. There is even some suggestion that the two effects are distinct, since the effect on the conductance occurs with little delay after the application of IBMX while the effect on the time constants takes some time to become apparent. These observations are consistent with the idea that cyclic GMP maintains light-sensitive channels in the open state, and they suggest that the effects of light adaptation on the time course of the light response are mediated (whether directly or indirectly) by cyclic nucleotide metabolism.

Light adaptation has the additional effect, not shared by IBMX, of decreasing the gain of at least one stage of the chain. One possible explanation is that IBMX may be non-specific, but a more interesting possibility is that light may have effects not mediated by cyclic nucleotide metabolism.

Inhibiting the Ca efflux from the cell should cause a suppression of the light-sensitive current if Ca is the internal transmitter. To this extent the action of La is consistent with the Ca hypothesis, but other observations pose difficulties for at least a simple form of the Ca hypothesis. Inhibiting the Ca pump with La slows one of the time constants of the chain of events initiated by a flash of light, an effect which is in the opposite direction to that of light. Another objection is that if Ca is the internal transmitter then the time constant of removal of Ca from the cell should appear as one of the time constants of the chain. But the rate of activation of current in low Ca concentration, which probably provides an index of the Ca pumping activity, can be slowed by La to the point where it is slower than any of the time constants of the light response (Fig. 7). These observations argue against a simple scheme involving Ca as internal transmitter, though they do not rule out more complex schemes in which Ca is involved in the light response, possibly in conjunction with cyclic GMP.

The notch component in the bright flash response

The plateau phase in the response to a bright flash always exhibits a small decaying inward current, the notch, which is small in Ringer solution and is greatly increased by IBMX (Fig. 1*C* and Table 2). In the absence of external Na the notch disappears (Fig. 10*A*). The idea that the notch might reflect the electrogenic extrusion of Ca in exchange for 3 or more Na ions was originally suggested by A. L. Hodgkin (personal communication). A light-insensitive current with the properties expected of a Na–Ca exchange has recently been reported in rods (Yau & Nakatani, 1984, 1985; Cook *et al.* 1984) which strengthens the identification of the notch with an electrogenic Na–Ca exchange.

In Ringer solution the notch seldom exceeds 1 pA in amplitude and decays with a time constant of about 0·5 s (Table 2), corresponding to a charge transfer of 0·5 pC or 3×10^6 Ca ions if the pump operates as a 3 Na–1 Ca exchange. The corresponding change in the intracellular concentration of Ca would be 5 μM if distributed over an outer segment volume of 1 pl, though probably only a small fraction of this would be ionized.

The pumping activity observed as the notch could represent a Ca transport activated by light, as a result either of a direct action of light on the pump or of a release of Ca inside the cell. Alternatively the pump might be active in darkness,

operating to extrude the Ca influx through the light-sensitive channels, and the decay of the pumping current on the plateau would represent the shut-off of pumping when the Ca influx is reduced by light. If the latter possibility is correct then about 2 pA of the total dark current of 20 pA is carried by Ca, and the exchangeable Ca in the cell turns over with a time constant of 0·5 s.

The charge transferred during the notch increases to 25 pC in 500 μM-IBMX, which corresponds to a change in the intracellular Ca concentration of 0·25 mM if Ca extrusion takes place as a 3 Na–1 Ca exchange. The increase in the notch area is much greater than the increase in light-sensitive current (see Table 2). In a simple system in which the Ca influx is proportional to the current through the light-sensitive channel, and where the pump rate in darkness is proportional to the intracellular Ca concentration, one would expect the area of the notch to increase in proportion to the dark current. The observed increase in the area of the notch in IBMX, relative to the dark current, could be explained either if a larger fraction of the dark current were carried by Ca in the presence of IBMX, or if the pump rate saturates as internal Ca rises. The time constant of relaxation of the notch is unaffected by IBMX in spite of the large changes in the dark current, which argues against a direct effect of cyclic GMP on the Na–Ca exchange.

Interactions between La and the light-sensitive channel

La ions appear to carry current through the light-sensitive channel, and the affinity of La for the channel must be high since significant current is observed in the presence of low concentrations of La (Fig. 10*C*). La has in addition the ability to block a current carried by Na (Fig. 8), and the observation that this block becomes more prominent in low [Ca] implies that Ca and La compete for the blocking site. The channel thus appears to be composed of two separate functional entities, namely a binding site, at which both Ca and La compete strongly with Na, and a translocation step, in which Ca and La substitute poorly for Na. Thus when the Ca or La concentrations are elevated the rapid suppression of current (Hodgkin *et al.* 1985; this paper) would be due to a displacement of Na from the binding site and a consequent inhibition of current because of the slower translocation of Ca or La across the membrane. In other circumstances Ca and La can carry current, so both ions must be capable of passing through the light-sensitive channel. These observations, while interesting, take us no further towards understanding whether ion translocation occurs via an ion-permeable pore or via a carrier mechanism.

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