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THE IONIC SELECTIVITY OF THE LIGHT-SENSITIVE CURRENT IN ISOLATED RODS OF THE TIGER SALAMANDER

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SUMMARY

1. Using the method of Hodgkin, McNaughton & Nunn (1985) for rapidly changing the extracellular medium, we analysed the effect of divalent cations on the photocurrent of isolated retinal rods of the tiger salamander.

2. When the extracellular NaCl was replaced by equiosmolar amounts of $BaCl_2$, $SrCl_2$, $CaCl_2$, $MgCl_2$ and $MnCl_2$ the efficacy in carrying the photocurrent at early times was $Ba^{2+} > Sr^{2+} > Ca^{2+} > Mg^{2+} > Mn^{2+}$. At early times Ba^{2+} could carry a photocurrent similar to or larger than that carried by Na^+.

3. The photocurrent carried by Ba^{2+} increased by about 50% when $[Ca^{2+}]_o$ was reduced from 1 to 0·1 mM. In the presence of 0·1 mM-Ca^{2+} in the extracellular medium the photocurrent carried by Ba^{2+} saturated when $[Ba^{2+}]_o$ was close to 50 mM and was half-activated at 15 mM $[Ba^{2+}]_o$.

4. The photocurrent which can be carried by Sr^{2+} is not larger than that carried by Ba^{2+} and does not saturate for $[Sr^{2+}]_o$ up to 70 mM.

5. When extracellular Na^+ is replaced by the impermeant organic ion choline it is possible to observe a transient photocurrent which is carried by Ca^{2+}. This current has a maximal value of about 11 pA and has a half-activation constant of about 50 μM.

6. Movements of Mg^{2+} across the light-sensitive channel can be seen only when extracellular Ca^{2+} is reduced below 10 μM. Under these conditions the maximal photocurrent which can be carried by Mg^{2+} at early times is about 8 pA and has a half-activation of about 2 mM. Under normal conditions Mn^{2+} is hardly permeable through the light-sensitive channel.

7. It is concluded that the selectivity of the light-sensitive channel in the low ionic concentration range is $Ca^{2+} > Sr^{2+} > Ba^{2+} > Mg^{2+} > Na^+$.

INTRODUCTION

In recent years basic features of phototransduction have been understood. It is now clear that internal Ca^{2+} is not the internal transmitter (Hodgkin *et al.* 1985;

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Matthews, Torre & Lamb, 1985; Lamb, Matthews & Torre, 1986) and that its intracellular concentration decreases during exposure to light (Yau & Nakatani, 1985; McNaughton, Cervetto & Nunn, 1986). However, in contrast, it has been shown that cyclic guanosine 3′,5′-monophosphate (cyclic GMP) directly gates light-sensitive channels (Caretta & Cavaggioni, 1983; Fesenko, Kolesnikov & Lyubarsky, 1985; Zimmerman & Baylor, 1986; Haynes, Kay & Yau, 1986; Owen, 1987).

There is now a general agreement that a variety of different monovalent and divalent cations permeate through the light-sensitive channel (Torre, Pasino, Capovilla & Cervetto, 1981; Yau, McNaughton & Hodgkin, 1981; Capovilla, Caretta, Cervetto & Torre, 1983; Yau & Nakatani, 1984*a*, 1985; Hodgkin *et al.* 1985). This article is primarily concerned with the analysis of the movement of divalent cations through the light-sensitive channel. The observation that calcium ions can block the photocurrent and at the same time can carry a substantial fraction of the photocurrent is still puzzling. We show that the movement of divalent cations through the light-sensitive channel does not follow the independence principle and that competition between cations is an essential feature of the light-sensitive channel. Divalent cations bind rather strongly to a site in the channel and their flow shows saturation.

Preliminary results have already been presented (Cervetto, Menini, Rispoli & Torre, 1987).

Further properties of the light-sensitive channel, showing that it may exist in two different conductive states, will be presented elsewhere (L. Cervetto, A. Menini, G. Rispoli & V. Torre, in preparation).

METHODS

Recordings of suction-pipette current were made from individual rods mechanically isolated from the dark-adapted retina of the larval tiger salamander *Ambystoma tigrinum* (Lawrence Waterdog Farm).

Apparatus

The apparatus for suction-electrode recording and optical stimulation of rods was similar to that described by Lamb *et al.* (1986). Experiments were performed at room temperature (17–25 °C). Unpolarized light of wavelength 498 nm was used for all stimuli. Flash intensities are given in photoisomerizations (Rh*, activated rhodopsin molecules), estimated from an assumed collecting area of 20 μm^2 (see Lamb *et al.* 1986).

Isolated rods

A dark-adapted tiger salamander was decapitated and pithed and the eyes were enucleated under dim red light, and subsequent procedures were carried out using an infra-red image converter. The retina from a piece of eyecup was gently removed under Ringer solution, and was finely chopped on Sylgard using a small piece of razor blade; no enzymes were used. An aliquot (240 μl) containing many cells was then transferred to the chamber.

Recording procedure

After allowing the cells to settle for a few minutes, slow perfusion was applied to remove debris from the chamber. The inner segment of an individual rod was drawn into a close-fitting suction pipette, and the light response tested. Experiments were performed only on rods whose saturating responses were sufficiently large ($\geqslant$ 25 pA) and stable.

Suction pipettes

The methods for making suction pipettes were similar to those of Baylor, Lamb & Yau (1979) except that each polished suction pipette was bent at an angle of about 90 deg roughly 0·5 mm from

its tip, using the method of Hodgkin *et al.* (1985). As before the pipettes were coated with silane (tri-*N*-butylchlorosilane, Pfaltz & Bauer) to prevent the cells from sticking.

Recording system

The reference electrodes in the bath and in the suction-pipette holder were silver wires coated with a Ag–AgCl matrix (E255, Clark Electromedical). Electrical transients during perfusion were

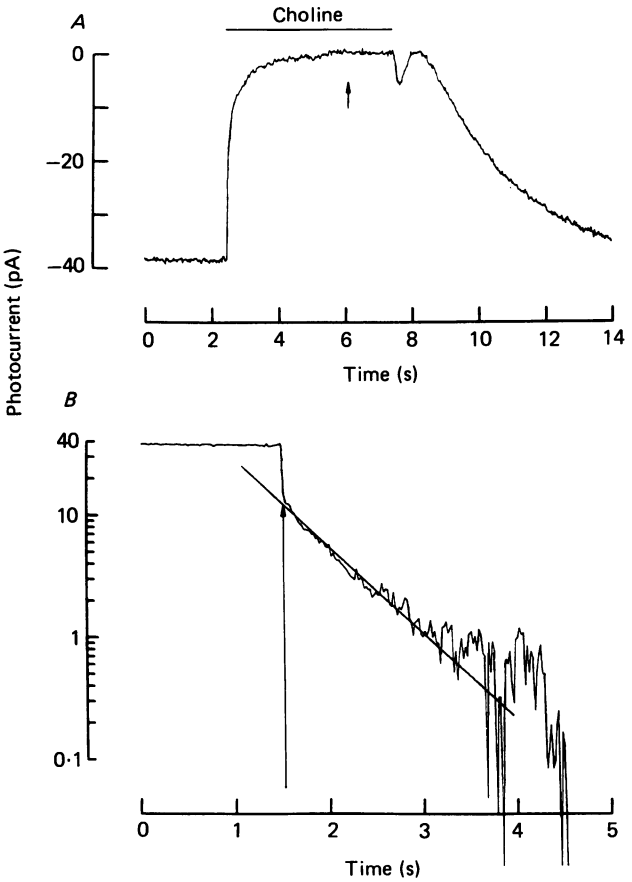

Fig. 1. Separation of the slow and fast component. *A*, the effect of the replacement of extracellular Na^+ with choline. Top trace shows timing of solution changes. A bright flash equivalent to 8700 Rh* (photoisomerizations) was delivered at the time indicated by the arrow. The trace is the average of five individual trials. *B*, same trace as in *A* plotted on a semilogarithmic scale. The straight line through the slow component was drawn by eye. The amplitude of the slow component is indicated by the arrow.

minimized by using active grounding of the bath (Hodgkin, McNaughton, Nunn & Yau, 1984). The suction-pipette signal was stored on a magnetic tape (TEAC R-80) for back-up and digitized on line at 50 Hz sampling rate by an A/D system kindly provided by Dr D. Bertrand. The digital data was subsequently transferred to a computer (IBM AT) for the analysis, which was performed using the program DATAC kindly provided by Drs C. Bader and D. Bertrand.

Solutions

The Ringer solution was the same as used by Baylor *et al.* (1979) and contained (in mM): NaCl, 110; KCl, 2·5; $CaCl_2$, 1; $MgCl_2$, 1·6; HEPES, 3; EDTA, 0·01; glucose, 5; buffered to pH 7·5 with tetramethylammonium hydroxide (TMAOH). The solutions with low Ca^{2+} were prepared as in Hodgkin *et al.* (1984). The composition of different test solutions is given in Figure legends.

Perfusion system

The perfusion system was based on that described by Hodgkin *et al.* (1985). In early experiments two different solutions were fed side-by-side down the two halves of a glass 'theta' tube into the back of the fluid-filled recording chamber. An isolated rod was held in one of the two flowing solutions by the recording pipette and could be exposed to the second solution by moving the microscope stage sideways. The rod was held within 300 μm of the mouth of the 'theta' tube near the boundary between the two solutions.

For most of the experiments the 'theta' tube was replaced by four cylindrical perfusion pipes at the back of the recording chamber, where different solutions flowed. The stage of the microscope was moved by a stepping motor, located on the microscope itself, which could be programmed for a large variety of movements backward and forward. The stepping motor acted directly on the microscope stage and not through a tube full of water as in Hodgkin *et al.* (1985). In this way the solution change was faster and more violent, but the solution around the outer segment could be changed more rapidly.

The pipette faced the solution inlet and was bent at 90 deg near the tip so that the rod pointed in a direction orthogonal to the direction of the flow. The solutions in the pipes were each fed by gravity through a system of taps described by Hodgkin *et al.* (1984) and could be changed independently in 200–300 ms using two-position exchange taps close to the recording chamber. One of up to twelve different solutions could be selected to flow down each pipe. The flow rates of the solutions were adjusted to be equal and smooth to avoid turbulence. The suction pipette was filled with Ringer solution all the time.

Determination of light-sensitive currents

The light-sensitive current was determined using the method of Hodgkin *et al.* (1985) as the current in darkness minus the current recorded during a saturating steady light. This last current represents the junction current or the artifact due to changes of solutions with different ionic mobilities.

Separation of the fast and slow component

When extracellular Na^+ is substituted by another cation the photocurrent usually changes in two phases showing a fast and a slow component. Figure 1*A* illustrates the effect on the photocurrent of the replacement of Na^+ with choline. The presence of the two components is clearer in Fig. 1*B* where the same trace reproduced in Fig. 1*A*, is plotted on a semilogarithmic scale. From this plot we can take as the amplitude of the slow component (or the amplitude of the photocurrent at early times after the ionic substitution) the quantity indicated by the arrow, which is obtained by tracing a straight line through the second component and measuring the current after the termination of the fast component.

RESULTS

The main goal of this article is to present experiments describing the movement of divalent cations, such as Ca^{2+} and Mg^{2+}, through the light-sensitive channel and to explain how Ca^{2+} can block the photocurrent whilst at the same time carry a noticeable fraction of it.

The family of records in Fig. 2*A* illustrates the results of an experiment in which the Na^+ present in the normal Ringer solution was completely substituted by equimolar amounts of Li^+ in the presence of six different concentrations of Ca^{2+}. In normal Ringer solution the light-suppressible current was 25 pA and increased when Na^+ was completely replaced by Li^+. In normal Ca^{2+} (1 mM), however, the current increase was not maintained and the current was inactivated. These observations confirm and extend previous results of Hodgkin *et al.* (1985). As clearly shown in Fig. 2*A*, when the normal Ringer solution is replaced by Li^+ solutions containing low levels of Ca^{2+} two main effects can be observed. Firstly, the early rise of photocurrent

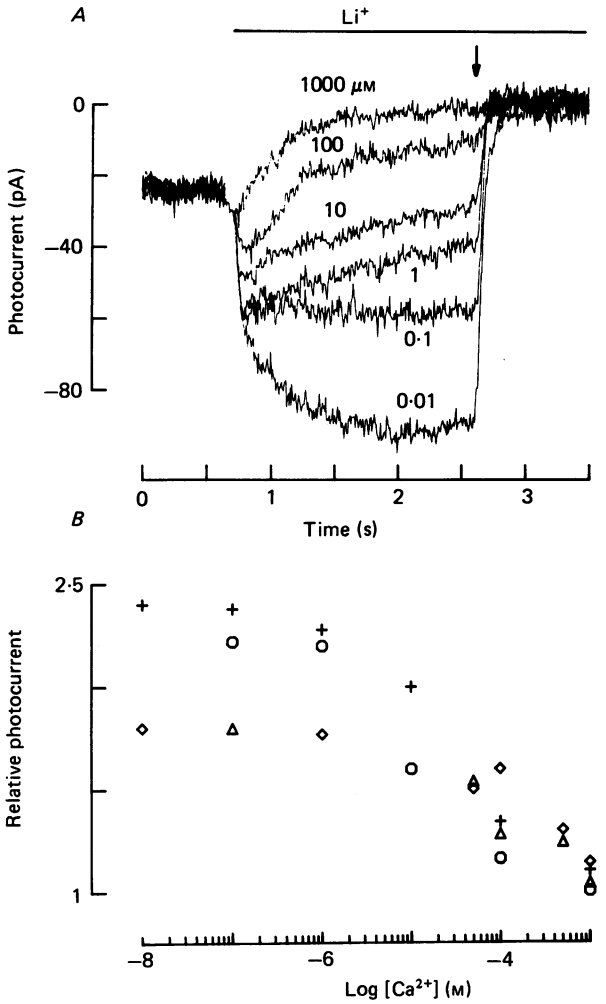

Fig. 2. Effect of the replacement of Na^+ by Li^+ in the presence of different amounts of $[Ca^{2+}]_o$. *A*, figures above each trace indicate the Ca^{2+} concentration present in the Li^+-Ringer solution. Ca^{2+} in test solutions was buffered as in Hodgkin *et al.* (1984). Top trace shows timing of solution changes. A bright flash equivalent to 8700 Rh* (photoisomerizations) was delivered at the time indicated by the arrow. *B*, relative amplitude of the Li^+ photocurrent at early times against $[Ca^{2+}]_o$ for four different cells. The relative amplitude of the Li^+ photocurrent was measured as the ratio of the photocurrent immediately after solution change and the photocurrent in normal conditions. The dark current in the physiological medium was: +, 23 pA; ○, 30 pA; △, 28 pA; ◇, 33 pA.

which can be observed when Li^+ substitutes for Na^+ can increase by 2–3 times. The dependence of this effect on the external Ca^{2+} is illustrated in Fig. 2*B* where the photocurrent recorded from four rods is plotted as a function of log $[Ca^{2+}]_o$. It is seen that when $[Ca^{2+}]_o$ is reduced to about 50 μM the photocurrent carried by Li^+ increases by 1–1·5 times, which is half of the maximal increase observed when $[Ca^{2+}]_o$ is reduced to 10^{-8} M. The second effect occurs at later times and when $[Ca^{2+}]_o$ is greater than 10^{-6} M consists of a decline in the photocurrent to smaller values. The decline

of the photocurrent has a time constant of 0·37 s in 10^{-3} M $[Ca^{2+}]_o$ and of 0·4 s when $[Ca^{2+}]_o$ is 10^{-4} M. This time constant becomes longer when $[Ca^{2+}]_o$ is reduced below 10^{-4} M. When $[Ca^{2+}]_o$ is below 10^{-7} M the photocurrent slowly increases to larger values.

The simplest explanation for these two distinct effects occurring at early and late times is that they are reflecting respectively an external and an internal action of reducing extracellular Ca^{2+} (Lamb, Matthews & Torre, 1985). The external action is likely to reflect the binding of Ca^{2+} to a site near or within the light-sensitive channel with a dissociation constant between 10 and 100 μM. The internal action is likely to be mediated by an effect of $[Ca^{2+}]_i$ on the cyclic GMP metabolism. As proposed by Yau & Nakatani (1984*b*) and Hodgkin *et al.* (1985), when Na^+ is removed from the extracellular medium, intracellular Ca^{2+} is likely to increase, because it cannot be extruded by Na^+–Ca^{2+} exchange. An increase in intracellular Ca^{2+} is likely to interfere with the cyclic GMP cascade either by inhibiting the cyclase (Lolley & Racz, 1982; Pepe, Panfoli & Cugnoli, 1986) or activating the phosphodiesterase (PDE) (Robinson, Kawamura, Abramson & Bownds, 1980; Stryer, 1986). The results shown in Fig. 2 suggest that when $[Ca^{2+}]_o$ is reduced below 10^{-7} M, intracellular Ca^{2+} decreases, leading to a slow activation of the photocurrent. When $[Ca^{2+}]_o$ is reduced only to 10^{-5} M and Na^+ is substituted by Li^+, intracellular Ca^{2+} is expected to rise because of a substantial influx of Ca^{2+}, which cannot be extruded by Na^+–Ca^{2+} exchange.

As proposed by Hodgkin *et al.* (1985) a viable method with which relative selectivity can be measured, is to compare photocurrents carried by different cations at very early times after ionic substitution, since at later times changes of the photocurrent are likely to reflect changes of the intracellular medium.

The ionic selectivity of divalent cations at equiosmolar levels

The simplest way to evaluate the selectivity of the light-sensitive channel to divalent cations is to replace extracellular NaCl with equiosmolar amounts of XCl_2 where X^{2+} is the tested divalent cation. In the experiments illustrated in Fig. 3 110 mM-NaCl was replaced by 73·3 mM-XCl_2 where X^{2+} was Ba^{2+}, Sr^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}. The extracellular Ca^{2+} was kept at the usual level of 1 mM (except when NaCl is replaced by $CaCl_2$).

In each panel of Fig. 3 we illustrate the photocurrent obtained when Na^+ was replaced by one of the tested divalent cations. A bright flash equivalent to about 8700 Rh* (photoisomerizations) was delivered to the rod at the time indicated by the arrow, to evaluate the photocurrent flowing at that time. When Ba^{2+} replaces Na^+, the photocurrent, after an initial increase, relaxes to a steady level of about 15 pA. When Na^+ is replaced with Sr^{2+} the early phase of current activation is not observed; instead there is quick reduction followed by a slower inactivation. Upon restoration of normal external Na^+ after exposure to the Sr^{2+} solution a light-insensitive current of about 10 pA is observed, which is likely to reflect electrogenic Sr^{2+} extrusion by a mechanism similar to the Na^+–Ca^{2+} exchange (Yau & Nakatani, 1984*b*; Hodgkin, McNaughton & Nunn, 1987; Hodgkin & Nunn, 1987). The reactivation of the photocurrent occurs only after the termination of light-insensitive current, suggesting that high internal Sr^{2+} blocks light-sensitive channels, in a way similar to

Ca^{2+}. When Ca^{2+} or Mg^{2+} replace Na^{+} the photocurrent is quickly reduced from 35 to 9 pA for Ca^{2+}, and to 8 pA for Mg^{2+}. This residual photocurrent disappears within 2 s. Upon restoration of the usual physiological extracellular medium, a light-insensitive current of about 5 pA is observed, which is likely to originate from the electrogenic activity of the Na^{+}–Ca^{2+} exchanger. Replacing Na^{+} with Mn^{2+} causes a

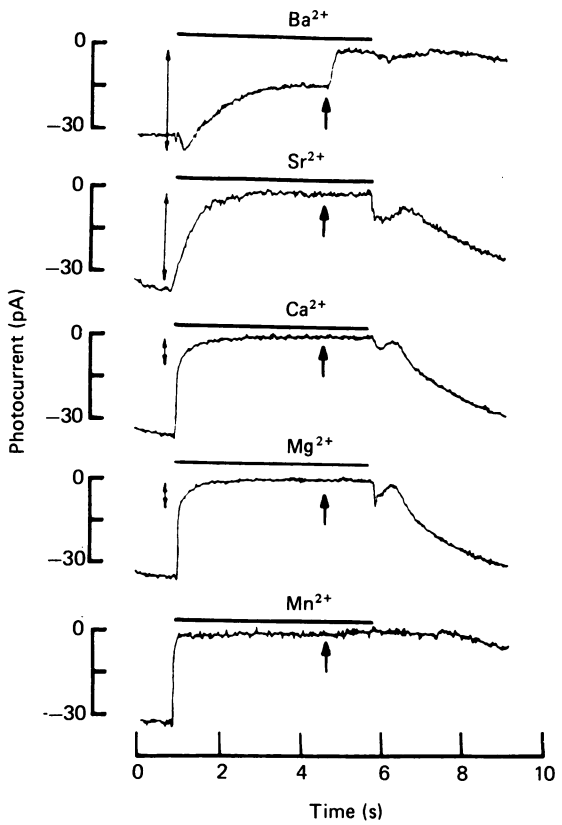

Fig. 3. Comparison of light-sensitive currents carried by different divalent cations. From top down 110 mM-NaCl was replaced by $BaCl_2$, $SrCl_2$, $CaCl_2$, $MgCl_2$ and $MnCl_2$ (73·3 mM). Top trace shows timing of solution changes. A bright flash equivalent to 8700 Rh* was delivered at the time marked by the arrow. Each trace is the average of at least two individual trials. The amplitude of the slow component, measured as described in the Methods section, is indicated by the arrow with double heads.

quick and almost complete suppression of the photocurrent suggesting that Mn^{2+} is very poorly permeant through the light-sensitive channel.

By measuring the photocurrent flowing at early times immediately after the solution change (see Methods and Fig. 1), the sequence of efficacy in carrying photocurrent at equiosmolar level is $Ba^{2+} > Sr^{2+} > Ca^{2+} > Mg^{2+} > Mn^{2+}$.

As we will see in later sections this sequence does not reflect the ionic selectivity in the low activity range because of the saturation of the photocurrent carried by divalent cations.

The effect of prolonged exposure to Ba^{2+}

The results shown in Fig. 3 suggest that Sr^{2+}, but not Ba^{2+}, can be extruded electrogenically from the rod. Therefore it is interesting to know whether Ba^{2+} can be extruded from the cell and by which mechanism. In fact when Ba^{2+} is applied

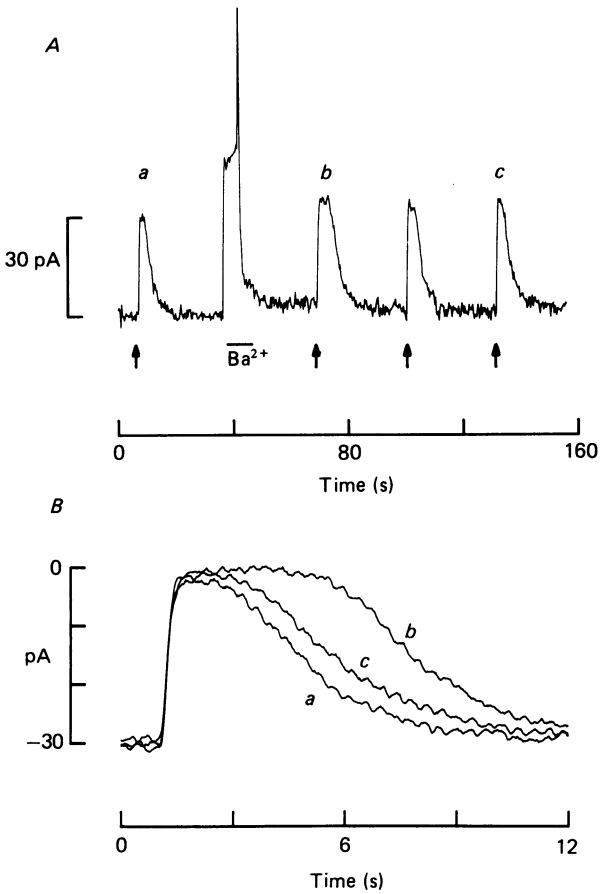

Fig. 4. Delayed effect of the exposure to Ba^{2+} on the time course of photoresponses. *A*, recording of the current obtained with the suction pipette. The bar indicates the time when 110 mM-NaCl was replaced by 73·3 mM-$BaCl_2$. The recording of the suction current during the exposure to Ba^{2+} is the combination of the change of the photocurrent and of the junction current (see Methods). Bright flashes equivalent to 7600 Rh* were delivered at the times indicated by arrows. *B*, the time course of photoresponses indicated by different letters in *A* reproduced in greater details. Observe the longer duration of photoresponse indicated by *b*, obtained after the exposure to Ba^{2+}. Slow oscillations superimposed on traces were introduced by flutter of the magnetic tape, during off-line digitization.

many times to the same rod, we do not see a complete recovery to the previous state. For instance, in two rods (out of approximately fifty cells tested), after several exposures to the same isotonic Ba^{2+} Ringer solution, a strong flash caused an inverted response (i.e. an outward current).

Figure 4*A* illustrates the chart recording of an experiment where bright flashes

were delivered to a rod at the times indicated by the arrow, while exposing the rod to a Ba^{2+} Ringer solution for the period indicated by the bar. The chart trace shows the original recording obtained during the exposure to Ba^{2+}, which is the sum of changes in the photocurrent and in the junction current (see Methods). It is interesting to observe that the photoresponse to a bright flash is reversibly prolonged

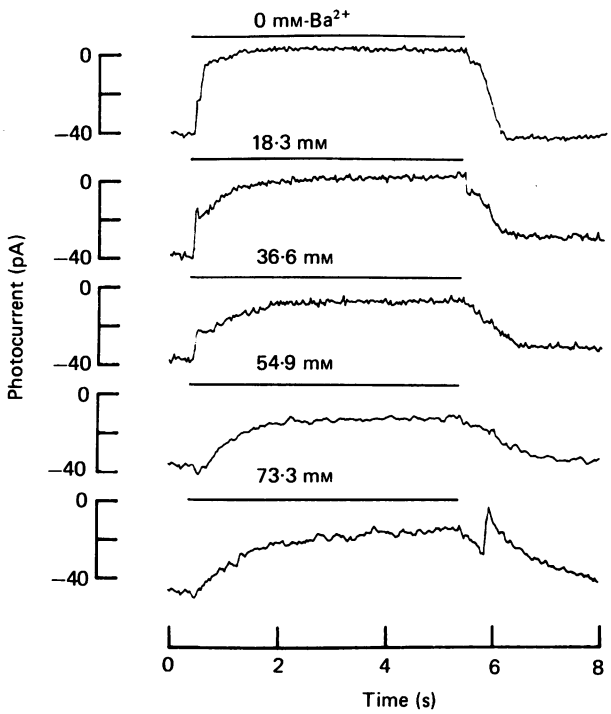

Fig. 5. Effect on the light-sensitive current of the substitution of Na^+ with different amounts of Ba^{2+} in the presence of 1 mM-Ca^{2+} in the bathing medium. Bars indicate the timing of solution changes. Each trace is the average of at least two individual trials.

after the exposure to Ba^{2+}, as illustrated in Fig. 4*B*. These results, which are typical, show that the entry of Ba^{2+} into the cell is likely to alter some metabolic functions, which can be restored only after many seconds.

We do not know whether Ba^{2+} can be extruded from the cell or can only be bound to internal sites. If the latter occurs, when these binding sites have been saturated we can expect a poisoning of the cell by further exposure to Ba^{2+}. In many cells, however, we have measured photocurrents carried by Ba^{2+} equivalent to the entry of a few mM, without leading to any damaging effect.

The dependence of the Ba^{2+} photocurrent on $[Ba^{2+}]_o$

Figure 5 illustrates the effect of exposure to solutions containing increasing amounts of Ba^{2+} on the current flowing through the light-sensitive channel. Three main effects are present in the traces reproduced in the Figure. The photocurrent flowing through the light-sensitive channels immediately after Na^+ replacement increases as the Ba^{2+} concentration in the bathing medium is increased. This current,

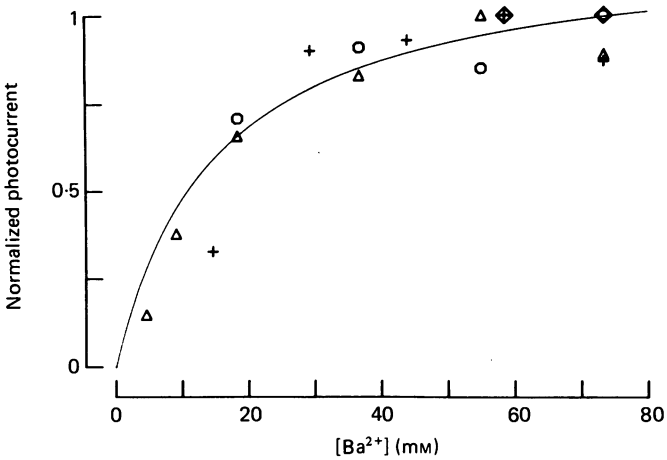

Fig. 6. Dependence of the light-sensitive Ba^{2+} current on the Ba^{2+} concentration in the bathing medium and in the presence of 0·1 mM-Ca^{2+}. Collected data from four cells. The dark photocurrent in the physiological medium was: ○, 22 pA; ◇, 34 pA; +, 21 pA; and △, 36 pA. The maximal photocurrent carried by 73·3 mM $[Ba^{2+}]_o$ at early times (see Methods) was: ○, 36 pA; ◇, 44 pA; +, 31 pA; and △, 40 pA. Continuous line obtained from eqn (1) with $K_{\frac{1}{2}} = 15$ mM, I_{max} equal to 1·2 times the maximal photocurrent recorded in the presence of 73·3 mM $[Ba^{2+}]_o$. The average maximal photocurrent recorded in the presence of 73·3 mM $[Ba^{2+}]_o$ was 38 pA.

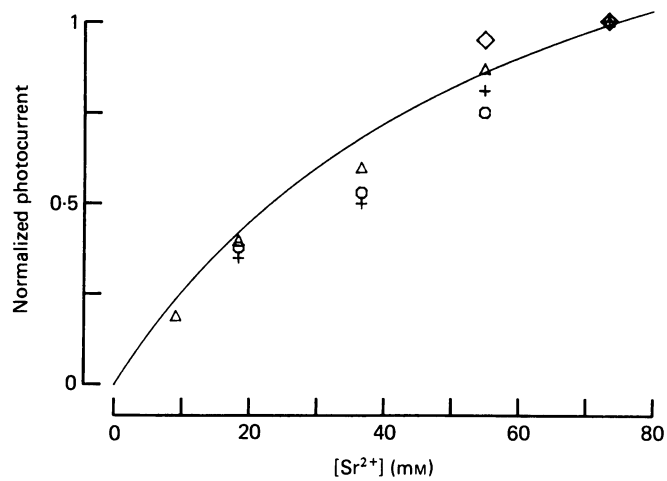

Fig. 7. Dependence of the light-sensitive Sr^{2+} current on the Sr^{2+} concentration in the bathing medium and in the presence of 1 mM $[Ca^{2+}]_o$ and 1·6 mM $[Mg^{2+}]_o$. Collected data from four cells. The dark current in the physiological medium was: ○, 30 pA; ◇, 28 pA; +, 40 pA; and △, 38 pA. The maximal photocurrent carried by 73·3 mM $[Sr^{2+}]_o$ at early times was: ○, 25 pA; △, 22 pA; +, 30 pA; and ◇, 33 pA. Continuous line obtained from eqn (1) with $K_{\frac{1}{2}} = 60$ mM, I_{max} equal 1·8 times the maximal photocurrent recorded in the presence of 73·3 mM $[Sr^{2+}]_o$. The average maximal photocurrent recorded in the presence of 73·3 mM $[Sr^{2+}]_o$ was 27·5 pA.

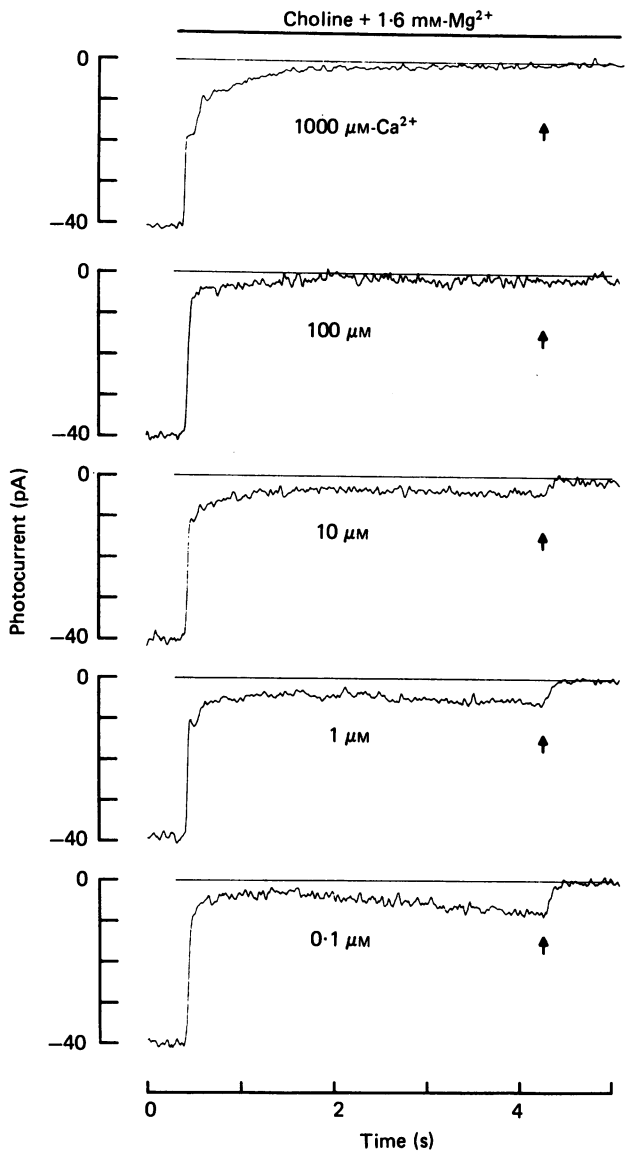

Fig. 8. Effect of the replacement of Na^+ with choline in the presence of different amounts of $[Ca^{2+}]_o$. Ca^{2+} in test solutions was buffered as in Hodgkin *et al.* (1984). Top trace shows timing of solution changes. A bright flash equivalent to 8700 Rh* was delivered at the time indicated by the arrow. Each trace was the average of at least three individual trials.

however, does not increase further when $[Ba^{2+}]_o$ is raised above 50 mM. When $[Ba^{2+}]_o$ is larger than 40 mM the photocurrent, after an initial increase, relaxes to a smaller value as already seen in Fig. 3. Upon restoration of the usual medium the photocurrent remained inactivated for a few seconds, probably because of Ba^{2+} accumulation within the cell. In the lowest trace of Fig. 5, when Na^+ is reintroduced in the bathing medium a transient suppression of the photocurrent is observed. This

transient suppression of the recorded photocurrent occurs about 300 ms after the solution change. We have no conclusive explanation for this phenomenon which may also reflect events occurring at the inner segment. Figure 6 reproduces collected data from four cells showing the dependence of the photocurrent at early times on $[Ba^{2+}]_o$, when extracellular Ca^{2+} was reduced to 0·1 mM in order to decrease the competition between Ba^{2+} and Ca^{2+} for the channel.

The experimental points can be fitted by a Michaelis–Menten equation with a half-activation $K_{\frac{1}{2}}$ and maximal current I_{max}:

$$\frac{I}{I_{max}} = \frac{[Ba^{2+}]_o}{K_{\frac{1}{2}} + [Ba^{2+}]_o}, \quad (1)$$

where $K_{\frac{1}{2}}$ is equal to 15 mM and I_{max} is equal to 1·2 times the maximal photocurrent recorded with 73·3 mM $[Ba^{2+}]_o$ (continuous line). The average value of the maximal photocurrent recorded with 73·3 mM $[Ba^{2+}]_o$ for four cells was 38 pA. Equation (1) can be obtained for a channel with one site which can be occupied by a single ion (Hille, 1984). The half-activation $K_{\frac{1}{2}}$ coincides with the dissociation constant of Ba^{2+} for the site in the absence of other ions competing for the same site.

The movement of Sr^{2+} through the light-sensitive channel

Sr^{2+} permeates through the light-sensitive channel in a similar way to Ba^{2+}. In the presence of 1 mM-Ca^{2+} and 1·6 mM Mg^{2+}, for a similar amount of salt dissolved in the extracellular medium, Sr^{2+} carries a smaller photocurrent than Ba^{2+}. As shown in Fig. 7, the dependence of the Sr^{2+} current at early times on $[Sr^{2+}]_o$ can be fitted by an equation analogous to eqn (1), with a value of $K_{\frac{1}{2}}$ equal to 60 mM and a value of I_{max} equal to 1·8 times the maximal photocurrent recorded with 73·3 mM $[Sr^{2+}]_o$. Collected data from four cells show that the average value of the maximal photocurrent carried by 73·3 mM $[Sr^{2+}]_o$ is about 27·5 pA.

The movement of Ca^{2+} and Mg^{2+} through the light-sensitive channel

It is not easy to see clear movements of Ca^{2+} through the light-sensitive channel under normal conditions. As shown in Fig. 3, when extracellular Na^+ is replaced by Ca^{2+} the photocurrent is quickly reduced and is abolished within 1 or 2 s. As shown by Hodgkin *et al.* (1985), when extracellular Na^+ is replaced by choline the photocurrent is not abolished instantaneously, but decreases quickly to a smaller value and then fades away. Figure 8 illustrates experiments in which extracellular Na^+ was replaced by choline in the presence of different levels of extracellular Ca^{2+}.

When the substitution was performed in the presence of 1 mM $[Ca^{2+}]_o$ the amplitude of the slow component of current (see Methods) was about 10 pA. The amplitude of this current was reduced to 6 pA when extracellular Ca^{2+} was reduced to 100 μM, suggesting that this current is carried by Ca^{2+}. This tail current vanishes completely within 2 s, when $[Ca^{2+}]_o$ is above 100 μM. Upon reduction of $[Ca^{2+}]_o$ below 10 μM, the amplitude of the initial photocurrent increased and a sustained photocurrent could be observed for several seconds. With $[Ca^{2+}]_o$ at 1 or 0·1 μM the sustained photocurrent could be as large as 10 pA.

The experiment shown in Fig. 9 helps us in the understanding of the ionic nature

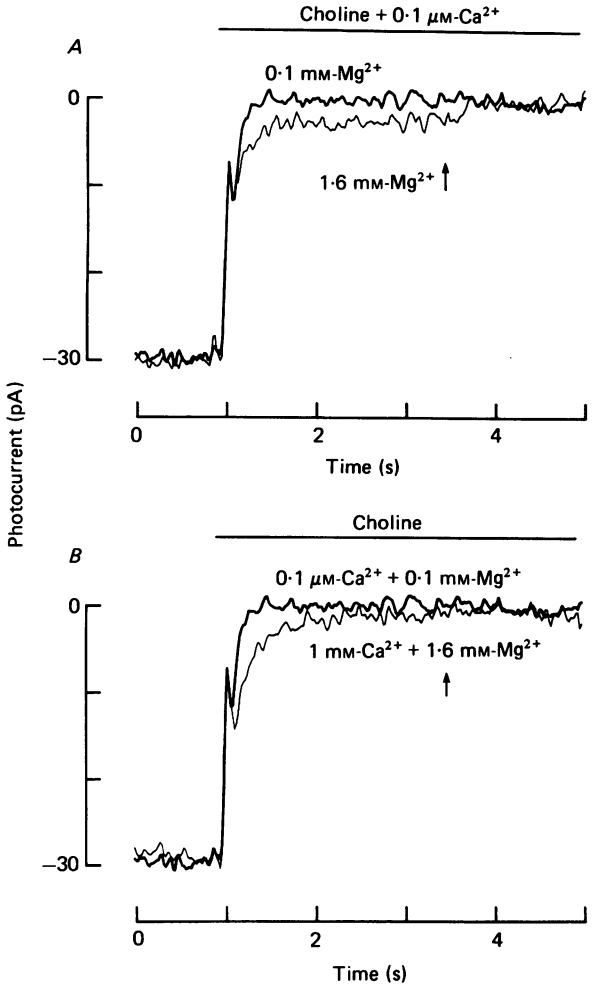

Fig. 9. Ionic nature of the residual photocurrent present when Na^+ is replaced by choline. *A*, comparison of the recordings when Na^+ is replaced by choline in the presence of 0·1 μM $[Ca^{2+}]_o$ and 0·1 mM $[Mg^{2+}]_o$ (thick trace) and in the presence of 0·1 μM $[Ca^{2+}]_o$ and 1·6 mM $[Mg^{2+}]$ (thin trace). *B*, comparison of recordings when Na^+ is replaced by choline in the presence of 0·1 μM $[Ca^{2+}]_o$ and 0·1 mM $[Mg^{2+}]_o$ (thick trace) and in the presence of 1 mM $[Ca^{2+}]_o$ and 1·6 mM $[Mg^{2+}]_o$ (thin trace). Top trace shows timing of solution changes. A bright flash equivalent to 8700 Rh* was delivered at the time indicated by the arrow. Each trace was the average of at least three individual trials.

of the photocurrent seen when Na^+ is replaced by choline. In Fig. 9*A* we compare the traces obtained upon replacing extracellular Na^+ with choline in the presence of 0·1 μM $[Ca^{2+}]_o$ and 1·6 mM $[Mg^{2+}]_o$. The sustained photocurrent present with the usual level of extracellular Mg^{2+} disappears when the concentration of this ion is reduced to 0·1 mM. It is, therefore, very likely that this residual current is carried by Mg^{2+}. In Fig. 9*B* we compare the trace obtained in the presence of the usual levels of $[Ca^{2+}]_o$ and of $[Mg^{2+}]_o$ with the trace obtained with 0·1 μM $[Ca^{2+}]_o$ and 0·1 mM $[Mg^{2+}]_o$. The current obtained with low divalent cations shuts off more

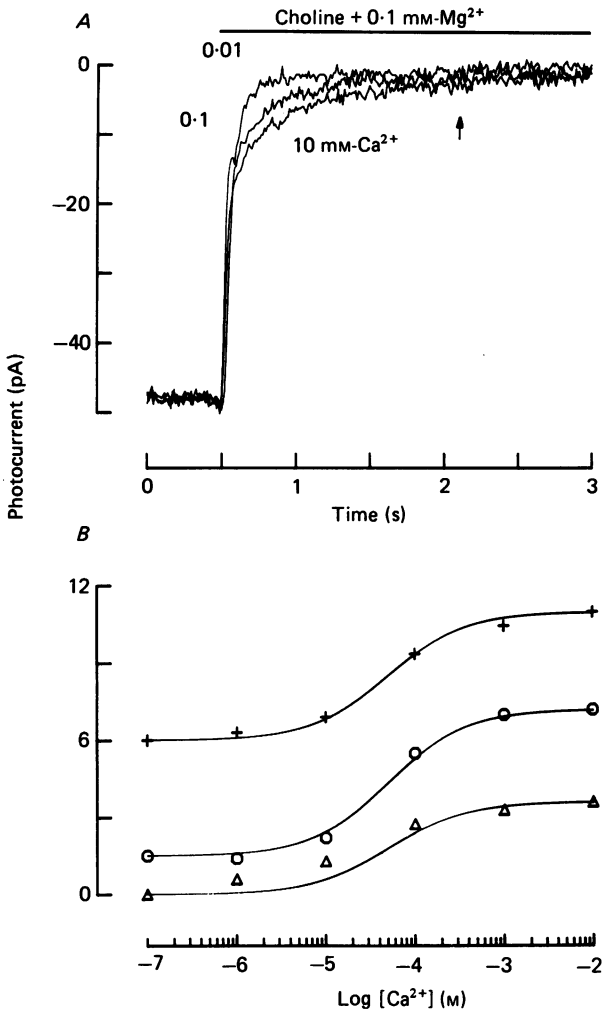

Fig. 10. Activation of the photocurrent carried by $[Ca^{2+}]_o$ when Na^+ was replaced by choline in the presence of different amounts of $[Ca^{2+}]_o$ and with 0·1 mM $[Mg^{2+}]_o$. *A*, comparison of recordings obtained in the presence of 0·01, 0·1 and 10 mM $[Ca^{2+}]_o$. Top trace shows timing of solution changes. A bright flash equivalent to 8700 Rh* was delivered at the time indicated by the arrow. Each trace was the average of at least three individual trials. *B*, amplitude of the Ca^{2+} current against $[Ca^{2+}]_o$ at three different times after the solution change (+, 125 ms; ○, 250 s; and △, 500 ms). Continuous curves were obtained from eqn (2) with $K_{\frac{1}{2}} = 50\ \mu M$ and an appropriate value of I_{max} plus the photocurrent which is observed in the presence of 10^{-7} M $[Ca^{2+}]_o$.

quickly than the current obtained with physiological divalent cations, but not instantaneously, suggesting that this tail current is carried by divalent cations.

As shown in Fig. 8 it is important to notice that we consistently observed a reduction of the initial residual photocurrent when $[Ca^{2+}]_o$ was reduced from 1 to 0·1 mM (in the presence of 1·6 mM-Mg^{2+}) but this tail current increased when $[Ca^{2+}]_o$ was reduced further. This behaviour is not to be expected if Ca^{2+} and Mg^{2+}

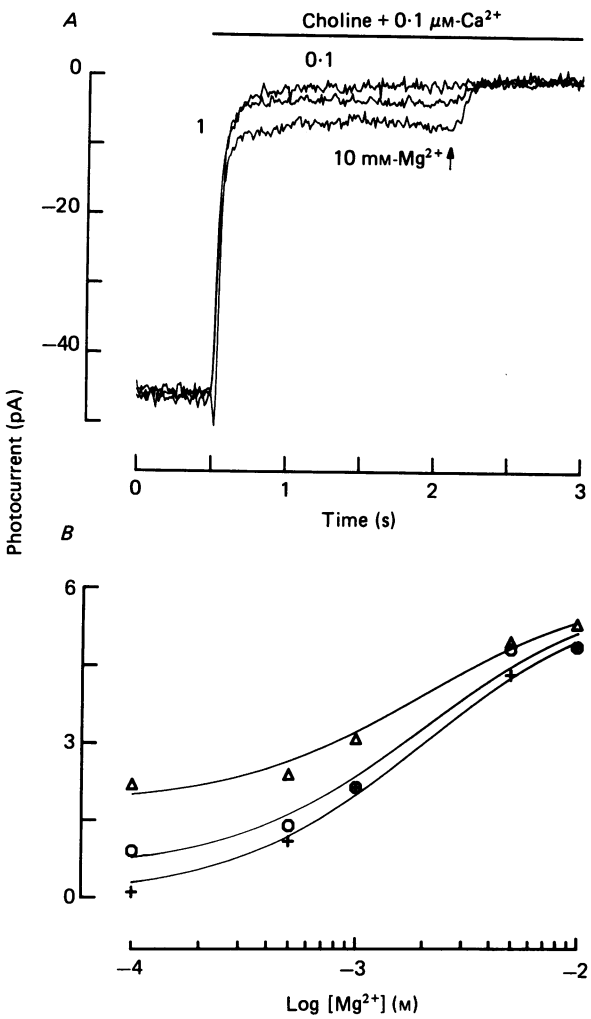

Fig. 11. Activation of the photocurrent carried by $[Mg^{2+}]_o$ when Na^+ was replaced by choline in the presence of different amounts of $[Mg^{2+}]_o$ and with 0·1 μM $[Ca^{2+}]_o$. Ca^{2+} was buffered with EGTA. *A*, comparison of recordings obtained in the presence of 0·1, 1 and 10 mM $[Mg^{2+}]_o$. Top trace shows timing of solution changes. A bright flash equivalent to 8700 Rh* was delivered at the time indicated by the arrow. Each trace was the average of at least three individual trials. *B*, amplitude of the Mg^{2+} current plotted against $[Mg^{2+}]_o$ at three different times after solution changes (△, 250 ms; ○, 500 ms; +, 1 s). Continuous curves were obtained with $K_{\frac{1}{2}} = 2$ mM and an appropriate value of I_{max} plus the photocurrent which is observed in the presence of 10^{-4} M $[Mg^{2+}]_o$.

move independently through the channel and it is reminiscent of the anomalous mole fraction effect in Ca^{2+} channels with mixtures of Ca^{2+} and Ba^{2+} (Almers & McCleskey, 1984; Hess & Tsien, 1984). These observations are better explained if we assume that Ca^{2+} and Mg^{2+} compete against each other within the channel and that Mg^{2+} is able to permeate only when Ca^{2+} is removed from the extracellular medium.

A way to test this idea is to measure the apparent dissociation constant of Ca^{2+} and

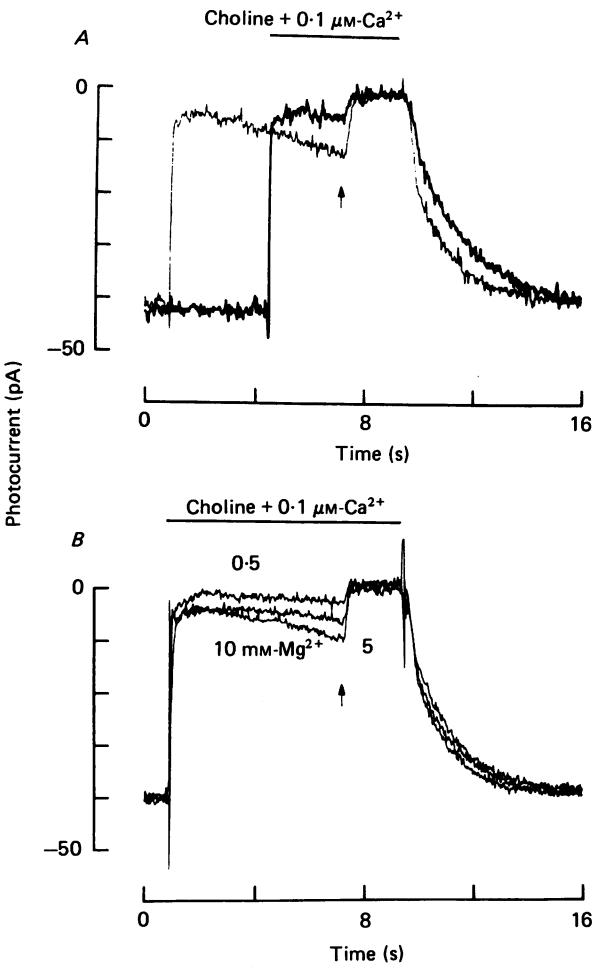

Fig. 12. Time-dependent activation of the light-sensitive current carried by Mg^{2+}. *A*, comparison of a brief (thick trace) and of a long exposure (thin trace) to a choline solution containing 0·1 μM-$CaCl_2$ and 10 mM-$MgCl_2$. Top traces show timing of solution changes. A bright flash equivalent to 8700 Rh* was delivered at the time marked by the arrow. *B*, comparison of a prolonged exposure to a choline solution containing 0·1 μM-$CaCl_2$ and different amounts of $MgCl_2$ (0·5, 5 and 10 mM). Top trace shows timing of solution changes. A bright flash equivalent to 8700 Rh* was delivered at the time marked by the arrow. Each trace was the average of at least two individual trials.

Mg^{2+} for the channel. In order to measure currents carried solely by Ca^{2+} or Mg^{2+} we measured photocurrents in the presence of different amounts of Ca^{2+} with low Mg^{2+} and in the presence of different amounts of Mg^{2+} with low Ca^{2+}.

Figure 10*A* shows experiments where extracellular Na^+ was replaced by choline in the presence of 0·1 mM $[Mg^{2+}]_o$ and with different $[Ca^{2+}]_o$ concentrations. The maximal size of the initial tail current is about 11 pA and is suppressed when $[Ca^{2+}]_o$ is reduced to 0·01 mM. This current, which is likely to be carried by Ca^{2+} ions, does not increase significantly when $[Ca^{2+}]_o$ in the bathing medium is raised to 10 mM. By removing permeant cations such as Na^+, Ca^{2+} and Mg^{2+} from the bathing medium,

we have not been able to obtain a complete suppression of the photocurrent within 250 ms after the solution change (see Figs 9 and 10). The most likely explanation for the presence of this persistent current is the existence of unstirred layers around the rod outer segment, which by forming a diffusional barrier to the test solution can be a source of permeant cations. In Fig. 10*B* we plot the amplitude of the tail current against log $[Ca^{2+}]_o$ at 125, 250 and 500 ms after the completion of the solution change. The experimental points can be fitted by a Michaelis–Menten equation with a half-activation constant of approximately 50 μM, plus a constant current, which reflects the residual persistent current previously mentioned. This measure of the half-activation of the Ca^{2+} current suggests a dissociation constant of approximately 50 μM of Ca^{2+} for the channel. These results indicate that the Ca^{2+} influx through the light-sensitive channel is already saturated in the usual physiological medium.

The movement of Mg^{2+} through the light-sensitive channel is best analysed when $[Ca^{2+}]_o$ is kept low. Figure 11 shows experiments in which in the choline solution $[Ca^{2+}]_o$ was kept constant at 0·1 μM while changing extracellular Mg^{2+}. The maximal amplitude of the photocurrent carried by Mg^{2+} was about 8 pA and was completely abolished when $[Mg^{2+}]_o$ was reduced to 0·1 mM. As shown in Fig. 11*B* this Mg^{2+} current did not appreciably change when $[Mg^{2+}]_o$ was increased from 5 to 10 mM. In Fig. 11*B* we plot the Mg^{2+} current against log $[Mg^{2+}]_o$ concentration in the extracellular medium 250 ms, 500 ms and 1 s after the solution change. The experimental points can be fitted by a Michaelis–Menten equation with a half-activation constant of 2 mM plus a constant current, similar to the one also present in the experiments illustrated in Figs 9 and 10. These results suggest that the affinity of Mg^{2+} for the light-sensitive channel is about 2 mM, approximately 40 times lower than that of Ca^{2+}.

These results are consistent with the idea that Ca^{2+} binds more strongly to the channel than Mg^{2+}, and that Ca^{2+} competes with Mg^{2+} during permeation through the light-sensitive channel.

The photocurrent carried by Mg^{2+}, which can be seen when Na^+ is replaced with choline and when $[Ca^{2+}]_o$ is kept low, builds up with time. Figure 12*A* illustrates an experiment in which a rod was exposed to a choline solution containing 0·1 μM-Ca^{2+} and 10 mM-Mg^{2+} for 4 s (thick trace) and for 8 s (thin trace) before a bright flash of light was delivered. Eight seconds after the exposure to the test solution the residual photocurrent was about 16 pA, when initially it was only 8 pA. The current which is suppressed by a bright flash of light is promptly reactivated when Na^+ is restored in the bathing medium. The rate of reactivation of the photocurrent is faster after a longer exposure to the test solution. These two observations are consistent with the idea that during the exposure to the test solution intracellular Ca^{2+} drops, leading to a larger photocurrent and to a faster reactivation following a flash of light.

Figure 12*B* shows an experiment supporting the notion that the photocurrent activated with time in the choline solution is carried by Mg^{2+}. When Mg^{2+} in the choline solution is reduced from 10 to 5 mM and to 0·5 mM the time-dependent activation of the photocurrent is reduced.

DISCUSSION

The main purpose of this study was to establish the ionic selectivity of the light-sensitive channel. The photocurrent I carried by a divalent cation X^{2+} depends on the ionic concentration $[X^{2+}]_o$ approximately, but not exactly, as

$$\frac{I}{I_{X,max}} = \frac{[X^{2+}]_o}{K_{\frac{1}{2}} + [X^{2+}]_o}, \tag{2}$$

where $I_{X,max}$ is the maximal photocurrent carried by cation X^{2+} and $K_{\frac{1}{2}}$ is the half-activation constant of the current. The half-activation $K_{\frac{1}{2}}$ of the current carried by a given ion is related to, but does not coincide with the dissociation constant K_X of the ion for the channel. When the ion is the only one permeating then the two quantities become identical. In the presence of competing ions $K_{\frac{1}{2}}$ is always larger than K_X.

TABLE 1. Values of I_{max} and $K_{\frac{1}{2}}$ for Ba^{2+}, Sr^{2+}, Ca^{2+} and Mg^{2+}

	I_{max} (pA)	$K_{\frac{1}{2}}$ (mM)	K_X (mM)	I_{max}/K_X (pA/mM)
Ca^{2+}	11·0	0·05	0·05	220
Mg^{2+}	8·0	2	2	4
Ba^{2+}	45·6	15	3·9	11·7
Sr^{2+}	49·5	60	2·7	18·3

I_{max} was measured for Ca^{2+} and Mg^{2+} and was estimated for Ba^{2+} and Sr^{2+} from the data shown in Figs 6 and 7. The half-activation constant $K_{\frac{1}{2}}$ was estimated for Ca^{2+} and Mg^{2+} in the absence of other permeant cations. $K_{\frac{1}{2}}$ for Ba^{2+} was obtained from experiments with 0·1 mM $[Ca^{2+}]_o$ and 1·6 mM $[Mg^{2+}]_o$; $K_{\frac{1}{2}}$ for Sr^{2+} was obtained from experiments with 1 mM $[Ca^{2+}]_o$ and 1·6 mM $[Mg^{2+}]_o$. The dissociation constant K_X for Ba^{2+} and Sr^{2+} was obtained from eqn (8) as described in the text.

Equation (2) can be obtained by a model with one site and one ion. Some observations reported in this paper could suggest the existence of two sites within the channel, namely the experiment of Fig. 8 reminiscent of the anomalous mole fraction effect. The decrease of the photocurrent when $[Ca^{2+}]_o$ is reduced from 1 to 0·1 mM and the subsequent increase when $[Ca^{2+}]_o$ is further reduced below 0·1 mM cannot be explained by a simple model with one site and ion. These results could, in principle, suggest the existence of two sites within the channel but other explanations are conceivable. Even if the model with one site is unable to account for all the observed results, it gives a reasonable explanation of the main features of the ionic permeation through the light-sensitive channels and at present it seems to be a simple and useful scheme.

Table 1 summarizes the values of I_{max} and $K_{\frac{1}{2}}$ for Ba^{2+}, Sr^{2+}, Ca^{2+} and Mg^{2+}, either measured or estimated. The low dissociation constant of Ca^{2+} for the light-sensitive channel suggests the existence of strong electrostatic interactions within the channel and therefore the presence of a site with a high field strength which can bind ions and control permeation. The existence of a site with high field strength is in agreement with the selectivity sequence for monovalent cations, as measured by Hodgkin *et al.*

(1985). The dissociation constants of the different cations for this site can be assumed to be of the same order as the half-activation constants ($K_{\frac{1}{2}}$). Because of the competition between divalent cations the actual values of K_{Ba} and K_{Sr} will be smaller than 15 and 60 mM.

The competition of cations in the light-sensitive channel

The Michaelis–Menten equation, eqn (2), describes only the saturation of the ionic current through the light-sensitive channel but not the competition between different cations. Competition between divalent cations is evident in the experiments shown in Figs 8 and 9.

It is evident that Mg^{2+} permeates through the channel only when Ca^{2+} is absent from the bathing medium. A simple way to describe this behaviour is to assume the existence of a site within the channel which can be occupied either by Ca^{2+} or by Mg^{2+}. This simple one-site model leads to the equations

$$I_{Ca} = I_{Ca,\max}\left(\frac{[Ca^{2+}]_o}{K_{Ca}}\Big/\left[1+\frac{[Ca^{2+}]_o}{K_{Ca}}+\frac{[Mg^{2+}]_o}{K_{Mg}}\right]\right) \tag{3}$$

and

$$I_{Mg} = I_{Mg,\max}\left(\frac{[Mg^{2+}]_o}{K_{Mg}}\Big/\left[1+\frac{[Ca^{2+}]_o}{K_{Ca}}+\frac{[Mg^{2+}]_o}{K_{Mg}}\right]\right). \tag{4}$$

From the experiments shown in Figs 10 and 11 we obtain $I_{Ca,\max} = 11$ pA, $K_{Ca} = 50\ \mu$M, and $I_{Mg,\max} = 8$ pA, $K_{Mg} = 2$ mM. With these values and from eqn (4) it is evident that with the usual level of extracellular Ca^{2+} and Mg^{2+} the light-sensitive current carried by Mg^{2+} is less than 1 pA. In agreement with this conclusion, the experiments illustrated in Fig. 8 show that a Mg^{2+} current is observed only when $[Ca^{2+}]_o$ is reduced below 100 μM.

In this study we did not obtain any evidence suggesting that Ca^{2+} and Na^{2+} do not permeate through the same channel. Therefore the same one-site model can be applied to the movements of Na^+ and Ca^{2+}, giving the equations

$$I_{Ca} = I_{Ca,\max}\left(\frac{[Ca^{2+}]_o}{K_{Ca}}\Big/\left[1+\frac{[Ca^{2+}]_o}{K_{Ca}}+\frac{[Na^{+}]_o}{K_{Na}}\right]\right) \tag{5}$$

and

$$I_{Na} = I_{Na,\max}\left(\frac{[Na^{+}]_o}{K_{Na}}\Big/\left[1+\frac{[Ca^{2+}]_o}{K_{Ca}}+\frac{[Na^{+}]_o}{K_{Na}}\right]\right), \tag{6}$$

which describe the competition between Ca^{2+} and Na^+ for the same site within the channel. Given the absence of any saturation of I_{Na} when $[Na^+]_o$ is increased from 0 to 110 mM (Capovilla, Cervetto, Pasino & Torre, 1981; A. Menini, G. Rispoli & V. Torre, unpublished observations) we can assume that $K_{Na} \gtrsim 100$ mM. If $K_{Na} \approx 100$ mM, then, in order to obtain $I_{Na} = 40$ pA under normal conditions, $I_{Na,\max}$ is around 840 pA. With these values we can conclude that a light-sensitive channel under normal physiological conditions is occupied by Ca^{2+} 20/21 of its time and in the residual time is occupied by Na^+. Therefore, in essence, this is the simplest explanation of how Ca^{2+} can block the Na^+ current and can carry a large fraction of the usual photocurrent.

The ionic selectivity in the low-activity range

In view of the fact that the current carried by divalent cations shows saturation it is of some interest to compare the relative selectivity of divalent cations in the low activity range, where

$$I \approx \frac{I_{X,max}}{K_X}[X]_o, \tag{7}$$

and K_X is the dissociation constant of the cation for the channel.

From Table 1 we have values of $I_{X,max}$ for Ca^{2+}, Ba^{2+}, Mg^{2+} and Sr^{2+} and we have measured the half-activation constant of the photocurrent carried by these ions in a variety of conditions. Because of the existence of competition we cannot use these values for the dissociation constant of the ion for the channel. The half-activation constants, $K_{\frac{1}{2}}$, for Ca^{2+} and Mg^{2+} were obtained in the absence of any competing ion and can be assumed to be similar to the dissociation constant of Ca^{2+}, K_{Ca}, and Mg^{2+}, K_{Mg}, for the channel. The half-activation constant for the Ba^{2+} current, $K_{\frac{1}{2}}$, is related to the Ba^{2+} dissociation constant K_{Ba}, as

$$\frac{K_{\frac{1}{2}}}{K_{Ba}} = 1 + \frac{[Ca^{2+}]_o}{K_{Ca}} + \frac{[Mg^{2+}]_o}{K_{Mg}}. \tag{8}$$

Since, in the presence of 0·1 mM-Ca^{2+} and 1·6 mM-Mg^{2+}, $K_{\frac{1}{2}}$ for Ba^{2+} was about 15 mM, we obtain $K_{Ba} \approx 3{\cdot}9$ mM. For Sr^{2+}, in the presence of 1 mM-Ca^{2+} and 1·6 mM-Mg^{2+}, $K_{\frac{1}{2}}$ was about 60 mM, and similarly we obtain $K_{Sr} \approx 2{\cdot}7$ mM.

Now, assuming that the selectivity ratio P_X/P_Y between ion X and Y is

$$\frac{P_X}{P_Y} = \frac{Z_Y I_X}{Z_X I_Y}, \tag{9}$$

where Z_X and Z_Y are the valences of ion X and Y respectively, I_X and I_Y are the currents carried, in the low activity range, by ions X and Y. From the data obtained, we have $P_{Ca}:P_{Sr}:P_{Ba}:P_{Mg} = 55:4{\cdot}5:2{\cdot}9:1$.

In order to obtain the selectivity ratio P_{Ca}/P_{Na} in the low activity range it is necessary to know the dependence of I_{Na} on $[Na^+]_o$ in the absence of extracellular Ca^{2+} and Mg^{2+}. When extracellular Ca^{2+} is buffered at 10^{-7} M and extracellular Mg^{2+} is about 10^{-4} M in the presence of 10 mM $[Na^+]_o$ a photocurrent of about 35 pA can be observed (A. Menini, G. Rispoli & V. Torre, unpublished observation), so that from eqn (9) we can estimate

$$\frac{P_{Ca}}{P_{Na}} \approx \frac{220}{2 \times 3{\cdot}5} = 31. \tag{10}$$

We conclude that in the low activity range the light-sensitive channel is primarily selective for Ca^{2+} and divalent cations, similarly to usual Ca^{2+} channels (Hagiwara & Byerly 1981; Almers & McCleskey, 1984; Hess & Tsien, 1984). A major difference between usual Ca^{2+} channels and the light-sensitive channel is the ability of Mg^{2+} to permeate through the latter.

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