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

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SUMMARY

1. By using the method of Hodgkin, McNaughton & Nunn (1985) for rapidly changing the extracellular medium, we analysed the effect of the organic compound IBMX (3-isobutyl-1-methylxanthine) on the movement of divalent cations through the light-sensitive channels of isolated retinal rods of the tiger salamander.

2. When the rod is treated with 0·5 mM-IBMX it is possible to observe photocurrents larger than 50 pA carried by Ba^{2+}, Sr^{2+}, Ca^{2+}, Mg^{2+} and Mn^{2+}. Under these conditions Ca^{2+}, Mg^{2+} and Mn^{2+} carry photocurrents of similar amplitude, while Ba^{2+} and Sr^{2+} usually carry larger photocurrents.

3. The movement of Mn^{2+} through the light-sensitive channel, which is hardly detected under normal conditions, can also be observed after treating the rod for a few seconds with a solution containing 35 mM $[Na^+]_o$ and 10^{-7} M $[Ca^{2+}]_o$. Under these conditions the photocurrent carried by Mn^{2+} is fully saturated in the presence of 1 mM-extracellular Mn^{2+}.

4. When the rod is pre-treated with an extracellular solution containing 0·5 mM-IBMX the maximal photocurrent which can be carried by 10 mM $[Ca^{2+}]_o$ increases from about 10 pA to approximately 200 pA. In these conditions the half-activation of the Ca^{2+} current is between 1 and 10 mM, that is 20–50 times higher than in normal conditions (Menini, Rispoli & Torre, 1988).

5. When the rod is pre-treated with an extracellular solution containing 0·5 mM-IBMX the half-activation of the photocurrent which can be carried by Mg^{2+}, Ba^{2+} and Sr^{2+} is equivalent to or greater than 10 mM. In the absence of pre-treatment with IBMX the half-activation of the photocurrent carried by Mg^{2+}, Ba^{2+} and Sr^{2+} is less than 5 mM.

6. We conclude that the light-sensitive channel can exist in at least two distinct open states. The selectivity of the channel in the first open state is as described in a previous paper (Menini *et al.* 1988). Mn^{2+}, which is hardly permeable through the

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light-sensitive channel in the first open state, can move through the light-sensitive channel in the second open state. Ca^{2+}, Mg^{2+}, Ba^{2+} and Sr^{2+} permeate more freely through the light-sensitive channel in the second open state, probably because the electrostatic interactions between these ions and the channel are less strong.

INTRODUCTION

The light-sensitive current in vertebrate rods is controlled by the cytoplasmic concentration of cyclic guanosine 3′,5′ monophosphate (cyclic GMP) which directly gates ionic channels (Caretta & Cavaggioni, 1983; Fesenko, Kolesnikov & Lyubarsky, 1985; Zimmerman & Baylor, 1986; Haynes, Kay & Yau, 1986; Matestic & Liebman, 1987; Matthews & Watanabe, 1987). In excised patches of outer segment membrane the current induced by cyclic GMP depends on the cube of the agonist concentration, therefore suggesting that at least three molecules of cyclic GMP are required to open a channel. This notion raises the question of whether a channel bound to a different number of cyclic GMP molecules can be characterized by distinct conductive states. The hypothesis receives some support from the observation of two sets of unitary current events recorded in excised patches of rod outer segments exposed to micromolar amounts of cyclic GMP (Zimmerman & Baylor, 1986; Haynes *et al.* 1986).

According to this view, increased cytoplasmic concentration of cyclic GMP, in addition to recruiting more channels to the open state, would change the conductive properties of the single channel. One way of testing this possibility is to analyse the ionic selectivity of the light-sensitive current at different internal concentrations of cyclic GMP. Increased cytosolic levels of cyclic GMP can be obtained in rods with external application of 3-isobutyl-1-methylxanthine (IBMX) (Capovilla, Caretta, Cavaggioni, Cervetto & Sorbi, 1983*b*), which inhibits the phosphodiesterase (PDE) responsible for breaking down the cyclic nucleotide (Beavo, Rogers, Crofford, Hardman, Sutherland & Newman, 1970; Wells, Wu, Baird & Hardman, 1975; Kakiuchi, Yamazaki, Teshima, Nenishi, Miyamoto, 1975). Higher levels of cyclic GMP are also caused by lowering intracellular Ca^{2+}, which stimulates the synthesis of cyclic GMP (Cohen, Hall & Ferrendelli, 1978; Lolley & Racz, 1982; Pepe, Panfoli & Cugnoli, 1986; Koch & Stryer, 1988; Rispoli, Sather & Detwiler, 1988). A drop of intracellular Ca^{2+} can be induced by exposing the rod outer segment to an extracellular solution containing very low free Ca^{2+}, so that the extrusion of internal Ca^{2+} by the Na^{+}–Ca^{2+} exchanger (Yau & Nakatani, 1984; Hodgkin, McNaughton & Nunn, 1987) is not balanced by a Ca^{2+} influx.

In the present study we have investigated both the effect of IBMX and of low external Ca^{2+} on the ionic selectivity of the light-sensitive current. In the presence of IBMX or of low external Ca^{2+} the light-sensitive current carried by divalent cations increases to large values. Particularly striking is the case of Mn^{2+} which is negligibly permeant in normal conditions, but becomes highly permeant when the internal cyclic GMP is increased. Analysis of the light-sensitive current carried by different concentrations of divalent cations indicates that the increased values of current measured in the above conditions are associated with a large increase in the apparent dissociation constant of the channel for the tested cations. This is

consistent with the idea that the light-sensitive channel may exist in more than one open state depending on the level of cyclic GMP.

METHODS

Light-sensitive currents were recorded with suction pipettes from individual rods mechanically isolated from the dark-adapted retina of the larval tiger salamander *Ambystoma tigrinum* (Lawrence Waterdog Farm) after decapitation and pithing. In all the experiments the inner segment was drawn into a suction pipette, leaving the outer segment exposed to the bathing medium, which could be rapidly changed.

Recording apparatus, optical stimulation

The apparatus for suction-electrode recording and optical stimulation of rods was similar to that described by Lamb, Matthews & Torre (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 isomerizations, estimated assuming a collecting area of 20 μm^2 (see Lamb *et al.* 1986). The isolation of rods and the recording apparatus were as described in Menini *et al.* (1988).

Solutions

The Ringer solution was the same as used by Baylor, Lamb & Yau (1979) and contained (in mM): NaCl, 110; KCl, 2·5; $CaCl_2$, 1; $MgCl_2$, 1·6; HEPES, 3; EDTA, 0·1; glucose, 5; buffered to pH 7·5 with tetramethylammonium (TMAOH). The solutions in low Ca^{2+} were prepared as in Hodgkin, McNaughton, Nunn & Yau (1984). The composition of different test solutions is given in figure legends. The perfusion system was similar to that described by Menini *et al.* (1988).

Determination of the light-sensitive currents

The light-sensitive current, usually called photocurrent, was determined (see Hodgkin *et al.* 1985) as the difference of the current in darkness minus the current recorded during a saturating steady light. This procedure allows the subtraction from the current recording of changes of the junction current due to solution changes and of the mechanical artifact.

RESULTS

The selectivity to divalent cations in the presence of IBMX

Figure 1*A* reproduces the results of an experiment in which the extracellular NaCl was replaced by 73·3 mM of XCl_2 where X^{2+} is a divalent cation such as Ba^{2+}, Sr^{2+}, Ca^{2+}, Mn^{2+} or Mg^{2+}. The usual level of Ca^{2+} and Mg^{2+} was present in the extracellular medium, except when Na^+ was replaced by Ca^{2+} or Mg^{2+}, and 0·5 mM-IBMX, a known inhibitor of phosphodiesterase (PDE) (Beavo *et al.* 1970; Kakiuchi *et al* 1975; Wells *et al.* 1975) was also added to the bathing medium. This drug is able to cross the plasma membrane and is expected to promptly inhibit the activity of PDE and ultimately to lead to an increase of intracellular cyclic GMP.

When Na^+ is replaced by Ca^{2+}, Mn^{2+} and Mg^{2+} the photocurrent is initially reduced, but it reactivates to a level of about 60 pA at a later time. The delayed activation occurs only in the presence of IBMX. When Ba^{2+} replaces Na^+ in the bathing medium the photocurrent at early times is not greatly affected. After 2 or 3 s the photocurrent increases to about 120 pA but then it starts to decline while the recording trace becomes noisy. This delayed inactivation of the Ba^{2+} photocurrent

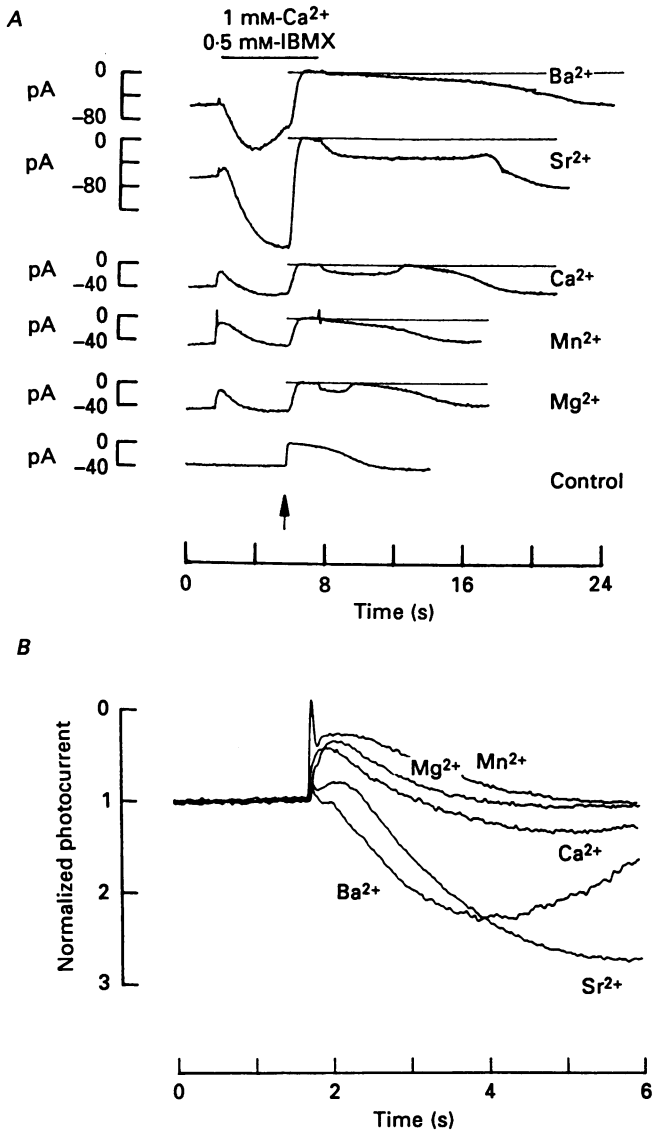

Fig. 1. *A*, the effect of 0·5 mM-IBMX on the selectivity to divalent cations. 110 mM-NaCl was replaced with 73·3 mM of $BaCl_2$, $SrCl_2$, $MnCl_2$, $CaCl_2$ or $MgCl_2$. The usual level of Ca^{2+} and Mg^{2+} was present in all test solutions, with the exception of those in which $CaCl_2$ or $MgCl_2$ replaced NaCl. Top trace shows timing of solution changes. A bright flash equivalent to 8700 Rh* was delivered at the time marked by the arrow. The rod was exposed to each of the five test solutions twice before exposure to the test solutions containing no Ca^{2+} (see Fig. 2) and then was exposed once again to the previous test solutions. Each trace is the average of three individual trials. *B*, activation of the divalent cation photocurrent by IBMX. Traces were scaled to the circulating current before the solution change, for exposure to Ba^{2+}, Sr^{2+}, Ca^{2+}, Mg^{2+} and Mn^{2+} this was 59, 68, 42, 48 and 48 pA respectively.

was observed in three other rods and is likely to reflect an accumulation of Ba^{2+} inside the rod. When Sr^{2+} replaces Na^+, the photocurrent, after an initial small suppression, increases to about 200 pA. At the time indicated by the arrow a bright flash quickly abolished the circulating photocurrent. After exposure to IBMX the duration of the light response is clearly prolonged in agreement with previous

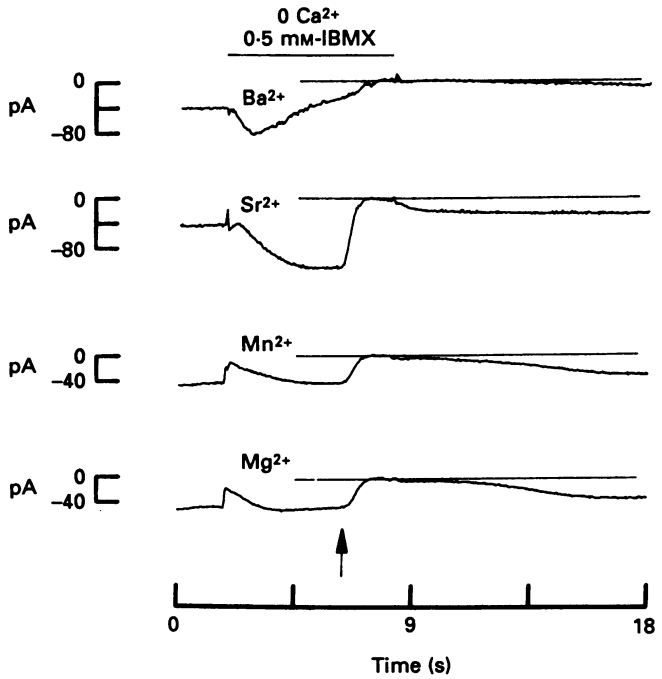

Fig. 2. The effect of 0·5 mM-IBMX on the selectivity to divalent cations in the absence of added extracellular Ca^{2+}. 110 mM-NaCl was replaced with 73·3 mM-$BaCl_2$, $SrCl_2$, $MnCl_2$ and $MgCl_2$. No Ca^{2+} was added to the test solutions and the usual level of 1·6 mM-Mg^{2+} was present in all test solutions, with the exception of that in which $MgCl_2$ replaced NaCl. Top trace shows timing of solution change. A bright flash equivalent to 8700 Rh* was delivered at the time marked by the arrow. Recordings from the same rod as in Fig. 1. Each trace is the average of two individual trials.

observations (Capovilla, Cervetto & Torre, 1983*c*; Cervetto & McNaughton, 1986). The time course of the light response in control conditions is illustrated at the bottom of Fig. 1*A*, and its duration is approximately 4 s. After exposure to IBMX the duration of the light response also depends on the divalent cation substituting for Na^+. When Mn^{2+} or Mg^{2+} substitute for Na^+ the photoresponse lasts about 8 s. When Ba^{2+}, Sr^{2+} or Ca^{2+} were used the light response was prolonged to about 16, 12 and 10 s respectively. This different duration of the light response is likely to be caused by the different time required for buffering or extruding these ions from the cell interior.

When Na^+ was restored in the bathing medium a light-insensitive current was observed after the exposure to solutions containing IBMX with Ca^{2+}, Sr^{2+} or Mg^{2+}. This light-insensitive current is probably due to the electrogenic nature of the Na^+–

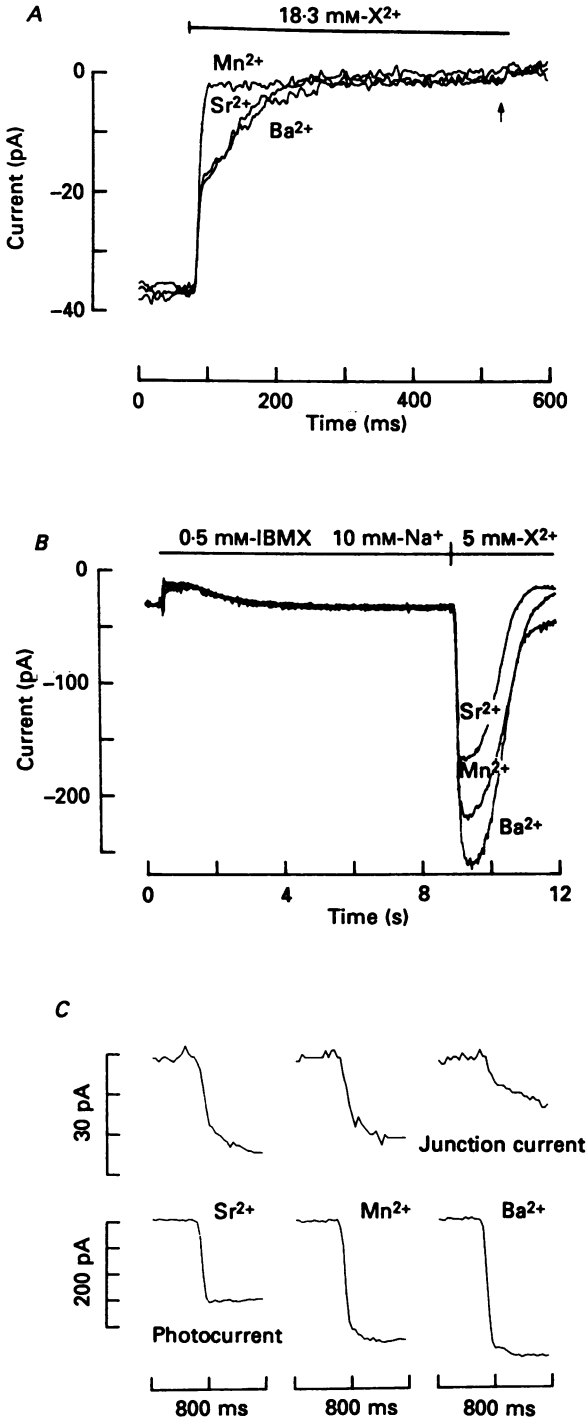

Fig. 3. For legend see facing page.

Ca^{2+} exchange (Yau & Nakatani, 1984; Hodgkin *et al.* 1987). The ionic nature of this current will be discussed later.

Figure 1*B* compares the effects of IBMX on the photocurrent carried by various divalent cations. The photocurrent was normalized to the circulating current prior to the ionic change for a better comparison. The time course of the current carried by Ca^{2+}, Mn^{2+} or Mg^{2+} after the initial suppression is similar and the three traces appear vertically shifted. The Ba^{2+} photocurrent increases more rapidly and reaches large values during the first 2 s then starts to inactivate, while the Sr^{2+} current is still increasing.

The presence of a light-insensitive current initiated by the restoration of Na^+ in the extracellular medium after exposure to solutions containing IBMX and Sr^{2+} or Mg^{2+} (Fig. 1*A*) could suggest that these ions are also extruded from the rod by an electrogenic mechanism, dependent on extracellular Na^+. In a previous paper (Menini *et al.* 1988) we showed that in normal conditions Mg^{2+} permeates through the light-sensitive channel only in the absence of extracellular Ca^{2+} and it is also conceivable that in the presence of IBMX, millimolar amounts of Ca^{2+} in the extracellular medium block the entry of Mg^{2+}. Therefore it is important to check whether the photocurrent recorded when Na^+ is replaced by Mg^{2+} in the presence of 1 mM-extracellular Ca^{2+}, is carried by Mg^{2+} or by Ca^{2+}. Figure 2 illustrates the results of exposure to 0·5 mM-IBMX and to Ba^{2+}, Sr^{2+}, Mn^{2+} and Mg^{2+} when no $CaCl_2$ was added to the test solution.

The recordings in Fig. 2, obtained from the same rod as Fig. 1, show that, following exposure to Mg^{2+} in the absence of added external Ca^{2+}, no light-sensitive current is observed upon restoration of normal Na^+. These experiments suggest that Mg^{2+}, Ba^{2+} or Mn^{2+} are poorly extruded by an electrogenic Na^+-dependent exchange mechanism and that Ba^{2+} and Mn^{2+} greatly reduce the entry of Ca^{2+} into the cell.

From the results reported in Figs 1 and 2 it is evident that the presence of Ca^{2+} in the extracellular medium has little effect on the movement of Mn^{2+} through the light-sensitive channel. The photocurrent recorded when Na^+ was replaced by Ba^{2+} or Sr^{2+} in the absence of extracellular Ca^{2+} was slightly smaller than when the same experiment was repeated in the presence of 1 mM $[Ca^{2+}]_o$. The rod from which the recordings shown in Figs 1 and 2 were obtained, was exposed several times to solutions containing IBMX and large amounts of divalent cations. Therefore it is possible that the smaller photocurrents recorded with Ba^{2+} and Sr^{2+} in the absence

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Fig. 3. Comparison of the relative permeability of Ba^{2+}, Sr^{2+} and Mn^{2+} in normal conditions and in the presence of 0·5 mM-IBMX. *A*, recordings of the photocurrent when 110 mM-NaCl was replaced by 82·5 mM-choline chloride and 18·3 mM-$BaCl_2$, $SrCl_2$ or $MnCl_2$. Top trace shows timing of solution changes. A bright flash equivalent to 8700 Rh* was delivered at the time indicated by the arrow. *B*, recordings of the photocurrent carried by Ba^{2+}, Sr^{2+}, Mn^{2+} after pre-treatment with IMBX. Initially the bathing medium was replaced by a solution containing 10 mM-NaCl, 100 mM-choline chloride, no added Ca^{2+} or Mg^{2+} and 0·5 mM-IBMX. At the time indicated by the bar 10 mM-NaCl was substituted by 5 mM-$BaCl_2$, $SrCl_2$ or $MnCl_2$. The order of exposure was Mn^{2+}, Ba^{2+}, Sr^{2+}. Each trace is the average of two individual trials. *C*, comparison of the time course of the junction current and photocurrent. Upper row shows recordings of the junction current during a steady saturating light. Lower row shows recordings of the photocurrent. Same experiment and recordings as those shown in *B*.

of added Ca^{2+} may be caused by an unusual accumulation of divalent cations inside the cell. Results obtained from other rods showed that the presence of millimolar amounts of Ca^{2+} in the extracellular medium had little effect on the amplitude of photocurrents carried by Ba^{2+} or Sr^{2+} in the presence of 0·5 mM-IBMX, suggesting that the blocking effect of Ca^{2+} was reduced by IBMX.

The effect of pre-treatment with IBMX

In previous reports it was proposed that the addition of the PDE inhibitor, IBMX, to the extracellular medium changes the selectivity of the light-sensitive channel (Torre, Pasino, Capovilla & Cervetto, 1981; Capovilla, Caretta, Cervetto & Torre, 1983*a*; Borsellino, Cervetto & Torre, 1985). But in the experiments supporting this view the solution changes were not fast enough to separate possible external and internal effects of divalent cations. For instance in rods treated with 0·5 mM-IBMX, Mn^{2+} appeared the most permeant cation, but since Mn^{2+} is known to be a potent activator of the cyclase (Krishnan, Fletcher, Chader & Krishna, 1978) it was difficult to separate direct effects on the channel from metabolic effects on cytoplasmic proteins. In the experiments shown in Figs 1 and 2 the slow activation of the photocurrent can be ascribed to the development of the IBMX effects or to more complex events in which the entry of divalent cations is affecting the cyclase or the phosphodiesterase. These difficulties may be circumvented by testing the effects of divalent cations on rods primed in IBMX sufficiently long to reach a steady level of the photocurrent, as shown in Fig. 3*B*.

Figure 3*A* illustrates an experiment in which 110 mM-NaCl was substituted with 82·5 mM-choline chloride and 18·3 mM of $BaCl_2$, $SrCl_2$ or $MnCl_2$. The NaCl usually present in the extracellular medium was not completely replaced by equiosmolar amounts of divalent cations salts in order to avoid saturation of the photocurrent. The Ba^{2+} and Sr^{2+} photocurrents are similar and decline with the same time course, while Mn^{2+} permeates very poorly through the light-sensitive channel. However, when the same rod was pre-treated for 7 s with a solution containing 0·5 mM-IBMX, 10 mM-Na^+ and no added Ca^{2+} or Mg^{2+}, switching from 10 mM-Na^+ to 5 mM-Ba^{2+}, Sr^{2+} or Mn^{2+} caused the appearance of a large photocurrent (Fig. 3*B*) of about 200 pA. Under these conditions Ba^{2+}, Sr^{2+} and Mn^{2+} permeate almost equally well through the light-sensitive channel. This observation is remarkable in view of the fact that in some preparations Mn^{2+} can block Ca^{2+} channels (Lansman, Hess & Tsien, 1986) but in other preparations Mn^{2+} can substitute for Ca^{2+} in generating action potentials (Anderson, 1983; Ochi, 1986). Figure 3*C* compares the amplitude and time course of the solution change, as measured by the junction current, with the appearance of the photocurrent carried by Sr^{2+}, Mn^{2+} and Ba^{2+}. As shown in Fig. 3*C* there is no appreciable delay between the rise of the photocurrent carried by divalent cations and the rise of the junction current. We conclude therefore that the appearance of photocurrent carried by Mn^{2+} is unlikely to be caused by any effect of Mn^{2+} on the cell interior.

In the presence of IBMX the large inward currents carried by Ba^{2+}, Mn^{2+} and Mg^{2+} are not associated with a light-insensitive exchange current. It is conceivable that the massive influx of these ions can be initially buffered by intracellular sites, but eventually the intracellular level of these ions must increase to unphysiological

levels. This may explain the difficulties encountered in establishing the selectivity sequence in the low activity range of Mn^{2+}, Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+} in the presence of IBMX: the progressively smaller currents recorded, after several exposures to Mn^{2+} or Ba^{2+}, may reflect the cytoplasmic accumulation of these ions, and also a poisoning of the cell.

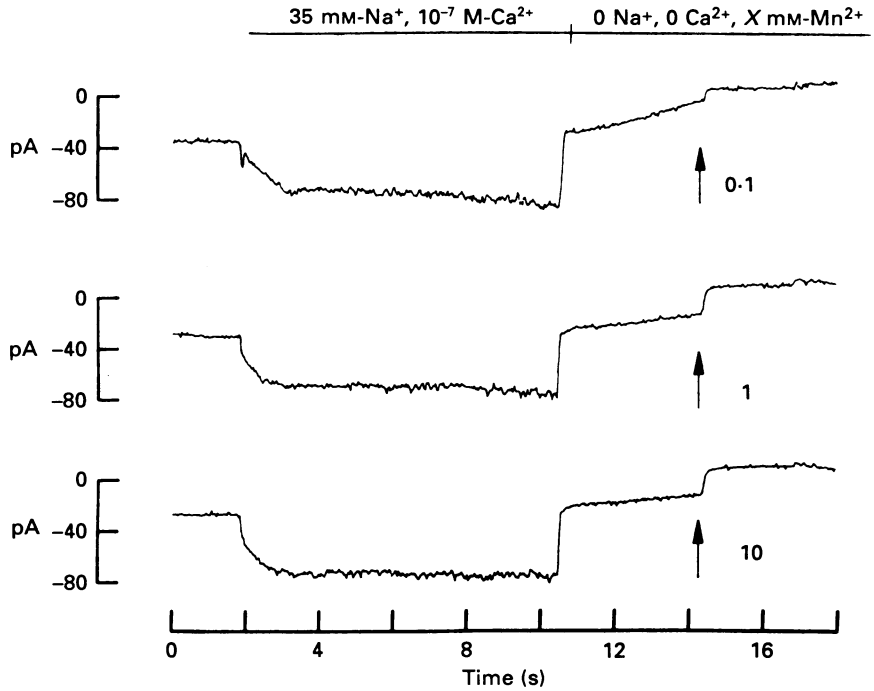

Fig. 4. Effect of pre-treatment with reduced $[Na^+]_o$ and 10^{-7} M $[Ca^{2+}]_o$ on the movement of Mn^{2+}. In each trial the rod was primed for 8 s in a solution containing 35 mM-Na^+, no added Mg^{2+}, and with $[Ca^{2+}]_o$ buffered to 10^{-7} M; the tonicity was adjusted by adding 75 mM-choline chloride. The test solution contained 0·1, 1 or 10 mM-$MnCl_2$, 110 mM-choline chloride and no added Ca^{2+} or Mg^{2+}. In the presence of 10 mM-$MnCl_2$, 10 mM-choline chloride was removed from the test solution. 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 two individual trials.

The effect of pre-treatment with 35 mM $[Na^+]_o$ and 10^{-7} M $[Ca^{2+}]_o$

The results presented in previous sections show that in the presence of IBMX, Mn^{2+} can easily permeate through the light-sensitive channel. Although the main effect of IBMX is to elevate internal cyclic GMP, it is possible that the effects observed on the photocurrents are caused by a direct action of the drug on the channel itself or by some other secondary effects. An independent way of elevating intracellular cyclic GMP, is to expose the rod outer segment to an extracellular medium containing a very low amount of free Ca^{2+}. In these conditions intracellular Ca^{2+} is expected to drop to very low levels, because the Ca^{2+} extrusion mediated by the Na^+–Ca^{2+} exchange is not balanced by a Ca^{2+} influx. Extracellular Na^+ must also be reduced to prevent excessive Na^+ loads of the cell resulting from the increased

dark current. A major consequence of the reduced intracellular Ca^{2+} is the elevation of the level of cyclic GMP via stimulation of the cyclase or inhibition of the phosphodiesterase (Robinson, Kawamura, Abramson & Bownds, 1980; Lolley & Racz, 1982; Capovilla *et al.* 1983*b*; Hodgkin *et al.* 1985; Pepe *et al.* 1986; Koch & Stryer, 1988; Rispoli *et al.* 1988).

Figure 4 illustrates an experiment in which the outer segment of a rod was primed in 35 mM-Na^+ and 10^{-7} M-Ca^{2+} before exposure to various concentrations of Mn^{2+}. Perfusion with the low-Na^+ and low-Ca^{2+} solution causes the photocurrent to increase. Substituting Na^+ with Mn^{2+} leads to a rapid decrease of the photocurrent after which a residual current of about 28 pA probably carried by Mn^{2+} is clearly observed for several seconds. The amplitude of this current does not increase when the extracellular Mn^{2+} is raised from 1 to 10 mM, indicating that the half-activation of this current by Mn^{2+} occurs below 1 mM. The result of the experiment illustrated in Fig. 4 supports the idea that the appearance of a photocurrent carried by Mn^{2+} in the presence of IBMX is caused by an elevation of intracellular cyclic GMP and not by other effects of the compound.

The Ca^{2+} photocurrent in the presence of IBMX

The larger Ca^{2+} photocurrent observed in the presence of IBMX could be explained by the opening of more light-sensitive channels and/or by conformational changes in the structure of the channel. In order to understand better the permeation of Ca^{2+} through the light-sensitive channels in the presence of IBMX, we have measured the photocurrent carried by different amounts of Ca^{2+}.

Figure 5 illustrates an experiment in which a rod was first exposed to a solution containing 0·5 mM-IBMX, 10 mM-Na^+, 0·1 mM-Mg^{2+} and 10^{-7} mM-Ca^{2+}. The photocurrent was first rapidly suppressed and later inverted its polarity, probably because extracellular Ca^{2+} was buffered at 10^{-7} M (compare with the experiment reported in Fig. 3, where Ca^{2+} was not buffered and the stray Ca^{2+} was probably around 10^{-5} M). At the time indicated by the bar Na^+ was removed from the bathing medium and concomitantly 10, 1 or 0·01 mM-Ca^{2+} were introduced in the extracellular medium. In order to avoid possible effects of Ca^{2+} accumulation, the exposure to 1 and 10 mM-Ca^{2+} was alternated, and consistent results were observed. The photocurrent carried by Ca^{2+} can be as large as 200 pA and does not show any sign of inactivation within 3 s. Under normal conditions, the Ca^{2+} concentration in the extracellular medium $K_{\frac{1}{2}}$ leading to a photocurrent with half the maximal amplitude is around 50 μM (Menini *et al.* 1988). It is evident that in the experiment shown in Fig. 5, 10 mM-Ca^{2+} carries a much larger current than 1 mM-Ca^{2+} and the half-activation $K_{\frac{1}{2}}$ of the Ca^{2+} photocurrent is likely to be between 1 and 10 mM. The increase of $K_{\frac{1}{2}}$ induced by IBMX can be taken as a decrease of the affinity of Ca^{2+} for the light-sensitive channel.

Dependence of the photocurrent carried by divalent cations on ionic concentration after a pre-treatment with IBMX

When the concentration of a permeable ion is increased in the extracellular medium, the current carried by this ion usually increases and then reaches saturation. The amplitude of the maximal current carried by the ion depends on the amplitude of the single-channel current, on the number of open channels and on their

average open time. The shape of the dependence of amplitude of the current on the ionic concentration, and the half-activation constant $K_{\frac{1}{2}}$ are set by the interactions occurring between the permeating ion and the channel. As a consequence changes of the value of the half-activation constant $K_{\frac{1}{2}}$, when cyclic GMP supposedly increases, can be taken as evidence of a modification of the structure of the light-sensitive

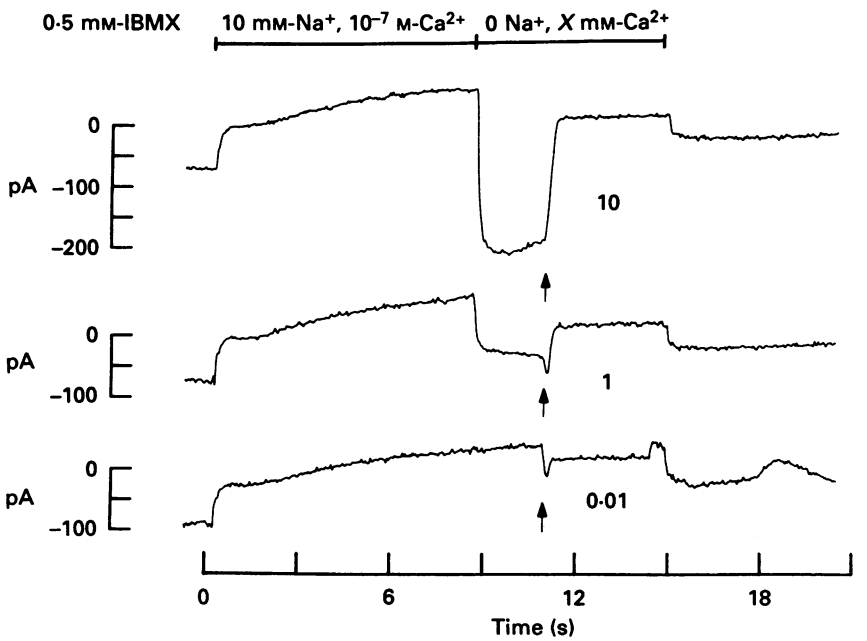

Fig. 5. The concentration dependence of the Ca^{2+} light-sensitive current in the presence of 0·5 mM-IBMX. The rod was initially exposed to a bathing medium containing 10 mM-NaCl, 100 mM-choline chloride, 0·5 mM-IBMX, 0·1 mM-$MgCl_2$, and with $[Ca^{2+}]_o$ buffered at 10^{-7} M. At the time indicated by the bar 10 mM-NaCl was substituted by 10, 1 or 0·01 mM-$CaCl_2$. A bright flash equivalent to 8700 Rh* was delivered at the time indicated by the arrow. The order of exposure of test solutions was 1, 10, 0·1, 1 and 10 mM-$CaCl_2$. The traces obtained with 1 and 10 mM-$CaCl_2$ were the average of the two individual trials.

channel. We measured on a given rod the photocurrent carried by 0·1, 1, 10 and 73·3 mM of XCl_2 where X^{2+} was Ca^{2+}, Mg^{2+}, Ba^{2+} or Sr^{2+}, after the rod had been pre-treated with an extracellular solution containing no added Na^+, Ca^{2+} or Mg^{2+}, with or without 0·5 mM-IBMX. When the rod is pre-treated with IBMX intracellular cyclic GMP is expected to increase.

Figure 6 illustrates the stoichiometry of the photocurrent carried by Ca^{2+} (*A*), by Mg^{2+} (*B*), by Ba^{2+} (*C*) and by Sr^{2+} (*D*) after a pre-treatment of 8·6 s with a solution containing no added Na^+, Ca^{2+} or Mg^{2+}. Under these conditions a transient photocurrent of about 17 pA carried by Ca^{2+} can be observed. In agreement with a previous report (Menini *et al.* 1988) the half-activation of the photocurrent carried by Ca^{2+} occurs at a concentration below 0·1 mM. The photocurrent carried by Mg^{2+}, Ba^{2+} and Sr^{2+} is stable for a few seconds and is promptly abolished by a flash of light delivered at the time indicated by the arrow. The half-activation of the photocurrent carried by these ions is approximately 1 mM and the amplitude of the current carried

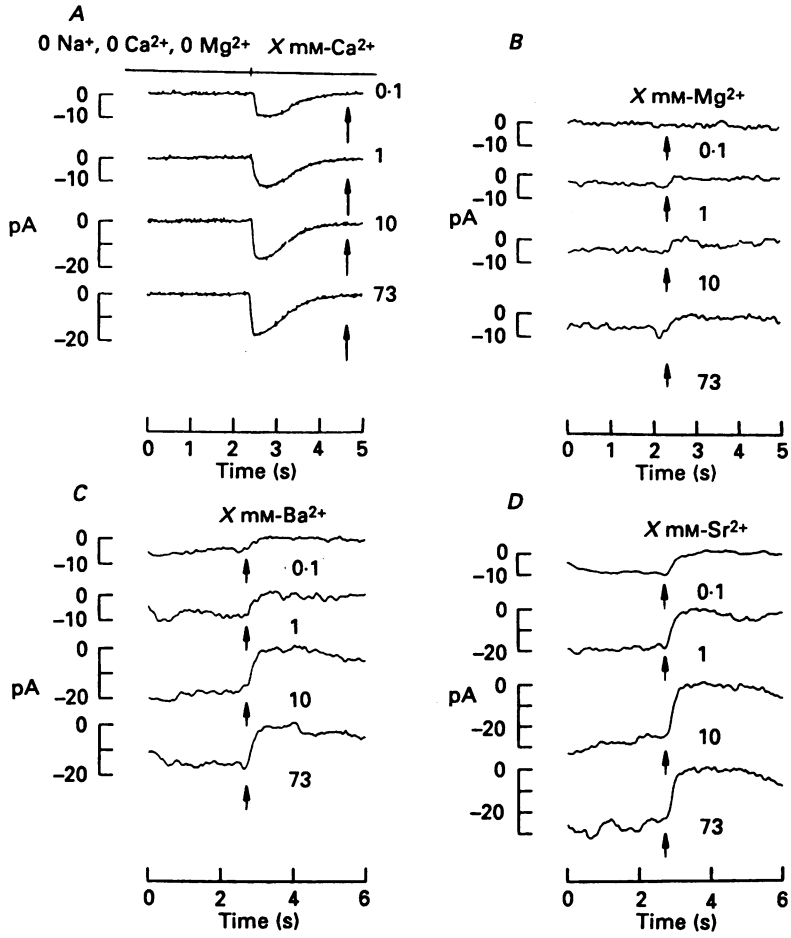

Fig. 6. The concentration dependence of the photocurrent carried by Ca^{2+}, Mg^{2+}, Ba^{2+} and Sr^{2+} under normal conditions. In each experiment the rod was exposed for 8·6 s to a solution containing 110 mM-choline chloride, 2·5 mM KCl, 3 mM-HEPES, 100 μM-EDTA buffered to pH 7·5 with TMAOH. Top trace in panels *B*, *C* and *D* shows timing of solution change. A bright flash equivalent to 8700 Rh* was delivered at the time marked by the arrow. *A*, the activation of the photocurrent carried by Ca^{2+}. *B*, the photocurrent carried by Mg^{2+} with $[Ca^{2+}]_o$ buffered to 10^{-7} M. Photocurrent carried by Ba^{2+} (*C*) and by Sr^{2+} (*D*) in the absence of added Ca^{2+} and Mg^{2+}. Each trace is the average of at least two individual trials.

by 10 mM is almost identical to the current observed in the presence of 73·3 mM of the permeating divalent cation.

When the same experiments illustrated in Fig. 6 were repeated with a priming solution also containing 0·5 mM-IBMX the recordings shown in Fig. 7 were obtained.

Larger photocurrents can now be observed, for a bright flash of light delivered at the time indicated by the arrow, which caused a detectable suppression of the photocurrent. The maximal photocurrent carried by Ca^{2+} is now 55 pA and half-saturation occurs at around 1 mM $[Ca^{2+}]_o$; that is, approximately 10 times higher

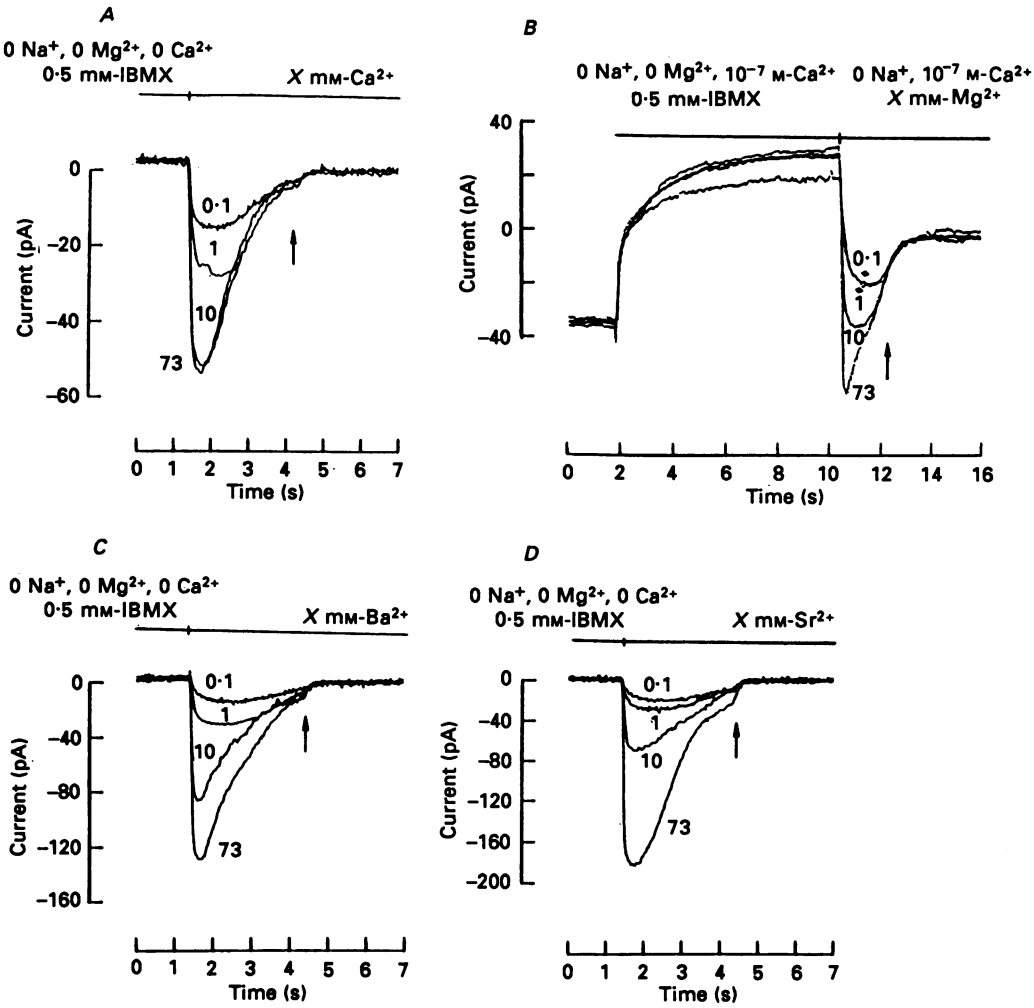

Fig. 7. The concentration dependence of the photocurrent carried by Ca^{2+}, Mg^{2+}, Ba^{2+} and Sr^{2+} after a pre-treatment with IBMX. In each experiment the rod was exposed for 8·6 s to a priming solution containing 110 mM-choline chloride, 2·5 mM-KCl, 3 mM-HEPES, 100 μM-EDTA buffered to pH 7·5 with TMAOH and 0·5 mM-IBMX. In the priming solution in *B* $[Ca^{2+}]_o$ was buffered to 10^{-7} M. Top trace in each panel shows timing of solution change. A bright flash equivalent to 8700 Rh* was delivered at the time marked by the arrow. *A*, the activation of Ca^{2+} photocurrent. *B*, the Mg^{2+} photocurrent with $[Ca^{2+}]_o$ buffered to 10^{-7} mM. Photocurrents carried by Ba^{2+} (*C*) and Sr^{2+} (*D*) in the absence of added Ca^{2+} and Mg^{2+}. Each trace is the average of at least two individual trials. Recordings in each panel (*A*, *B*, *C* and *D*) of Figs 6 and 7 were obtained from the same rod.

than in the experiment shown in Fig. 6. Because of the high affinity of Ca^{2+} for the light-sensitive channel the movement of Mg^{2+} is best seen when $[Ca^{2+}]_o$ is buffered to 10^{-7} M. As shown in Fig. 7, when the rod is primed with a solution containing 0·5 mM-IBMX, $[Ca^{2+}]_o$ buffered to 10^{-7} M and no added Na^+ or Mg^{2+}, the photocurrent was promptly abolished and was then reversed in polarity (Capovilla *et al.* 1983*a*;

Hodgkin *et al.* 1984). Upon removal of IBMX and addition of Mg^{2+} to the bathing medium the photocurrent flowed in its usual direction. Under these conditions it was possible to observe photocurrents carried by Mg^{2+} as large as 60 pA. The amplitude of the photocurrent recorded with 10 mM $[Mg^{2+}]_o$ was consistently smaller than the photocurrent recorded with 73·3 mM $[Mg^{2+}]_o$, suggesting that the half-activation of

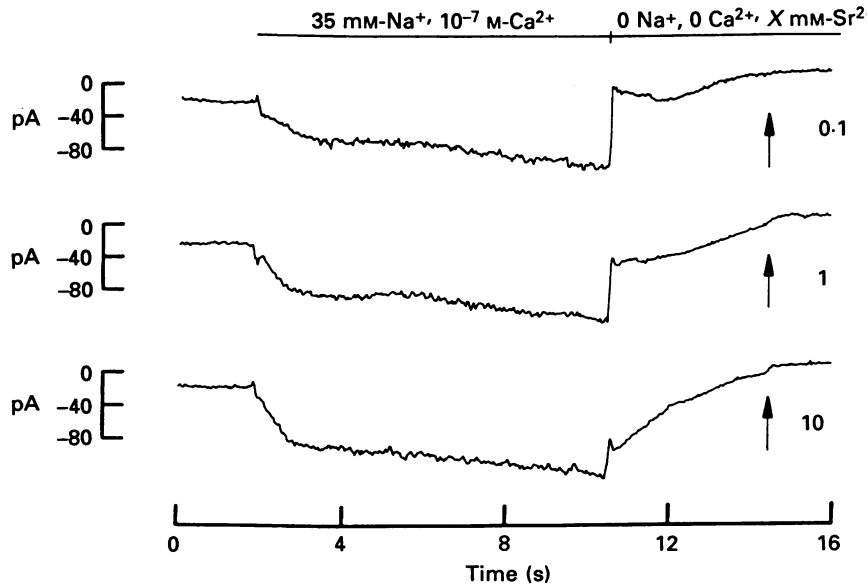

Fig. 8. The effect of pre-treatment with reduced $[Na^+]_o$ and 10^{-7} M $[Ca^{2+}]_o$ on the concentration dependence of the photocurrent carried by Sr^{2+}. In each trial the rod was exposed for 8 s to a priming solution containing no added Ca^{2+} or Mg^{2+} and in which 75 mM-NaCl was substituted with 75 mM-choline chloride. The test solution contained 0·1, 1 or 10 mM-$SrCl_2$, 110 mM-choline chloride, no added Mg^{2+} and $[Ca^{2+}]_o$ buffered to 10^{-7} M. In the presence of 10 mM-$SrCl_2$, 10 mM-choline chloride was removed from the test solution. Top trace shows timing of solution change. A bright flash equivalent to 8700 Rh* was delivered at the time marked by the arrow. Each trace is the average of two individual trials.

these currents occurred at a Mg^{2+} concentration higher than 10 mM. In this rod and in another two cells the photocurrent observed in the presence of 0·1 and 1 mM-$[Mg^{2+}]_o$ had similar amplitude. This unexpected behaviour, which has also been observed when the permeating ion was Ca^{2+}, Ba^{2+} or Sr^{2+} shows that after priming the rod with IBMX the relation between the amplitude of the photocurrent and the extracellular concentration of the permeating ion cannot be described by a simple Michaelis–Menten relation as in normal conditions (Menini *et al.* 1988). The maximal photocurrent carried by Ba^{2+} or Sr^{2+} is larger than 100 pA and the photocurrent carried by 73·3 mM is larger than the photocurrent carried by 10 mM. In the case of Sr^{2+} the half-activation occurs at a concentration higher than 10 mM, while with Ba^{2+} it probably occurs around 10 mM.

The dependence of the photocurrent carried by divalent cations on the ionic concentration after a pre-treatment with 35 mM $[Na^+]_o$ and 10^{-7} mM $[Ca^{2+}]_o$

It is interesting to check whether the increase of the half-activation constant seen after priming with IBMX can also be observed after pre-treatment with 35 mM $[Na^+]_o$ and 10^{-7} mM $[Ca^{2+}]_o$. Figure 8 illustrates an experiment, similar to the one shown in Fig. 4, in which different concentrations of Sr^{2+} were added to the bathing medium after priming in reduced Na^+ and low Ca^{2+}. In this experiment the amplitude of the photocurrent carried by Sr^{2+} increased, upon increasing $[Sr^{2+}]_o$ from 0·1 to 10 mM, indicating a half-activation constant not lower than 5 mM, significantly higher than that observed in the experiment shown in Fig. 6. A similar increase of the half-activation constant was also observed with Ca^{2+}, Ba^{2+} and Mg^{2+}. Experiments with Mn^{2+} are shown in Fig. 4.

The current activated by divalent cations after priming with reduced Na^+ and low Ca^{2+} was usually lower than that observed after priming with IBMX. The results obtained by the two priming procedures were otherwise similar, suggesting a common action mediated by an increase of intracellular cyclic GMP.

DISCUSSION

The experiments described in this paper show that conditions which elevate the concentration of cyclic GMP into the rod cause large increases in the light-sensitive current and modify its ionic selectivity for divalent cations. These observations suggest that an alteration of the internal levels of cyclic GMP modifies the conductive properties of the light-sensitive channel. These results suggest the existence of a novel mechanism of ionic permeation whereby the substance responsible for gating the channel also modulates its conductance.

The effect of IBMX and of low external Ca^{2+}

A crucial assumption for the interpretation of the present results is that both IBMX and low external Ca^{2+} act effectively in rods by increasing the cytosolic concentration of cyclic GMP. Although both IBMX and low Ca^{2+} are likely to exert multiple effects on rods, a well-documented consequence of applying these two conditions is an increased level of cyclic GMP. The results of Capovilla *et al.* (1983*b*) show that concentrations of IBMX and Ca^{2+} similar to those used in the present study induce a marked increase in cyclic GMP in rods of both frog and toad. Moreover, the effects of IBMX on the electrical properties of rods are shared by a variety of other PDE inhibitors (Capovilla *et al.* 1983*c*), thus suggesting that the main action of IBMX is to increase the internal level of cyclic GMP. Finally, it has been shown that direct incorporation of exogenous cyclic GMP in isolated rods reproduces many of the effects of IBMX (Matthews, Torre & Lamb, 1985). It seems therefore reasonable to assume that priming a rod in IBMX or low Ca^{2+} leads to increased levels of cyclic GMP. However, we do not have any quantitative estimates of the size of the cyclic GMP increases from its resting level of about 1 μM (see Stryer, 1986).

The results of the experiments of Figs 1 and 2 show that the amplitude of

photocurrent carried by Ba^{2+}, Sr^{2+}, Mg^{2+} and Mn^{2+} in the presence of IBMX does not increase when Ca^{2+} is removed from the bathing solution. This result is somewhat unexpected, because an entry of Ca^{2+} into the rod cytoplasm should inhibit the activity of the cyclase (Lolley & Racz, 1982; Pepe *et al.* 1986; Koch & Stryer, 1988), thereby decreasing the level of intracellular cyclic GMP. The simplest way to reconcile this observation with the present knowledge of the cyclic GMP metabolism is to assume that the cyclase activity is already greatly inhibited by the usual physiological level of intracellular Ca^{2+}. Consequently, when 0·5 mM-IBMX causes a substantial inhibition of the phosphodiesterase, a large increase of intracellular Ca^{2+} leads only to a small additional inhibition of the cyclase and therefore to an elevation of cyclic GMP, which is almost independent of extracellular Ca^{2+}.

Effect of increased levels of cyclic GMP on the interactions of the divalent cations with the light-sensitive channel

Under normal conditions the relation between the amplitude of the photocurrent, I, and the external concentration of the permeant cation $[X^{2+}]_o$ can be described by the equation:

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

where I_{max} is the maximal current and $K_{\frac{1}{2}}$ is the external concentration of the ion X^{2+} for which half of the maximal current is obtained. The number of open channels or their average open time determines I_{max}, but not $K_{\frac{1}{2}}$ (see Hille, 1984).

In the absence of any other permeating ion $K_{\frac{1}{2}}$ is close to or equal to the dissociation constant of the complex ion channel. Assuming this model is true, the apparent dissociation constants estimated for the tested cations are 50 μM for Ca^{2+}, 2 mM for Mg^{2+}, 2·7 mM for Sr^{2+} and 3·9 mM for Ba^{2+} (Menini *et al.* 1988). After treatment of the rod with either IBMX or low Ca^{2+} the value of the $K_{\frac{1}{2}}$ for various cations increases more than tenfold. This observation can be explained assuming that elevated cyclic GMP lowers the affinity of the divalent cation for the channel. Therefore one may expect, as a consequence of the reduced affinity, that the blocking action of the divalent cations on the channel will be reduced. A difficulty, however, arises from the additional observation that in IBMX the relation between current and external concentration of the permeating cation is more complex than predicted by eqn (1). From Fig. 7 it is evident, for instance, that the current is little affected by increasing the concentration of the permeant ion from 1 mM. This feature might be accounted for by assuming the existence of multiple affinity sites within the same channel or of two populations of channels with different affinity for the divalent cations. Our data, however, are too qualitative to attempt a further analysis of this point.

The permeation of divalent cations through the light-sensitive channel

The permeation of divalent cations through the light-sensitive channel in normal conditions can be simply described as movements of ions through an energy profile consisting of two barriers and one well (Menini *et al.* 1988). If one chooses to describe the ionic permeation as a sequence of interactions through a channel whose energy

profile includes two barriers and one well, many selectivity properties can be seen as consequences of the height of barriers and the depth of wells. Accordingly, the movement of Mn^{2+}, which in normal conditions does not appreciably permeate the channel, but antagonizes the Ca^{2+} movements, can be explained by assuming that Mn^{2+} remains trapped within the channel in a very deep well. In the presence of high levels of cyclic GMP the depth of the well is not as great and Mn^{2+} can move more freely.

In this model the half-activation constant of the current carried by an ion is mainly determined by the depth of the well inside the channel. The increase of the half-activation constant of the current carried by divalent cations can also be explained by a reduction in the well's depth. The depth of a well in the energy profile for a permeating ion is usually ascribed to electrostatic interactions between the ion and charged groups within the channel. In the absence of a detailed molecular description of the light-sensitive channel, we may simply suggest that as a consequence of the elevation of intracellular cyclic GMP, the channel undergoes a conformational change whereby the electrostatic interactions between divalent cations and negatively charged groups of the channel are reduced.

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