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The blocking effect of *l-cis*-diltiazem on the light-sensitive current of isolated rods of the tiger salamander

G. Rispoli* and A. Menini**

Dipartimento di Fisica, Universitá di Genova, Via Dodecaneso 33, I-16146 Genova Italy

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Abstract. The effect of the organic compound *l-cis*-diltiazem on the light-sensitive current of isolated rods of the tiger salamander was analysed by rapidly changing the extracellular medium using the method of Hodgkin et al. (1985). Addition to the extracellular medium of small amounts of *l-cis*-diltiazem rapidly inhibits the photocurrent. Complete suppression of the current was observed with 1 m*M* *l-cis*-diltiazem. Half blockage of the photocurrent occurred with about 150 μ*M* *l-cis*-diltiazem. The blocking effect of *l-cis*-diltiazem was enhanced by light and by a reduction of extracellular Na. A concentration of *l-cis*-diltiazem of 140 μ*M*, which suppresses one third of the photocurrent, was able to completely suppress the photocurrent carried by Ba. It is suggested that *l-cis*-diltiazem blocks the light-sensitive channel, possibly competing with cyclic guanosine-3′-5′-monophosphate (cGMP) for an internal regulatory site.

Key words: Rods, phototransduction, light-sensitive current, *l-cis*-diltiazem

Introduction

Monovalent and divalent cations permeate through light-sensitive channels in vertebrate rods (Yau et al. 1981; Capovilla et al. 1983; Yau and Nakatani 1984a; Hodgkin et al. 1985; Torre et al. 1987; Owen 1987; Menini et al. 1988), but it is not known whether monovalent and divalent cations permeate through the same channel, through different channels, or through the same channel in different configurations.

In order to test the idea of distinct light-sensitive pathways for different cations it is useful to establish possible selective effects of blockers of the light-sensitive current on the movements of monovalent and of divalent cations. In this paper we analyse the effect of a known blocker of light-sensitive channels, *l-cis*-diltiazem (Koch and Kaupp 1984; Stern et al. 1986; Matesic and Liebman 1987). We will show that a careful analysis of the blocking effect of *l-cis*-diltiazem suggests that monovalent and divalent cations permeate through the same channel.

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 isomerizations, estimated from an assumed collecting area of 20 μm^2 (see Lamb et al. 1986).

Isolated rods

A dark-adapted animal was decapitated and pithed and the eyes were enucleated under dim red light, 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.

* Present address: Department of Physiology and Biophysics, University of Washington, Seattle, WA 98195, USA

** To whom offprint requests should be sent

Recording procedure

The inner segment of an individual rod was drawn into a close-fitting suction pipette, and the light response tested. Experiments were performed by selecting only rods where the saturating response was sufficiently large (≥ 25 pA) and stable. The suction pipette was filled with Ringer solution all the time.

Suction pipettes

The methods for current measurements with suction pipettes were similar to those of Baylor et al. (1979) except that each polished suction pipette was bent at an angle of about 90° roughly 0.5 mm from its tip, using the method of Hodgkin et al. (1985). As before the pipettes were coated with silane 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 (E 255, Clark Electromedical Instruments). Electrical transients during perfusion were minimized by using active grounding of the bath (Hodgkin et al. 1984). The suction pipette signal was stored on a magnetic-tape (TEAC R-80) and digitized on line at 50 Hz sampling rate by an A/D system kindly provided for us by Dr. D. Bertrand. The digital data were subsequently transferred to a computer (IBM AT) for the analysis, which was performed using the program DATAC kindly provided by Dr. C. Bader and Dr. D. Bertrand.

Solutions

The Ringer solution was the same as that used by Baylor et al. (1979) and contained (in m*M*) 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). *L-cis*-diltiazem was a generous gift from Dr. Tanino (Tanabe Seiyaku Co.).

Perfusion system

The perfusion system was based on that described by Hodgkin et al. (1985). We used a perfusing chamber with four cylindrical holes in the back, each of them connected to a pipe, where different solutions flowed. An isolated rod was held in one of the flowing solutions by the recording pipette and could be exposed to a different solution by moving the microscope stage sideways using a stepping motor. One of up to 12 different solutions could be selected to flow down each pipe using the system of taps described by Hodgkin et al. (1984).

Determination of light-sensitive currents

The determination of the light-sensitive current was as described by Hodgkin et al. (1985), the light-sensitive current was obtained as the difference of the current in darkness minus the current recorded with saturating steady light. This last current represents the junction current or the artifact due to changes of solutions with different ionic mobilities. In many instances we did not subtract the artifact from the original trace, because it was very small and it could be used to monitor the exact timing of solution changes.

Results

The effect of l-cis-diltiazem

The organic compound *l-cis*-diltiazem inhibits the cGMP dependent Ca efflux from rod outer segment vesicles (Koch and Kaupp 1984), blocks the cGMP dependent current recorded from excised patches of the rod plasma membrane (Stern et al. 1986) and ionic movements through cGMP dependent channels from retinal rod outer segments (Matesic and Liebman 1987). Because cGMP is now considered as the internal transmitter in phototransduction and is an agonist of light-sensitive channels (Fesenko et al. 1985; Haynes et al. 1986; Zimmerman and Baylor 1986), it is useful to analyse the effect of *l-cis*-diltiazem on the intact cell and to establish whether it blocks the photocurrent more powerfully when it is carried by monovalent or divalent cations.

The effect of an exposure of the outer segment to 140 μ*M* *l-cis*-diltiazem is illustrated in Fig. 1. As shown by the thick trace in A the addition of the compound to the extracellular medium causes a reduction of the photocurrent, which is completely suppressed by a bright flash of light delivered during the exposure to *l-cis*-diltiazem (thin trace). The action of *l-cis*-diltiazem in intact rods is not instantaneous and develops with time as shown in Fig. 1 B, where the junction current, present during the solution change, can be used to monitor the timing of the arrival of the compound in the bathing medium. The delayed action of the compound is probably caused by the time required to cross the plasma membrane or by some other intracellular events.

Figure 2 shows that *l-cis*-diltiazem is able to block the photocurrent completely. Here the addition of 1 m*M* of the compound to the bathing medium caused

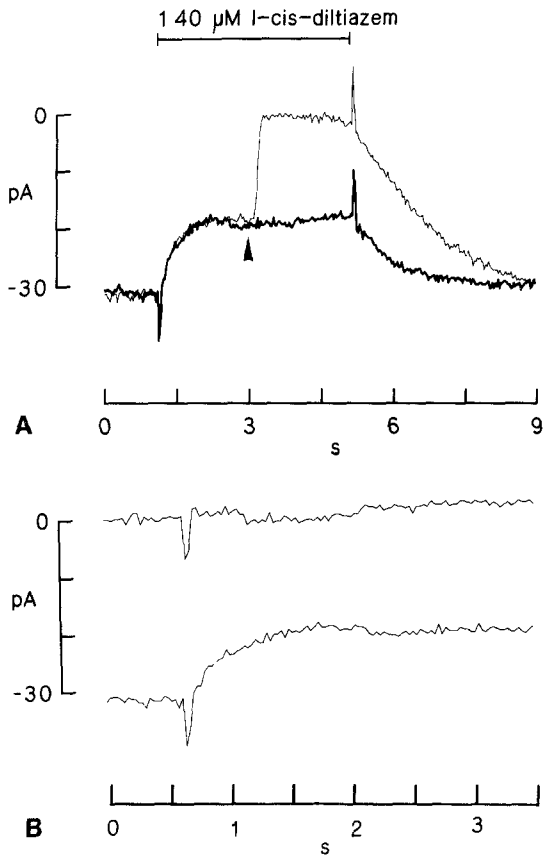

Fig. 1A and B. The effect of 140 μ*M* *l-cis*-diltiazem. **A** *Thick trace* is a recording obtained in darkness; thin trace is a recording while delivering a bright flash equivalent to 7,800 Rh at the time indicated by the *arrow*. The artifact due to changes in junction current was not subtracted, because it consisted of only two brief spikes. **B** Comparison of the time course of the effect of *l-cis*-diltiazem and the timing of solution change, as monitored by the junction current artifact

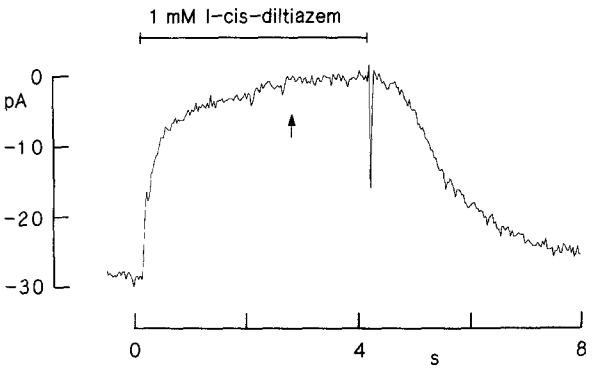

Fig. 2. The complete blockage of the photocurrent by the addition of 1 m*M* *l-cis*-diltiazem in the bathing medium. *Top trace* indicates the timing of solution changes. A bright flash equivalent to 7,800 Rh was delivered at the time marked by the *arrow*

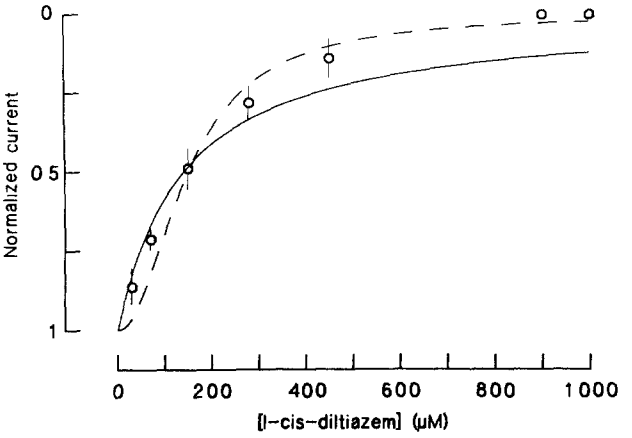

Fig. 3. Stoichiometry of the blocking effect of *l-cis*-diltiazem. Fraction of suppressed photocurrent against concentration of *l-cis*-diltiazem. Collected data from 28 cells. *Continuous curve* was obtained with Eq (1) with $K_D = 140\,\mu M$, *broken line* was obtained with Eq (2) with $K_D = 150\,\mu M$

a quick suppression of the photocurrent. A bright flash, delivered at the time marked by the arrow, did not evoke any photoresponse indicating that the photocurrent was already completely suppressed. As the compound was removed from the bathing medium, the effect of *l-cis*-diltiazem was quickly reversible.

Figure 3 shows data collected from 28 cells. Here the fraction of suppressed photocurrent is plotted against the concentration of *l-cis*-diltiazem in the extracellular medium. Half blockage of the photocurrent was obtained using approximately 140 μ*M* of the compound. The continuous curve through the experimental points was obtained with the equation

$$\frac{I_{max} - I}{I_{max}} = \frac{[l\text{-}cis\text{-dilt}]}{K_D + [l\text{-}cis\text{-dilt}]}, \tag{1}$$

where I is the circulating current in the presence of the compound, I_{max} is the circulating current in the absence of the compound and K_D has the value of 140 μ*M*.

The broken line was obtained using the equation

$$\frac{I_{max} - I}{I_{max}} = \frac{[l\text{-}cis\text{-dilt}]^2}{K_D^2 + [l\text{-}cis\text{-dilt}]^2}, \tag{2}$$

with K_D equal to 150 μ*M*. Equation (1) implies a one to one binding of *l-cis*-diltiazem to the channel, while equation (2) can be derived by assuming that two molecules of *l-cis*-diltiazem are necessary in order to block a channel.

As is evident from Fig. 3 the fit of the experimental points with a Michaelis relation is not very good, suggesting one of two possibilities: either two molecules of the compound are necessary to block a channel or another mechanism may be operating in addition to the direct blockage of light-sensitive channels. A possible additional mechanism is inhibition of Na/Ca exchange.

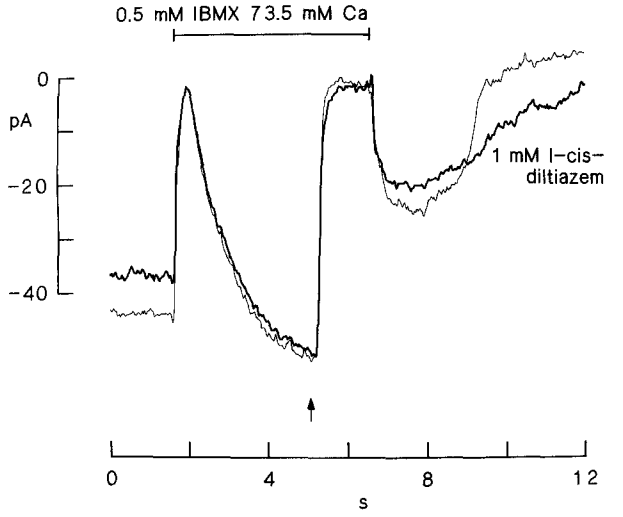

Fig. 4. The effect of 1 m*M* *l-cis*-diltiazem on Na/Ca exchange. The rod was first exposed to a test solution where NaCl was replaced by an equiosmolar amount of $CaCl_2$ in the presence of 0.5 m*M* IBMX. The large light-insensitive current recorded on restoring Na to the bathing medium (*thin trace*) reflects the activity of the Na/Ca exchange. When Na is restored in the bathing medium in the presence of 1 m*M* *l-cis*-diltiazem (*thick trace*) the amplitude of the light-insensitive current is slightly reduced and its duration is prolonged. A bright flash equivalent to 7,800 Rh was delivered at the time marked by the *arrow*

The effect of l-cis-diltiazem on the Na/Ca exchange

In excised patches half blockage of the cGMP activated current occurs at the much lower concentration of *l-cis*-diltiazem of around 10 μ*M* (Stern et al. 1986). It is conceivable that the compound has some additional effects when used at higher concentrations, such as an inhibition of Na/Ca exchange (Yau and Nakatani 1984b; Hodgkin et al. 1987), which is likely to cause a suppression of the photocurrent (Hodgkin et al. 1985; Menini et al. 1988). We have analysed the effect of *l-cis*-diltiazem on Na/Ca exchange using the experimental protocol illustrated in Fig. 4.

We first loaded the cell interior with Ca, by exposing the rod to a solution in which NaCl was substituted by equiosmolar amounts of $CaCl_2$ in the presence of 0.5 m*M* 3-isobutyl-1-methylxanthine (IBMX). This treatment causes a large influx of Ca into the cell, where it remains trapped because it cannot be extruded by Na/Ca exchange. When Na is restored in the bathing medium a large light-insensitive current can be observed which reflects Ca extrusion mediated by the electrogenic activity of Na/Ca exchange (Yau and Nakatani 1984b).

The effect of *l-cis*-diltiazem on the activity of Na/Ca exchange was analysed by loading the rod with Ca and restoring the usual Na in the presence of different amounts of *l-cis*-diltiazem. In the presence of 70 μ*M* *l-cis*-diltiazem no change in the amplitude and time course of the light-insensitive current associated with Na/Ca exchange was observed. Increasing the concentration of the compound up to 280 μ*M* caused an inhibition of about 30% of the activity of the exchange. Figure 4 illustrates the effect of 1 m*M* *l-cis*-diltiazem on Na/Ca exchange. This concentration of the compound, which is able to completely suppress the photocurrent, slightly inhibits but does not suppress the current produced by the electrogenic activity of the exchanger. Since *l-cis*-diltiazem takes some time to enter the cell it is not fair to conclude from this experiment that there is little effect on Na/Ca exchange activity, since the inhibition might take some time to develop. To check this possibility we applied *l-cis*-diltiazem soon after the flash of light, but we delayed the return to normal Na by 4 s. Using this experimental protocol we obtain results similar to those previously described. We conclude, therefore, that *l-cis*-diltiazem at high concentrations can partially inhibit Na/Ca exchange, but its effect on the photocurrent is not primarely mediated by an action on the exchanger.

The effect of l-cis-diltiazem during steady illumination and in the presence of low [Na]

In an attempt to gain a better understanding of the mechanism by which *l-cis*-diltiazem blocks the light-sensitive channel we analysed its effect during steady illumination and in the presence of reduced extracellular Na.

Figure 5A illustrates the blocking effect of 140 μ*M* *l-cis*-diltiazem in the dark and under conditions of steady illumination. In Fig. 5B, responses to *l-cis*-diltiazem were scaled to the same circulating current, for a better visualization of its blocking effect. It is evident that the same concentration of the compound blocks a larger fraction of the circulating photocurrent in the light: in the experiment shown in Fig. 5 the blocking potency of *l-cis*-diltiazem increased by about 7 times with constant illumination halving the photocurrent. In four other cells we observed similar but smaller effects.

Figure 6 illustrates the effect of simultaneously halving the extracellular Na and adding 70 μ*M* *l-cis*-diltiazem to the extracellular medium. In a normal bathing medium that concentration of the compound blocked about 1/8 of the photocurrent (Fig. 6A). When extracellular Na was halved, the same concentration of the compound blocked slightly less than 50% of the circulating current. We conclude therefore that the blocking potency of *l-cis*-diltiazem is enhanced by light and by reducing extracellular Na.

An explanation can be envisaged by considering that light directly reduces the concentration of cGMP (see Stryer 1986 for a review) and that removal of extracellular Na is also likely to cause a fall in cGMP

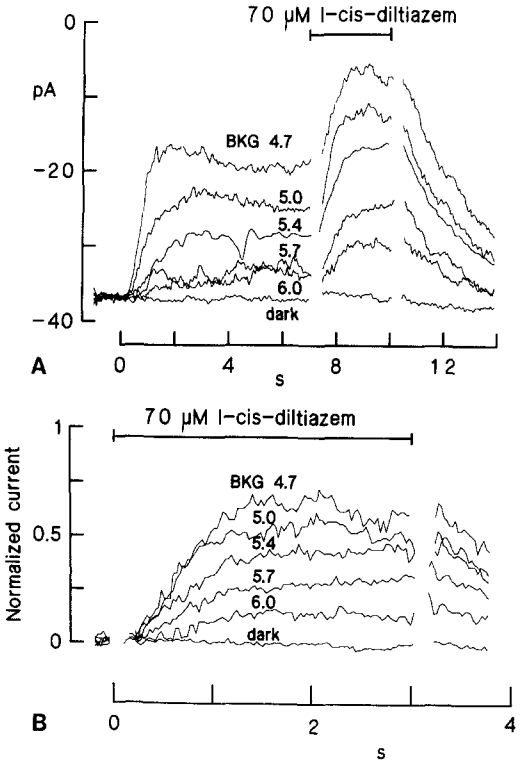

Fig. 5 A and B. The effect of constant illumination on the blocking effect of 70 μ*M l-cis*-diltiazem. **A** Recordings obtained in darkness and in the presence of constant illumination equivalent to 98 Rh/s (6.0), 196 Rh/s (5.7); 390 Rh/s (5.4); 980 Rh/s (5.0); 1980 Rh/s (4.7). The constant illumination was turned on at the time marked 0 and lasted 10 s. **B** Recordings as in **A** normalized to the circulating current

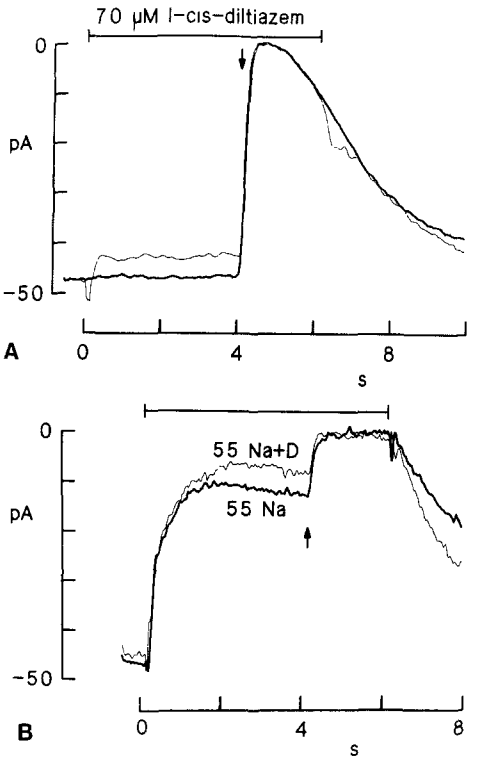

Fig. 6 A and B. The blocking effect of 70 μ*M l-cis*-diltiazem in low Na. **A** Thick trace is a recording obtained in normal conditions, thin trace is a recording obtained in the presence of 70 μ*M l-cis*-diltiazem. **B** *Thick trace* is a recording obtained in a solution where 55 m*M* NaCl was substituted with 55 m*M* choline chloride; *thin trace* is a recording obtained in the same ionic solution by adding 70 μ*M l-cis*-diltiazem. *Top trace* indicates the timing of solution changes. A bright flash equivalent to 7,800 Rh was delivered at the time marked by the *arrow*

concentration, by inhibiting Na/Ca exchange (Robinson et al. 1980; Hodgkin et al. 1985). As a consequence it seems likely that the blocking effect of *l-cis*-diltiazem is enhanced when cGMP is low, suggesting a competition between *l-cis*-diltiazem and cGMP for the same regulating site. Several kinetic schemes can describe this mechanism. For example if two molecules of the internal transmitter cGMP activate the current and one molecule of *l-cis*-diltiazem inhibits it, then the Michaelis constant of inhibition by *l-cis*-diltiazem in the presence of cGMP is

$$K = K_D\left[1 + \frac{[\mathrm{cGMP}]^2}{K_g}\right], \qquad (3)$$

where K_g is the constant describing the second-order cooperative activation by cGMP. This equation predicts the observed increase in apparent inhibitory affinity as cGMP is reduced.

The blocking effect of l-cis-diltiazem on the movement of divalent cations

In this section we examine the effect of *l-cis*-diltiazem on the photocurrent carried by divalent cations.

Figure 7 compares the effect of 140 μ*M* diltiazem on the photocurrent carried by Na and by Ba. In Fig. 7 A we see that when the compound is added to the normal medium it blocks approximately one third of the photocurrent. When Ba replaces Na in the bathing medium the photocurrent, at early times, is nearly unchanged but declines at later times, to a smaller value. At the steady state there is always a noticeable photocurrent carried by Ba (see thin trace of Fig. 7 B). When the same amount of *l-cis*-diltiazem is added to a solution where Na was replaced by Ba, a complete suppression of the photocurrent was observed. The time course of the action of *l-cis*-diltiazem on the photocurrent carried by Na and by Ba is similar (compare Fig. 7 A and B).

The increased efficacy of *l-cis*-diltiazem in blocking the photocurrent carried by Ba can be explained either by a higher affinity of the compound for a population of channels selective for Ba, or by a mechanism similar to that observed with background illumination or with reduced extracellular Na (see Figs. 5 and 6).

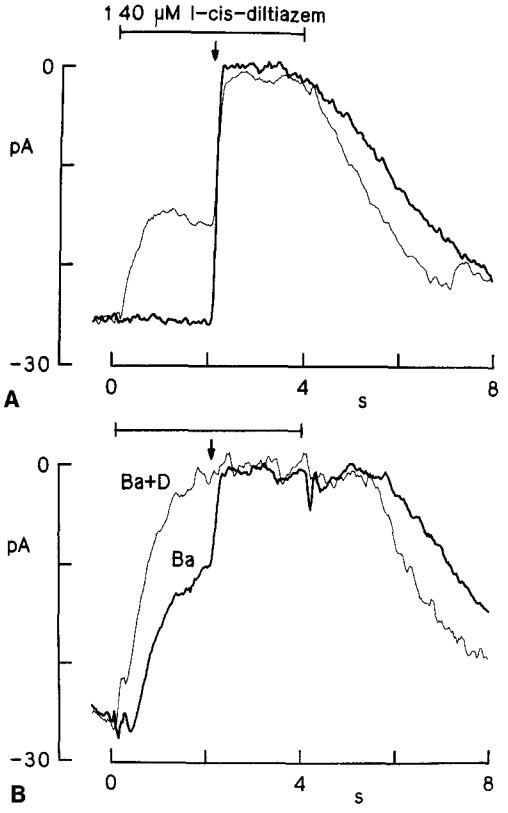

Fig. 7 A and B. The blocking effect of 140 μ*M* *l-cis*-diltiazem on the photocurrent carried by Ba. **A** *Thick trace* is a recording obtained in normal conditions, *thin trace* is a recording obtained in the presence of 140 μ*M* *l-cis*-diltiazem. **B** *Thick trace* is a recording obtained in a solution where 110 NaCl was substituted with 73.5 m*M* $BaCl_2$, *thin trace* is a recording obtained with a solution where 110 m*M* NaCl was substituted with 73.5 m*M* $BaCl_2$ and in the presence of 140 μ*M* *l-cis*-diltiazem. *Top traces* indicate timing of solution changes. A bright flash equivalent to 7,800 Rh was delivered at the time marked by the *arrow*

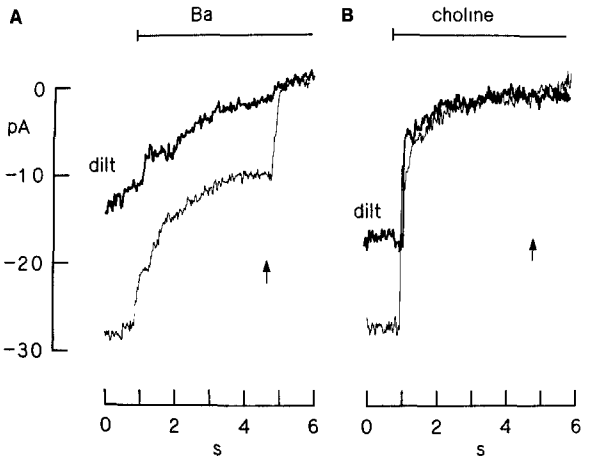

Fig. 8 A and B. The effect of a pretreatment with *l-cis*-diltiazem on the light-sensitive current carried by Ba and Ca. **A** *Thin trace* is a recording obtained by replacing 110 m*M* NaCl with 73.5 m*M* $BaCl_2$, *thick trace* is a recording obtained by a similar solution but with a pretreatment of 8 s with 280 μ*M* *l-cis*-diltiazem. **B** *Thin trace* is a recording obtained by replacing Na with choline, *thick trace* is a recording obtained by a similar solution but with a pretreatment of 8 s with 280 μ*M* *l-cis*-diltiazem. *Top traces* indicate the timing of solution changes. A bright flash equivalent to 7,800 Rh was delivered at the time marked by the *arrow*

If in normal conditions *l-cis*-diltiazem blocks channels selective for Ba more powerfully, when a rod is first exposed to *l-cis*-diltiazem and only at a later time is Ba substituted for Na one would not expect a delay in the suppression of the photocurrent carried by Ba. Figure 8 A shows an experiment where the effect of substituting Na with Ba in a normal medium or in the presence of 280 μ*M* *l-cis*-diltiazem is compared. It is evident that pretreating the rod with *l-cis*-diltiazem does not eliminate the slow phase in the shutting off of the photoresponse. It is interesting to see the effect of *l-cis*-diltiazem on the movement of Ca through light-sensitive channels. When Na is replaced with choline, the photocurrent is not instantaneously abolished (Hodgkin et al. 1985; Menini et al. 1988), but a tail current of about 6–10 pA carried by Ca can be observed for 1 or 2 s, as shown in Fig. 8 B. Figure 8 B shows that pretreatment with 280 μ*M* *l-cis*-diltiazem for 7 s does not abolish the tail current, carried by Ca which is observed on replacing Na with choline. We conclude, therefore, that *l-cis*-diltiazem does not selectively block the photocurrent when carried by monovalent or divalent cations.

Discussion

The blocking action of l-cis-diltiazem

The experiments reproduced in Figs. 1–3 show that *l-cis*-diltiazem completely blocks the photocurrent with an apparent dissociation constant at about 150 μM. Experiments performed in excised patches where the membrane interior was exposed to different concentrations of cGMP and *l-cis*-diltiazem indicated a higher affinity (≈10 μ*M*) of the compound for the cGMP activated channels (Stern et al. 1986). Since the blocking effect of *l-cis*-diltiazem can be increased up to 7 times by constant illumination (see Fig. 5), the discrepancy of the efficacy of the blocking action of *l-cis*-diltiazem in our preparation and in excised patches (Stern et al. 1986) can be simply explained by different intracellular conditions.

Many of our results can be best explained by supposing that cGMP and *l-cis*-diltiazem compete for the same regulatory site. If two cGMP molecules open the channel and one *l-cis*-diltiazem molecule blocks it we have Eq. (3). This explanation is in agreement with our results on the effect of *l-cis*-diltiazem in the presence of constant illumination and low extracellular Na.

The apparent increased blocking effect of *l-cis*-diltiazem on the photocurrent carried by Ba in normal

conditions can be explained in two ways. The first possibility is the existence of two populations of channels, one selective for monovalent and the other selective for divalent cations. If *l-cis*-diltiazem has a higher affinity for channels selective for divalent cations it would explain the result reported in Fig. 7. In this view when a rod is pretreated with *l-cis*-diltiazem for a time sufficient for the completion of its effect, as Ba replaces Na we expect an almost instantaneous blockage of the photocurrent, because Ba selective channels are already closed. The experiments reproduced in Fig. 8 do not support this view. Therefore the apparent increased efficacy of *l-cis*-diltiazem on the photocurrent carried by Ba is most easily explained as mediated by a reduction of intracellular cGMP, caused by the removal of extracellular Na, and the competition of cGMP and the compound for the same site. This mechanism is identical to that used to explain the effect of light.

Do monovalent and divalent cations permeate through the same channel?

In Ca channels, when extracellular Ca is removed, it is possible to observe the movement of alkali cations. At millimolar concentrations of extracellular Ca, these movements can hardly be seen (Almers and McCleskey 1984; Hess and Tsien 1984). In rods, monovalent cations permeate through the light-sensitive channel in the presence of millimolar amounts of extracellular Ca. Here is a basic difference between Ca channels and light-sensitive channels in vertebrate rods.

The question arises as to whether Ca and Na permeate under normal conditions through the same channel in the same conductive state. The experiments on the movement of monovalent and divalent cations (Menini et al. 1988) and the effect of *l-cis*-diltiazem on the photocurrent carried by Na, Ba and Ca in normal conditions do not support the view of different pathways for Na and Ca.

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