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Control of the light-regulated current in rod photoreceptors by cyclic GMP, calcium, and *l-cis*-diltiazem

(light response/intracellular messenger/membrane conductance/excised patch/intracellular dialysis)

JEFFREY H. STERN*, U. BENJAMIN KAUPP†, AND P. R. MACLEISH*

*Laboratory of Neurobiology, The Rockefeller University, 1230 York Avenue, New York, NY 10021; and †Fachbereich Biologie/Chemie, Abteilung Biophysik, Universität Osnabrück, Osnabrück, Federal Republic of Germany

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ABSTRACT **The effect of calcium ions on the cGMP-activated current of outer segment membrane was examined by the excised-patch technique. Changes in the extracellular calcium concentration had marked effects on the cGMP-activated current, while changes in intracellular calcium concentration were ineffective. Changes in calcium concentration in the absence of cGMP had little, if any, effect on membrane conductance. These results suggest that both intracellular cGMP and extracellular calcium can directly affect the conductance underlying the light response in rod cells. The pharmacological agent *l-cis*-diltiazem reversibly inhibited the cGMP-activated current when applied to the intracellular side of an excised patch. When superfused over intact rod cells, *l-cis*-diltiazem reversibly blocked much of the normal light response. The isomer, *d-cis*-diltiazem, did not significantly affect either patches or intact rod cells. Thus, the light-regulated conductance has binding sites for both calcium and cGMP that may interact during the normal light response in rod cells and a site specific for *l-cis*-diltiazem that can be used to identify and further study the conductance mechanism.**

The hyperpolarizing response to light of the rod photoreceptor cell is caused by a decrease in the transmembrane current that enters its outer segment in the dark. This coupling of light to the membrane conductance of photoreceptors is likely to be mediated by intracellular messenger(s) (1). Experiments designed to decide between the two main intracellular messenger candidates, calcium (see ref. 2) and cyclic GMP (see ref. 3), have been complicated by the finding that the activities of these two putative messengers are interrelated (4). This has raised an important question about the phototransduction process: which internal messengers directly affect the membrane conductance that underlies the light response and which serve a regulatory role?

Recent electrophysiological and biochemical experiments indicate that cGMP directly increases the cation permeability of the rod membrane. Fesenko *et al.* (5), using the excised-patch technique (6), described a direct effect of cGMP on the membrane conductance of frog rod outer segments. This conductance had an ion selectivity resembling that of the conductance underlying the response to light of the rod cell, and its activation was relatively independent of the calcium concentration at the intracellular side of the membrane. This result suggested that cGMP is the internal messenger of visual transduction in the rod cell (5). However, while it is clear that cGMP can directly affect the outer segment conductance, the mechanism of cGMP action is unknown. For example, is it sufficient for cGMP to interact with the intracellular side of the membrane to activate the conductance or are coregulators involved? Of particular interest is the role of extracellular calcium ions. Yoshikami and Hagins (2) first described a pronounced increase of the light-regulated current upon lowering the concentration of external calcium. This effect of external calcium on the rod cell led to the hypothesis that light causes an increase in the activity of cytoplasmic calcium, which in turn reduces the light-regulated membrane conductance (2). While an intracellular route of action is indicated by the effect of extracellular calcium on cGMP metabolism (4), there is evidence for both intracellular (7) and extracellular sites of action for calcium (8). The excised patch technique provided an opportunity to examine the sensitivity of the cGMP-activated current to changes in the extracellular calcium concentration to determine its possible role in the suppression of the light-regulated current.

An understanding of the molecular mechanism of the conductance underlying the light response is an obvious goal and would be facilitated by the availability of specific pharmacological agents that interact with the conductance. One candidate is the substance, *l-cis*-diltiazem, which blocked (9) a cGMP-activated permeability in suspensions of rod outer segment membranes (10). The sensitivity to *l-cis*-diltiazem of the cGMP-activated currents in patches and in intact rod cells as well as the effect on the normal light response were investigated.

METHODS

Solitary rod photoreceptors were dissociated from the retina of the tiger salamander *Ambystoma tigrinum* by using the enzyme papain (11, 12). Dissociated cells were maintained at 10°C in an extracellular solution (12) of 108 mM NaCl/1.5 mM KCl/0.5 mM $MgSO_4$/0.5 mM $MgCl_2$/1.0 mM $NaHCO_3$/0.5 mM NaH_2PO_4/1.0 mM Na pyruvate/16 mM glucose/2.0 mM Hepes/1.8 mM $CaCl_2$/0.001 mM phenol red/0.1 mM choline chloride/0.014 mM bovine serum albumin. The pH was adjusted to 7.3 with NaOH.

Experiments were carried out under normal fluorescent room light unless otherwise indicated. Dark-adapted rods were obtained by maintaining an animal in the dark for ≈12 hr and then carrying out subsequent manipulations under infrared illumination.

Patch-clamp pipettes were from Corning type 7740 Pyrex or Drummond 100-μl microcap tubing with a BB-CH pipette puller (Geneve, Switzerland) and were used directly from the puller. The pipettes had a tip inner diameter of ≈1 μm and a resistance of 2–10 MΩ. Seal resistances were typically >5 GΩ. Patches of membrane were excised by moving the pipette from the cell to which it was sealed.

In excised-patch experiments, the pipette contained extracellular solution without bovine serum albumin, while the bathing solution was extracellular solution lacking calcium and bovine serum albumin. The patch membrane was determined to have its intracellular side facing the bath through an examination of the cGMP sensitivity at each side of the membrane, as described further in *Results*. To vary the solutions at the intracellular side of the membrane, excised

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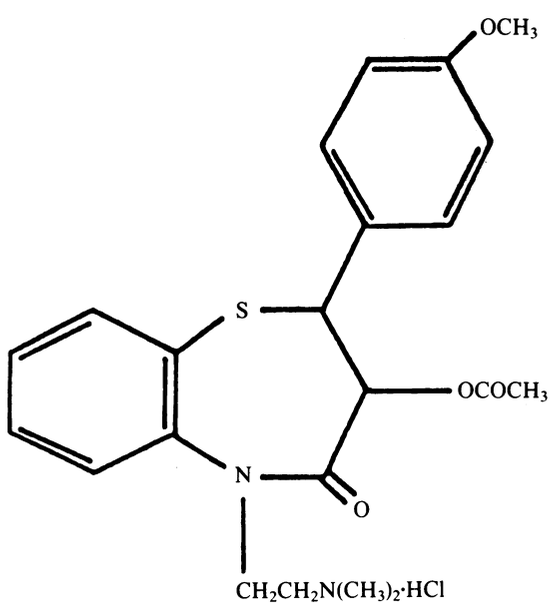
patches were superfused by using a flat array of fused pipettes, each having a tip diameter of ≈100 μm. Solutions flowed from each port independently and a patch could be exposed to a particular solution within a few seconds by moving the patch to the mouth of the port. The bath solution was continuously exchanged at a rate of 1 ml/min. The effects of changing the composition of the extracellular solution were determined by averaging the responses from different patches exposed to the same extracellular solution. For intracellular dialysis, the pipette was filled with an intracellular solution (13) of 90 mM K aspartate/30 mM sucrose/5 mM NaH_2PO_4/3 mM $MgCl_2$/1 mM Na_2ATP/0.05 mM EGTA. The pH of the internal solution was adjusted to 7.5 with KOH.

Current was measured with a virtual ground current-to-voltage transducer. Current flowing from the pipette into the bath was taken as positive. Membrane potentials are given as pipette interior voltage minus bath voltage. Voltage ramps used to obtain current–voltage (*I–V*) curves were delivered at a slew rate of 1 mV/20 msec. Data were filtered by using a low-pass cutoff frequency of 50 Hz.

Cyclic GMP was obtained as the sodium salt from P-L Biochemicals (Milwaukee, WI). β,γ-Imidoadenosine 5′-triphosphate [App(NH)p] as the sodium salt, β,γ-imidoguanosine 5′-triphosphate [Gpp(NH)p] as the lithium salt, and bovine serum albumin were obtained from Sigma. *l-cis*-Diltiazem and *d-cis*-diltiazem were the generous gift of Gödecke (Freiburg, F.R.G.). The structure of *l-cis*-diltiazem is shown below.



RESULTS

Excised Patches. *Cyclic GMP-activated currents.* The current–voltage relationship of an excised patch of outer segment membrane was measured in the presence of different bath concentrations of cGMP. The result of one such experiment is shown in Fig. 1, where the cGMP-induced current—that is, the total current in the presence of cGMP minus the current in the absence of cGMP—is plotted against membrane potential. The cGMP-activated current was approximately described by the relationship $I(V) = I_0(e^{V/k} - 1)$, where I = the cGMP-activated current, I_0 = the limiting current for large positive pipette voltages, V = the voltage across the membrane, and k = a constant having a minimum value near 25 mV. This relationship also approximated the voltage dependence of the light-regulated current measured from whole cells (14).

The amplitude of the cGMP-activated current depended on the concentration of cGMP in the bath, as shown in Fig. 1. Consistent with the findings of Fesenko *et al.* (5), the membrane current was most sensitive to changes in cGMP concentration between 20 and 50 μM. The activation by cGMP was reversible and as rapid as the change in the applied solution (<3 sec). The hydrolysis-resistant cGMP analog, 8-bromo-cGMP, was at least as effective as cGMP in activating the membrane conductance. The activation of membrane conductance by cGMP was little affected by adding either 1 mM ATP, 1 mM App(NH)p (a hydrolysis-resistant ATP analog that inhibits many protein kinases), 1 mM GTP, or 1 mM Gpp(NH)p to the bathing medium. These results provide evidence that the hydrolysis of high-energy phosphate bonds is not necessary for the activation of the membrane by cGMP and extend the results of Fesenko *et al.* (5).

cGMP (100 μM) inside the patch pipette had no apparent effect on membrane conductance. This is taken to indicate that the membrane was oriented with its intracellular side exposed to the bath solution because intracellular, but not extracellular, cGMP activated the membrane conductance in rod cells (unpublished data).

The cGMP-activated conductance was observed in >90% of the patches excised from positions along the length of the outer segment. This result suggests that the conductance mechanism was not localized in large discrete compartments. The magnitude of the current among active patches, however, was variable. Discrete fluctuations in the cGMP-activated current were not observed at a resolution of 0.5 pA at a low-pass cutoff frequency of 1 KHz.

Blockade by calcium. One consistent finding over the years has been an increase in the light-regulated conductance of rod cells upon decreasing the extracellular calcium concentration (2). The study of excised patches provided an opportunity to determine whether the membrane is directly sensitive to calcium. We found that decreasing the calcium concentration at the extracellular face of excised membranes markedly increased the cGMP-activated current, as is shown in Fig. 2. Each trace is the average from five different patches and shows the current activated by 100 μM cGMP with the indicated concentrations of calcium in the pipette. The calcium concentration in the bath was kept constant at 10 μM. When the external calcium concentration was lowered from 1 mM to 10 μM, the cGMP-activated current increased by a factor of ≈5 at −50 mV. The average currents (±SEM) recorded at −40 mV and +40 mV for the three concentrations of extracellular calcium were, respectively, as follows: −17.4 (±2.9) pA and 33.6 (±5.7) pA for 10 mM calcium, −13.0 (±1.4) pA and 32.2 (±3.0) pA for 10 mM calcium, and −54.6 (±3.2) pA and 113 (±9.9) pA for 10 μM calcium. The current–voltage curves obtained in the presence of high and low extracellular calcium had different shapes. The curves obtained in the presence of low extracellular calcium rectified less than those obtained under conditions of high extracellular calcium within the voltage range shown in Fig. 2. There was little change in the current when the external calcium concentration was increased from 1 to 10 mM. One conclusion from these experiments is that a substantial part of the increase in the light-regulated current observed upon decreasing the external calcium concentration in whole cells may involve a direct action of calcium on the external face of the outer segment membrane.

In agreement with published results (5), the cGMP-activated current was relatively insensitive to changes in the calcium concentration at the intracellular side of the membrane in the presence of 1 mM magnesium. In addition, the leak current—that is, the current through the patch membrane in the

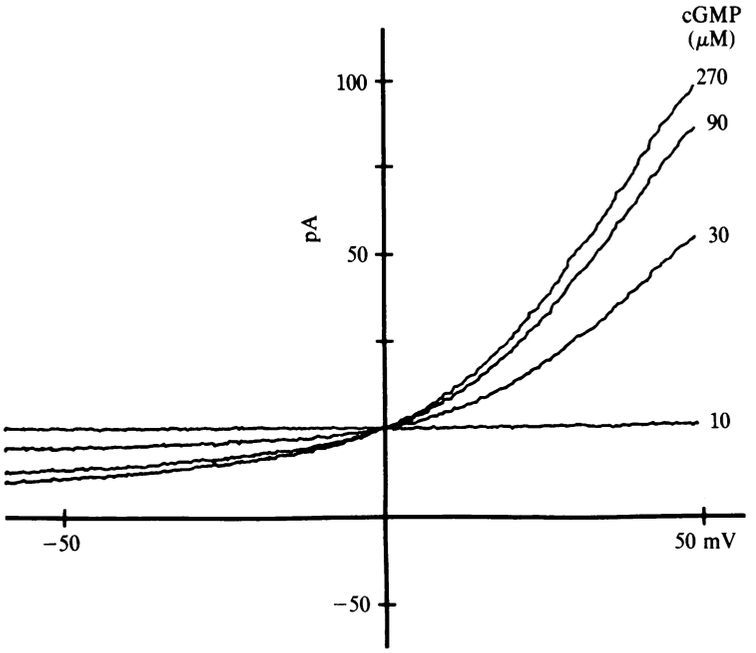


FIG. 1. cGMP-activated currents in an excised patch. Each trace shows the voltage dependence of the current activated by bath application of the cGMP concentrations indicated to the right of each curve. The current recorded in the absence of cGMP has been subtracted from the total current. The conductance recorded in the absence of cGMP was ≈200 pS. Membrane patches were excised from the outer segments of light-adapted rod cells into a medium containing ≈10 μM calcium. The pipette contained the same extracellular solution but with 1 mM calcium. The activated current was most sensitive to changes in cGMP concentration between 20 and 50 μM and showed a voltage dependence that closely resembled that of the light-regulated current measured from intact rod cells. The average of nine current–voltage curves is shown for each concentration of cGMP. The electrode used in this experiment had a resistance of 2 MΩ and an inner diameter slightly greater than 1 μm. The abscissa in this figure is drawn through −25 pA to clearly illustrate the currents recorded in the presence of 10 μM cGMP.

absence of cGMP—was relatively unaffected by changing the calcium concentration at either side of the membrane under these conditions.

Blockade by l-cis-*diltiazem*. The cGMP-activated conductance in excised patches was blocked by application of *l-cis*-diltiazem to the intracellular side of the membrane. Each trace in Fig. 3 shows membrane current as a function of membrane voltage when an excised patch was exposed to the concentrations of cGMP and *l-cis*-diltiazem indicated. The current activated by 90 μM cGMP was greatly inhibited by 10 μM *l-cis*-diltiazem and was little affected by 1 μM *l-cis*-diltiazem. The current through the patch in the presence of 10 μM *l-cis*-diltiazem was less than that in the absence of cGMP. This decrease in patch conductance below the control value was not observed in all patches. The blocking effect of *l-cis*-diltiazem was reversible and as rapid as solution changes (<3 sec). The stereoisomer, *d-cis*-diltiazem, had little effect on the cGMP-activated conductance when concentrations up to 1 mM were applied to the intracellular side of excised patches. The concentration dependence and stereo selectivity of *cis*-diltiazem effects on excised patches resembled those observed in experiments measuring ion fluxes from suspensions of outer segment membrane (9).

In experiments to further characterize the effects of *l-cis*-diltiazem, two results suggestive of interaction with calcium were found. When 40 μM *l-cis*-diltiazem and 1 mM calcium

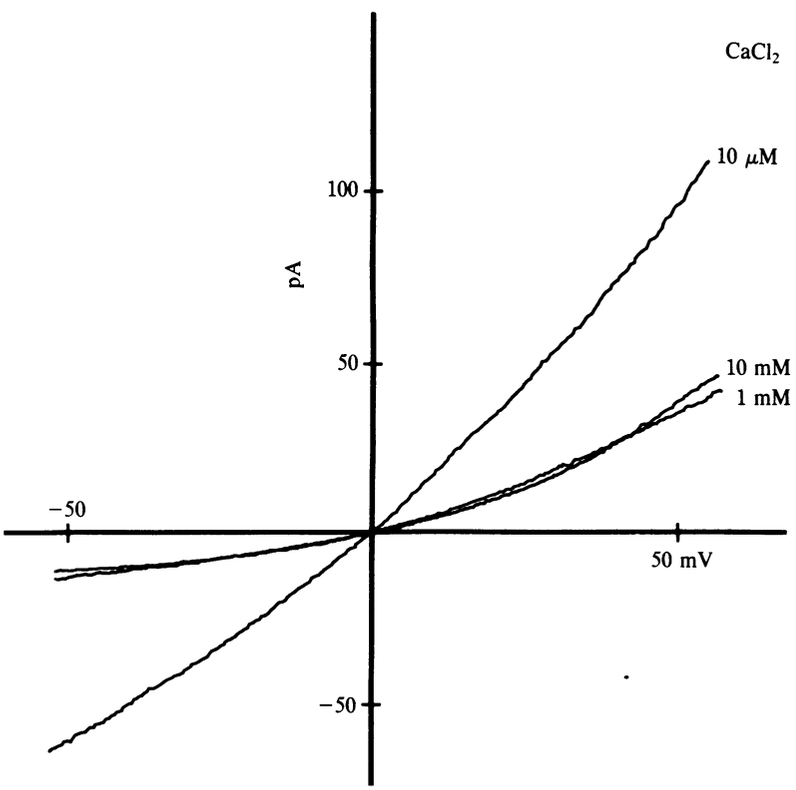


FIG. 2. Extracellular calcium effects on the average cGMP-activated conductance in excised patch membranes. Excised patches of outer segment membrane were exposed to 100 μM cGMP on the bath (intracellular) side of the membrane. The cGMP-activated current—that is, the difference between the current recorded in the presence and absence of cGMP—has been plotted against membrane potential. The calcium concentration inside the pipette (facing the extracellular side of the membrane) was as indicated. Decreasing the extracellular calcium concentration from 1 mM to 10 μM increased the cGMP-activated current severalfold, whereas increasing extracellular calcium from 1 mM to 10 mM had little effect. The average results obtained from five different patches are shown for each calcium concentration. The standard error for each average was ≈10% of the mean value. The calcium concentration in the bath solution (facing the intracellular side of the membrane) was 10 μM.

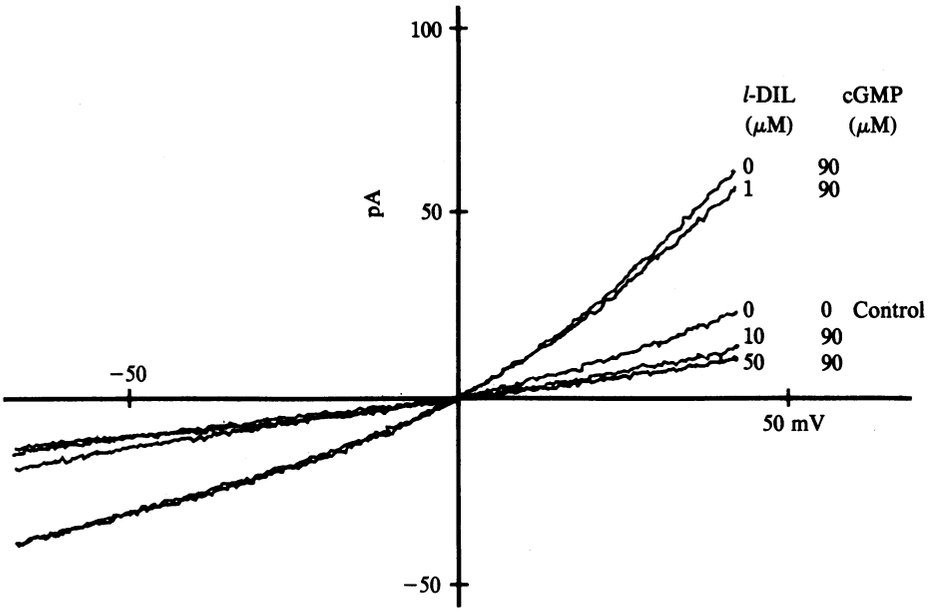


FIG. 3. The effect of *l-cis*-diltiazem on the cGMP-activated current in an excised patch. The total current through an excised patch of outer segment membrane is plotted against the membrane potential. Patches were excised into a bath solution containing 10 μM calcium in the absence of cGMP and the I–V curve (control) was recorded. The patch was then superfused with four different solutions each containing 90 μM cGMP, and *l-cis*-diltiazem (*l*-Dil) at the concentration indicated. At *l-cis*-diltiazem concentrations >10 μM, the cGMP-activated current and part of the current recorded in the absence of cGMP were blocked. The average of results obtained from three ramps is represented by each curve.

were applied to the extracellular side of the patch membrane, the current activated by 100 μM cGMP was slightly larger than that observed in the presence of 1 mM external calcium. An increase in current would be expected if *l-cis*-diltiazem had a competitive action with calcium without itself blocking the conductance. In another series of experiments, the *l-cis*-diltiazem block of the cGMP-activated current was slightly reduced by lowering the intracellular divalent cation concentration. These effects were subtle compared to the blocking action of *l-cis*-diltiazem applied to the intracellular side of excised patches.

Intact Rod Cells. Given the effects of *l-cis*-diltiazem on excised patches, it seemed obvious to investigate the effect of this pharmacological agent on the light response and on the cGMP-activated current in rod cells.

Extracellular effects of l-cis-*diltiazem.* The response of

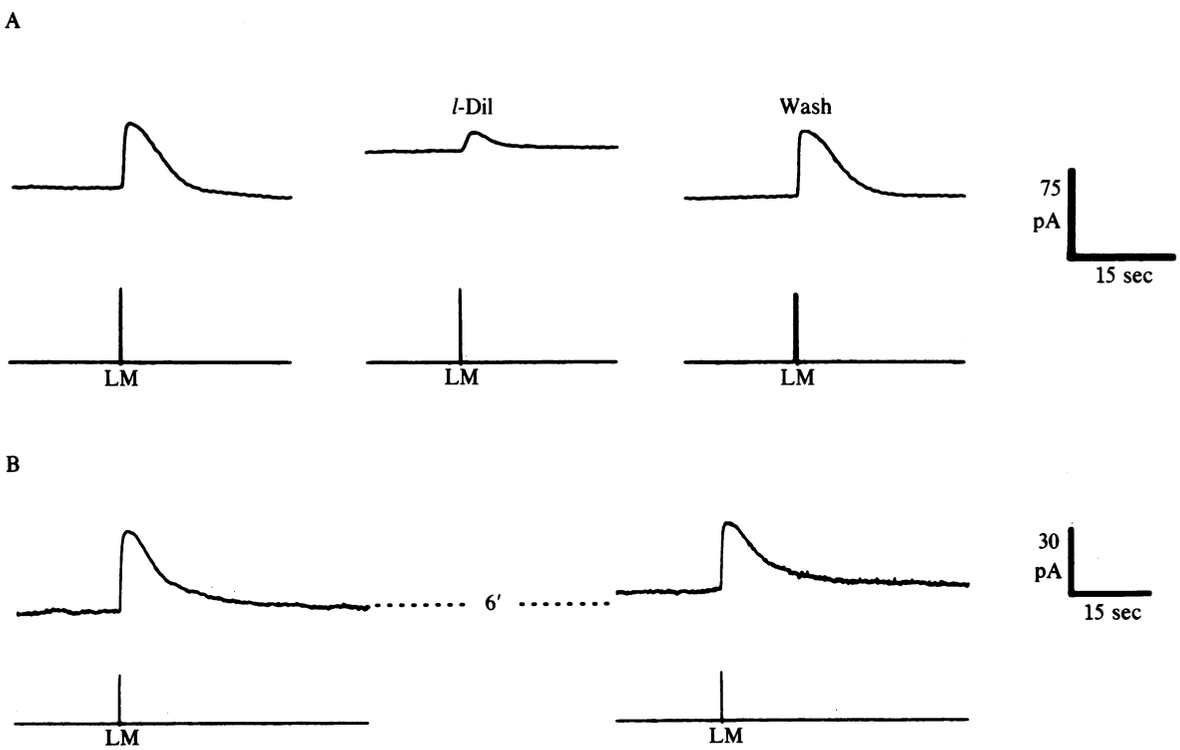


FIG. 4. Effect of *l-cis*-diltiazem on the light response of dark-adapted rod cells. The response of dark-adapted rods to brief light flashes was recorded by using intracellular dialysis electrodes. The intensity of the light flash was adjusted to induce maximal or saturating responses. Light flashes occurred during the times indicated by the upward deflections in the light monitor record (LM). (*A*) Responses recorded before (*Left*), during (*Center*), and after (*Right*) bath application of 20 μM *l-cis*-diltiazem (*l*-Dil). The effect of *l-cis*-diltiazem was to reduce the light response amplitude and the baseline current recorded in the dark by equal amounts. The time between the records was ≈2 min, which was also the time required to change the bathing solution. The extracellular solutions contained 0.1 mM calcium. (*B*) Responses recorded immediately after and 6 min after beginning intracellular dialysis with an internal solution containing 2.3 mM *l-cis*-diltiazem. There was little change in the photoresponse during intracellular dialysis with *l-cis*-diltiazem.

dark-adapted rods to light was partially blocked by extracellular application of *l-cis*-diltiazem. Fig. 4 shows responses to light flashes recorded under voltage-clamp conditions before and after bath application of 20 μM *l-cis*-diltiazem in an extracellular solution that contained 100 μM $CaCl_2$. The suppression of the light response by *l-cis*-diltiazem occurred with the time course of the solution change (2 min) and reversed as rapidly. The baseline current recorded in the dark decreased and recovered with a similar time course. The decrease in baseline current caused by *l-cis*-diltiazem was approximately equal to the accompanying decrease in the light response.

Intracellular application of l-cis-*diltiazem.* The light response of the dark-adapted rod cell was not blocked by intracellular dialysis with an internal solution containing *l-cis*-diltiazem. This result was unexpected given the results obtained from the excised-patch experiments. Fig. 4*B* shows the light response, recorded under voltage-clamp conditions, of a dark-adapted rod at the beginning and after 6 min of dialysis with an internal solution containing 2.3 mM *l-cis*-diltiazem. The small decrease in the baseline current and in the amplitude of the light response is attributed to the typical rundown observed under internal dialysis recording conditions. The rate of exchange between the soluble cytoplasm and internal solution during dialysis was estimated to be faster than 90% exchange per 10 sec by observing the current induced by including 60 μM 8-Br-cGMP in the internal solution both in the presence and in the absence of *l-cis*-diltiazem. There was little effect of *l-cis*-diltiazem on either the light response or on the cGMP-activated current many minutes after the effect of dialysis with 8-Br-cGMP had reached a steady level. Several possible interpretations of this negative result will be discussed.

DISCUSSION

The finding that extracellular calcium can reduce the cGMP-activated current suggests that external calcium may have an important role in controlling the light-regulated current. One possible mechanism for activation of the light-regulated conductance is allosteric interaction whereby intracellular cGMP relieves the effect of extracellular calcium. In this way, both cGMP and calcium can directly control the conductance by interacting on the membrane. This is one alternative to the hypothesis that cGMP acts alone to control the conductance. In addition to the direct effect of calcium on membrane conductance, there is good evidence that extracellular calcium can indirectly regulate the conductance through regulation of intracellular cGMP metabolism (4). Thus, calcium can have at least two effects on the light-regulated conductance, one direct and the other indirect through regulation of intracellular cGMP.

The block by *l-cis*-diltiazem of the cGMP-activated currents in excised patches occurred in the same concentration range as that in experiments measuring ion fluxes in membrane suspensions (9). The block was stereoselective and specific for the cGMP-activated conductance. In this regard, *l-cis*-diltiazem is a useful diagnostic reagent for identifying this particular conductance mechanism. For example, the similarities of the block by *cis*-diltiazem in excised patches and membrane suspensions, combined with other evidence (5, 9), strongly suggest that the cGMP-activated membrane permeabilities in the two preparations are closely related. We have not been able to reconcile fully the *l-cis*-diltiazem effects on patches with those on whole cells. The dramatic effects, observed by applying *l-cis*-diltiazem to the intracellular side of the membrane, strongly suggest that the conductance mechanism has a binding site for *l-cis*-diltiazem, which when occupied reduces the activation by cGMP. These findings suggested that the light response of the rod cell would be blocked when *l-cis*-diltiazem was dialyzed into the rod; however, this did not occur. It is possible that *l-cis*-diltiazem passed through the rod membrane or was metabolized by intracellular enzymes rapidly enough to prevent an increase in the intracellular concentration. Alternatively, the intracellular concentration of *l-cis*-diltiazem increased but the cGMP-activated conductance in the intact cell was different or less accessible than the one present in excised patches. Similar reasons might explain why *l-cis*-diltiazem partially blocked the light response of rods when added to the bathing medium if one assumes that, as in excised patches, *l-cis*-diltiazem acts on the intracellular side of the membrane.

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