Block of the Cyclic GMP–gated Channel of Vertebrate Rod and Cone Photoreceptors by *l-cis*-Diltiazem

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ABSTRACT Inside-out patches were excised from catfish rod or cone outer segments. Single channel and macroscopic currents were recorded from GMP-gated channels activated by 1 mM cGMP in low divalent buffered saline. Currents were blocked by the application of micromolar concentrations of *l-cis*-diltiazem to the cytoplasmic side of the patch. The concentration dependence of block indicated that a single molecule was sufficient to block a channel and that all channels were susceptible to block. The dissociation constant for the rod channel was an order of magnitude smaller than for the cone channel, but the voltage dependence of block was nearly identical. The macroscopic current–voltage relation in the presence of blocker was inwardly rectifying and superficially resembled voltage-dependent block by an impermeant blocker occluding the ion-conducting pore of the channel. Block by diltiazem acting from the extracellular side of the channel was investigated by including 5 μM diltiazem in the recording pipette solution. The macroscopic current–voltage relation again showed inward rectification, inconsistent with the idea that diltiazem acts by occluding the pore at the external side. The kinetics of block by diltiazem applied to the intra- and extracellular side were measured in cone patches containing only a single channel. The unbinding rates were similar in both cases, suggesting a single binding site. Differences in the binding rate were consistent with greater accessibility to the binding site from the cytoplasmic side. Block from the cytoplasmic side was independent of pH, suggesting that the state of ionization of diltiazem was not related to its ability to block the channel in a voltage-dependent fashion. These observations are inconsistent with a pore-occluding blocker, but could be explained if the hydrophobic portion of diltiazem partitioned into the hydrophobic core of the channel protein, perhaps altering the gating of the channel.

INTRODUCTION

The cGMP-gated channels of rod and cone outer segments are nonspecific cation channels that close in response to light (for review, see Yau and Baylor, 1989). Under physiological conditions, the current–voltage relation of the rod channel shows

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strong outward rectification, while that of the cone channel shows "inward and outward" rectification, i.e., an exponential increase in current at both positive and negative potentials. Since rectification in both channels is the result of a voltage-dependent block by permeant divalent cations (Haynes, Kay, and Yau, 1986; Yau and Haynes, 1986; Zimmerman and Baylor, 1986; Haynes and Yau, 1990a, b), there must be a difference between the rod and cone channels in the ion-conducting pathway. The use of channel blockers is one way of probing the structure of the ion permeation pathway of a channel. l-cis-Diltiazem, a benzothiazapine, is one of the few pharmacological agents available that blocks the cGMP-gated channels of rod and cone photoreceptor outer segments. Understanding the mechanism of block by diltiazem may, therefore, shed light upon the internal structure of the pore and upon the differences in the permeation pathway between the rod and cone channels.

The experiments described here will show that the cGMP-gated channels in excised patches from either rod or cone photoreceptors are qualitatively similar in that each undergoes voltage-dependent block by diltiazem, each constitutes a single pharmacological type that can be completely blocked by diltiazem, unlike the results previously reported for higher vertebrates (Koch, Cook, and Kaupp, 1987; Kaupp, Niidome, Tanabe, Terada, Bönigk, Stühmer, Cook, Kangawa, Matsuo, Hirose, Miyata, and Numa, 1989; Quandt, Nicol, and Schnetkamp, 1991), and a single diltiazem molecule is sufficient to block either channel. Quantitatively, the results will also show that the affinity of the rod channel for diltiazem is ~10-fold higher than for the cone channel, and that both the binding and unbinding rates are voltage dependent. Experiments will be described that seek to determine the location of the diltiazem binding site. Application of diltiazem to the extracellular side of the channel produced block that is qualitatively similar to block by diltiazem applied to the cytoplasmic side of the channel, suggesting that the diltiazem does not block from the extracellular side and that the binding site is on the cytoplasmic side of the channel. The extent of block by diltiazem applied to the cytoplasmic side of the patch was unchanged even when the charge of diltiazem was altered by changing pH, suggesting that the binding site does not lie within the voltage drop of the pore itself. Diltiazem may therefore act not by occluding the pore but by altering the gating of the channel.

This work has previously been described briefly in abstract form (Haynes and Yau, 1988; Haynes, 1991).

METHODS

Preparation

Recordings were made from excised, inside-out patches of plasma membrane obtained from the rod or cone outer segments of the channel catfish, Ictalurus punctatus. Each fish was dark-adapted for 3 h or more before it was killed by decapitation and the eyes were removed under dim red illumination. Under infrared illumination, each eye was hemisected and the posterior portion was bisected near the optic nerve. The resulting pieces were placed in a small petri dish filled with Ringer's solution (see below) and the retina of each piece was gently pulled from the pigment epithelium with the aid of fine forceps. The isolated pieces of retina were transferred to a dish containing 0.5 mg ml^{-1} hyaluronidase (type IV; Sigma Chemical Co., St.

Louis, MO) and 0.05 mg ml^{-1} collagenase (type IV; Sigma Chemical Co.) for 30 min, and then to a second identical enzyme bath for an additional 30 min. After completion of the enzyme treatment, the pieces of retina were stored on ice in the dark in Ringer's solution. All subsequent procedures were performed under room illumination.

As needed, an individual piece of retina was placed in a Sylgard-bottomed petri dish filled with buffered saline (see below). A small slip of razor blade was used to triturate the retina, yielding a dispersion of isolated cells and broken outer segments which were collected, transferred to the experimental chamber, and allowed to settle. Cells that did not settle, and any residual traces of Ringer's solution, were washed from the chamber by the saline solution before the first patch pipette was introduced.

Solutions

The retina was isolated, treated, and stored in a Ringer's solution consisting of (mM): 110 NaCl, 2.5 KCl, 1.6 MgCl$_2$, 1.0 CaCl$_2$, 5.0 glucose, and 5.0 NaHEPES (pH 7.6). The pipette and bath solutions were identical and contained (mM): 120 NaCl, 0.1 NaEGTA, 0.1 NaEDTA, and 5.0 NaHEPES (pH 7.6). With the ions symmetrically distributed across the patch of plasma membrane, the driving force for current flow came only from the holding potential. A second bath solution containing an additional 1 mM of the sodium salt of guanosine 3',5' cyclic monophosphate (cGMP; Sigma Chemical Co.) was used to fully activate all of the channels in the patch. *l-cis*-Diltiazem was added to either the bath solution or the pipette solution to a final concentration of 0.5, 1, 2, 5, 10, 20, 50, or 100 μM as required. The bath solution could be rapidly changed ($\tau < 300$ ms) to expose the cytoplasmic surface of the patch to cGMP, diltiazem, or both. The perfusion system and the chamber were essentially the same as those that have been described previously (Nakatani and Yau, 1988). All experiments were done at room temperature (22–24°C). Diltiazem concentrations >20 μM often produced a current offset which was corrected for by first applying diltiazem in the absence of cGMP and nulling the offset.

Electrical Recordings

Patch pipettes were fabricated from thick-walled borosilicate glass (Corning 7740; A-M Systems, Everett, WA), coated with Sylgard 184 (Dow Corning Corp., Midland, MI), and fire polished. The pipette tip inside diameter was ~0.5 μm and resistances were typically 20–30 MΩ when filled with the saline solution described above. Gigaohm seals were obtained by pressing the pipette against the tip of a cone or the side of a rod outer segment and applying gentle suction. The extent of invagination of the patch into the pipette during seal formation was observed through the microscope (Axiovert 10 with DIC, ×1,000; Carl Zeiss, Inc., Thornwood, NY) and in all cases was <1 μm. A tap on the micromanipulator holding the pipette sheared the pipette and its attached patch of plasma membrane away from the outer segment, yielding an excised, inside-out patch whose cytoplasmic surface was exposed to the bath solution.

Current flowing across the patch was measured under voltage clamp by an Axopatch 1D (Axon Instruments, Inc., Foster City, CA) patch clamp amplifier with a bandwidth of DC to 20 kHz (−3 dB, 4-pole Bessel filter). The signal from the patch clamp was then low-pass filtered by an 8-pole Bessel filter to 5 kHz (−3 dB) and recorded on videotape using a PCM adapter. Current–voltage relations were obtained under computer control by generating 1- or 2-s steps to the desired voltage from a holding potential of 0 mV. The resulting currents were filtered at 250 Hz (−3 dB, 8-pole Bessel), digitized on line at 1,000 Hz, and stored on the computer's hard disk. The standard physiological conventions were followed in these recordings, with currents from the cytoplasmic side to the extracellular side considered positive and positive potentials referring to positive voltages on the cytoplasmic side of the patch.

Data Analysis

For single channel recordings, the recorded signal was low-pass filtered to 2.5 kHz (−3 dB, 8-pole Bessel) during playback and digitization. Continuous stretches of record were digitized at 10 kHz and stored on the computer's hard disk. The digitized signal was digitally filtered to 1 kHz and analyzed by the IPROC2 program (Axon Instruments, Inc.). Log-binned histograms of the open or closed durations were fitted with single or multiple exponential curves using the method of maximum likelihood.

Current–voltage relations were determined by averaging the digitized points during each voltage step, after allowing the block to relax to a new steady-state level. The leakage current was determined for each voltage before and after the application of cGMP, averaged together, and then subtracted from the current in the presence of cGMP to obtain the net cGMP-dependent current. The currents at each voltage and concentration of diltiazem were simultaneously fitted according to an equation describing block by an impermeant blocker (Woodhull, 1973):

$$I_{V,Blocked} = \frac{I_{V,Unblocked}}{\left(1 + \frac{[Diltiazem]}{K_{D,0}e^{(-V/V_0)}}\right)} \tag{1}$$

where $I_{V,Unblocked}$ is derived from Eq. 3 below, $K_{D,0}$ is the apparent dissociation constant at 0 mV, and V_0 is the voltage range over which $K_{D,0}$ changes by e-fold. For an impermeant blocker of valence z, V_0 can be related to the (electrical) distance, δ, crossed by the blocker to reach its binding site by

$$\delta = \frac{RT}{zFV_0} \tag{2}$$

One complicating factor in predicting the current in the presence of a blocker is that the open probability of the cGMP-gated channel is weakly voltage dependent, being higher at depolarized potentials (Haynes et al., 1986; Zimmerman and Baylor, 1986; Karpen, Zimmerman, Stryer, and Baylor, 1988; Haynes and Yau, 1990a). This results in slightly more current at depolarized potentials than at hyperpolarized potentials and would result in Eq. 1 predicting too much current at positive potentials. To account for this, the current–voltage relations in the absence of diltiazem were fitted using a least-squares method with the equation

$$I_{V,unblocked} = I_{max} [e^{((1-\delta)(V-V_{Rev})zF/RT)} - e^{(-\delta(V-V_{Rev})zF/RT)}] \tag{3}$$

which is simply the single barrier model of ion permeation (Jack, Nobel, and Tsien, 1975). In this equation, δ is the fractional distance of the barrier across the membrane with respect to the outside, V is the membrane potential, V_{Rev} is the reversal potential (0 mV here), and I_{Max} is a scaling constant. This equation was used simply as an empirical description of the observed current–voltage relation in order to calculate the expected current during block, rather than as a description of permeation or of the block itself.

Throughout, data are presented as mean ± SD. All statistical tests of means used the (one-sided) Student's t test with the indicated degrees of freedom (d.f.). Rate constants and dissociation constants at 0 mV and the voltage dependence of those constants were determined by linear regression of semi-logarithmic plots of the data as a function of voltage.

RESULTS

The block produced by l-cis-diltiazem in the cGMP-gated channel from both rods and cones was rapid and reversible. An example of the time course of activation and

subsequent block of the channels in an inside-out cone patch clamped at +30 mV is illustrated in Fig. 1. The upper trace is a plot of the membrane current over time on a slow time scale. The introduction of 1 mM cGMP to the bath superfusing the cytoplasmic surface of the patch produced a rapid increase in current as all six channels in the patch became maximally activated. After ~12 s, 5 μM diltiazem was added to the bath. This reduced the mean current by about two-thirds, from 6 pA to ~2 pA, and also produced a large increase in the current noise due to the random occurrence of blocking events. The current rapidly recovered to nearly the initial
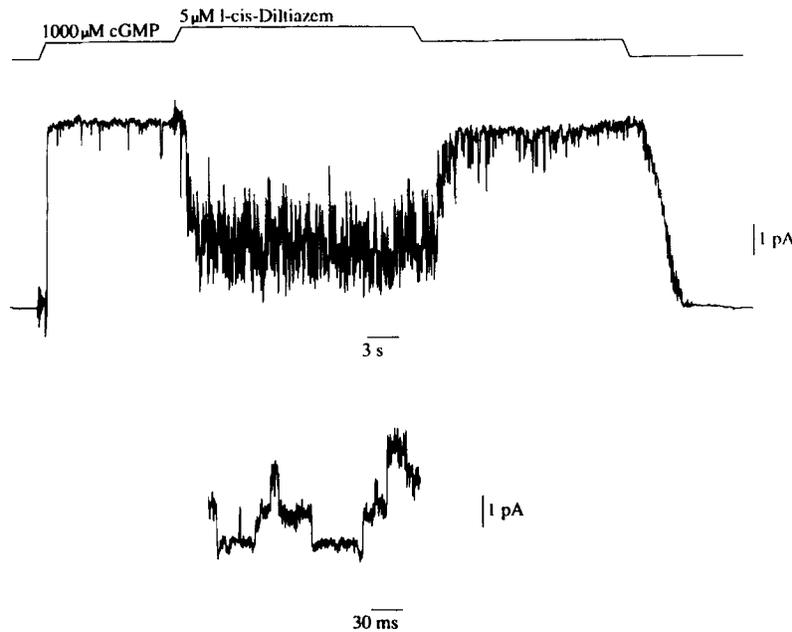

FIGURE 1. Block of cone channels by diltiazem. (*Upper trace*) Current recorded from a cone patch containing six channels held at +30 mV. The line above indicates the time course of solution changes. The initial application of 1 mM cGMP produced a large outward current, two-thirds of which was blocked by 5 μM *l-cis*-diltiazem. The block was reversible, although occasional blocking events can be observed many seconds after diltiazem was washed out. (*Lower trace*) Current recorded during diltiazem block on faster time scale. The amount of current blocked during each blocking event was approximately equal to that of a single channel.

value when diltiazem was removed. However, blocking events were still observed for many seconds after removal, suggesting that not all of the diltiazem was immediately removed from the vicinity of the channel. This was probably due to diltiazem repartitioning out of the membrane and pipette (see below). On a 100-fold more rapid time scale (lower trace in Fig. 1), the noise in the current in the presence of diltiazem can be resolved into discrete jumps with an amplitude of ~1.25 pA, approximately the same as the amplitude of the current contributed by a single

channel under these conditions. Qualitatively similar results were obtained with patches from rod outer segments.

Dose–Response Relation

The extent to which the rod and cone channels were blocked by diltiazem was both concentration and voltage dependent. As the concentration of diltiazem was increased, the degree of block increased. Fig. 2 A shows a plot of the fraction of the current blocked by diltiazem as a function of the concentration of diltiazem applied via the bath to the cytoplasmic side of the same cone patch at +30 mV (*circles*) and at
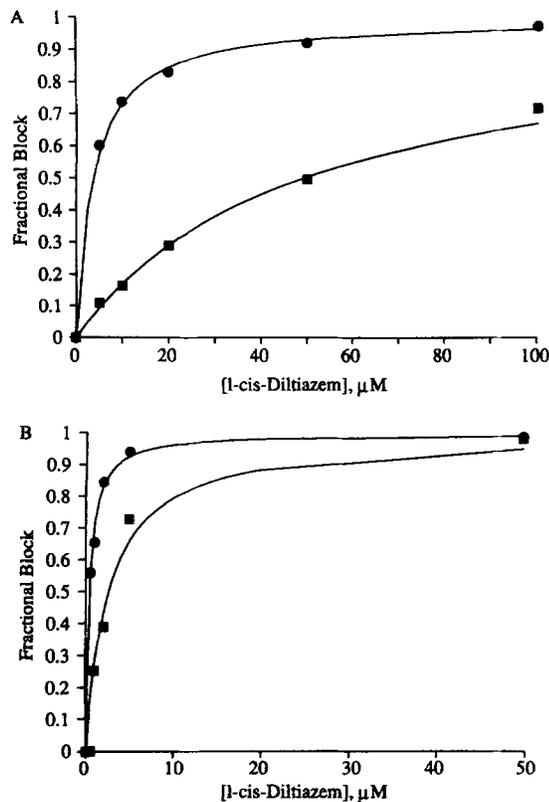

FIGURE 2. Concentration dependence of diltiazem block. (*A*) Cone patch data at +30 mV (*circles*) and −30 mV (*squares*). Curves were derived from the Michaelis relation with dissociation constants of 3.7 and 49 μM at +30 and −30 mV, respectively, giving an *e*-fold change in the dissociation constant over 23 mV. (*B*) Rod patch data at +30 mV (*circles*) and −30 mV (*squares*). Curves were derived from the Michaelis relation with dissociation constants of 0.4 and 2.6 μM at +30 and −30 mV, respectively, giving an *e*-fold change in the dissociation constant over 33 mV.

−30 mV (*squares*). The degree of block at a given voltage could be fitted by a simple Michaelis relation (curves in Fig. 2), indicating that a single diltiazem molecule was sufficient to block the channel. In all cases, the degree of block was greater at positive potentials than at negative potentials. In this example, the dissociation constant at +30 mV was 3.7 μM and increased to 49 μM at −30 mV. This shift with voltage corresponds to an *e*-fold change in the dissociation constant over a 23-mV change in voltage. The average dissociation constant from 19 patches at +30 mV was 8.7 ± 9.8 μM (mean ± SD) and increased to 40 ± 36 μM in 18 patches at −30 mV. In 13

patches, data were obtained at both voltages and the dissociation constant changed e-fold by an average of 52 ± 24 mV.

Block by diltiazem of the cGMP-gated channels from rod outer segments was similar to that from cones, except that the dissociation constant was lower by about a factor of 10. Fig. 2 B shows an example of a dose–response relation obtained from a rod patch at +30 mV (circles) and −30 mV (squares). In this experiment, the dissociation constant at +30 mV was only 0.4 μM and increased to 2.6 μM at −30 mV, corresponding to an e-fold change in the dissociation constant over 33 mV. In 17 patches at +30 mV and 13 patches at −30 mV, the dissociation constants averaged 0.9 ± 0.6 and 3.4 ± 2.1 μM, respectively. Both values were significantly lower than their counterparts in cones ($P < 0.005$; d.f. = 34 and 29, respectively). The average e-fold change in the dissociation constant was not significantly different from that of cones, averaging 61 ± 29 mV in 10 patches where data were obtained at both voltages.

The dose–response relations in Fig. 2 both asymptotically approach a value of 1, indicating complete block of both rod and cone channels at a sufficiently high concentration of diltiazem. This is in contrast to previous observations in both the native (Koch et al., 1987; Quandt et al., 1991) and cloned (Kaupp et al., 1989) cGMP-gated rod channel from higher vertebrates. There does not appear to be a subpopulation of either rod or cone channels from catfish that is insensitive to diltiazem.

Current–Voltage Relations: Cytoplasmic Diltiazem

The voltage dependence of block by diltiazem applied to the cytoplasmic side of the rod and cone channels is clearly evident in the current–voltage relations shown in Fig. 3. The smooth curves fitted to the data in the absence of diltiazem (filled squares) were derived from Eq. 3 (see Methods) in order to account for the slight outward rectification of the macroscopic current–voltage relation caused by the weak voltage dependence in the gating of the fully liganded channel (Haynes et al., 1986; Zimmerman and Baylor, 1986; Karpen et al., 1988; Haynes and Yau, 1990a). These curves were then scaled according to Eq. 1 (see Methods), which describes the voltage-dependent block of a channel by a blocker unable to permeate through the channel. This relation is characterized by the dissociation constant of the blocker at 0 mV ($K_{D,0}$) and a voltage constant (V_0) which is the change in voltage necessary to produce an e-fold change in the dissociation constant. For the cone patch illustrated in Fig. 3 A, $K_{D,0}$ was 6.1 μM and V_0 was 45 mV. In 12 cone patches, the average value for $K_{D,0}$ was 21 ± 24 μM and V_0 averaged 52 ± 20 mV. Assuming that diltiazem enters the pore to block the channel, the fraction (δ) of the electric field crossed by the univalent diltiazem to its binding site is 0.49, on average.

Consistent with the concentration dependence of block shown in Fig. 2, the current at any given voltage in a rod patch was substantially lower than in a cone patch in the presence of an equal concentration of diltiazem. For the rod patch in Fig. 3 B, the value for $K_{D,0}$ was 0.6 μM, or ~10-fold lower than in the cone patch, and V_0 was 50 mV. In nine rod patches, $K_{D,0}$ averaged 2.9 ± 1.4 μM and V_0 averaged 60 ± 21 mV. The fraction of the electrical distance crossed by diltiazem (assuming that diltiazem enters the pore) is 0.43. These values are consistent with the values obtained from the

dose–response relations and also with those obtained by McLatchie and Matthews (1992) in salamander rods ($K_{D,0}$ = 2.4 µM, V_0 = 50 mV, δ = 0.5), and indicate that the rod channel has a significantly higher affinity for diltiazem (P < 0.025, d.f. = 19), but a similar voltage dependence when compared with the cone channel.

While the data obtained in the presence of a low concentration of diltiazem and at negative potentials with higher concentrations could by fitted adequately by Eq. 1, the fit was often poor at positive potentials and higher diltiazem concentrations where the degree of block was greater than expected. For example, the data points
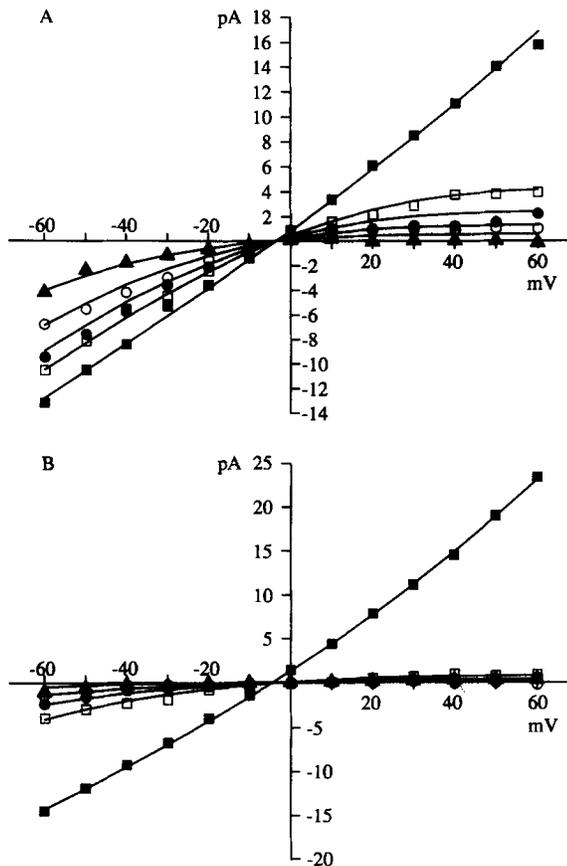

FIGURE 3. Net current–voltage relations for diltiazem applied to the cytoplasmic surface. Symbols show the net cGMP-induced current in the presence of 0 (■), 5 (□), 10 (●), 20 (○), and 50 (▲) µM diltiazem applied to the cytoplasmic side of a cone (A) and rod (B) patch. The curve fitted to data in the absence of diltiazem was fitted using Eq. 3, while the curves fitted to data in the presence of diltiazem were fitted using Eq. 1 (see Methods) with $K_{D,0}$ = 6.1 µM and V_0 = 45 mV for the cone channels and $K_{D,0}$ = 0.6 µM and V_0 = 50 mV for the rod channels.

for block by 10, 20, and 50 µM diltiazem in the cone patch in Fig. 3 A at positive potentials all fall below the line predicted by Eq. 1. This equation assumes both that diltiazem does not permeate through the channel and that diltiazem itself enters and directly blocks the pore. Since the equation fails to fit the data, either the supposition that the blocker is impermeant is incorrect or the blocker may act by a mechanism other than direct occlusion of the pore. Three points argue against the first possibility. First, if the blocker were able to permeate through the channel, one would expect more rather than less current at positive potentials. Second, the current–

voltage relations from both rod and cone patches often show a negative slope conductance at voltages more positive than +50 mV, consistent with block by a charged molecule unable to permeate through the channel pore. Finally, given that molecules such as TEA and TMA, which are much smaller than diltiazem (414.52 mol wt), are impermeant (rods: Menini, 1990; cones: Haynes, L. W., manuscript in preparation), it seems unlikely on the grounds of steric hinderance that diltiazem would be able to pass through the channel. The possibility remains, however, that diltiazem does not directly occlude the pore but may act by another mechanism, indirectly resulting in obstruction of the channel pore.

Current–Voltage Relations: Extracellular Diltiazem

If diltiazem directly occludes the pore of the cGMP-gated channels of rods and cones, then the direction of rectification of the current–voltage relation should depend on the side of the channel to which diltiazem is applied. That is, since diltiazem applied to the cytoplasmic side of the channel blocked current flowing from the cytoplasmic side to the extracellular side of the channel and thereby produced inward rectification, one might expect that application of diltiazem to the extracellular side of the channel should block current flowing from the extracellular side to the intracellular side of the channel and produce outward rectification. The results of an experiment testing this prediction, illustrated in Fig. 4, indicate that this is not the case. In fact, diltiazem applied to the extracellular side of the patch via the pipette produced inward rectification in patches from both cones (Fig. 4 *A*) or rods (Fig. 4 *B*), a result qualitatively similar to diltiazem applied from the cytoplasmic side. Unfortunately, this result cannot be made quantitative because the difficulty of changing the solution inside the pipette during the course of the experiment is compounded by the physical fragility of rod and cone patches. Furthermore, the possibility of obtaining outside-out patches from rod or cone outer segments is precluded by their structure. Fragmentation of the rod disks that are tightly anchored to the plasma membrane and the laminated nature of cone outer segment "disks" makes it impossible to reseal a patch of membrane after the whole-cell configuration has been attained.

Kinetics of Block: Single Channel Analysis

One conclusion from the experiments with extracellularly applied diltiazem might be that there are two binding sites for diltiazem, one intracellular and one extracellular, which somehow produce a similar pattern of rectification. A second, simpler possibility would be that there is only a single site that diltiazem can reach from either side of the membrane by virtue of its lipid solubility. One test that might distinguish between these possibilities would be to measure the microscopic kinetics of block. A single class of binding site should have a single unbinding rate, but might have different binding rates because of different accessibility from either side.

The lower channel density in cones, about one-tenth that of rods (Haynes and Yau, 1990*a*), makes it possible on rare occasions to obtain patches from cone outer segments that contain only a single active channel. Using such a preparation, it is possible to measure directly the rates of blocking and unblocking of the channel in the steady state by measuring the durations of the blocked and unblocked periods. An example of currents from a single cone channel undergoing block by diltiazem

applied to the cytoplasmic side of the patch via the bath is shown in Fig. 5. The current traces show a continuous 2-s stretch of data excerpted from a 28-s record. The patch was clamped at +30 mV and openings are indicated by upward current deflections, following the usual physiological convention that outward current is positive. The presence of a concentration of cGMP (1 mM) high enough to saturate all of the cGMP binding sites simplifies the gating model to a (relatively) simple three-state system consisting of two closed states and one open state. The open probability of the channel is ~0.9 (Haynes and Yau, 1990a), with the channel rapidly
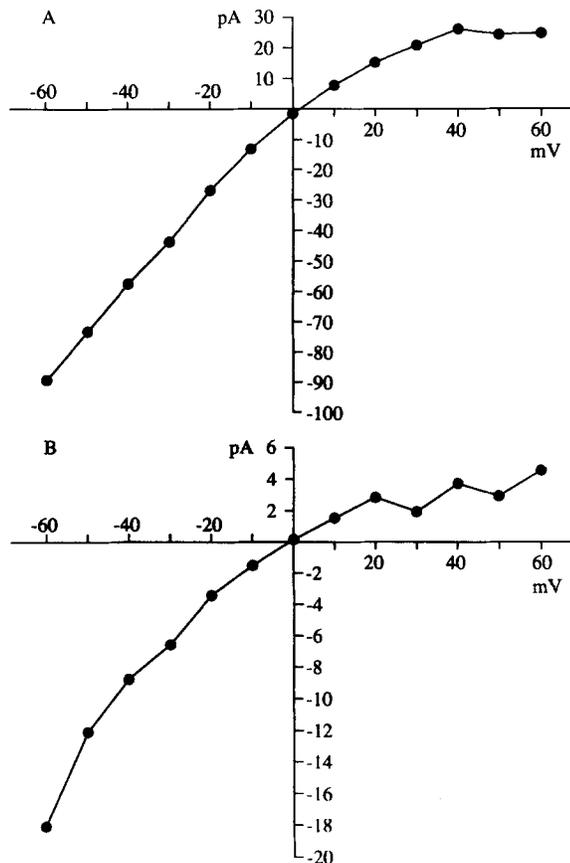

FIGURE 4. Net current–voltage relations for diltiazem applied to the extracellular surface. (A) Cone; (B) rod. In both cases, the channels were activated with 1 mM cGMP applied to the cytoplasmic side of the patch. The pipette solution, facing the extracellular side of the patch, contained 5 μM diltiazem. Block by extracellular diltiazem produced inward rectification.

alternating between the fully liganded closed state and the open state on a time scale of ~100 μs, and with occasional sojourns in a somewhat longer-lived closed state (~5 ms) whose nature is not well understood (Haynes and Yau, 1990a). The long silent periods, therefore, represent periods when the channel is blocked.

The histogram of closed and blocked durations required the sum of three exponentials for the best fit. The shortest time constants (0.4 and 5.3 ms) represent the intervals between individual openings and the rarely occurring long-lived closed state (described above) that are seen in the absence of blocker. Diltiazem adds a

much longer third component representing the duration of block. This third component had a time constant of 63.2 ms in this experiment, which gives a rate constant for the unbinding of diltiazem of 15.8 s^{-1}. It was possible to apply more than one concentration of diltiazem in the case of five patches. Not unexpectedly, in all of these cases the rate constant for unbinding diltiazem was concentration
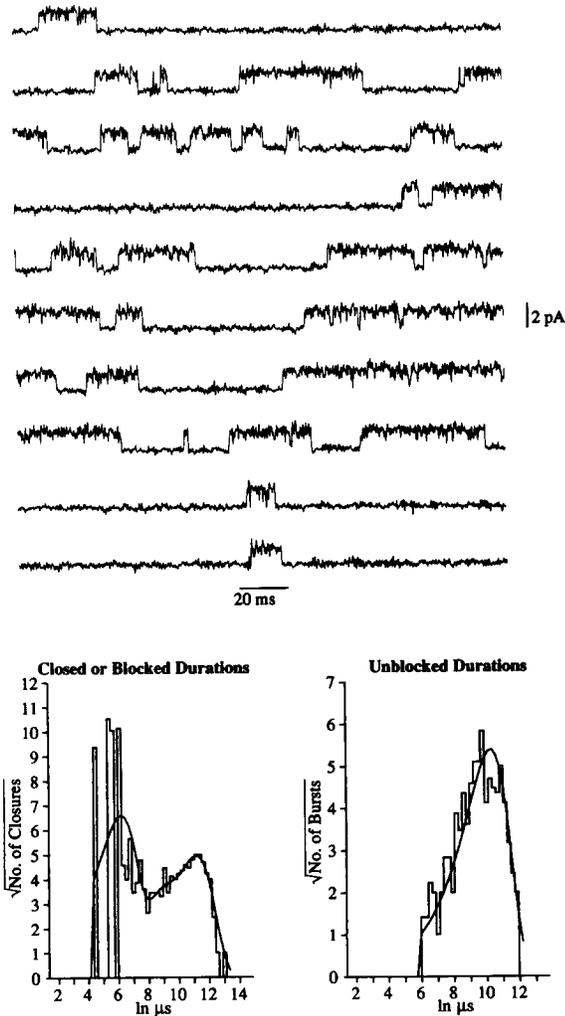

FIGURE 5. Kinetics of block in a single cone channel by diltiazem applied to the cytoplasmic surface. The traces show a continuous 2-s record of current recorded from a single cone channel clamped at +30 mV in the presence of 1 mM cGMP and 5 µM diltiazem. The bandwidth of the recording was DC to 2.5 kHz, but the data were digitally filtered to 1 kHz before analysis. The rapid flicks (often incomplete) between the open and closed levels are the normal gating behavior of the fully liganded channel. The longer closed periods are due to the presence of diltiazem and represent blocked periods. The histograms show the distribution of blocked (or closed) and unblocked durations from the complete 28-s recording. Three exponentials were required to fit the distribution of closed and blocked durations. The longest time constant was 63.2 ms and represents the blocked durations (τ_B). A single exponential with a time constant (τ_o) of 26.5 ms was sufficient to fit the open duration histogram.

independent. The average rate constant for unbinding diltiazem in six patches at +30 mV was 16.7 ± 4.3 s^{-1}. In three patches clamped at −30 mV, this increased to 43.2 ± 6.1 s^{-1}. When plotted together and analyzed by linear regression, these data show a rate constant for unbinding diltiazem at 0 mV of 26.4 s^{-1} with an *e*-fold increase over 62 mV (r = 0.92, d.f. = 7).

The rate constant for the binding of diltiazem was derived from the histogram of unblocked durations. As with the closed periods, the individual brief openings (~ 100 μs) of the channel have been ignored because they represent the normal gating of the channel. Instead, the unblocked durations are actually the durations of clusters (or bursts) of these individual openings. The histogram of unblocked durations in Fig. 5 was fitted by a single exponential with a time constant of 26.5 ms, corresponding to a rate constant for the binding of diltiazem of 7.5 μM^{-1} s^{-1} at +30 mV. The rate constant for the binding of diltiazem depended on the first power of concentration in all five patches where more than one concentration was tested. This is consistent with the conclusion from the concentration dependence of block of the macroscopic current (Fig. 2) that a single diltiazem is sufficient to block the channel. As an additional check, the dissociation constant of 2.1 μM calculated from the rate constants in this case is in good agreement with the observed dissociation constant of 2.3 μM. Similar agreement was obtained at -30 mV in this patch (15 μM observed vs. 17 μM calculated), and in another patch that was held only at +30 mV (2.3 μM observed vs. 2.7 μM calculated). The average rate constant for binding diltiazem in six patches at +30 mV was 8.7 $\pm$ 3.6 μM^{-1} s^{-1}. Like the unbinding rate constant, the rate constant for binding diltiazem was voltage dependent, but in the opposite direction. In three patches at -30 mV, the rate constant for binding diltiazem dropped to 2.1 $\pm$ 0.7 μM^{-1} s^{-1}. This gives a rate constant for binding diltiazem at 0 mV of 4.1 μM^{-1} s^{-1} and an e-fold decrease over 43 mV ($r = 0.9$, d.f. $= 7$).

When diltiazem was applied via the pipette to the extracellular side of the patch under conditions otherwise identical to those used for application to the intracellular surface, qualitatively similar results were obtained. The 2-s current trace in Fig. 6 shows long blocked durations similar to those in Fig. 5, although the frequency of block appears to be lower. As before, the histogram of closed or blocked durations could be fitted by the sum of three exponentials, the longest of which had a time constant of 55.6 ms at +30 mV. This corresponds to an unbinding rate of 18 s^{-1}. In two patches at +30 mV, the mean rate constant for unbinding diltiazem was 21.7 $\pm$ 7.6 s^{-1}. Data were obtained from only a single patch at -30 mV, which had a unbinding rate constant of 45 s^{-1}. Values at neither voltage were significantly different from those with diltiazem applied to the cytoplasmic surface of the patch ($P > 0.1$, d.f. $= 6$ and $P > 0.2$, d.f. $= 2$, respectively). The rate constant for unbinding diltiazem at 0 mV was 30.7 s^{-1}, and this decreased e-fold over 78.8 mV ($r = 0.87$, d.f. $= 3$). Again, the values are not very different from those for diltiazem applied to the cytoplasmic surface of the patch. This suggests that there is only a single class of binding site that can be occupied from either side of the membrane.

The histogram of unblocked durations could be fitted by a single exponential that, in this case, had a value of 129 ms. For a diltiazem concentration of 5 μM, this gives a binding rate constant of only 1.6 μM^{-1} s^{-1}. The mean binding rate constant for the two patches at +30 mV was 2.1 $\pm$ 0.6 μM^{-1} s^{-1} and the single patch at -30 mV had a binding rate constant of 0.9 μM^{-1} s^{-1}. Only the value at +30 mV was significantly lower ($P < 0.05$, d.f. $= 6$) than the binding rate constant for diltiazem applied to the cytoplasmic surface. At 0 mV, these values give a rate constant of 1.4 μM^{-1} s^{-1} which increased e-fold over 68 mV. Thus, the rate constant for binding diltiazem from the

extracellular surface is somewhat lower and shows perhaps slightly less voltage dependence.

It is impossible to determine if the difference in the voltage dependence of the binding rate constant is real or an artifact because of the limited quantity of data available. The smaller magnitude of the binding rate constant, on the other hand,
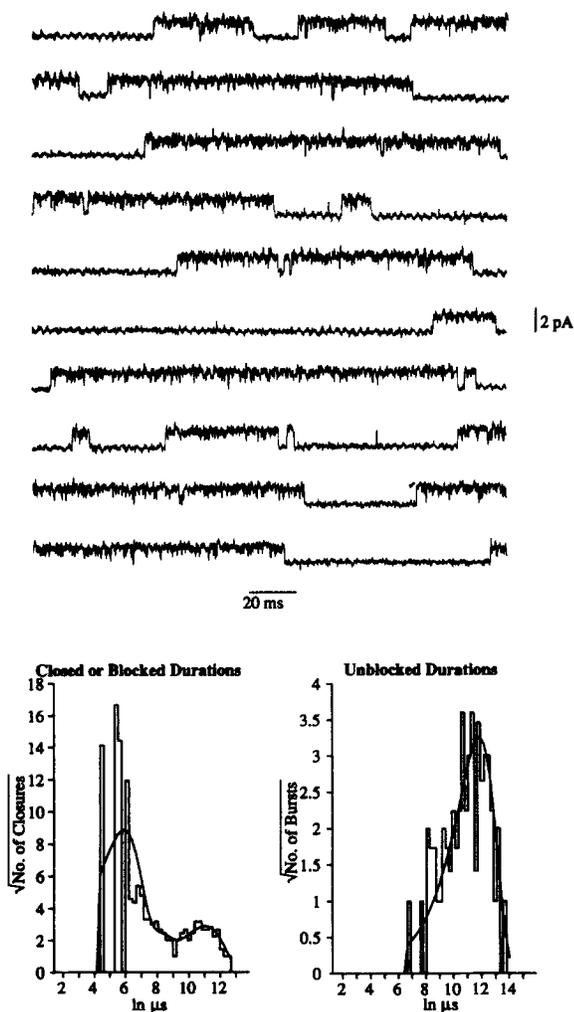

FIGURE 6. Kinetics of block in a single cone channel by diltiazem applied to the extracellular surface. The traces show a continuous 2-s record of current recorded from a single cone channel clamped at $+30$ mV in the presence of 1 mM cGMP in the bath and 5 μM diltiazem in the pipette. The bandwidth of the recording was DC to 2.5 kHz, but the data were digitally filtered to 1 kHz before analysis. The histograms show the distribution of blocked (or closed) and unblocked durations from the complete 22-s recording. Three exponentials were required to fit the distribution of closed and blocked durations. The longest time constant was 55.6 ms and represents the blocked durations (τ_B). A single exponential with a time constant (τ_o) of 129 ms was sufficient to fit the open duration histogram.

may be real. This could mean that there are, in fact, two separate binding sites, one on each surface, which by happenstance have the same rate constants and voltage dependence for unbinding, but which have different binding rates. A more likely possibility is that there is a single binding site that is not equally accessible from either side. With an octanol to water partition coefficient of 200:1 (Hermann and

Morselli, 1985), diltiazem is a highly lipophilic molecule. Thus, the most likely explanation for its behavior is that diltiazem partitions into and across the plasma membrane, a conclusion also reached by others (Caretta, Sorbi, Stein, and Tirindelli, 1991; McLatchie and Matthews, 1992). While the volume of the bath solution flowing across the cytoplasmic surface of the patch is essentially infinite and provides a sink for the diltiazem flux across the plasma membrane, Karpen et al. (1988) observed a diffusion barrier near the cytoplasmic surface due to the remnants of disk membranes, or perhaps the geometry of the patch within the pipette. There should, therefore, be a gradient of diltiazem across the patch resulting in a lower, but nonzero, concentration near the cytoplasmic surface of the patch. If this were the case, then the binding rate constant, but not the unbinding rate constant, would be underestimated and results similar to those above would be obtained. The data are therefore consistent with a single binding site on the cytoplasmic side of the channel.

Location of the Binding Site

Two tests were performed to determine if diltiazem partitions into and across the plasma membrane, as suggested above. The first test was to alter the flow rate of the control bath perfusate while applying diltiazem to the extracellular side of the patch in the expectation that eliminating convective removal of diltiazem that crossed the membrane would raise the local concentration. Increasing the flow rate 1.6-fold had no measurable effect, but in some patches stopping the flow completely produced a small increase in block. Other patches showed no change. Fig. 7 shows a current–voltage relation from a cone patch with 5 μM diltiazem on the extracellular side of the patch. The circles show the average of two current–voltage relations before the normal bath flow of 1.4 ml min^{-1} was turned off and after it was resumed. When the flow of the bath was turned off (squares), the block at positive voltages increased slightly. This suggests that diltiazem does, in fact, cross the membrane and that the site of action for extracellular diltiazem is on the cytoplasmic side of the membrane. It also suggests that convection is probably not the major path by which diltiazem is lost from near the surface of the patch. Rather, it is likely that loss by diffusion is the major route and that a significant diffusion barrier exists. The application of 5 μM diltiazem via the bath to the cytoplasmic side of the same patch (triangles) resulted in a substantial increase in block. This implies that diltiazem on the extracellular surface of the membrane produces a concentration at the cytoplasmic surface that is substantially lower (~50%) than that at the extracellular surface as a result of diffusion into the bath.

Diltiazem, with a pK$_A$ of 7.7, exists in a mix of neutral (44%) and positively charged (56%) forms at the pH of 7.6 used in these experiments. It is generally assumed that charged molecules ought not to be able to partition across the plasma membrane (but see Lempereur, Sautereau, Tocanne, and Laneelle, 1984), assuming that the conditions near the membrane are similar to those of the bulk solution. Increasing the fraction of ionized diltiazem on the extracellular side of the patch should reduce the extent of block by reducing the ability of diltiazem to diffuse across the membrane. This experiment is complicated by the fact that the pH of the solution inside the pipette cannot be changed during the experiment, making it impossible to quantify the extent of block. Attempts to change the pH of the pipette solution
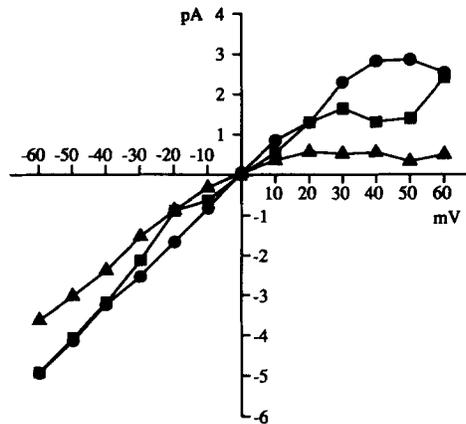
FIGURE 7. Reducing bath flow rate or simultaneous application of diltiazem to cytoplasmic and extracellular surfaces of a patch increases block. Net current–voltage relation for a cone patch with 5 μM diltiazem bathing the extracellular surface (●). Mean of two trials, before and after reducing the flow of control bath perfusate from 1.4 ml min⁻¹ to 0. This produced a slight increase in block at positive potentials (■) which was reversed when the flow resumed. The addition of 5 μM diltiazem to the bath facing the cytoplasmic surface reduced the outward current substantially (▲).

during the experiment using a membrane-permeant buffer, acetate, uniformly resulted in rupture of the patch. The experiment was nonetheless tried at a single pH, 6.7, where 91% of the diltiazem is in the ionized form, in the hope that block would be nearly abolished. However, block was still observed under these conditions, probably due to the diffusion of the remaining neutral diltiazem. Attempts to decrease the external pH further were hampered by proton block of the channel and instability in the patches.

If the ionized form of diltiazem enters and blocks the pore, as suggested by the voltage dependence of block, increasing the fraction of ionized diltiazem on the cytoplasmic side of the patch should increase the extent of block since the concentration of the putative blocking agent (diltiazem⁺) is increased. Unexpectedly, however, neither increasing the pH to 8.63 (9% diltiazem⁺) nor decreasing pH to 6.76 (91% diltiazem⁺) on the cytoplasmic side of the membrane affected the ability of diltiazem to block. An experiment in which diltiazem was applied at three pH's to the
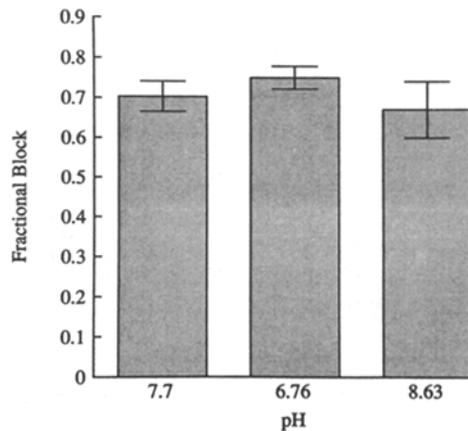

FIGURE 8. pH dependence of diltiazem block. Altering pH did not alter the ability of diltiazem to block the channel when applied to the cytoplasmic side of the patch. Three trials were done at pH 7.7 and two each at 6.76 and 8.63. The error bars show standard deviations. All data are from the same cone patch held at +30 mV.

cytoplasmic side of a cone patch held at +30 mV is shown in Fig. 8. In this experiment, three determinations were made at pH 7.7 and two each at 6.76 and 8.63. While there is some scatter in the data, there is virtually no difference between the results at any of the pH's. Similar results were found in three other patches. As shown above, the blocking and unblocking rates are voltage dependent, as if diltiazem must cross some fraction ($\sim$0.5) of the electrical distance across the channel before reaching its binding site. If this were so, then altering the proportion of diltiazem that is charged should alter the effective concentration of the putative blocking species (diltiazem$^+$). The expected result, that the degree of block should increase at acidic pH and decrease at basic pH, was not observed. This implies that diltiazem can block the channel independent of its own charge and that the apparent voltage dependence of block must arise from an event subsequent to diltiazem binding. On the other hand, if the binding site for diltiazem were completely lipophilic (e.g., inside the membrane itself), then the degree of block should decrease with increasing ionization of diltiazem. This, too, was not observed. Taken together, these results imply that the binding site does not interact with the ionized "tail" of the molecule and that the tail remains hydrated. They also imply that the simple notion of the quaternary ammonium side chain of diltiazem slipping into the pore only to be restrained by the bulk of the rest of the molecule is untenable.

DISCUSSION

While several investigators have used diltiazem as a tool to characterize the cGMP-gated channel of rod photoreceptors (Koch and Kaupp, 1985; Stern, Kaupp, and MacLeish, 1986; Koch et al., 1987; Nicol, Schnetkamp, Saimi, Cragoe, and Bownds, 1987; Kaupp et al., 1989; Caretta et al., 1991; Hurwitz and Holcombe, 1991; Quandt et al., 1991), the mechanism by which l-cis-diltiazem blocks the cGMP-gated channels of rods and cones remained unknown. The experiments presented here suggest that the rod and cone cGMP-gated channels each possess a single class of binding site for diltiazem, located on the cytoplasmic surface of the channel, and that the occupation of a single site is sufficient to block the channel. The simplest model of block, that the quarternary ammonium side chain enters the ion-conducting pore of the channel but is restrained by the remainder of the diltiazem molecule from passing through the pore and thereby plugging the channel, is not consistent with the observations presented here. The presence of chiral sites in the hydrophobic portion of the molecule and the known difference in efficacy between d-cis- and l-cis-diltiazem (Koch and Kaupp, 1985) suggest that the hydrophobic portion of the molecule is involved in binding to the channel. Since the dissociation constant of diltiazem from the binding site is independent of the charge of the molecule, as shown by the pH independence of block, the site must be relatively shallow so that the charged "tail" of diltiazem can remain hydrated, but the site itself must be hydrophobic in order to interact with the hydrophobic portion of diltiazem. One possible location for such a site would be at the mouth of the channel, where the quaternary ammonium "tail" could interfere in some way with the movement of ions through the channel. Such a site cannot, however, be within the electric field drop of the channel, since diltiazem can block the channel independent of its charge. Likewise, the "tail" must be able to block the channel regardless of its charge. The voltage dependence of block must

then arise from some voltage dependence in the conformation of the binding site which changes its accessibility and stability. Alternatively, the site could be external to the pore at the aqueous solution interface where diltiazem could disrupt channel gating by intercalating itself into the channel or between channel subunits. It is unlikely that this binding site is the cGMP binding site, since there is a clear difference between rods and cones in their affinity for diltiazem, but not for cGMP (Haynes et al., 1986; Zimmerman and Baylor, 1986; Haynes and Yau, 1990a). Caretta et al. (1991) reached this same conclusion based on the binding of the fluorescent cGMP analogue SAF-cGMP in the presence of diltiazem.

The voltage dependence of the microscopic binding and unbinding rates seen in single channel recording and the macroscopic dissociation constant seen in the current–voltage and dose–response relations is difficult to explain in light of the pH independence of block if diltiazem directly blocks the pore, since the uncharged molecule is apparently affected by voltage. However, the gating of the cGMP-activated channels of both rods and cones shows a weak voltage dependence (e-fold over ~ 200 mV; Haynes et al., 1986; Zimmerman and Baylor, 1986; Karpen et al., 1988; Haynes and Yau, 1990a) and a region similar to the S4 voltage sensor of voltage-gated channels has been described (Jan and Jan, 1990). Rather than block the channel directly in response to the transmembrane electric field, diltiazem may bind hydrophobically to a binding site on the channel that undergoes a slight voltage-dependent conformational change. This may be similar to the charge-independent but voltage-dependent block of sodium channels by saxitoxin and tetrodotoxin (Moczydlowski, Hall, Garber, Strichartz, and Miller, 1984). In binding to such a site, diltiazem may simply alter the gating of the channel, enhancing its voltage dependence. It would seem, then, that diltiazem is not a good candidate as a tool for probing the structure of the ion-conducting pore of the channel since it does not interact with the channel as an ion per se. Diltiazem may, however, prove to be a useful probe of the channel's gating behavior or of the structure of the mouth of the channel.

A recent paper by McLatchie and Matthews (1992) reaches a somewhat different conclusion. As here, they found that block by diltiazem was voltage dependent, that it could be described using a model for block by an impermeant ion, and that diltiazem could partition into the membrane. To show that diltiazem could partition into the membrane, McLatchie and Matthews (1992) altered the charge of diltiazem using changes in pH (5.6, 7.6, 8.6). However, after loading the membrane with diltiazem at various pH's, they measured block by diltiazem repartitioning out of the membrane only at a single pH (7.6), and so failed to detect that the block itself was independent of diltiazem's state of ionization. If they had done so, they would have been forced to conclude, as here, that voltage-dependent block by diltiazem cannot be explained by supposing that diltiazem enters and blocks the ion-conducting pore of the channel.

While low concentrations of diltiazem block the channels in excised patches quite effectively, several factors suggest that block of the dark current and the light response in the intact cell should not occur until much higher concentrations are reached. First, photoreceptors operate at hyperpolarized potentials and diltiazem is not as good a blocker of inward current at negative potentials as it is of outward current at positive potentials. More importantly, the large number of channels (90%)

that are closed at normal resting levels of cGMP under physiological conditions provides a pool of "spare" channels that can replace those blocked by diltiazem. The number of open channels is maintained at an optimal level by the phototransduction cascade via the calcium feedback mechanism used in light adaptation. If some fraction of the open channels is blocked, the calcium influx will decrease and recoverin will release its bound calcium and activate guanylate cyclase. This will quickly result in an increased synthesis of cGMP and the opening of more channels. These channels will themselves be blocked part of the time, but the total number of unblocked channels at any instant will be the same as in the absence of diltiazem and so the total current will be maintained. This mechanism acts to maintain the total influx of calcium constant by increasing the level of free cGMP to recruit "spare" channels which are themselves partially blocked. For example, if 10% of the channels are open in the dark and enough diltiazem is applied to block 50% of these channels, the cell need only open 20% of the total number of channels to get the same amount of current as in the absence of diltiazem and it will appear that diltiazem was without effect. In short, the total number of effective channels should remain constant until a substantial fraction of the channels have been recruited. The requirement for 10–100-fold higher concentrations of diltiazem to block current in the intact cell (Stern et al., 1986; Rispoli and Menini, 1988) can be explained by this mechanism. Likewise, the inability of diltiazem applied via whole-cell patch pipettes (Stern et al., 1986) can be explained by a combination of this mechanism and the rapid diffusion of the lipid-soluble diltiazem from the cell.

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