In Intact Cone Photoreceptors, a Ca^{2+}-dependent, Diffusible Factor Modulates the cGMP-gated Ion Channels Differently than in Rods

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ABSTRACT We investigated the modulation of cGMP-gated ion channels in single cone photoreceptors isolated from a fish retina. A new method allowed us to record currents from an intact outer segment while controlling its cytoplasmic composition by superfusion of the electropermeabilized inner segment. The sensitivity of the channels to agonists in the intact outer segment differs from that measured in membrane patches detached from the same cell. This sensitivity, measured as the ligand concentration necessary to activate half-maximal currents, $K_{1/2}$, also increases as Ca^{2+} concentration decreases. In electropermeabilized cones, $K_{1/2}$ for cGMP is 335.5 ± 64.4 μM in the presence of 20 μM Ca^{2+}, and 84.3 ± 12.6 μM in its absence. For 8Br-cGMP, $K_{1/2}$ is 72.7 ± 11.3 μM in the presence of 20 μM Ca^{2+} and 15.3 ± 4.5 μM in its absence. The Ca^{2+}-dependent change in agonist sensitivity is larger in extent than that measured in rods. In electropermeabilized tiger salamander rods, $K_{1/2}$ for 8Br-cGMP is 17.9 ± 3.8 μM in the presence of 20 μM Ca^{2+} and 7.2 ± 1.2 μM in its absence. The Ca^{2+}-dependent modulation is reversible in intact cone outer segments, but is progressively lost in the absence of divalent cations, suggesting that it is mediated by a diffusible factor. Comparison of data in intact cells and detached membrane fragments from cones indicates that this factor is not calmodulin. At 40 μM 8Br-cGMP, the Ca^{2+}-dependent change in sensitivity in cones is half-maximal at $K_{Ca} = 286 \pm 66$ nM Ca^{2+}. In rods, by contrast, K_{Ca} is $\sim$50 nM Ca^{2+}. The difference in magnitude and Ca^{2+} dependence of channel modulation between photoreceptor types suggests that this modulation may play a more significant role in the regulation of photocurrent gain in cones than in rods.

KEY WORDS: retina • phototransduction • teleost • cyclic nucleotides • light adaptation

INTRODUCTION

In complete darkness, rod photoreceptors respond to single photons, and cones are 10–100-fold less sensitive (Baylor et al., 1979; reviewed by Miller et al., 1994). However, the absolute sensitivity of the photoreceptor response is continuously adjusted to the ambient illumination, a process known as adaptation. In general, absolute sensitivity to light decreases in proportion to the increase in background illumination, and photoreceptors, therefore, convey information on the contrast between an object and its background, rather than on the absolute brightness of the object (Shapley and Enroth-Cugell, 1984). Retinal cones adapt over a much wider range of background intensities than do rods in the same species (Normann and Werblin, 1974; Malchow and Yazulla, 1986; Miller and Korenbrot, 1993b). Indeed, mechanisms of adaptation in cones are so effective that the sensitivity of the photoresponse can be adjusted to maintain cell responsiveness even when background intensities change over seven orders of magnitude (Normann and Perlman, 1979; Burkhardt, 1994). Cytoplasmic Ca^{2+} in the outer segment plays a role in adaptation in both rods and cones (reviewed by Fain and Matthews, 1990). While it is now established that light causes a fall in intracellular Ca^{2+} in the photoreceptor outer segment (McNaughton et al., 1986; Gray-Keller and Detwiler, 1994; McCarthy et al., 1996), the mechanisms of the action of Ca^{2+} are not fully understood. Ca^{2+} modulates the physiological activity of several of the biochemical events underlying phototransduction. A reduction in Ca^{2+} concentration activates guanylyl cyclase (Lolley and Racz, 1982; Pepe et al., 1986; Koch and Stryer, 1988), accelerates rhodopsin phosphorylation (Kawamura and Murakami, 1991), and reduces the light-induced catalytic activity of rhodopsin (Lagnado and Baylor, 1994).

Ca^{2+} in the cytoplasm of the photoreceptor outer segment also modulates the sensitivity of the cGMP-gated ion channels to their agonist. Sensitivity is measured by the value of $K_{1/2}$, the ligand concentration necessary to activate half maximal current. In rod outer segment membrane vesicles, a soluble factor, well mimicked by calmodulin, lowers the value of $K_{1/2}$ by $\sim$1.5-fold as Ca^{2+} concentration decreases (Hsu and Molday, 1993; Bauer, 1996). In membrane patches detached

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from outer segments of either rods or cones, exposure to a solution free of divalent cations also reduces the $K_{1/2}$ for cGMP by ~1.5-fold (Gordon et al., 1995; Hackos and Korenbrot, 1997). Since in the membrane patches the modulation is irreversibly lost after exposure to solutions free of divalent cations, the modulation has also been presumed to arise from the action of a soluble, endogenous modulator (Gordon et al., 1995; Bauer, 1996; Hackos and Korenbrot, 1997). This irreversible loss of modulation poses two serious experimental limitations. First, it is possible that the endogenous modulator is partially lost upon membrane isolation and, therefore, that modulation in the intact cell differs from that in the membrane patch. Second, the equilibrium Ca^{2+} dependence of the modulation cannot be investigated in membrane patches because stationary conditions never exist once Ca^{2+} concentration begins to be lowered. For rods, these limitations have been overcome in studies of channel modulation in truncated rod outer segments (Nakatani et al., 1995; Sagoo and Lagnado, 1996), a preparation in which the cytoplasmic content of a single, intact outer segment can be rapidly controlled while measuring currents through the cGMP-gated channels. We report here on the development of a functionally comparable preparation to study transduction in cones: an intact outer segment with an electropermeabilized inner segment (ep-cones).

In truncated rod outer segments, the maximum Ca^{2+}-dependent shift in $K_{1/2}$ for cGMP activation is ~1.5-fold, the same as that observed in detached membrane patches from the same cell. Half-maximal shift occurs at ~50 nM free Ca^{2+} (Nakatani et al., 1995; Sagoo and Lagnado, 1996). We report here that the Ca^{2+}-dependent modulation of ligand sensitivity in the intact cone outer segments is larger in extent than that observed in intact rod outer segments or in detached membrane patches of either receptor type. Moreover, in cones, unlike rods, this modulation occurs over the full physiological range of Ca^{2+} concentrations expected in the intact cell. The extent of Ca^{2+} modulation in intact cones and its Ca^{2+} dependence provides a mechanism that may contribute to the light adaptation characteristics of cones and explains, in part, the significant difference in transduction and adaptation between the two photoreceptor types.

MATERIALS AND METHODS

Materials

Striped bass were obtained from Professional Aquaculture Services (Chico, CA). Fish were maintained in an aquarium in the laboratory for up to 4 wk under 10:14 h dark:light cycles. Tiger salamanders were obtained from Charles Sullivan (Memphis, TN). They were maintained in the laboratory in an aquarium at 8°C under 12:12 h dark:light cycles. The UCSF Committee on Animal Research approved protocols for the maintenance and killing of the animals.

Cell Isolation and Solutions

Single cones were isolated from the dark-adapted retina of striped bass (*Morone saxitilis*) and rods from that of tiger salamander (*Ambystoma tigrinum*). Retinas were isolated under infrared illumination, and retinas and photoreceptors were dissociated as previously described (Miller and Korenbrot, 1993a, 1994). Dissociated cones were maintained in Ringer's solution consisting of (mM): 136 NaCl, 2.4 KCl, 5 NaHCO$_3$, 1 NaH$_2$PO$_4$, 1 CaCl$_2$, 1 MgCl$_2$, 10 glucose, 10 HEPES, pH 7.5, 0.1 mg/ml BSA supplemented with MEM vitamins and amino acids. Dissociated rods were maintained in Ringer's solution consisting of (mM): 100 NaCl, 2 KCl, 5 NaHCO$_3$, 1 NaH$_2$PO$_4$, 1 CaCl$_2$, 1 MgCl$_2$, 10 glucose, 10 HEPES, pH 7.4, 0.1 mg/ml BSA supplemented with MEM vitamins and amino acids.

A suspension of dissociated photoreceptor cells was added to a recording chamber held on the fixed stage of an inverted microscope. Under microscopic observation, using infrared illumination, the outer segment of a single photoreceptor was drawn into a suction electrode. The suction electrode was filled with a solution in all respects identical to the Ringer's solution, except that the free Ca^{2+} concentration was 1 μM. The cell held within the suction electrode was superfused with a solution delivered through a 75-μm diameter capillary positioned within 50 μm of the cell. The 75-μm capillary was the single output from a micromanifold that allowed the superfusing solution to be rapidly changed (<30 ms) with the use of electronically controlled valves (DAD-12; ALA Scientific Instruments, Inc., Westbury, NY). The superfusion solutions for single cones always consisted of (mM): 140 cholineCl, 10 HEPES, pH 7.5, 5 glucose, 0.4 Zaprinast. The solutions could contain divalent cations (20 μM Ca^{2+} and 0.5 mM Mg^{2+}) or not (no added cations, with 2 mM HEDTA). Solutions of known Ca^{2+} concentration were prepared using EGTA in the absence of Mg^{2+}, as described (Hackos and Korenbrot, 1997). Cyclic guanine monophosphate nucleotides were added to the solutions as needed. The same superfusion solutions were used with rods, but with 110 cholineCl. After electropermeabilization, the superfusion solution became the intracellular solution. Because the cGMP-gated channels are impermeable to choline, their activation under these ionic conditions and at 0-mV holding voltage generated an inward current.

Electropermeabilization

Two sharp tungsten microelectrodes, electrically insulated except at their tip (1 μm) (A-M Systems, Everett, WA), were mounted on separate micromanipulators. Under microscopic observation, they were positioned opposite each other and pressed against the surface of the inner segment of a photoreceptor cell; the outer segment of the cell was held within a suction electrode (see Fig. 1). Single, brief (1–10 ms) voltage pulses 1–5 V in amplitude for cones (1–5 kV/cm) and up to 8 V for rods (1–5 kV/cm) were applied between the metal electrodes. Successful electropermeabilization was apparent by a sudden and subtle change in the visual appearance of the inner segment membrane (see Fig. 1). Experimental results with electropermeabilized rods are similar to those obtained in truncated rod outer segments (see Fig. 4), indicating that outer segments are not harmed by electropermeabilization any more than by truncation.

Electrophysiological Recording and Data Analysis

Suction electrodes were produced from 1752 capillary glass (1.5 × 1.0 mm, o.d. × i.d.; Corning Glass Works, Corning, NY) and were

rendered hydrophobic by coating with *bis*-dimethyl-amino dimethyl-silane. Individual cone outer segments were drawn by gentle suction and the electrode sealed against the surface of the slightly wider inner segment. Seal resistances were between 0.5 and 3 MΩ. Membrane currents were recorded at zero holding voltage with a patch-clamp amplifier (EPC-7; List Electronic, Darmstadt, Germany). Analogue signals were low pass filtered (50 Hz) through an eight-pole Bessel filter and digitized online (100 Hz; Axon Instruments, Foster City, CA) with a computer-based data acquisition system (FastLab; Indec Instruments, Capitola, CA).

Because the suction electrode/cell combination is low in impedance, there is an unavoidable drift in the current data recorded experimentally. To minimize uncertainties due to this drift, we used a track-and-hold circuit before data digitization. The data acquisition computer controlled both this circuit and the valves in the superfusion system, such that current was zeroed immediately before superfusion of the ep-cone commenced. In addition, the superfusing solutions were changed at fixed intervals, 6–8-s long. These intervals were sufficiently long for current to reach a nearly unvarying value, but we did not always obtain an absolute steady state. To asses the error that may arise from this experimental compromise, we measured the current drift in the presence of constant cyclic nucleotide (a "typical bad" drift is illustrated in Fig. 3). Current drift was no worse than ±10%/min. Considering that superfusing solutions were exchanged at intervals of 6–8 s, in any given data set the error in the value of $K_{1/2}$ due to drift can be calculated to be no worse than 1%. This error is negligible when compared with the variance from cell to cell, which is the limiting source of error in our data. Mathematical functions were fit to experimental data with the use of a nonlinear, least square minimization algorithm (N-Fit, Galveston, TX). Experimental error is presented throughout as mean ± SD.

RESULTS

We investigated the modulation of the cGMP-gated channels in single cones isolated from a fish retina. We measured outer segment currents with suction electrodes in cones whose inner segment was rendered freely permeable by electropermeabilization (ep-cones) (Fig. 1). The cytoplasmic composition of the outer segment was controlled by continuous superfusion of the electropermeabilized inner segment. Activation of the cGMP-gated ion channels (the only channels in the outer segment membrane; Miller and Korenbrot, 1993*a*) by superfusion with varying concentrations of cGMP caused an inward current. The dependence on cGMP of the steady state amplitude of the current was well described by the Hill equation (Fig. 2).

$$I_{\mathrm{m}} = I_{\max} \frac{[\mathrm{cGMP}]^{\mathrm{n}}}{[\mathrm{cGMP}]^{\mathrm{n}} + [K_{1/2}]^{\mathrm{n}}}, \qquad (1)$$

where I_{m} is the membrane current, $I_{\max}$ is the maximum, saturated amplitude, [cGMP] is the concentration of the agonist, $K_{1/2}$ is the cGMP concentration that activates half maximal current, and n is an adjustable parameter that measures a cooperative action of cGMP molecules. $I_{\max}$ varied widely among electropermeabilized cones, from a few hundred picoamperes up to one nanoampere. This range likely reflects varying
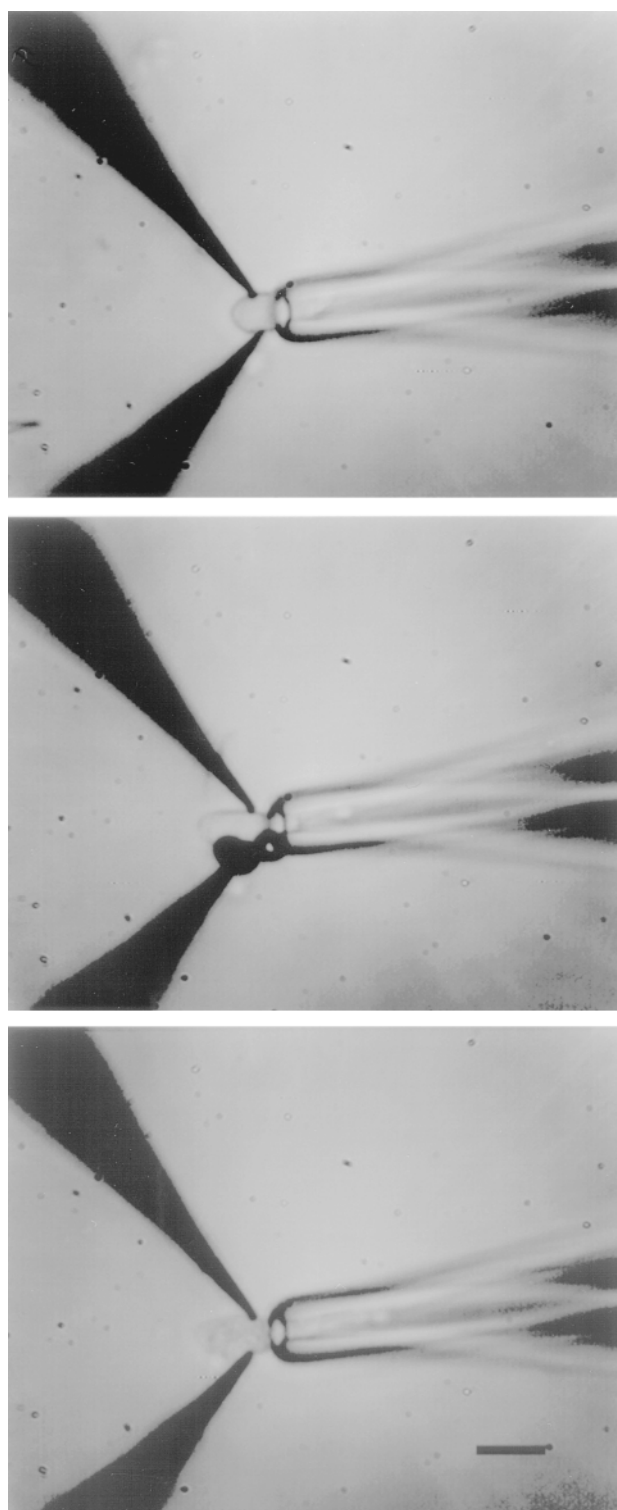
FIGURE 1. Photomicrograph illustrating the method to record outer segment currents and electropermeabilize the inner segment of a single cone isolated from the retina of striped bass. The outer segment is drawn into a suction electrode and two tungsten microelectrodes are pressed against the surface of the inner segment (*top*). A brief voltage pulse is applied between the metal electrodes causing electrolysis and local dielectric breakdown of the inner segment membrane (*middle*). Superfusion of the electropermeabilized inner segment permits continuous control of the cytoplasmic composition of the outer segment (*bottom*, calibration bar is 10 μm).
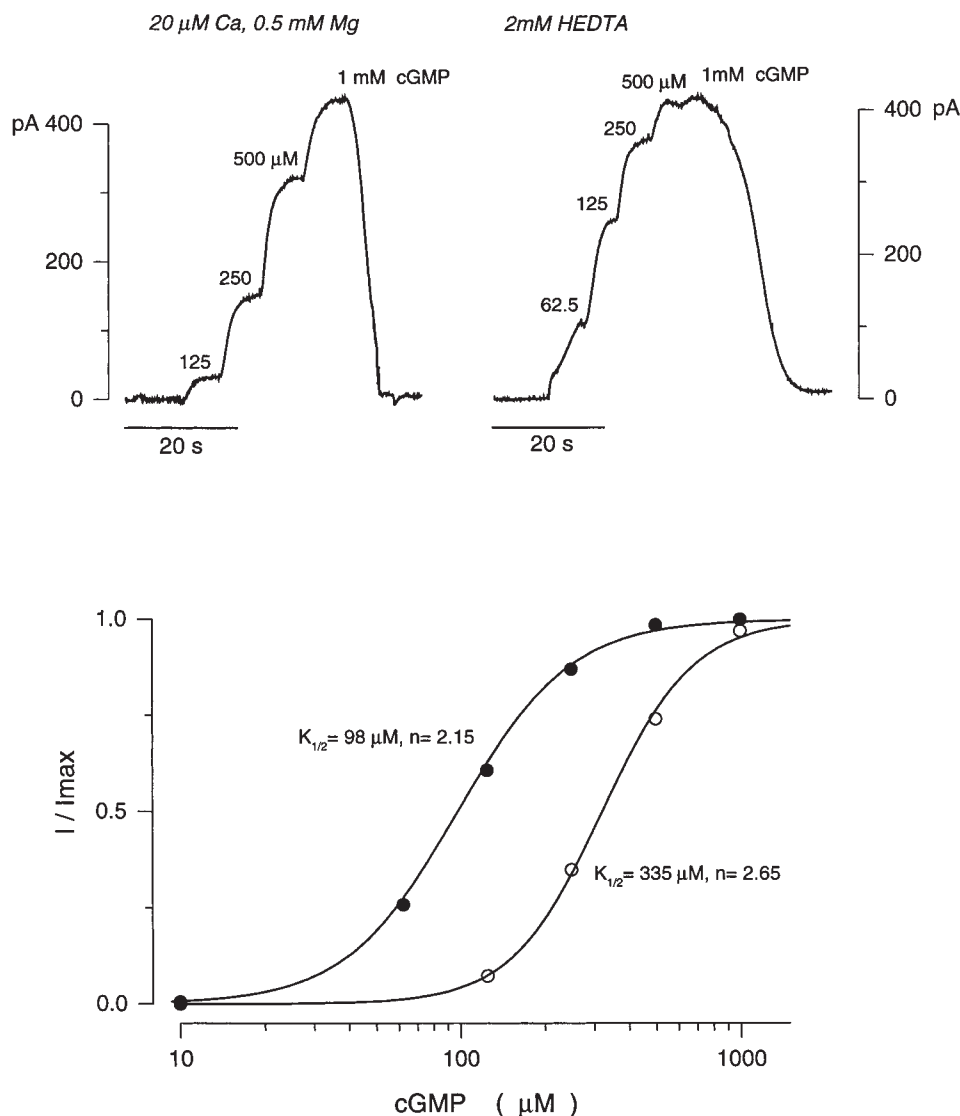
FIGURE 2. Activation of cGMP-dependent currents in the intact outer segment of electropermeabilized cones. (*top left*) Inward outer segment currents activated by increasing concentrations of cGMP in the presence of 20 μM Ca^{2+} and 0.5 mM Mg^{2+}. (*top right*) Currents measured in response to cGMP, but in the absence of divalent cations (with 2 mM HEDTA). (*bottom*) The dependence of normalized current amplitude on cGMP concentration. The continuous curves illustrate an optimized fit of the Hill equation (Eq. 1) to the experimental. For the data shown, $K_{1/2}$ = 335 μM cGMP and n = 2.65 in the presence of divalent cations (○), and $K_{1/2}$ = 98 μM cGMP and n = 2.15 in their absence (●).

lengths of functional outer segment within the suction electrode.

The parameters of the equation, $K_{1/2}$ and n, provide a measurement of the sensitivity of the channels to their ligand. Manipulation of the concentration of divalent cations caused dramatic shifts in this sensitivity (Fig. 2). In the presence of 20 μM Ca^{2+} and 0.5 mM Mg^{2+}, $K_{1/2}$ was 335.5 ± 64.4 μM cGMP (range 200–550, n = 28), and n was 2.58 ± 0.42 (range 1.66–3.52). However, in the absence of divalent cations, $K_{1/2}$ decreased to 84.3 ± 12.6 μM cGMP (range 67–103, n = 7), and n was 2.30 ± 0.37 (range 1.9–3). When Mg^{2+} was removed and Ca^{2+} maintained at 20 μM, we did not observe a shift in the cGMP dependence of current activation, though current amplitude increased due to the relief of channel block caused by Mg^{2+} at the concentration used. Thus, cytoplasmic Ca^{2+} specifically modu-

lates the ligand sensitivity of the cGMP-gated channels in the intact cone outer segment.

The experiments with cGMP in electropermeabilized cones were executed in the presence of Zaprinast, an effective inhibitor of phosphodiesterase (PDE)[1] in cones (Gillespie and Beavo, 1989). Nevertheless, to rule out the possibility that some component of channel modulation might reflect effects of residual PDE activity, we tested current activation by 8Br-cGMP in the presence of Zaprinast. 8Br-cGMP activates the channels but is poorly hydrolyzed by PDE (Zimmerman et al., 1985). Although the channel's absolute sensitivity to 8Br-cGMP is higher than to cGMP, as was previously known, the modulation by Ca^{2+} of this sensitivity is nearly the

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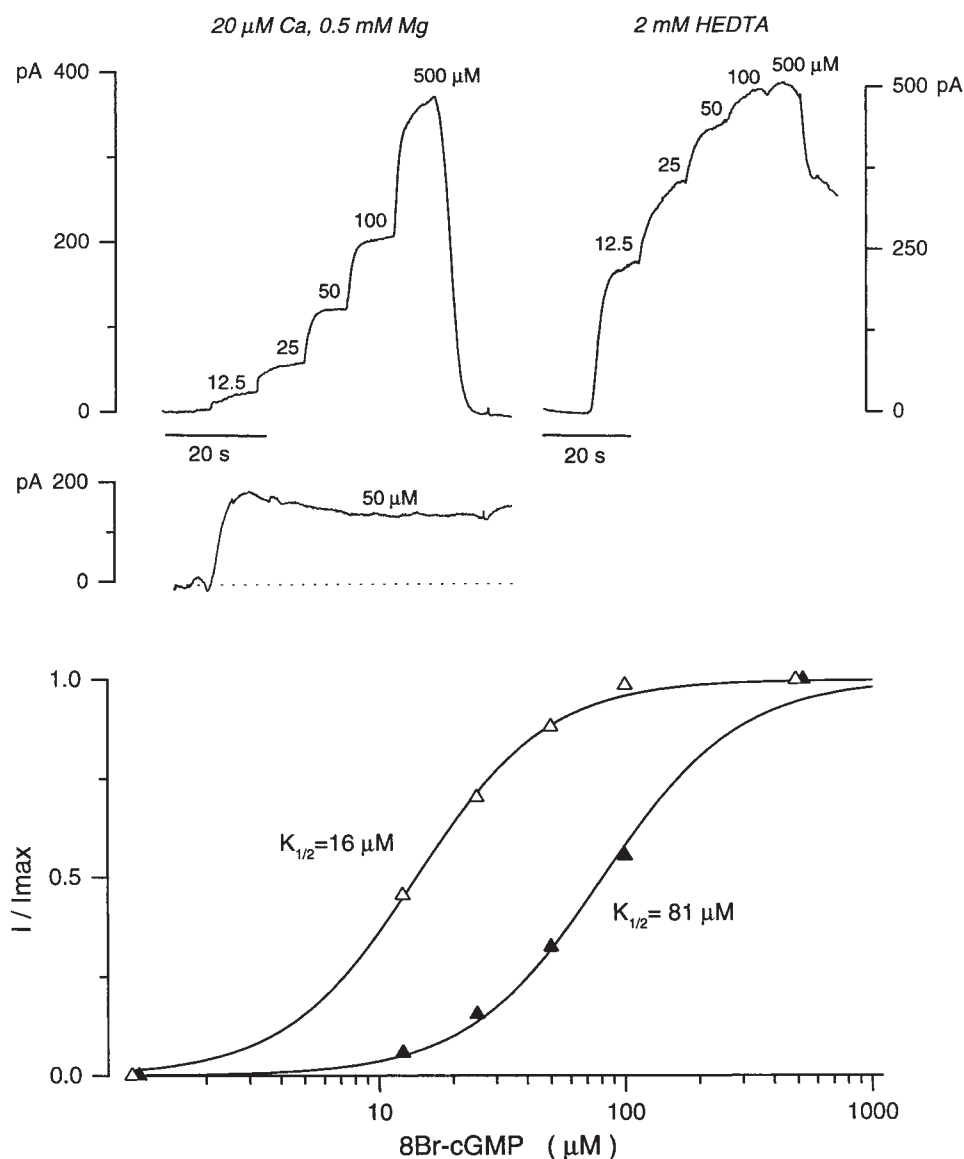[1]*Abbreviation used in this paper:* PDE, phosphodiesterase.

FIGURE 3. Ca^{2+}-dependent modulation of activation by 8Br-cGMP of the cyclic nucleotide-gated channels in electropermeabilized striped bass single cones. (*top left*) Outer segment currents measured in the presence of 20 μM Ca^{2+} and 0.5 mM Mg^{2+} in response to increasing nucleotide concentrations. Also shown is the continuous response of a different ep-cone to a single step of subsaturating 8Br-cGMP (50 μM). The example shown is atypical, but was selected to illustrate extreme drift in current amplitude and make evident its small contribution to experimental error in the determination of the values of $K_{1/2}$ and n in the Hill equation (Eq. 1). (*top right*) Currents activated by 8Br-cGMP in the absence of divalent cations. (*bottom*) The continuous curves illustrate the Hill equation that best fits the experimental data. For the data shown, $K_{1/2} = 81$ μM cGMP and n = 1.3 in the presence of divalent cations ($\blacktriangle$), and $K_{1/2} = 16$ μM cGMP and n = 1.1 in their absence ($\triangle$).

same for both cyclic nucleotides (Fig. 3). In the presence of 20 μM Ca^{2+} and 0.5 mM Mg^{2+}, $K_{1/2}$ was 72.7 ± 11.3 μM 8Br-cGMP (range 46–100, $n = 25$), and n was 1.55 ± 0.22 (range 1–1.75). Removal of divalent cations shifted $K_{1/2}$ to 15.3 ± 4.5 μM 8Br-cGMP (range 6.7–19, $n = 30$), and n to 1.26 ± 0.37 (range 1–1.69). These results indicate that PDE is inactive under our experimental conditions, and does not influence channel sensitivity to cyclic nucleotides or its modulation by Ca^{2+}.

As an additional control, we measured the response to a continuous step of subsaturating 8Br-cGMP concentration in the presence of Ca^{2+} and Mg^{2+}. If there were a "spontaneous" increase in channel sensitivity over the time scale of our observations, then the current activated by low nucleotide concentrations would be expected to increase dramatically and systematically

over the 60–100 s of a typical experimental trial. Fig. 3 illustrates the continuous response of an ep-cone to 50 μM 8Br-cGMP in the presence of 20 mM Ca^{2+} and 0.5 mM Mg^{2+}. The current changed rapidly to a new value and, in this example, it then drifted at a rate of $\sim$−30 pA/min. Drift, of course, is random and we selected this as an example of large drift. On average, drift was no worse than ±10%/min. To within this accuracy there is no evidence of spontaneous changes, and $K_{1/2}$ is in error by <±1% due to drift.

Comparison with Channel Modulation in Detached Membrane Patches

It is instructive to compare the present results with those obtained in studies of membrane patches detached from the same outer segments and studied un-
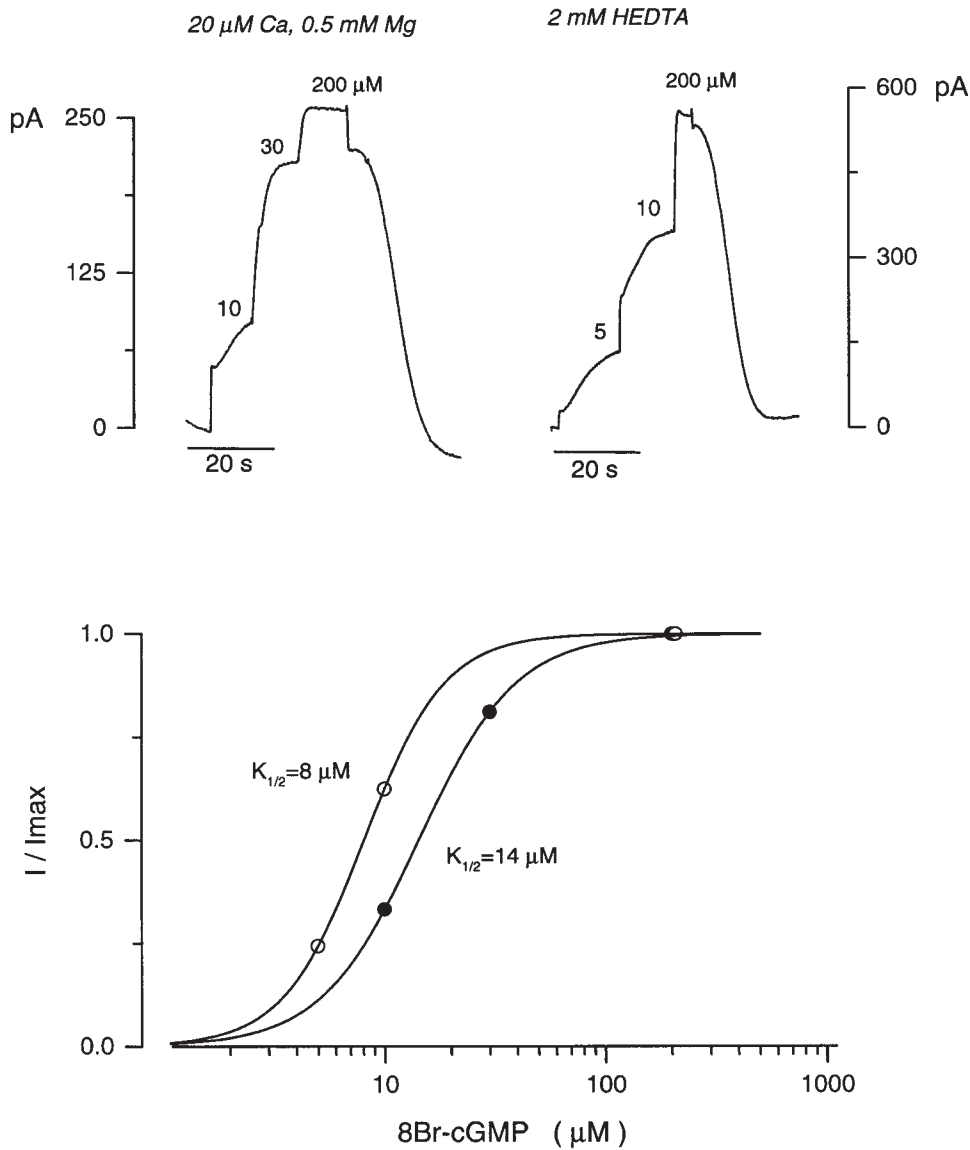
FIGURE 4. Ca^{2+}-dependent modulation of activation by 8Br-cGMP of the cGMP-gated channels in electropermeabilized tiger salamander rods. (*top left*) Outer segment currents measured in the presence of 20 μM Ca^{2+} and 0.5 mM Mg^{2+} in response to increasing nucleotide concentrations. (*top right*) Currents activated by 8Br-cGMP in the absence of divalent cations. (*bottom*) The continuous curves illustrate the Hill equation that best fits the experimental data. For the data shown, $K_{1/2} = 14$ μM cGMP and n = 2 in the presence of divalent cations (●), and $K_{1/2} = 8$ μM cGMP and n = 2.3 in their absence (○).

der similar experimental conditions. In the membrane patches at -40 mV and under 20 μM Ca^{2+} and 0.1 mM Mg^{2+}, the average values for cGMP activation were $K_{1/2} = 86$ μM and n = 2.57, and for 8Br-cGMP they were $K_{1/2} = 22$ μM and n = 2.29 (Hackos and Korenbrot, 1997). The high values of $K_{1/2}$ characteristic of intact cone outer segment, but not of detached patches, suggests that modulation of the channel sensitivity depends on a molecular process that is lost upon membrane isolation. On the other hand, the $K_{1/2}$ for both cGMP and 8Br-cGMP measured in the absence of divalent cations is essentially the same in the electropermeabilized cone and the detached membrane patch. In membrane patches in the absence of divalent cations, the average values for cGMP activation were $K_{1/2} = 58$ μM and n = 1.8, and for 8Br-cGMP they were $K_{1/2} = 16$ μM and n = 1.54 (Hackos and Korenbrot, 1997). Thus, the chan-

nels in the absence of the endogenous modulator are functionally similar in the membrane patch and the intact outer segment. Moreover, the similarity in channel sensitivity measured in the absence of endogenous modulator in intact cells and detached membrane fragments confirms the suggestion that PDE is essentially inactive in the presence of Zaprinast in our experiments.

Channel Modulation in Intact Rod Outer Segments with an Electropermeabilized Inner Segment

To compare channel modulation in the intact cone outer segment with that in the intact rod outer segments, we studied current modulation in electropermeabilized rods isolated from the tiger salamander retina (Fig. 4). In rods, removal of divalent cations caused a
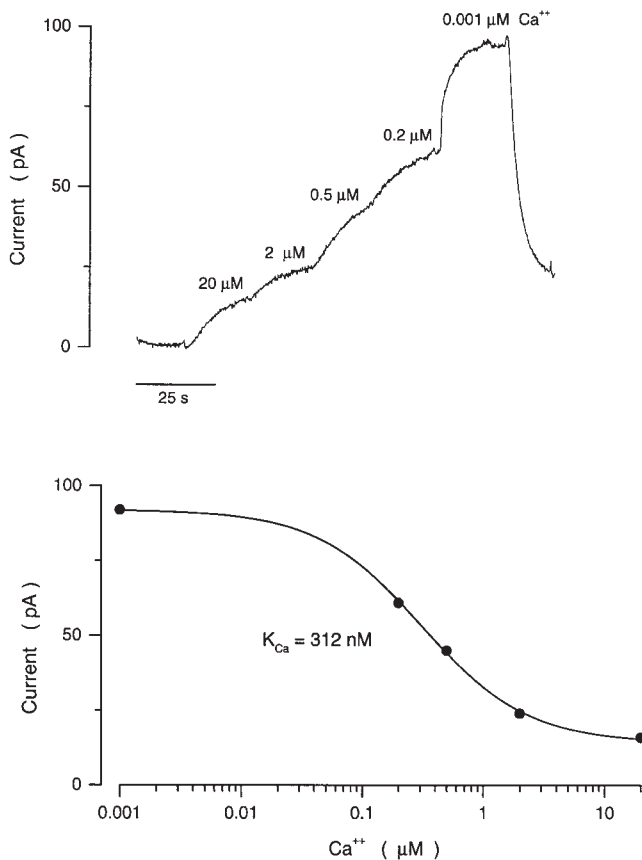
FIGURE 5. Ca^{2+} dependence of the 8Br-cGMP-activated current in an electropermeabilized single cone. The cell was electropermeabilized in the presence of 20 μM Ca^{2+} without Mg^{2+}, current was then activated with 40 μM 8Br-cGMP and the free concentration of Ca^{2+} was progressively reduced in steps to 1 nM. (*top*) Currents measured with this experimental protocol. We consistently observed that the response time constant to changes in Ca^{2+} concentration was slower than that to changes in cyclic nucleotide concentration. (*bottom*) The dependence of current amplitude on Ca^{2+} concentration. The continuous curve illustrates an optimum fit to the experimental data of the inverse Hill function (Eq. 2). For the data shown, K_{Ca} = 312 nM and n = 1.4.

Ca^{2+} Dependence of Channel Modulation

To investigate the Ca^{2+} dependence of the channel sensitivity to nucleotides, we measured the outer segment current in the presence of a constant cyclic nucleotide concentration and varying Ca^{2+} concentrations. We measured current activated by 40 μM 8Br-cGMP, a concentration at which we expected to observe currents at all Ca^{2+} concentrations of interest (see Fig. 3). We found that the current amplitude increased monotonically as Ca^{2+} decreased (Fig. 5). As Ca^{2+} was lowered, the current amplitude increased rapidly towards a new plateau value, but an absolute stationary state was not attained. Since Ca^{2+} dependence reflects the action of a diffusible factor (see below), the absence of an absolute steady state likely reflects the unavoidable and continuing loss of modulator from the ep-cone as Ca^{2+} concentration was lowered. There is an experimental compromise. We waited long enough to reach a reasonable plateau, but the plateau would never have been absolutely invariant. The dependence of the plateau on Ca^{2+} concentration was well described by an inverse Hill equation:

$$I_m = I_{max} \frac{(1/[Ca])^n}{(1/[Ca])^n + (1/K_{Ca})^n}, \qquad (2)$$

where I_m is current, I_{max} is the maximum current, [Ca] is the Ca^{2+} concentration, K_{Ca} is the Ca^{2+} concentration to reach half maximum current inhibition, and n is a parameter that indicates a cooperative interaction among Ca^{2+} ions. The average values of the adjustable parameters that best fit our data were K_{Ca} = 286 $\pm$ 66 nM Ca^{2+} (range 176–456, n = 15) and n = 1.0 $\pm$ 0.3 (Fig. 5). Our findings in cones are in sharp contrast to the results of similar experiments in intact rod outer segments where K_{Ca} is ~50 nM (Nakatani et al., 1995; Sagoo and Lagnado, 1996).

The Channel Modulator Is a Factor that Diffuses Away from the Cone Outer Segment

Channel modulation in cones likely reflects the interaction of the membrane-bound channel protein with a soluble factor. In detached membrane patches, Ca^{2+}-dependent modulation is irreversibly lost upon exposure to solutions free of divalent cations (Hackos and Korenbrot, 1997). The electropermeabilized cones, in contrast, could be exposed to divalent-free solutions, yet modulation recovered upon adding Ca^{2+} and Mg^{2+} (Fig. 6). Nonetheless, the extent of Ca^{2+}-dependent modulation was progressively and irreversibly lost with continuing exposure to solutions free of divalent cations (Fig. 6). The time course of loss of modulation caused by exposure to solutions free of divalent cations varied from cell to cell. Thus, modulation is probably mediated by a factor that binds reversibly to the chan-

modest shift in the channel sensitivity to cyclic nucleotides. We measured a $K_{1/2}$ for activation with 8Br-cGMP of 17.9 $\pm$ 3.8 μM and n of 2.06 $\pm$ 0.16 (n = 6) in the presence of 20 μM Ca^{2+} and 0.5 mM Mg^{2+} that shifted to 7.2 $\pm$ 1.2 and n to 2.1 $\pm$ 0.38 upon removal of the divalent cations. These results essentially reproduce those previously reported for truncated rod outer segments of tiger salamander (Sagoo and Lagnado, 1996) or frog (Nakatani et al., 1995) and confirm that the extent of Ca^{2+}-dependent channel modulation in intact cones is larger than that in intact rods. These results also indicate that electropermeabilization does not damage the outer segment any more than truncation does.
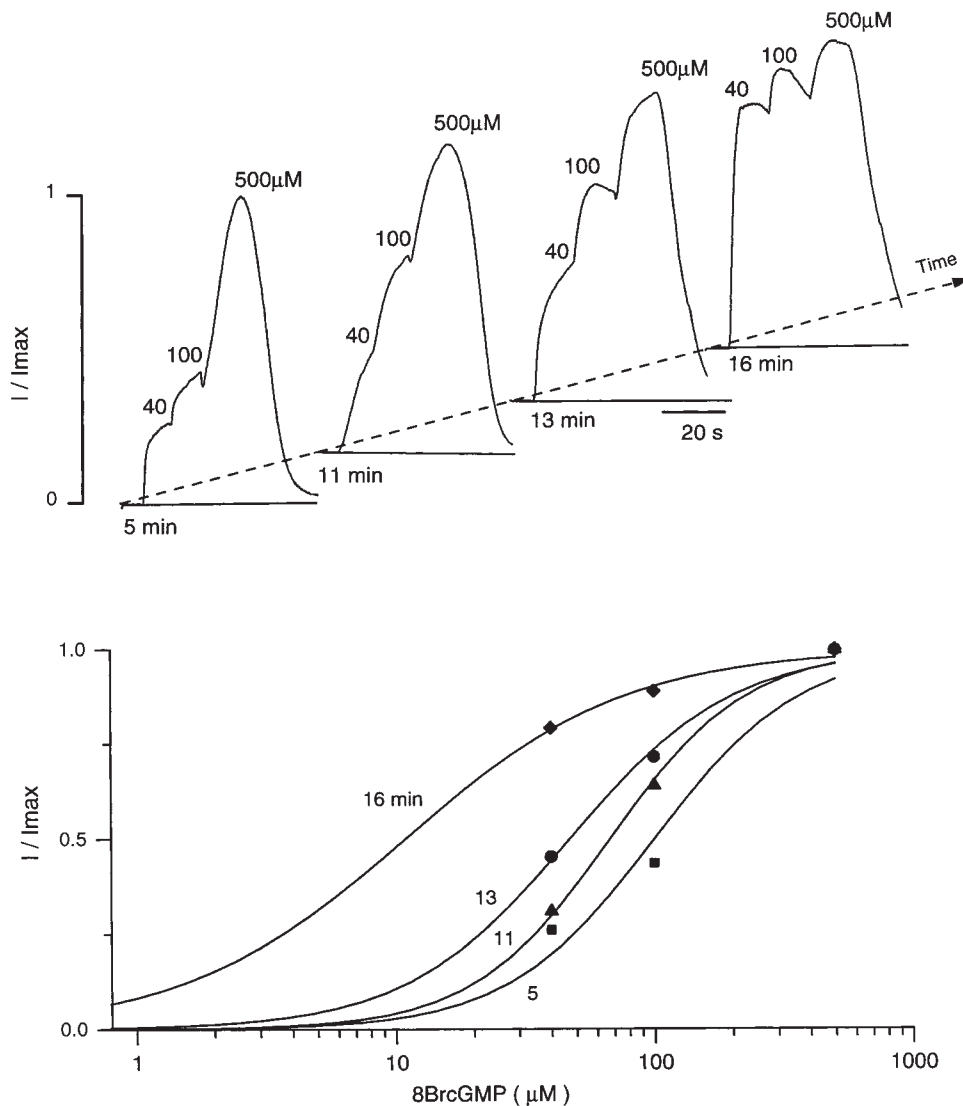
FIGURE 6. Modulation of the cGMP-gated current slowly washes out from the cone outer segment after intermittent exposure to Ca^{2+}-free solutions. The dependence on 8Br-cGMP of the outer segment current in the presence of 20 μM Ca^{2+} and 0.5 mM Mg^{2+} was measured in successive trials in the same cells. In between trials, the electropermeabilized cone was superfused with a solution free of divalent cations for 1–3 min. (*top*) The currents measured in the same cell in trials conducted at 5, 11, 13, and 16 min after the electropermeabilization. The continuous curves in the bottom panel illustrate Hill functions optimally fit to the experimental data. The values of $K_{1/2}$ and n at each successive trial are: 5 min (■) 100 μM, n = 1.52; 11 min (▲) 75 μM, n = 1.42; 13 min (●) 54 μM, n = 1.07; and 16 min (◆) 12 μM, n = 1.

nel in a Ca^{2+}-dependent manner. In the continuing absence of divalent cations, the factor is free and it slowly diffuses away from the electropermeabilized cell.

DISCUSSION

We have investigated the modulation of cGMP-gated ion channels in intact cone outer segments and have found that the absolute sensitivity of the channels to activation by cyclic nucleotides is a function of cytoplasmic Ca^{2+} concentration. The affinity of the channels for cGMP or 8Br-cGMP decreases as Ca^{2+} concentration rises. Ca^{2+}-dependent modulation of nucleotide sensitivity of cyclic nucleotide-gated ion channels has been previously observed in other sensory cells (reviewed in Molday, 1996). In olfactory sensory neurons, for example, the modulation is large, the sensitivity changes >100-fold between its extremes (Kramer and Siegelbaum, 1992; Chen and Yau, 1994; Lynch and Linder-

man, 1994; Liu et al., 1994; Balasubramanian et al., 1996), and the modulation plays an important role in adaptation to odors (Kurahashi and Menini, 1997). In rod photoreceptors, in contrast, the sensitivity changes by only ~1.5–2-fold (Nakatani et al., 1995; Sagoo and Lagnado, 1996; this report). The extent of modulation in cones, four- to fivefold, is in between that of the other two cell types. The quantitative features of the modulation of chemical sensitivity, then, are not universal; rather, they are likely optimized for the physiological role of the channels in the various sensory cells.

The extent of channel modulation in intact cone outer segments is not only larger than that in rods, but is also larger than that measured in membrane patches detached from the same cell (Hackos and Korenbrot, 1997) (Table I). The $K_{1/2}$ for activation by cGMP in the presence of Ca^{2+} in the intact cone, ~335 μM, is approximately five times larger than that measured in the membrane patches. Reassuringly, in the absence of

Agonist Sensitivity of Cyclic Nucleotide-gated Ion Channels in Intact Outer Segments or Detached Membrane Patches from Rods and Cones*

	Cone				Rod			
	Intact outer segment		Membrane patch		Intact outer segment		Membrane patch	
	$K_{1/2}$	n	$K_{1/2}$	n	$K_{1/2}$	n	$K_{1/2}$	n
cGMP								
High Ca²⁺‡	335.5 ± 64.4(1)	2.58 ± 0.42	86.1 ± 18 (2)	2.57 ± 0.34	37.8 ± 8 (3)	2.3 ± 0.5	41.1 ± 7 (2)	2.58 ± 0.43
					40 (4)	2.4		
0 Ca²⁺	84.3 ± 12.6	2.30 ± 0.37	58.8 ± 19.3	1.8 ± 0.23	28 ± 6	2.2 ± 0.6	27.5 ± 6.2	1.97 ± 0.28
					27	2		
8BrcGMP								
High Ca²⁺‡	72.7 ± 11.3(1)	1.55 ± 0.22	22.1 ± 3.1 (2)	2.29 ± 0.39	17.9 ± 3.8 (1)	2.06 ± 0.16		
0 Ca²⁺	15.3 ± 4.5	1.26 ± 0.37	16.4 ± 3.8	1.54 ± 0.35	7.2 ± 1.2	2.1 ± 0.38	4 (5)	2.5

*Data are taken from reports in which the modulatory effects of Ca^{2+} were investigated. Other thorough studies have reported the values of $K_{1/2}$ and n in the absence of divalent cations (reviewed in Zagotta and Siegelbaum, 1996). Data in those reports are in general agreement with those recapitulated in this table. ‡In the various reports quoted, High Ca^{2+} was anywhere between 1 and 20 μM, and Mg^{2+} was between 0.5 and 1 mM. 1. This report. 2. Hackos and Korenbrot (1997), striped bass, tiger salamander. 3. Sagoo and Lagnado (1996), tiger salamander. 4. Nakatani et al. (1995), bullfrog. 5. Gordon et al. (1995), frog.

Ca^{2+}, the $K_{1/2}$ is similar in the intact cone and detached membrane patches (Picones and Korenbrot, 1992; Hackos and Korenbrot, 1997). Thus, membrane patches detached in the continuing presence of 20 μM Ca^{2+} exhibit some modulation of $K_{1/2}$, but much less than that of the intact cell. This result suggests that modulation is mediated by a factor that is lost upon membrane isolation, even in the presence of 20 μM Ca^{2+}. The small extent of modulation observed in the detached patch could reflect the remaining presence of one modulator, but it is also possible that there exist two different modulators, each with a distinct membrane-binding behavior. Recent studies in cyclic nucleotide-gated channel from mammalian olfactory neurons indicate that Ca^{2+}-dependent modulation of $K_{1/2}$ in these cells likely reflects the activity of two different modulators. One is calmodulin and the other, as yet unidentified, is characterized by its insensitivity to calmodulin blockers, such as mastoparan, and its irreversible loss upon exposure to high Mg^{2+} (3 mM) (Balasubramanian et al., 1996).

The modulator in the intact cone outer segments is progressively lost, except when the Ca^{2+} and Mg^{2+} concentrations are kept high (20 μM and 0.5 mM, respectively). The extent of the loss can be limited by exposing the outer segment to Ca^{2+} and Mg^{2+} free solution for only brief periods of time (see Fig. 6). If outer segments are only briefly exposed to divalent free solutions, the $K_{1/2}$ for cGMP can be reversibly shifted from its high to its low value (Fig. 6). This reversibility observed in the absence of added triphosphate nucleotides suggests that the mechanism of modulation does not depend on the activity of a coupled system of phosphatase/kinase.

The molecular identity of the endogenous channel modulator is yet to be determined. It is likely to be a factor that binds reversibly to the channel in a Ca^{2+}-dependent manner. Calmodulin is a candidate channel modulator in rods (Hsu and Molday, 1993; Gordon et al., 1995; Bauer, 1996). In detached cone membrane patches, adding calmodulin fails to restore Ca^{2+}-dependent channel modulation with features that resemble those of the intact outer segments (Hackos and Korenbrot, 1997), or is altogether ineffective (Haynes and Stotz, 1997). This molecule, therefore, is almost certainly not the native modulator in cones. Nonetheless, because in cone membrane patches calmodulin modulates the channels to some extent (Hackos and Korenbrot, 1997), the authentic modulator probably shares structural homology with calmodulin. Other Ca^{2+}-binding proteins found in photoreceptors, such as GCAP1, GCAP2, and recoverin, also fail to mimic the effects of the endogenous modulator (Hackos and Korenbrot, 1997).

Differences between Rods and Cones

Table I summarizes the values of $K_{1/2}$ and n in the Hill equation that define the sensitivity to cyclic nucleotides of the channels in striped bass cones and tiger salamander rods and the extent of their modulation by Ca^{2+}. As was previously known, the absolute sensitivity to cyclic nucleotides is higher in rods than in cones, both in membrane patches and in the intact outer segment. In rods, there is a relatively small Ca^{2+}-dependent modulation of this sensitivity and the extent of modulation is similar in intact outer segments and detached membrane patches. In cones, the modulation in detached patches is small, comparable to that in rods, but it is much larger in the intact outer segment. In the intact outer segments, the Ca^{2+} dependence of the modulation has very different midpoints in rods and

cones. Because the modulator is continuously lost both from the ep-cones and the truncated or ep-rods, the value of this titration midpoint was measured only at a single cyclic nucleotide concentration and absolute equilibrium was not attained. This experimental caveat applies equally to measurements in rods (Nakatani et al., 1995; Sagoo and Lagnado, 1996), yet under similar experimental protocols the difference between rods and cones is striking.

Sagoo and Lagnado (1996) have suggested that modulation in rods may be initially larger than the 1.5–2.5-fold observed in a stationary state. They made this suggestion based on the observation that immediately after truncation of tiger salamander rods in low Ca^{2+}, there is an initial large current that decays spontaneously. We note, however, that these rapid changes were observed after truncation in the presence of ATP, GTP, and cGMP and without added PDE inhibitor. We have not observed this rapid, spontaneous change in current amplitude under our experimental conditions in electropermeabilized rods or cones. Since we electropermeabilize in the presence of the standard intracellular solution, which lacks nucleotides and contains Zaprinast, we speculate that the transient changes observed by Sagoo and Lagnado (1996) reflect other biochemical events, in addition to specific changes in the agonist sensitivity of the channels.

The differences between rods and cones discussed here are based on data from only a few species: modulation in rods has been studied in cells from bovine (Hsu and Molday, 1993; Bauer, 1996), frog (Gordon et al., 1995; Nakatani et al., 1995), and tiger salamander (Sagoo and Lagnado, 1996), and cones have been studied in only two species of fish (this report; Hackos and Korenbrot, 1997; Haynes and Stotz, 1997). To be certain of the generality of our observations, additional species must be investigated. Nonetheless, the fundamental differences in the biophysical features of the cGMP-gated channels of rods and cones are not only consistently different among the various species studied, but also exist in cloned alpha subunits of the channels of bovine rods and cones (Frings et al., 1995).

Physiological Implications of the Results

The photoresponse in retinal cones is less sensitive to light, faster in time course, and adapts over a much wider range of intensities than does that of rods in the same species (reviewed in Miller et al., 1994). The mechanisms that explain the difference in the transduction signal between receptor types are not understood in detail. However, we now know that the relative Ca^{2+} permeability of the cGMP-gated ion channels is higher in cones than in rods (Picones and Korenbrot, 1995; Frings et al., 1995). Also, the rate of Ca^{2+} clear-

ance from the outer segment is faster in the cone outer segment than in that of rods (Hestrin and Korenbrot, 1990; Perry and McNaughton, 1990). Since the cytoplasmic Ca^{2+} is controlled by the continuous balance between Ca^{2+} influx through cGMP-gated ion channels and its clearance through Na^+/Ca^{2+},K exchangers (Yau and Nakatani, 1985), cones can be expected to exhibit light-dependent changes in cytoplasmic Ca^{2+} concentration in the outer segment that are larger and faster than those in rods (Miller and Korenbrot, 1994; Korenbrot, 1995).

The link between changes in cytoplasmic Ca^{2+} and control of gain, kinetics, and adaptation reflects the action of Ca^{2+} on several biochemical events that underlie the phototransduction signal, among them the agonist sensitivity of the cGMP-gated ion channels. In rods, modulation of the cGMP-gated channels has been deemed to be of relatively little physiological importance because the maximum shift in agonist sensitivity is small in extent and it occurs at Ca^{2+} concentrations expected only at bright light levels (Koutalos and Yau, 1996). The same line of thought suggests that channel modulation in cones, unlike rods, is of significant importance since it is larger in extent and, particularly, its Ca^{2+} sensitivity overlaps the cytoplasmic Ca^{2+} concentration expected in the cone outer segment in darkness.

The difference in channel modulation between rods and cones may help explain the difference in the range of light intensities over which the photoreceptors adapt. In response to a step of light, the concentration of cGMP decreases to a new stationary value, the channels close and cytoplasmic Ca^{2+} declines. In cones, but not rods, the ligand sensitivity of the channels will rise. Consequently, it can then be expected that channels will reopen, in spite of the lowered cGMP level. That is, the standing outer segment current will fall at first, and then recover steadily as the channel sensitivity rises. The Ca^{2+} feedback on the channels opposes the direct action of light, thus recovering some of the operating range of the photocurrent. Constant intensity light flashes, when superimposed on increasing backgrounds of light, cause progressively smaller changes in cytoplasmic cGMP concentration. Because the channel sensitivity to cGMP increases as the background intensity rises, the constant flashes will close a proportionally larger number of channels in light (low Ca^{2+}) than might have been predicted from their behavior in darkness (high Ca^{2+}). This is the hallmark of light adaptation. Again, the feedback action of Ca^{2+} on channel sensitivity opposes the direct action of light and can cause the lowering of photocurrent gain characteristic of light adaptation.

The difference between rods and cones and the role that channel modulation by Ca^{2+} might play can be es-
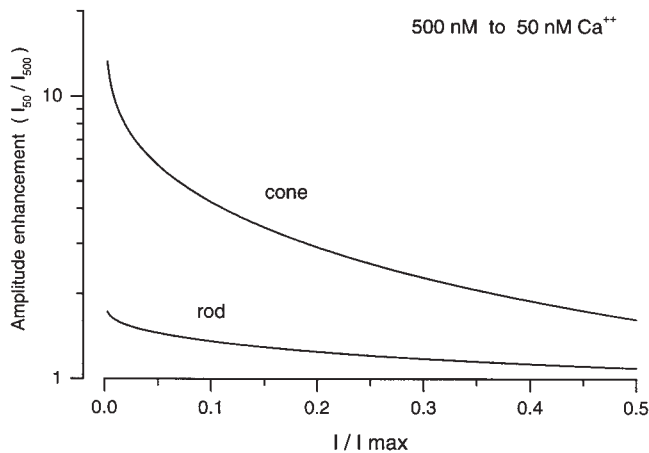
500 nM to 50 nM Ca⁺⁺

FIGURE 7. Computation of the extent of enhancement in the steady state current amplitude as a function of cGMP concentration caused by a fixed fall in cytoplasmic Ca^{2+}. Current enhancement is expressed as a ratio of the current at low over high Ca^{2+}, where low is 50 and high is 500 nM. To compare data for rods and cones, the cGMP concentration, using Eq. 1, is expressed in dimensionless units that correspond to the fraction of the maximum possible current in the outer segment. In the dark and under normal physiological conditions, the standing outer segment current is 1–3% of the maximum possible current in the outer segment of both rods and cones. Shown are predictions for single fish cones and tiger salamander rods based on the data presented in this report. In a dark-adapted rod, 500–50 nM is the likely excursion of cytoplasmic Ca^{2+} in response to a bright flash (Grey-Keller and Detwiler, 1994; McCarthy et al., 1996; Sampath et al., 1998).

timated through a simple calculation. This calculation does not attempt to predict differences on the comprehensive effects of Ca^{2+} in intact rods and cones, since such calculation requires the quantitative definition of all processes modulated by Ca^{2+}. The simple calcula-

tion summarizes the observations we have made. If we assume that the modulation by Ca^{2+} on the channels shifts only $K_{1/2}$ and n, then it is possible to estimate the extent of the current amplitude enhancement caused by the given decrease in Ca^{2+} concentration. The magnitude of the current enhancement is a function of the cGMP concentration at which it is tested, and is given by Hackos and Korenbrot (1997):

$$\frac{I_{lo}}{I_{hi}} = [cGMP]^{n_{lo}-n_{hi}}\left(\frac{[cGMP]^{n_{hi}} + K_{hi}^{n_{hi}}}{[cGMP]^{n_{lo}} + K_{lo}^{n_{lo}}}\right), \qquad (3)$$

where I_{lo} and I_{hi} are the currents at low and high Ca^{2+}, respectively, [cGMP] is the ligand concentration, K_{lo} and K_{hi} are the values for $K_{1/2}$, and n_{lo} and n_{hi} are the values for n at low and high Ca^{2+}, respectively. Results of such calculations for rods and cones are illustrated in Fig. 7 for a change in Ca^{2+} from 500 to 50 nM. This is the range of decline in Ca^{2+} concentration between darkness and bright light measured in rod outer segments (Gray-Keller and Detwiler, 1994; McCarthy et al., 1996; Sampath et al., 1998). To compare rods and cones, we converted cGMP concentration to the fraction of the maximum, saturated current in the outer segment (using Eq. 1). This calculation makes quantitatively evident that the effect of channel modulation over the physiologically expected range of Ca^{2+} changes is much more significant in cones than in rods.

It is also interesting to note that the effectiveness of modulation increases as the probability of channel opening decreases. This observation may provide an evolutionary advantage to the puzzling fact that in photoreceptors the entire transduction signal operates by changing the probability of opening of the cGMP-gated channel from essentially 0 to only 3–5% at its highest value.

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