

# In Intact Mammalian Photoreceptors, $\text{Ca}^{2+}$ -dependent Modulation of cGMP-gated Ion Channels Is Detectable in Cones but Not in Rods

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**ABSTRACT** In the mammalian retina, cone photoreceptors efficiently adapt to changing background light intensity and, therefore, are able to signal small differences in luminance between objects and backgrounds, even when the absolute intensity of the background changes over five to six orders of magnitude. Mammalian rod photoreceptors, in contrast, adapt very little and only at intensities that nearly saturate the amplitude of their photoreponse. In search of a molecular explanation for this observation we assessed  $\text{Ca}^{2+}$ -dependent modulation of ligand sensitivity in cyclic GMP-gated (CNG) ion channels of intact mammalian rods and cones. Solitary photoreceptors were isolated by gentle proteolysis of ground squirrel retina. Rods and cones were distinguished by whether or not their outer segments bind PNA lectin. We measured membrane currents under voltage-clamp in photoreceptors loaded with Diazo-2, a caged  $\text{Ca}^{2+}$  chelator, and fixed concentrations of 8Br-cGMP. At 600 nM free cytoplasmic  $\text{Ca}^{2+}$  the midpoint of the cone CNG channels sensitivity to 8Br-cGMP,  $^{8\text{Br-cGMP}}K_{1/2}$ , is  $\sim 2.3 \mu\text{M}$ . The ligand sensitivity is less in rod than in cone channels. Instantly decreasing cytoplasmic  $\text{Ca}^{2+}$  to  $<30 \text{ nM}$  activates a large inward membrane current in cones, but not in rods. Current activation arises from a  $\text{Ca}^{2+}$ -dependent modulation of cone CNG channels, presumably because of an increase in their affinity to the cyclic nucleotide. The time course of current activation is temperature dependent; it is well described by a single exponential process of  $\sim 480 \text{ ms}$  time constant at  $20\text{--}21^\circ\text{C}$  and  $138 \text{ ms}$  at  $32^\circ\text{C}$ . The absence of detectable  $\text{Ca}^{2+}$ -dependent CNG current modulation in intact rods, in view of the known channel modulation by calmodulin in-vitro, affirms the modulation in intact rods may only occur at low  $\text{Ca}^{2+}$  concentrations, those expected at intensities that nearly saturate the rod photoreponse. The correspondence between  $\text{Ca}^{2+}$  dependence of CNG modulation and the ability to light adapt suggest these events are correlated in photoreceptors.

**KEY WORDS:** retina • phototransduction • ion channel • signal transduction • light adaptation

## INTRODUCTION

Photoreceptors in the vertebrate retina signal over a remarkably wide range of stimuli intensities because they are able to “adapt”. Neural adaptation is a term used to denote the fact that, at light levels that do not bleach a significant fraction of the visual pigment, photoreceptor cells adjust the gain and kinetics of their transduction process to continue reporting small differences between an object and the background, regardless of the absolute intensity of that background (for reviews see Perlman and Normann, 1998; Fain et al., 2001). The range of stimuli intensities over which photoreceptors adapt, however, is not the same across species nor is it the same for rods and cones in the same species. In normal human subjects, for example, the response of cones to constant intensity test flashes is unchanged in amplitude when background intensity increases  $\sim 100$ -fold above darkness. Over the next 1,000-fold change in background brightness, response amplitude declines linearly with the log of intensity (Weber law), with a half-loss in response amplitude at  $\sim 3,000$  photopic trolands (Paupoo et al.,

2000). In rods, in contrast, response amplitude to a constant flash begins to decrease as soon as background intensity rises above darkness and it is reduced by half when background illumination is only  $\sim 70$  trolands (Thomas and Lamb, 1999). This feature allows the cone-driven visual system, but not the rod-driven one, to detect contrast between objects and background under natural luminance that can change in intensity as much as 10 orders of magnitude (Rodieck, 1998).

In nonmammalian vertebrates both rods and cones demonstrate significant light-adaptation, although even among these species cones adapt over a far wider range of background intensities than do rods (rods, Kleinschmidt and Dowling, 1975; Fain, 1976; Baylor et al., 1979; cones, Baylor and Hodgkin, 1974; Malchow and Yazulla, 1986; Burkhardt, 1994; Perlman and Normann, 1998). Electrophysiological studies of mammalian rods demonstrate that neural adaptation in these cells is very limited (Penn and Hagins, 1972; Baylor et al., 1984). In fact, it was believed not to occur at all and only relatively recent studies have shown that some adaptation does occur, but only at light intensities that nearly saturate the photocurrent amplitude (a few hundred photoexcited Rhodopsin molecules per cell) (Tamura et al., 1989; Nakatani et al., 1991). In contrast,

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mammalian cones readily light adapt (Kraft, 1988; Schnapf et al., 1990; Schneeweis and Schnapf, 1999).

The detailed molecular mechanisms that explain photoreceptor adaptation are not known, nor are the mechanisms of the specific differences between rods and cones. It is clear, however, that adaptation in both rods and cones requires light-dependent changes in cytoplasmic-free  $\text{Ca}^{2+}$  which, in turn, control and modulate several of the molecular events underlying signal transduction (for review see Pugh and Lamb, 2000). Among these, we have recently reported that in intact cones from a fish, CNG ion channels in the outer segment, those regulated by light, exhibit a large  $\text{Ca}^{2+}$ -dependent modulation of their sensitivity to cyclic nucleotide concentration.  $^{\text{cGMP}}K_{1/2}$ , the midpoint of the dependence of channel activation on cyclic nucleotide concentration, decreases about three-fold as  $\text{Ca}^{2+}$  concentration decreases, and the midpoint of this change is at  $\sim 860$  nM  $\text{Ca}^{2+}$  (Rebrik and Korenbrot, 1998; Rebrik et al., 2000). In intact nonmammalian rods the  $\text{Ca}^{2+}$ -dependent CNG channel modulation is smaller and occurs at much lower  $\text{Ca}^{2+}$  concentration:  $^{\text{cGMP}}K_{1/2}$  only decreases  $\sim 1.5$ -fold as  $\text{Ca}^{2+}$  concentration decreases and midpoint of this change is at  $\sim 50$  nM  $\text{Ca}^{2+}$  (Nakatani et al., 1995; Sagoo and Lagnado, 1996; Rebrik and Korenbrot, 1998). Because the effect of modulation is to reset the CNG channel sensitivity it is evident that it could play an important role in adaptation: as background light intensity increases, cytoplasmic  $\text{Ca}^{2+}$  concentration decreases proportionately (Gray-Keller and Detwiler, 1996) and therefore channels can be equally activated by progressively smaller changes in cGMP concentration (Rebrik and Korenbrot, 1998).

A thorough test of the physiological role of CNG channel modulation in cones will ultimately require the molecular identification of the modulator molecule, and unambiguous tests of its function, perhaps through transgenic manipulations. For now, we have taken advantage of the natural difference between mammalian rods and cones to investigate a role in light adaptation of the modulation of CNG ion channels. To this end, we report here on the development of a preparation of solitary rod and cone photoreceptors isolated from the retina of ground squirrel and amenable to electrophysiological investigation. We have assessed in both cell types whether the activity of CNG channels is modulated by changes in cytoplasmic  $\text{Ca}^{2+}$  and find that detectable modulation occurs in cones, but not in rods. We characterized some functional features of the mammalian cone CNG channel modulation.

## MATERIALS AND METHODS

### Materials

California ground squirrels were field trapped in the summer months and maintained in the laboratory for up to 48 h under

10:14 h dark:light cycles. The UCSF Committee on Animal Research approved protocols for the maintenance and sacrifice of the animals. Papain and collagenase were obtained from Worthington Biochemicals, DNaseI from Roche Applied Science, Di-azo-2 and bis-Fura-2 from Molecular Probes, FITC-labeled peanut agglutinin (FITC-PNA) from E-Y Labs, and Zaprinast from CalBiochem. Tissue culture media of standard composition were obtained through the tissue culture facility at UCSF. All other chemicals and enzymes were from Sigma-Aldrich.

### Cell Isolation

Animals were killed in the light, but all subsequent tissue processing was conducted under dim red light. Eyes were enucleated, the front half cut away and the remaining eyecup cut into three or four pieces that were then submerged in standard L-15 culture medium with 10 mM glucose. These eyecup pieces remained viable sources of solitary photoreceptors for as long as 12 h when maintained at  $4^{\circ}\text{C}$  in darkness. At room temperature the retina was separated from the eyecup by gently teasing it away from the pigment epithelium. The isolated retina was transferred to 7 ml of the L-15/glucose medium containing, in addition, 10 mM taurine and hyaluronidase, and 3 mg each of collagenase and DNaseI. Tissue was incubated 2 min at room temperature and was then transferred in a minimum volume into 200  $\mu\text{l}$  of L-15/glucose medium containing 19 U/ml papain maintained at  $32^{\circ}\text{C}$ . After a 7-min incubation at  $32^{\circ}\text{C}$ , the retina was again transferred in a minimum volume to the room temperature solution containing multiple enzymes. After 30 s, the retina was washed free of enzymes and glucose by successively transferring it in minimum volume through two 10 ml baths of L-15/pyruvate medium, in which glucose was replaced with pyruvic acid (5 mM).

Cells were dissociated by mechanically chopping the enzymatically treated retina. The tissue was submerged in 200  $\mu\text{l}$  of L-15/pyruvate and manually cut with a blade into  $\sim 0.5$ -mm thick sections along two orthogonal axes. Leaving discernable tissue bits behind, the suspending medium was carefully collected with a cut pipette plastic tip and transferred onto an electrophysiology recording chamber. The bottom of this chamber was a glass coverslip covalently coated with Concanavalin A (3 mg/ml) (Pi-cones and Korenbrot, 1992). Solitary photoreceptors firmly attached to the coverslip and after 15 min the cell-bathing solution was exchanged with L-15/glucose medium with 0.1 mM  $\text{Ca}^{2+}$  and 0.1 mg/ml BSA. This bath solution was intermittently perfused throughout the experimental session.

### Electrophysiological Recording

The electrophysiology recording chamber was held on the stage of an inverted microscope equipped with DIC contrast enhancement. Under deep red illumination ( $>620$  nm), and observing with the aid of a CCD camera and monitors, tight-seal electrodes were applied onto the side of photoreceptor inner segments. Electrodes were produced from borosilicate glass (N51A,  $1.5 \times 0.8$  mm od  $\times$  id; Garner Glass Company). We measured membrane currents under voltage-clamp with a patch clamp amplifier (Axopatch 1D; Axon Instruments, Inc.). Analogue signals were low pass filtered below 200 Hz with an eight pole Bessel filter (Kronh-Hite) and digitized on line at 500 Hz (pClamp; Axon Instruments, Inc.) or at 1 Hz (extended time records) (2800 Digital oscilloscope; A-M Systems).

All experiments were conducted at room temperature ( $20$ – $21^{\circ}\text{C}$ ), except those explicitly designed to test the effects of temperature. To control temperature, the recording chamber on the microscope stage was held on a specially designed copper stage. The temperature of the copper stage was controlled with Peltier cells powered by a feedback-regulated, adjustable DC power

source (PTC-10; ALA Instruments). Temperature in the recording chamber was maintained within  $\pm 0.5^\circ\text{C}$ .

Electrode filling solution was freshly prepared and used within 3 h or discarded. A salt solution consisting of (in mM):  $\text{K}^+$  gluconate (130), KCl (10), and HEPES (10), pH 7.22, was made thoroughly free of multivalent cations by passing it over Chelex100 resin (BioRad Labs). Under deep red light we added (final concentrations): 1 mM dark Diazo-2, 1.04 mM total  $\text{MgCl}_2$  (1 mM free), 183  $\mu\text{M}$  total  $\text{CaCl}_2$  (600 nM free), 400  $\mu\text{M}$  Zaprinast, and various concentrations of 8Br-cGMP. Dark Diazo-2 (caged BAPTA) is a  $\text{Ca}^{2+}$  chelator that shifts its  $\text{Ca}^{2+}$  binding constant upon bright flash illumination (Adams et al., 1989). Zaprinast is an effective inhibitor of photoreceptor phosphodiesterase (PDE). The solution lacked triphosphate nucleotides and, therefore, could not sustain endogenous synthesis of cGMP or phototransduction (Hestrin and Korenbrot, 1987; Rispoli et al., 1993).

### Fluorescence Imaging, Flash Uncaging, and $\text{Ca}^{2+}$ Concentration

The instrument used in these experiments was designed to operate as an inverted microscope with both DIC contrast and epifluorescent illumination (details in Ohyama et al., 2000). Cells were observed with a near-UV transmitting, high numerical aperture objective (Fluo 40 $\times$ /1.3 NA, Nikon Optics). Fluorescence was excited using a DC-operated Xe light source and fluorophor-specific optical cubes to select excitation and emission spectra (Chroma Technology). We captured images using a cooled, high-resolution interlined CCD camera (MicroMax, 1300(H)  $\times$  1030(V), 6.7  $\mu\text{m} \times 6.7 \mu\text{m}$  pixels, 5 MHz; Roper Scientific) operated with appropriate controller, image acquisition boards, and software (WinView3.2; Roper Scientific). The optical system could be readily switched to run either under near-IR DIC illumination (for electrophysiological recordings) or under epifluorescent illumination (to identify photoreceptor types).

To uncage Diazo-2 we designed into the inverted microscope a second epi-illumination pathway to deliver light to the cell under electrophysiological study. The uncaging flash was generated by a Xenon arc flash lamp (200 Joules, 170  $\mu\text{s}$  half bandwidth) (Chadwick-Helmuth Co.). The arc image was focused on the isolated photoreceptor using the microscope's Nikon objective and a quartz relay lens. Homogeneous, spectrally unfiltered uncaging flashes were delivered over the entire surface of the cell.

To determine the extent and speed of  $\text{Ca}^{2+}$  concentration change we filled tight-seal electrodes with the standard electrode-filling solution containing, in addition, 50  $\mu\text{M}$  bis-Fura-2, a ratiometric fluorescent  $\text{Ca}^{2+}$  indicator (Molecular Probes). We placed the electrode tip in the position normally occupied by photoreceptors in the recording chamber and measured the fluorescence intensity emitted by the sample when excited with identical photon flux of either 340 or 380 nm wavelength. Fluorescence intensity was measured using a micro-objective collecting lens, fiber optic bench, and photomultiplier as described in detail elsewhere (Ohyama et al., 2000). The main experimental challenge is to sample the  $\text{Ca}^{2+}$  concentration in the same limited volume in which the uncaging of Diazo-2 occurs, just as in the isolated photoreceptors. To meet this goal, we limited uncaging light and bis-Fura-2 excitation light to the same volume,  $\sim 8 \mu\text{m}$  in diameter and 16  $\mu\text{m}$  in height, by using an 8- $\mu\text{m}$  diameter pinhole in the epiillumination pathway and measuring the signal from the solution in the electrode tip at a point where its internal diameter was  $\sim 16 \mu\text{m}$ . To quantitate  $\text{Ca}^{2+}$  concentration from the measured ratiometric fluorescent signal, we calibrated the fluorescent signals in the same instrumental configuration using a standard in vitro calibration protocol (Williams and Fay, 1990). We measured bis-Fura-2 Kd for  $\text{Ca}^{2+}$  to be 373 nM in the absence of  $\text{Mg}^{2+}$ , a value that closely matches the published one.

### Cell Labeling

To recognize rod from cone photoreceptors we labeled isolated cells with FITC-PNA. After dissociation, the suspension of retinal cells in L-15/pyruvate (150  $\mu\text{L}$ ) was gently mixed with 30  $\mu\text{L}$  of 1 mg/ml FITC-PNA. The suspension was incubated in the dark for 15 min at room temperature. The suspension was then transferred onto the ConA-coated glass coverslip in the electrophysiological recording chamber, cells allowed to attach to the coverslip and unbound PNA washed away by extensive superfusing of the recording chamber.

### Data Analysis

Experimental uncertainty is presented as standard deviation. Selected functions were fit to experimental data using nonlinear, least square minimization algorithms (Origin, Microcal Software). WinMAXC ([www.stanford.edu/~cpatton](http://www.stanford.edu/~cpatton)) was used to compute free  $\text{Ca}^{2+}$  concentration in the presence of binding molecules (Diazo-2, BAPTA, and/or bis-Fura-2).

## RESULTS

To investigate CNG channel modulation in mammalian rods and cones we studied photoreceptors isolated from the retina of the California ground squirrel (*Speomophilus beecheyi*). In this species, cones outnumber rods by  $\sim 7:1$ , although the specific value of this ratio varies across the retinal surface (Kryger et al., 1998). Ground squirrel cones have been used in past biochemical (von Schantz et al., 1994; von Schantz et al., 1998; Weiss et al., 1998) and electrophysiological (Jacobs et al., 1976; Dawis and Purple, 1981; Blakeslee and Jacobs, 1987; Kraft, 1988) studies of phototransduction. We succeeded in isolating photoreceptors by gentle proteolysis of the retina, followed by fine tissue chopping. Only a minority of the isolated photoreceptors retain their outer segment; these cells, however, remain functional in short term cell culture and can be studied electrophysiologically. While the ultrastructure of squirrel cone and rod outer segment is characteristically distinct (Anderson and Fisher, 1976), the cells cannot be easily distinguished from each other with conventional light microscopy. Isolated rods and cones are similar in anatomical appearance and the outer segments in both cell types are typically 7–10  $\mu\text{m}$  long and  $\sim 1.5 \mu\text{m}$  in diameter (Fig. 1).

To distinguish rods from cones we took advantage of the fact that in ground squirrel retina, just as in other mammalian species, PNA lectin binds selectively to the cone outer segment plasma membrane, but not to the rod membrane (Szel et al., 1993). We succeeded in distinguishing rods from cones under epifluorescent illumination by their ability to bind, or not, FITC-labeled PNA. In cones, PNA brightly labeled the outer segment, a small region in the inner segment immediately under the mitochondrial bag, and the synaptic pedicle. In rods, we only observed diffuse labeling over the entire inner segment (Fig. 1).

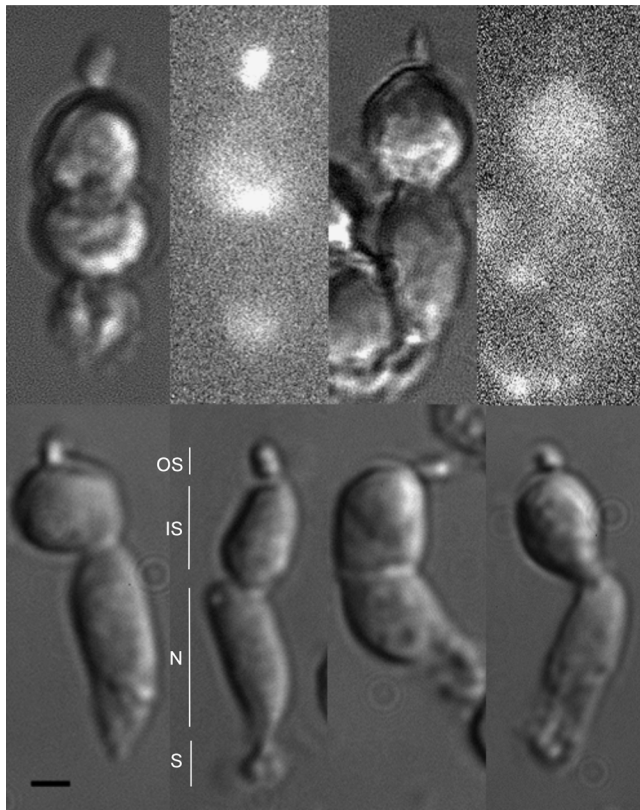


FIGURE 1. Micrographs of isolated ground squirrel photoreceptors. Side-by-side images of the same cells labeled with FITC-PNA captured either under DIC contrast enhancement or epifluorescent illumination. The top, left is a cone, characterized by a brightly fluorescent outer segment and a discrete fluorescent band in the inner segment. The top, right is of a rod, characterized by the absence of fluorescence in the outer segment and diffuse fluorescence throughout the inner segment. The images in the lower row were captured under DIC illumination and depict the range of cytological structure of isolated photoreceptors. OS outer segment IS inner segment, N nuclear region, and S synaptic terminal. Bar, 5  $\mu$ m.

#### *Sensitivity to Activation by 8Br-cGMP Is Higher in Channels of Cones than in Those of Rods*

Having first established an anatomical tool to identify rods from cones, we were then also able to establish a distinct rod versus cone electrophysiological characteristic. Under voltage clamp, we measured the membrane current of isolated photoreceptors with tight-seal electrodes in the whole-cell mode. The electrodes were filled with a solution containing Zaprinast, a phosphodiesterase (PDE) inhibitor, and a fixed concentration of 8Br-cGMP to activate a constant fraction of the cell's CNG ion channels. The solution also contained dark Diazo-2 titrated to yield 600 nM free  $\text{Ca}^{2+}$ , a concentration near that measured in dark-adapted cones (Sampath et al., 1999). After attaining whole-cell mode, the holding current at  $-40$  mV slowly drifted from a start-

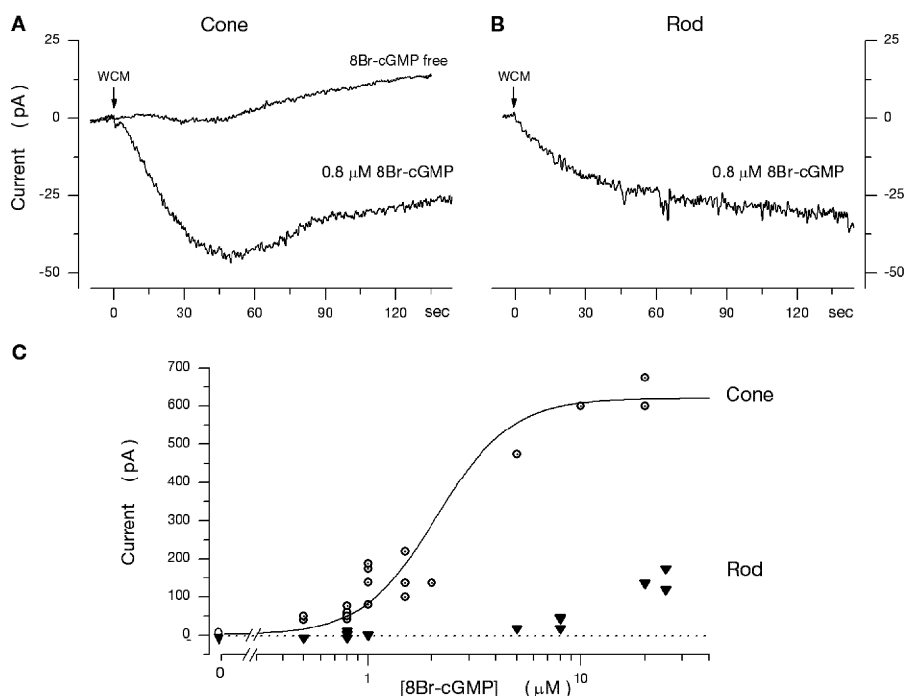
ing value near zero to a final, steady-state inward value reached after 60–90 s (Fig. 2). We distinguished cones from rods through the kinetics of their holding current and their sensitivity to the nucleotide. In cones, identified by their PNA labeling, the holding current reached to an initial peak and then relaxed to a final stationary value (Fig. 2 A). This time course reflects the time to exchange/replace the cell's cytoplasmic content by the electrode-filling solution and the cell's adjustment to the imposed  $\text{Ca}^{2+}$  and nucleotide concentrations. The inward current was activated at relatively low nominal 8Br-cGMP concentrations; for example, at 0.8  $\mu$ M 8Br-cGMP the mean steady current was  $-59 \pm 27.2$  pA ( $n = 8$ ). In rods, on the other hand, the inward holding current reached its final stationary position with a simple exponential time course and its amplitude was less sensitive to the nucleotide concentration (Fig. 2 B). For example, at nominal 8  $\mu$ M 8Br-cGMP the mean steady current was  $-31.5 \pm 15.6$  pA ( $n = 4$ ).

In both rods and cones, the steady-state inward current reflects the activity of CNG ion channels. In the absence of added 8Br-cGMP, the mean steady-state holding current at  $-40$  mV was outward,  $11.6 \pm 5$  pA in every single photoreceptor studied ( $n = 13$ ) (Fig. 2 A). The electrode filling solution lacked triphosphate nucleotides and, therefore, photoreceptors could not support endogenous cGMP synthesis (Hestrin and Korenbrot, 1987; Rispoli et al., 1993). In the absence of cyclic nucleotide (endogenous or exogenous), the steady-state membrane current is outward because of the activity of voltage-gated  $\text{K}^+$  channels known to exist in the inner segment membrane of both rods and cones (Bader et al., 1982; Barnes and Hille, 1989; Maricq and Korenbrot, 1990b).

We investigated the effects of various 8Br-cGMP concentrations on the holding current in both rods and cones. In Fig. 2 C we illustrate the dependence on nucleotide concentration of the steady-state current measured 2.5 min after breaking the membrane seal. The continuous line superimposed on the cone experimental results in Fig. 2 C depicts the Hill equation optimally fit to the data points.

$$I = I_{\max 8\text{Br-cGMP}} \frac{[8\text{Br-cGMP}]^n}{K_{1/2}^n + [8\text{Br-cGMP}]^n}. \quad (1)$$

The function that best fit all the experimental data in cones was one with  $I_{\max} = 620$  pA,  $n = 2.5$ ,  ${}^{8\text{Br-cGMP}}K_{1/2} = 2.3$   $\mu$ M. The experimental data collected in rods is not sufficient to confidently determine values for the parameters in Eq. 1. Although we do not know the precise ligand sensitivity of squirrel rod channels, it is certainly less than that in cones at the same  $\text{Ca}^{2+}$  concentration. Of relevance to the experiments reported here is that we know, in both rods and cones, the 8Br-cGMP concentration necessary to sustain inward currents be-



**FIGURE 2.** Time continuous record of membrane current at  $-40$  mV holding voltage in isolated ground squirrel cones (A) or rod (B). The arrow labeled WCM indicates the moment whole-cell mode was attained, defined as time zero. (A) Data from two different cones, one studied with a tight seal electrode filled with  $0.8 \mu\text{M}$  8Br-cGMP with  $400 \mu\text{M}$  zaprinast and  $600$  nM free  $\text{Ca}^{2+}$  and the other with a solution lacking the nucleotide, as labeled. In the absence of nucleotide, current slowly changed to a final, steady outward value. In the presence of 8Br-cGMP, the inward current reflects activation of CNG ion channels with a time course determined by the rate of exchange/replacement between the cell's cytoplasmic content and the electrode-filling solution, as well as the cell's adjustment to the new, imposed  $\text{Ca}^{2+}$  and nucleotide concentrations. (B) Membrane current in a rod studied with a tight seal electrode filled with  $8 \mu\text{M}$  8Br-cGMP with  $400 \mu\text{M}$  Zaprinast and  $600$  nM free  $\text{Ca}^{2+}$ . After reaching WCM, the current

changed with an exponential time course to a final, steady inward value. C illustrates the amplitude of the steady-state difference current measured in individual photoreceptors before and after attaining whole-cell mode in the presence of various 8Br-cGMP concentrations in the electrode filling solution. The continuous line depicts the Hill equation (text Eq. 1) optimally fit to the cone data with  $\text{Imax}$   $620$  pA,  $n = 2.5$  and  $^{8\text{Br-cGMP}}K_{1/2} = 2.3 \mu\text{M}$ . The concentration range tested is too small to accurately define the features of the Hill function for rods.

tween  $-50$  and  $-100$  pA at  $-40$  mV, the range of the dark current in intact cells. Our findings in ground squirrel are consistent with the features of channels formed from recombinant  $\alpha$  subunits. In channels of humans, for example,  $^{\text{cGMP}}K_{1/2}$  is  $\sim 20 \mu\text{M}$  in cones (Wissinger et al., 1997; Peng et al., 2003), but it is  $\sim 80 \mu\text{M}$  in rods (Dhallan et al., 1992). In native bovine rod membranes,  $^{\text{cGMP}}K_{1/2}$  is  $\sim 165 \mu\text{M}$  (Quandt et al., 1991).

Ground squirrels have both S and M cone photoreceptors (Kraft, 1988; Kryger et al., 1998) and in the intact eye the sheaths of pigment epithelium that wrap the two cone outer segment types bind PNA differently (Szel et al., 1993). Among isolated cones, however, we could not distinguish two different cell populations by either anatomical or electrophysiological criteria. This experimental limitation is likely without severe consequences because we expect the physiological properties of CNG channels to be the same in the two different cone types. In fish retina, the properties of CNG channels are the same in different cone types (Picones and Korenbrot, 1992).

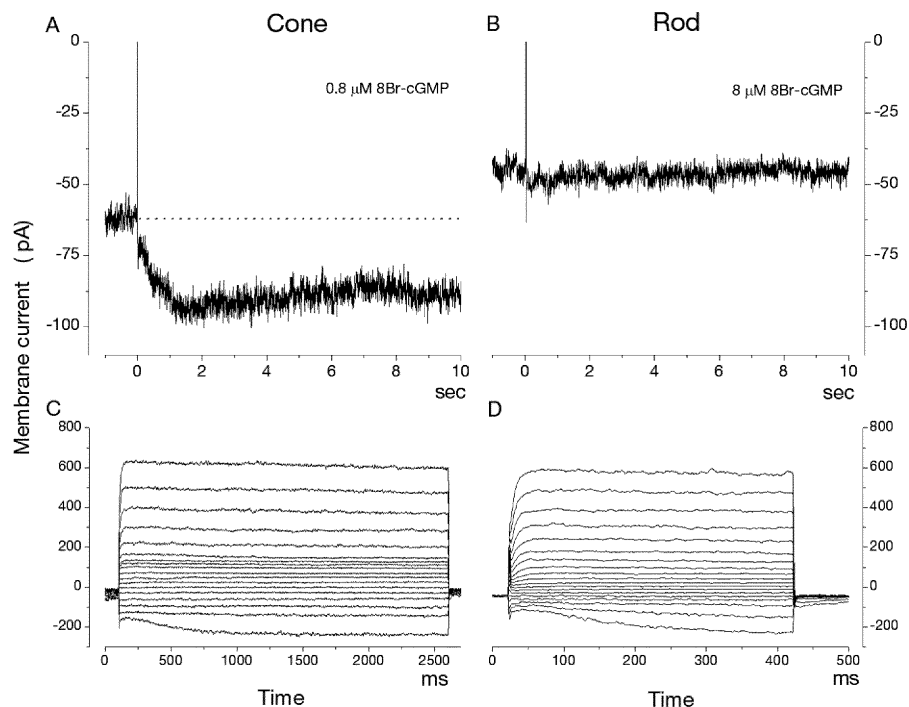
#### *Decreasing Cytoplasmic $\text{Ca}^{2+}$ Activates an Inward Current in Cones, but Not in Rods*

We assessed whether lowering cytoplasmic free  $\text{Ca}^{2+}$  modulates CNG ion channel in mammalian photoreceptors. Single cells were loaded with 8Br-cGMP and

dark diazo-2 titrated to yield an initial free  $\text{Ca}^{2+}$  concentration of  $600$  nM. Bright flash illumination uncaged Diazo-2 causing a sudden decrease in free cytoplasmic  $\text{Ca}^{2+}$ . Photometric measurements carried under the same experimental conditions as with the isolated cells (see MATERIALS AND METHODS) showed that in our instrument, flash uncaging Diazo-2 caused  $\text{Ca}^{2+}$  to decrease to  $\leq 30$  nM (the limit of bis-Fura-2 detection) in  $< 50$  ms (the limit of our temporal resolution).

Single cones, identified by their PNA labeling, loaded with  $0.8$ – $1.5 \mu\text{M}$  8Br-cGMP exhibited a mean holding current at  $-40$  mV of  $-87.3 \pm 44$  pA ( $n = 18$ )  $2.5$  min after attaining whole-cell mode. This magnitude of the nucleotide-gated current is similar to the amplitude of the outer segment dark-current in intact squirrel cones ( $-30$  to  $-70$  pA; Kraft, 1988).  $2.5$  min after attaining whole-cell mode we delivered an uncaging Xe flash that activated an inward current in every cell tested (Fig. 3 A). The current developed with an exponential time course to a peak, and then slowly drifted back toward its starting value. The peak mean amplitude of the  $\text{Ca}^{2+}$ -dependent current was  $-28.8 \pm 13.6$  pA ( $n = 12$ ). Thus, in mammalian cones, just as in fish cones (Rebrik et al., 2000), lowering cytoplasmic  $\text{Ca}^{2+}$  enhances the CNG current by  $\sim 30\%$ .

**FIGURE 3.** The effect of lowering cytoplasmic  $\text{Ca}^{2+}$  on cyclic nucleotide-dependent membrane currents. (A) The membrane current measured in a cone at  $-40$  mV holding voltage with a tight-seal electrode filled with  $0.8 \mu\text{M}$  8Br-cGMP,  $1 \text{ mM}$  dark Diazo-2, and  $600 \text{ nM}$  free  $\text{Ca}^{2+}$ .  $2.5 \text{ min}$  after attaining whole-cell mode, free  $\text{Ca}^{2+}$  was lowered by uncaging Diazo-2 with a bright Xe flash. The moment the flash was presented defines time zero in the graphs. The flash discharge caused a fast ( $<10 \text{ ms}$ ) electrical artifact observed even with the electrode alone. Rapidly lowering  $\text{Ca}^{2+}$  ( $<50 \text{ ms}$ ) increased the inward current to a peak followed by a slow drift back toward its starting, steady-state value, to which it eventually returned (not depicted). (B) The membrane current measured in a rod at  $-40$  mV with an electrode filled with  $8 \mu\text{M}$  8Br-cGMP,  $1 \text{ mM}$  total dark Diazo-2, and  $600 \text{ nM}$  free  $\text{Ca}^{2+}$ . Lowering cytoplasmic  $\text{Ca}^{2+}$  with the uncaging flash was without any effect on membrane current. (C and D) Membrane currents measured in the same cone and rod after flash illumination. Currents were activated by stepping membrane voltage to between  $-90$  and  $90 \text{ mV}$  in  $10\text{-mV}$  steps. The voltage- and time-dependent electrical behavior of rods and cones is indistinguishable and demonstrate both cells were normal and electrically intact.



The same experimental protocol when applied to rods yielded very different results. In rods, as pointed out above, higher nucleotide concentrations were needed to attain steady-state current amplitudes comparable to those measured in cones.  $2.5 \text{ min}$  after attaining whole-cell mode, the current at  $-40 \text{ mV}$  in rods loaded with  $5\text{--}8 \mu\text{M}$  8Br-cGMP was  $-31.5 \pm 15.6 \text{ pA}$  ( $n = 4$ ) and  $-143.7 \pm 28.3 \text{ pA}$  in those loaded with  $20\text{--}25 \mu\text{M}$  8Br-cGMP ( $n = 3$ ). At either concentration, uncaging Xe flashes that reduced free cytoplasmic  $\text{Ca}^{2+}$  to  $<30 \text{ nM}$  failed to cause any change in the amplitude of the holding current in every one of the rods tested (Fig. 3 B).

The consistency of our experimental results makes it unlikely that the rod cone difference arises from cell damage. Nonetheless, we ensured this possibility was not a problem by measuring the voltage-dependent currents in the same cells in which Xe flash effects were first tested. As we illustrate in Fig. 3, C and D, rods and cones exhibited much the same currents and electrical impedance. We did not explore in detail the identity of the voltage-gated currents, but the patterns are similar to those thoroughly investigated in rods and cones of several other species and they arise principally from the activity of three ion channels:  $I_h$ , a cationic-selective channel activated by hyperpolarization (Bader et al., 1982; Hestrin, 1987; Barnes and Hille, 1989; Maricq and Korenbrot, 1990a);  $I_{Ca}$ , a nifedipine-sensitive

$\text{Ca}^{2+}$ -selective channel (Bader et al., 1982; Maricq and Korenbrot, 1988; Barnes and Hille, 1989); and  $I_{Kdr}$ , a classical delayed rectifier  $\text{K}^+$  channel (Bader et al., 1982; Barnes and Hille, 1989; Maricq and Korenbrot, 1990b). That is, in healthy, intact isolated photoreceptors and under nucleotide concentrations and CNG channel activation comparable to those expected under dark physiological conditions, a decrease in cytoplasmic  $\text{Ca}^{2+}$  activates cyclic nucleotide-dependent currents in cones, but not in rods.

#### *In Cones, the Inward Current Activated by Lowering $\text{Ca}^{2+}$ Arises from the Modulation of CNG Ion Channels*

To prove that the inward current activated by uncaging Diazo-2 arises specifically from the activation of CNG channels we tested, and dismissed, alternative mechanisms. Possible mechanisms are: (1)  $\text{Ca}^{2+}$ -dependent activation of guanylate cyclase (GC), which in turn, synthesizes cGMP; (2) activation of  $\text{Ca}^{2+}$ -dependent currents that are not cyclic nucleotide-gated; (3) an unexpected effect of light. GC activation cannot be the causal mechanism because of the experimental design: the electrode-filling solution lacked GTP and, hence, the enzyme was without substrate. In addition, the absence of endogenous GC activity in both rods and cones is made evident by the fact that the steady holding current in the absence of added 8Br-cGMP was out-

ward and photoreceptors did not respond to light, however bright it might be.

As controls, we measured the effects of uncaging light flashes in cones loaded with electrode-filling solutions modified as follows: (1) free of 8Br-cGMP (to test whether a cyclic nucleotide-independent current is activated by lowering  $\text{Ca}^{2+}$ ) and (2) Diazo-2 replaced with 1 mM total BAPTA (to test whether in the absence of  $\text{Ca}^{2+}$  changes light causes channel activation). When replacing Diazo-2 with BAPTA, we maintained the same free  $\text{Ca}^{2+}$  concentration (600 nM) and  $\text{Ca}^{2+}$  buffering capacity in the cell cytoplasm. Typical results of these tests are shown in Fig. 4. In the absence of 8Br-cGMP, the steady holding current was outward and lowering  $\text{Ca}^{2+}$  with uncaging flashes did not generate changes in current (Fig. 4 A). In the absence of Diazo-2, the steady holding current was inward and, again, flashes failed to cause any change in membrane current (Fig. 4 B). We found the same results in every cone we tested (8Br-cGMP free,  $n = 4$ ; Diazo-2 free,  $n = 4$ ). Thus, control experiments demonstrate that the enhancement in inward current generated by uncaging Diazo-2 arise specifically from activation of CNG ion channels caused by lowering cytoplasmic  $\text{Ca}^{2+}$ .

#### *The Time Course of $\text{Ca}^{2+}$ -dependent CNG Current Modulation in Cones*

The results shown in Figs. 3 and 4 demonstrate that the time course of CNG channel modulation in squirrel cones is complex: it reaches exponentially to a peak and then slowly relaxes back to its starting value. This is qualitatively the same observation we made in striped bass cones (Rebrik et al., 2000). In bass cones we simultaneously measured membrane current and cytoplasmic outer segment  $\text{Ca}^{2+}$  change caused by Diazo-2 uncaging. Analysis of the simultaneous measurements showed that the kinetics of  $\text{Ca}^{2+}$ -dependent modulation reflects two successive events. First,  $\text{Ca}^{2+}$  concentration decreased nearly instantaneously and, in turn, activated the channels with a distinct exponential time course. Second, channel activation allowed  $\text{Ca}^{2+}$  influx through the open channels, which, alongside cytoplasmic replenishment by exchange/diffusion from the electrode, allowed the cell to slowly return to its starting, dark value. In the present experiments in ground squirrel, unfortunately, we could not resolve specifically the cone outer segment  $\text{Ca}^{2+}$  concentration because they are so small that any  $\text{Ca}^{2+}$  measurement (at sufficient time resolution) was dominated by the  $\text{Ca}^{2+}$  concentration of the much larger, adjacent inner segment. In mammalian cones, we then characterized only the kinetics of the  $\text{Ca}^{2+}$ -dependent current activation.

To accurately measure the time course of current activation, experimental data were acquired under two strict conditions: (1) the flash-activated current was

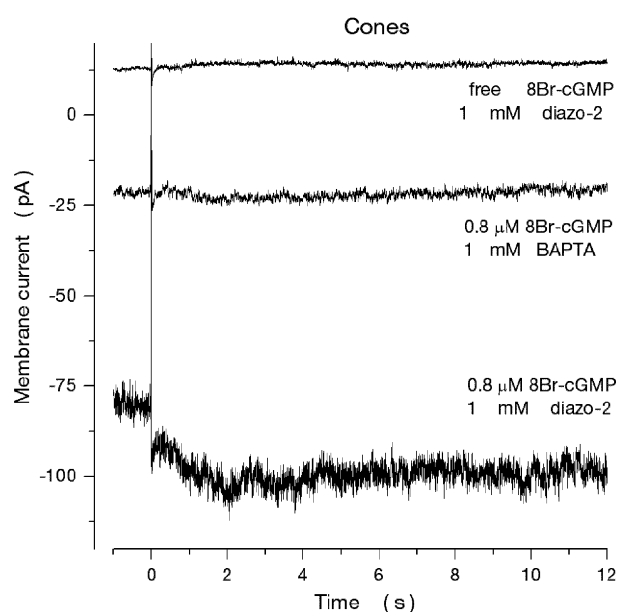
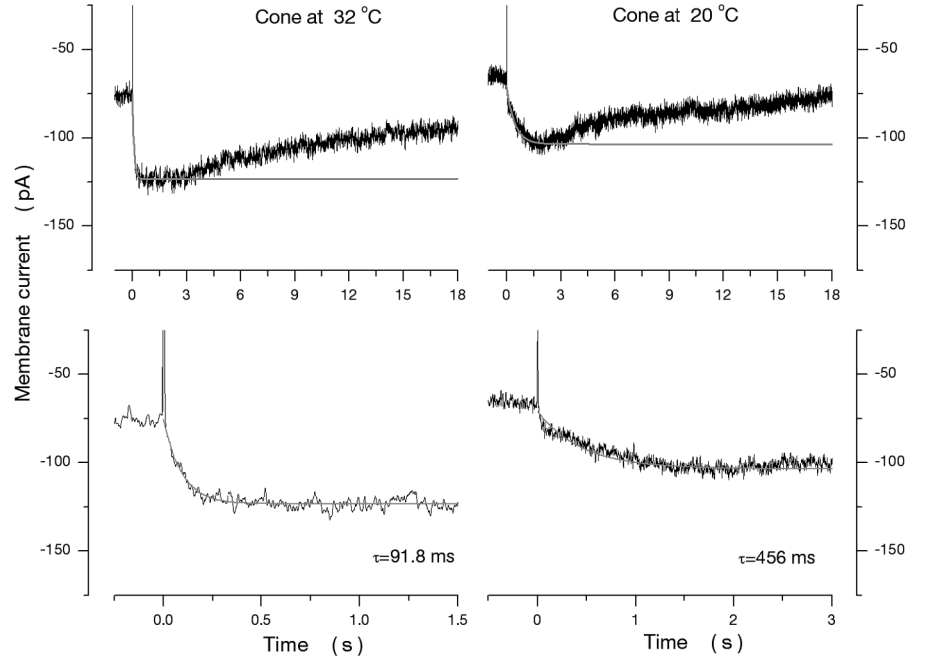


FIGURE 4. Current activation by flash uncaging Diazo-2 requires the presence of cyclic nucleotides and does not arise from an effect of light alone. Currents measured at  $-40$  mV holding voltage in three different cones. The currents shown were measured at the steady holding current reached 2.5 min after attaining whole-cell mode. At time zero an uncaging Xe flash was presented and the lamp discharge caused a transient artifact. Every cone shown was loaded with solutions that contained 600 nM free  $\text{Ca}^{2+}$  and 1 mM free  $\text{Mg}^{2+}$ , but differed in their 8Br-cGMP or Diazo-2 concentrations. The cone loaded with 0.8  $\mu\text{M}$  8Br-cGMP and 1 mM total dark Diazo-2 (as labeled) exhibited an exponential increase in the inward current amplitude upon flash illumination. The flash-dependent current enhancement was not observed if we omitted either Diazo-2 (replaced with 1 mM total BAPTA) or 8Br-cGMP from the cell-filling solution (as labeled).

measured 2.5 min after first attaining whole-cell mode. This is important because, as we show below, the extent of channel modulation is progressively lost from intact cells, but the loss is small at 2.5 min at room temperature. (2) Photoreceptors were not identified by PNA labeling to avoid exposure to epifluorescent illumination (and PDE activation), thus minimizing variance in the effective 8Br-cGMP concentration in the outer segment after loading. Nonetheless, we readily distinguished rods from cones by means of the electrophysiological data: at the low nucleotide concentrations used in these experiments (0.8–1.5  $\mu\text{M}$ ) the holding inward current was larger in cones than in rods (see Fig. 2) and cones exhibited current modulation which, of course, rods did not (Fig. 3).

We measured the kinetics of channel modulation both at room temperature (20–21°C) and at a more nearly physiological temperature for ground squirrel (32°C) in the presence of 0.8–1.5  $\mu\text{M}$  cytoplasmic 8Br-cGMP. At either temperature, the flash-induced decrease in cytoplasmic free  $\text{Ca}^{2+}$  activated an inward

FIGURE 5. Kinetics of  $\text{Ca}^{2+}$ -dependent modulation of CNG currents in cones. Cells studied in a temperature-controlled chamber at either 32°C (loaded with 1.5  $\mu\text{M}$  8Br-cGMP) or 20°C (loaded with 0.8  $\mu\text{M}$  8Br-cGMP). Currents measured at  $-40$  mV at the steady holding current reached 2.5 min after attaining whole-cell mode. At that moment, a flash to uncage Diazo-2 was delivered and the instant of delivery defined the origin of the time axis. At either temperature, the flash-induced decrease in cytoplasmic-free  $\text{Ca}^{2+}$  activated an inward current of comparable amplitude, but different time course. The upper and lower tracings in each column are the same data displayed at different time resolution. Superimposed on the experimental data are first order exponential decay curves optimally fit to the results (text Eq. 2). At 32°C,  $I_{\text{hold}} = -75.3$  pA,  $I_{\text{max}} = -123.3$  pA,  $\Delta I_{\text{Ca}} = 48$  pA,  $\tau = 91.8$  ms. At 20°C,  $I_{\text{hold}} = -71$  pA,  $I_{\text{max}} = -103.5$  pA,  $\Delta I_{text{Ca}} = 32.5$  pA,  $\tau = 456$  ms.



current of comparable amplitude but different time course (Fig. 5). We confirmed that at 32°C, just as at room temperature, rods did not exhibit current modulation (unpublished data). The initial current change in cones is well described as a first order exponential process with time constant  $\tau$  (Figs. 5 and 6) given by:

$$I(t) = I_{\text{max}} + \Delta I_{\text{Ca}} e^{-t/\tau}, \quad (2)$$

where  $I_{\text{max}}$  is the maximum amplitude of the enhanced current and  $\Delta I_{\text{Ca}}$  is the peak difference between holding and enhanced current. The holding current is simply:  $I_{\text{hold}} = I_{\text{max}} - \Delta I_{\text{Ca}}$ . Summary results for

FIGURE 6. CNG current modulation is lost over time from individual cells. Cones studied in a temperature-controlled chamber at either 32°C (loaded with 1.5  $\mu\text{M}$  8Br-cGMP) or 20°C (loaded with 0.8  $\mu\text{M}$  8Br-cGMP). (A and B) Currents repeatedly measured at  $-40$  mV in the same cell at various times after first attaining whole-cell mode, as labeled in minutes. At each time, a flash to uncage Diazo-2 was delivered and the instant of delivery defined the zero time in the scales in seconds. The holding current at  $-40$  mV drifted in value to the extent illustrated in C and D for each cell. To compare repeated flash measurements in the same cell and because the holding current drifts, currents in A and B were DC shifted so that the holding current immediately before a flash a zero value. Each uncaging flash caused a change in inward current, but the extent of the change was progressively smaller as time elapsed after establishing whole-cell mode. Superimposed on the current measured after 2.5 min are first order exponential decay curves optimally fit to the results (text Eq. 2). At 32°C,  $I_{\text{hold}} = -94$  pA,  $I_{\text{max}} = -134$  pA,  $\Delta I_{\text{Ca}} = 40$  pA,  $\tau = 85.6$  ms. At 20°C,  $I_{\text{hold}} = -65$  pA,  $I_{\text{max}} = -93.7$  pA,  $\Delta I_{\text{Ca}} = 28.8$  pA,  $\tau = 475$  ms. The rate of modulation loss, represented by the slope of the line that passes through the points in C and D, is higher at 32°C than 20°C.

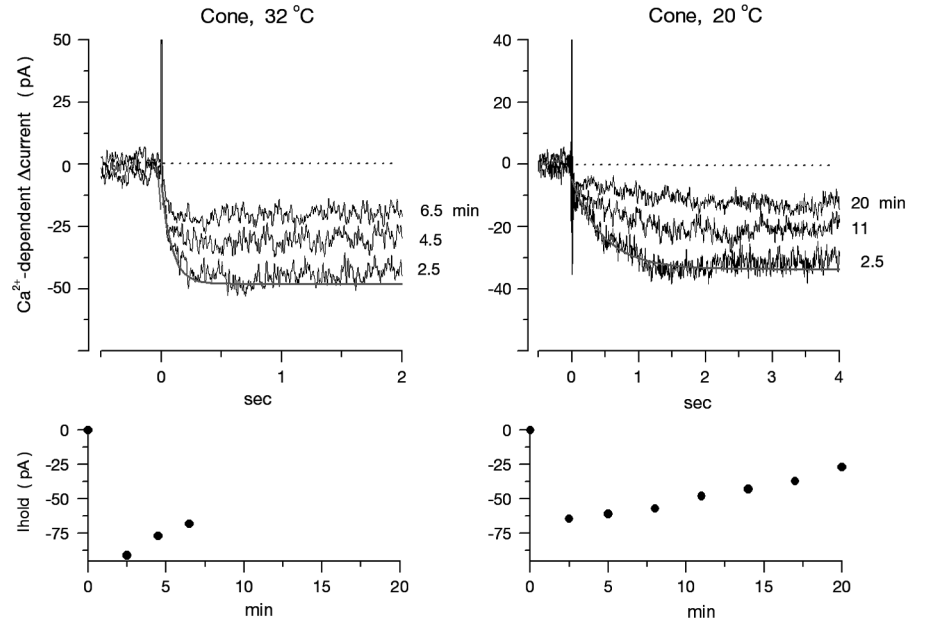


TABLE I  
*Kinetics of Ca<sup>2+</sup>-dependent Modulation in Ground Squirrel Cone CNG Ion Channels*

	Cytoplasmic 8Br-cGMP	I <sub>hold</sub>	I <sub>max</sub>	ΔI <sub>Ca</sub>	τ
	μM	pA	pA	pA	ms
20°C	0.8–1	−73.1 ± 9.3	−99.4 ± 5.11 ( <i>n</i> = 3)	26.3 ± 7.76	480.3 ± 27.4
32°C	1.5	−124.3 ± 65	−153.3 ± 68.4 ( <i>n</i> = 4)	29.0 ± 17.6	138.1 ± 63.7

all cells studied are presented in Table I. There is no distinguishable effect of temperature on dark holding inward current. The magnitude of current enhancement is also not affected by temperature. The kinetics, however, are significantly different, modulation is faster at higher temperatures and the average Q10 of the time constant is  $\sim 2.9$ .

*Channel Modulation Depends on the Activity of a Factor that Is Lost from the Cone Outer Segment*

The molecular mechanism of the Ca<sup>2+</sup>-dependent cone CNG channel modulation we describe here remains to be elucidated. The small modulation of CNG channel sensitivity in rods is almost certainly mediated by calmodulin (Hsu and Molday, 1993; Bauer, 1996), although some quantitative aspects of the modulation cannot be fully mimicked by exogenous calmodulin (Gordon et al., 1995). In cone CNG channels, in contrast, calmodulin is not likely the modulator of Ca<sup>2+</sup> sensitivity because when it is added to native COS membrane patches it induces Ca<sup>2+</sup>-dependent shifts in ligand sensitivity that are far smaller than those observed in the intact cells (Hackos and Korenbrot, 1997; Haynes and Stotz, 1997). Moreover, added calmodulin is without effect on recombinant cone channels formed from  $\alpha$  subunits alone (Muller et al., 2001), and its effect on recombinant channels formed from  $\alpha$  and  $\beta$  subunits is just as small, or smaller, than that observed in native channels (Peng et al., 2003). Finally, pharmacological blockers of calmodulin action are without effect on the channel modulation in intact cones (Rebrik et al., 2000).

A class of voltage-gated Ca<sup>2+</sup> channels exhibit Ca<sup>2+</sup>-dependent inactivation mediated by calmodulin (for review see Saimi and Kung, 2002). In these channels calmodulin is an integral constituent of the channel multimeric structure and, therefore, exogenously added calmodulin does not modulate the channels (Peterson et al., 1999; Qin et al., 1999). It is possible, then, that failure to observe cone CNG channel modulation by exogenous calmodulin arises from a similar mechanism. To test this possibility we repeatedly measured channel modulation in the same cone at various times after attaining whole-cell mode. We waited 2–5 min between successive flashes to allow the cell's holding current to reach a steady value. This steady holding

current, however, drifted slowly over time, reflecting the fact that the net sum current through the many active channels in the cells, including the CNG ones, never really was at equilibrium (Figure 6, C and D, data points stop when cell's seal was lost). To compare among the measurements in the same cell, we DC shifted the measured current and assigned it a zero value before flash delivery (Fig. 6, A and B). In all cells we tested (*n* = 6), the extent of change in current amplitude upon flashing was progressively smaller as time elapsed after attaining whole-cell mode (Fig. 6). The rate of loss of modulation was faster at 32°C than 20°C. Since the modulation is lost over time, it is unlikely that the modulator molecule is an integral component of the cone CNG channel.

#### DISCUSSION

Dawis and Purple (1981) first demonstrated that at light levels that do not bleach a significant fraction of the visual pigment, photoreceptors in ground squirrel exhibit neural light-adaptation. They analyzed photoreceptor features in the electroretinogram, but because they tested with long flashes of white light, it was impossible to resolve the adaptation features of individual photoreceptor types. Blakeslee and Jacobs (1987) used optic nerve single-unit recordings to demonstrate distinct light-adaptation features of rod- and cone-driven retinal circuits in ground squirrel. These measurements, again, could not discern the adaptation features of individual photoreceptor types. Electrical recordings from single cones by Kraft (1988) unequivocally demonstrate these photoreceptors adapt in a manner similar to that of cells in other mammals and, furthermore, signals from S and M cones are indistinguishable. Electroretinographic data also indicate, though less directly, that the function of ground squirrel photoreceptors is similar to that of other mammalian species (Jacobs et al., 1976; Blakeslee et al., 1988). Hence, we take it to be reasonable to expect that, just as in all other mammals, ground squirrel rods light-adapt very little and only near their amplitude saturation, whereas cones adapt over changes in background intensity that extend  $\sim 5$  orders of magnitude.

We report here that in isolated, intact ground squirrel photoreceptors lowering cytoplasmic free Ca<sup>2+</sup> from  $\sim 600$  nM to  $< 30$  nM activates cGMP-gated chan-

nels in cone outer segments, but not those in rod outer segments. The mechanism of this activation is not explicitly proven, but it is almost certainly caused by  $\text{Ca}^{2+}$ -dependent modulation of the channel's sensitivity to cGMP since our results in ground squirrel cones are similar to those we obtained previously in cones of fish under the same experimental paradigm (Rebrik et al., 2000). In fish cones we have demonstrated the existence of  $\text{Ca}^{2+}$ -dependent shifts in the channel's nucleotide sensitivity (Rebrik and Korenbrot, 1998). Demonstrating that the same mechanism operates in ground squirrel cones requires the application of a method in which CNG currents are measured in an intact cell while the cytoplasmic cGMP concentration is continuously and precisely controlled. Such has been achieved either by truncating the outer segment (Nakatani et al., 1995; Sagoo and Lagnado, 1996) or electroporabilizing the inner segment (Rebrik and Korenbrot, 1998) while recording outer segment membrane currents. The very small size of the cone outer segment technically precludes comparable studies in squirrel.

We found that at a fixed  $\text{Ca}^{2+}$  concentration, the cyclic nucleotide sensitivity of CNG channels in intact rods is less than in those of cones ( $^{8\text{Br}}\text{cGMPK}_{1/2} \sim 2.3 \mu\text{M}$ ). To obtain these results we combined data measured in different cells, rather than testing various concentrations in the same cells, a technical impossibility. The difference in nucleotide sensitivity between CNG channels of mammalian photoreceptors stands in contrast to findings in nonmammalian cells, where the sensitivity is higher in rod CNG channels than in those of cones. In the absence of  $\text{Ca}^{2+}$ ,  $^{8\text{Br}}\text{cGMPK}_{1/2}$  is  $\sim 5 \mu\text{M}$  in rods of tiger salamander, frog, and bullfrog (Zimmerman et al., 1985; Gordon et al., 1995; Hackos and Korenbrot, 1997), but  $^{8\text{Br}}\text{cGMPK}_{1/2}$  is  $\sim 60 \mu\text{M}$  in cones of striped bass and catfish (Haynes and Yau, 1990; Picones and Korenbrot, 1992). In spite of these relatively large differences in CNG channel sensitivity between mammals and nonmammals and rods and cones, the fraction of CNG channels open in darkness in both mammalian and nonmammalian rods and cones is within a narrow range of 2–5% of all channels present (for review see Pugh and Lamb, 2000). This means that photoreceptors adjust their outer segment cGMP concentration in darkness to various actual values as necessary to maintain a nearly constant number of CNG channels open. The kinetic balance of the activities of synthetic (GC) and hydrolytic (PDE) enzymes maintains the value of cGMP concentration in the dark. The constancy of number of open channels, rather than actual cGMP concentration, across species and photoreceptor types indicate that the enzymatic kinetic balance is set at various points in different photoreceptors. This suggests that, as photoreceptors evolved in their phylogenetic scale and CNG channels changed in their cyclic

GMP sensitivity, the transduction enzyme system was controlled not to maintain a given cytoplasmic cGMP concentration, but to adjust the concentration in order to maintain a constant fraction of channels open.

The time course of cone channel modulation is well described by a temperature-dependent, single-exponential process. This finding contrasts with in vitro measurements of the kinetics of interactions between  $\text{Ca}^{2+}$ , calmodulin, and bovine rod CNG channels, which cannot be described by a single-exponential process (Bauer, 1996). Whereas specific kinetic mechanism cannot be inferred from our results, we do know that the rate-limiting event in the modulation is not the rate of  $\text{Ca}^{2+}$  concentration change. In our experiments,  $\text{Ca}^{2+}$  level decreased to its minimum value in  $<50$  ms, yet the mean time constant of current modulation at  $32^\circ\text{C}$  was  $\sim 138$  ms.

The time constant of channel activation, especially at  $32^\circ\text{C}$ , varied in value in our experiment with a standard deviation of  $\sim 50\%$  of the mean (Table I). We don't think that modulation is that variable under physiological conditions, but rather our results vary because the conditions within the cell are changing in time, outside of our control. We have found that the extent of channel modulation (and its apparent rate) change with time after first attaining whole-cell mode (Fig. 6), caused by the loss of the soluble modulator molecule. In an ideal case, hence, we would wish to measure modulation immediately after establishing whole-cell mode. At that moment, however, modulation could not be observed under our experimental paradigm since we must first load the cell with Diazo-2 and cyclic nucleotide up to a steady value. We established an experimental compromise under which channel modulation was always measured exactly 2.5 min after rupturing the membrane seal; this time was sufficient to reach a stable Diazo-2 and cyclic nucleotide concentration, but we likely lost modulator to a variable extent. At room temperature this error is small (standard deviation is  $\sim 5\%$  of the mean value) because the loss of modulator is slow (Fig. 6), the error is larger at  $32^\circ\text{C}$  because the rate of loss of modulator is faster. That is, the rate of channel activation we measured at  $20^\circ\text{C}$  is, indeed, quite reproducible and we take it to be reasonably accurate. At  $32^\circ\text{C}$ , in contrast, the measured rate is less reproducible and less accurate.

The molecular mechanism of cone channel modulation remains to be discovered. This mechanism is unstable in time and is lost by exchange with the electrode lumen contents. By analogy to CNG channels in rods and olfactory neurons, the argument can be made that modulation in cones depends on the direct action of a single calmodulin-like, soluble factor that diffuses out from the cones outer segment in a temperature-dependent manner. Until the modulator is discovered,

however, it is also plausible that the factor lost from the cone outer segment is not the modulator itself, but another molecule (or enzyme) that primes or readies the channel for modulation.

### Physiological Significance

Difference in transduction signals between rods and cones in the same species generally reflects subtle, yet critical, differences in the quantitative performance of the molecular events underlying phototransduction.  $\text{Ca}^{2+}$ -dependent modulation of ligand sensitivity in CNG ion channels is one of the few molecular events that can now be recognized to be dramatically different in the two photoreceptor types. Because light adaptation is extensive in cones and occurs at all light levels, whereas it is small in rods and occurs only near their amplitude saturation, we suggest that  $\text{Ca}^{2+}$ -dependent CNG channel modulation contributes to the mechanisms of neural light adaptation in cones.

Light adaptation is not a molecular phenomenon distinct and apart from the events of transduction. In a complex molecular choreography yet to be fully understood, several of the biochemical events that define the gain and kinetics of the transduction signal are each modified as time progresses in the continuous presence of light. Each of them can be recognized to function differently in complete darkness than under background illumination. Similarly, the  $\text{Ca}^{2+}$ -dependent channel modulation cannot be thought to be relevant only to light adaptation; it contributes to determine the kinetics and sensitivity of the transduction signal under all conditions, including the response of a thoroughly dark-adapted cell.

We observed CNG channel activation when lowering cytoplasmic  $\text{Ca}^{2+}$  in ground squirrel cones, and none at all in rods. Our failure to observe channel activation in intact rods likely arises from its quantitative features, not its absolute absence: rod channels exhibit  $\text{Ca}^{2+}$ -dependent sensitivity modulation mediated by calmodulin (for review see Kaupp and Seifert, 2002; Saimi and Kung, 2002). Thorough quantitative studies by Bauer (1996) show that the  $\text{Ca}^{2+}$ -calmodulin interaction with the bovine rod CNG channel in the presence of cGMP has an affinity constant of 1 nM. In our experiments, we measured 30 nM as the upper limit of the  $\text{Ca}^{2+}$  concentration achieved by uncaging Diazo-2, while calculations indicate that if all Diazo-2 had been photoconverted to BAPTA the lowest  $\text{Ca}^{2+}$  could have been is 18 nM. If we assume the  $\text{Ca}^{2+}$  dependence of ligand sensitivity in squirrel rod channels is the same as in bovine rods, then the expected shift in  $^{8\text{BrcGMP}}K_{1/2}$  is from say 40  $\mu\text{M}$  (an upper limit) at 600 nM  $\text{Ca}^{2+}$  to 37.3  $\mu\text{M}$  at 18 nM. The expected enhancement in current is dependent on 8BrcGMP concentration and is given by:

$$\frac{I_{lo}}{I_{hi}} = \frac{[8\text{BrcGMP}]^n + K_{hi}^n}{[8\text{BrcGMP}]^n + K_{lo}^n} \quad (3)$$

where  $I_{lo}$  and  $I_{hi}$  are the currents at low and high  $\text{Ca}^{2+}$ , respectively,  $[8\text{BrcGMP}]$  is the ligand concentration, and  $K_{lo}$  and  $K_{hi}$  are the values for  $K_{1/2}$  and  $n$  at low and high  $\text{Ca}^{2+}$ , respectively (Eq. 1). Under our experimental conditions (8  $\mu\text{M}$  8BrcGMP), the expected current enhancement is, at most, 4.8%. Thus, if calmodulin-mediated sensitivity modulation of rod CNG channels occurs in ground squirrel photoreceptors, the current would be so small as to be outside the detection limit of our experiments. If CNG channel sensitivity modulation is necessary for light adaptation, its small amplitude and its activation only at very low  $\text{Ca}^{2+}$  levels, may explain why light adaptation in mammalian rods is small and occurs only at light levels that nearly saturate the photocurrent amplitude.

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