Analysis of Fluctuations in the cGMP-Dependent Currents of Cone Photoreceptor Outer Segments

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ABSTRACT We measured cGMP-dependent currents, under voltage clamp, in membrane patches detached from the outer segment of single-cone photoreceptors isolated from the retina of striped bass. We analyzed the variance of the current about its mean and the spectral density distribution of the current fluctuations. From the analysis of variance, we determined that the cGMP-gated channels increase their probability of opening with increasing cGMP up to a maximum value of 0.87 ± 0.03. The dependence on cGMP of the probability of opening is well described by a Hill equation with K_m= 60.2 ± 3.7 μM and n = 2.33 ± 0.32 at −50 mV. At the same voltage, the spectral density distribution is well fit by the sum of two Lorentzians with corner frequencies at 26 ± 18 and 318 ± 58 Hz. The single-channel conductance calculated from the current noise by two different methods suggests that the most frequently occupied conductance state has an amplitude of about 18 pS.

INTRODUCTION

Characterization of the kinetics and probability of closed to open transitions in single-ion channels provides a powerful tool to understand the mechanics of ligand-gated channels. Although the unitary activity of the cGMP-gated channels of rod photoreceptors has been measured, disagreement exists as to the functional behavior of the channel. cGMP-dependent single-channel activity measured in black-lipid membranes with "incorporated" bovine rod membrane fragments is reported to exhibit multiple conductance states that change in unitary amplitude and in probability of opening as a function of cGMP concentration (Ildefonse and Bennett, 1991). In intact rod photoreceptors, on the other hand, single channels measured in patches detached from the outer segment plasma membrane most often display rapid open to closed transitions to a single conductance state, but occasionally the transitions are slow and well resolved in time (Zimmerman and Baylor, 1986; Haynes et al., 1986; Karpen et al., 1988). Based on studies of both fluctuations in macroscopic currents and single channels, Torre et al. (1992) have recently suggested that in rod photoreceptors there exist two classes of cGMP-gated channels: one rare class with slow kinetics and well resolved closed to open transitions and a second class, representing the vast majority of channels, whose kinetics are so fast as to be poorly resolved in time. The kinetics of these fast channels are more accurately characterized through analysis of their ensemble behavior as reflected in current noise.

The cGMP-gated channels in cone photoreceptors are similar, but not identical with those of rods (Picones and Korenbrot, 1992*a*; Haynes, 1992; Bonigk et al., 1993). The kinetics and conductance of the cGMP-gated channels in cones have been characterized only in studies of single-channel activity. In membrane patches detached from fish cone outer segments, Haynes and Yau (1990) recognized that channels exist in more than one conductance state, but they restricted their analysis to the properties of the single highest conductance state, about 50 pS in magnitude. Watanabe and Murakami (1991) analyzed the unitary conductance of single channels also in fish cones and, in contrast, detected only one conductance state about 14 pS in amplitude. It is possible that in cones, as in rods, the channel population may not be homogeneous. To investigate this possibility we undertook a statistical analysis of fluctuations in the cGMP-dependent currents measured in cone outer segment membranes. This analysis reflects the properties of the most frequently occupied channel state and avoids the limitations of sample bias when the channel population is not uniform or is complex in its behavior (Patlak, 1993). Comparison of our results to those reported in studies of unitary activity suggests that in cones the channels are homogeneous in their kinetics, but not in their conductance level.

MATERIALS AND METHODS

Materials

Striped bass (*Morone saxatilis*) were obtained from a commercial hatchery (Professional Aquaculture Services, Chico, CA) and maintained in the laboratory for up to 4 weeks under 14–10-h light-dark cycles with daily feeding. Wheat germ agglutinin was purchased from E-Y Labs (San Mateo, Ca), other chemicals were from Sigma (St-Louis, MO).

Cell preparation, solutions and electrical recordings

We studied single and twin cones isolated from the retina of striped bass. We have previously described methods to isolate the photoreceptors and maintain them in short-term culture (Picones and Korenbrot, 1992*a*). Isolated cells were immobilized on wheat germ agglutinin-coated coverslips placed on the bottom of a shallow recording chamber. The recording chamber consisted of two compartments that could be separated by a small, movable gate. Cells were held in one of the compartments, about 1 ml in volume, in which they were intermittently perfused with a fish-Ringer's

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solution of the composition (in millimolar): 136 NaCl, 2.4 KCl, 5 $NaHCO_3$, 1 NaH_2PO_4, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose, and 10 HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.5 and 309 mOsM. Under microscopic observation, using DIC optics, we applied tight-seal electrodes onto the side of the distal third of the cone outer segments. After giga-seal formation, we excised inside-out membrane patches by rapidly withdrawing the electrode from the outer segment surface. For unexplained reasons, some cells (~20%) yielded patches that were unresponsive to cGMP, perhaps because of formation of closed vesicles. After obtaining a detached membrane patch, the electrode tip was slowly moved under the solution in the recording chamber from the compartment that contained the cells to a second compartment. This second compartment was 0.5 ml in volume and was continuously perfused with the same solution filling the tight-seal electrode. In this compartment, the tip of the tight-seal electrode was placed within 150 μm of the opening of a 100-μm-diameter glass capillary used to deliver test solutions to the cytoplasmic surface of the membrane patch.

Tight-seal electrodes (Hamill et al., 1981) were fabricated from Corning 1724 glass capillaries (aluminosilicate, 1.5/1.0 mm outside diameter/inside diameter; Garner Glass Co., Claremont, CA) and were coated to within 100 μm from the tip with Sylgard (Silicon elastomer 184, Dow Corning Co., Midland, MI). The composition of the electrode-filling solution was (in millimolar): 157 NaCl, 1 EGTA, 1 EDTA, 10 HEPES, pH 7.5 and 305 mOsM. Test solutions were of the same ionic composition as the electrode-filling solution, but contained varying concentrations of cGMP. In a typical experiment, the membrane patch was exposed to test solutions of cGMP concentrations increasing from 5 to 1000 μM. In between each concentration tested, the membrane was bathed with cGMP-free solution. Data were accepted for analysis only if the conductance in the absence of cGMP changed by ≤10% during the course of measurements.

At room temperature, we measured membrane currents under voltage clamp using a patch clamp amplifier (EPC-7, List Instruments, Darmstadt, Germany). We compensated membrane and electrode capacitance. The analog signal was low-pass filtered below 2 kHz with a Butterworth filter (Frequency Devices, Haverhill, MA) and digitized on line with 12-bit accuracy at 5-kHz acquisition rate. Data were fit with selected functions using a nonlinear, least-squares minimization algorithm (NFit, Galveston, TX).

Data analysis

Analysis of fluctuations in stationary current demands that the mean current amplitude be constant during the period of sampling. We obtained this condition by holding membrane voltage at 0 mV and sampling currents activated by 150-ms duration step changes to −50 mV (extracellular surface defined as ground). The nucleotide-dependent inward currents in cones are time-independent; they do not exhibit desensitization (Haynes and Yau, 1990) and they do not decay in time, as large outward currents sometimes do, because of restricted diffusion in the cytoplasmic face of the excised outer segment patch (Zimmerman et al., 1988).

Computation of cGMP-dependent mean current and variance

To compute these numbers, we measured the mean and variance of the current during the 150-ms hyperpolarizing voltage pulse. We measured these parameters in successive trials; first, 10 trials in the absence of cGMP, and then 10 trials in the presence of the test cGMP solution. Individual trials, one with and one without nucleotide, were randomly paired and the difference between their mean and variance computed. This yielded one pair of values for the mean and variance of the cGMP-dependent currents. For each membrane patch and at each concentration tested, the values of mean and variance were the average of the 10 individual pairs of values. This procedure eliminated the contribution of sources other than the activation of the cGMP-gated channels such as leakage of the electrode-membrane seal or ligand-independent activity of the cGMP-gated channels (Picones and Korenbrot, 1992*b*).

Spectral density distribution

Current sweeps of 2048 points (409.4 ms at 0.2 ms per point) were acquired and power density spectra calculated by averaging the power spectrum of each of 20 different current sweeps. In the graphic display of power spectra data, points below 50 Hz are individual data points, points between 50 and 500 Hz are the average of two consecutive data points, and those between 500 Hz and 2 kHz are the average of 10 consecutive data points.

RESULTS

We analyzed the fluctuations of cGMP-dependent currents in inside-out membrane patches detached from the outer segment of single cones. Under voltage clamp, we measured cGMP-dependent currents and characterized the unitary conductance, probability of opening, and kinetics of the channels whose activity underlies the macroscopic currents.

cGMP dependence of macroscopic mean currents

The dependence of mean current amplitude on cytoplasmic cGMP concentration in cone outer segment membranes at −50 mV is illustrated in Fig. 1. This dependence is well described by a cooperative activation function of the form

$$\frac{I}{I_{max}} = \frac{[cGMP]^n}{K_m^n + [cGMP]^n} \quad (1)$$

where I is the mean current amplitude measured at some cGMP concentrations, I_{max} is the maximum amplitude of this

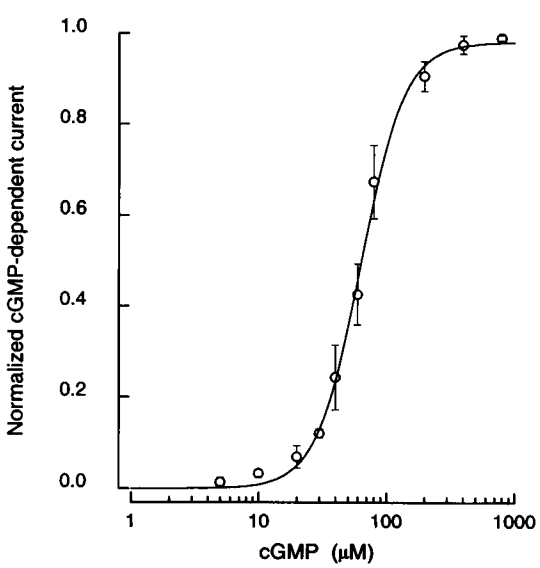

FIGURE 1 cGMP dependence of currents in inside-out membrane patches detached from cone outer segments. Currents amplitudes were measured at −50 mV in the presence of varying cGMP concentrations and their amplitude normalized by the maximum amplitude observed. Data points and the vertical bars are the mean ± SEM, respectively, computed from four to nine different patches. The continuous line is an optimized fit to the data of text Eq. 1 (Hill's equation). The values of the parameters of Eq. 1 estimated from the optimized fit are: K_m= 62.9 ± 2.0 μM (± SD) and n = 2.53 ± 0.19.

current, and K_m and n are adjustable parameters. K_m measures the binding constant of cGMP to the ion channels, and n is a coefficient of cooperativity. The continuous curve in Fig. 1 is a least-squares fit of Eq. 1 to the average of data from nine different patches. The optimum fit yields the following average values (± SD): K_m= 62.9 ± 2.0 μM and n = 2.53 ± 0.19. The value of K_m is significantly different from that measured at +80 mV, K_m=42.6 ± 5.9 μM (Picones and Korenbrot, 1992*a*), reflecting a voltage dependence of the cGMP binding or of the channel lifetime.

Analysis of mean current and variance

Single channel current and number of channels in a patch

We determined the number of cGMP-gated channels and their unitary current in individual membrane patches through analysis of the mean current amplitude and its variance (Stevens, 1972; Sigworth, 1980; Neumcke, 1982). In a patch with N ionic channels, each with single-channel current i, the mean current amplitude (I) and its variance (σ^2) are given by

$$I = iNP_0 \tag{2}$$

$$\sigma^2 = i^2NP_0(1 - P_0) \tag{3}$$

where P_o is the probability of a channel being in the open state. The fluctuations in I, which originate in the changes in probability of channel opening, are less pronounced at small values of I, when openings are less probable, and at high values of I, when closures are less favored and P_o approaches 1. The single channel current i, can be calculated by combining Eqs. 2 and 3

$$i = \frac{\sigma^2}{I(1 - P_0)}. \tag{4}$$

Combining Eqs. 3 and 4 to eliminate the explicit dependence on P_o yields

$$\sigma^2 = iI - \frac{I^2}{N}. \tag{5}$$

Because the value of I varies as a funtion of the concentration of cGMP (Eq. 1), we measured in each membrane patch the mean current amplitude and its variance at each of various cGMP concentrations in the range between 0 and 800 μM. In Fig. 2 we illustrate cGMP-dependent currents measured in the same membrane patch in response to 150-ms step hyperpolarization to −50 mV. Inspection of the records in Fig. 2 *A* reveals that current fluctuations are largest near 40 μM cGMP and that they decline at both higher and lower concentrations. Fig. 2 *B* illustrates the dependence of variance on the mean current for the data shown in Fig. 2 *A*. This dependence is well described by Eq. 5, and least-squares fit of the equation to the data indicates that in this patch i = −0.77 pA and N = 79.6 channels. The same analyses were made in 14 patches and the averages (± SD) of values measured were i= −0.87 ± 0.24 pA and N = 388.0 ± 291.8 channels.

cGMP dependence of probability of channel opening

Analysis of variance also allowed us to calculate the dependence on cGMP of the probability of channel openings. In

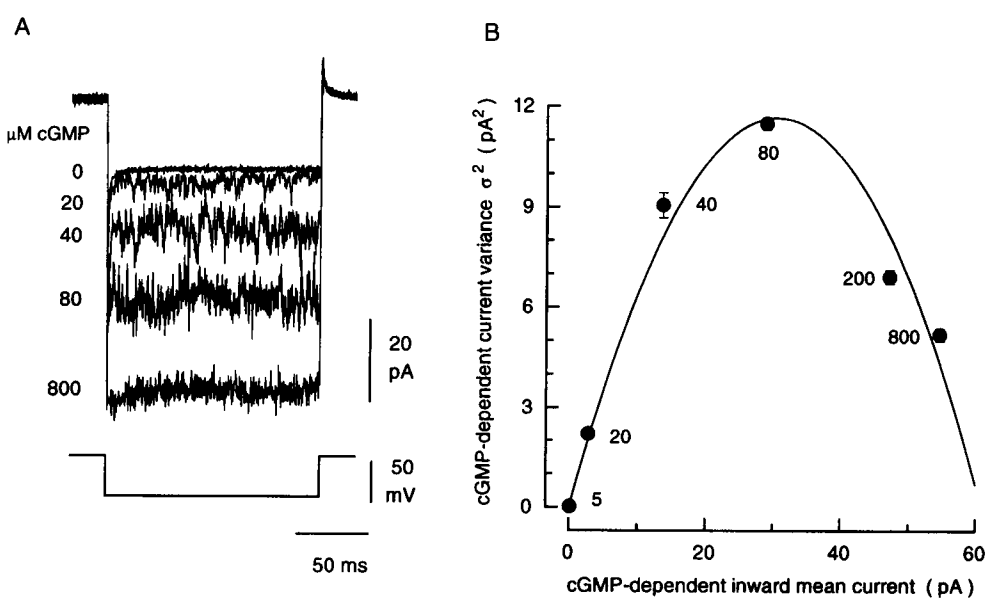

FIGURE 2 Fluctuations in the cGMP-dependent currents in a cone outer segment membrane patch. Panel *A* illustrates single sweeps of currents recorded in response to −50 mV voltage pulses in the presence of varying concentrations of cGMP (identified by the label on each trace). Panel *B* demonstrates the dependence of current variance on the mean amplitude of the cGMP-dependent current for the data shown in *A*. Data points are the averages (± SEM) of 10 repeats at each cGMP concentration tested. The continuous line is the optimum fit to the data of text Eq. 5. The fit yields values of unitary current i = −0.77 ± 0.08 pA and N = 79.6 ± 10.8 channels.

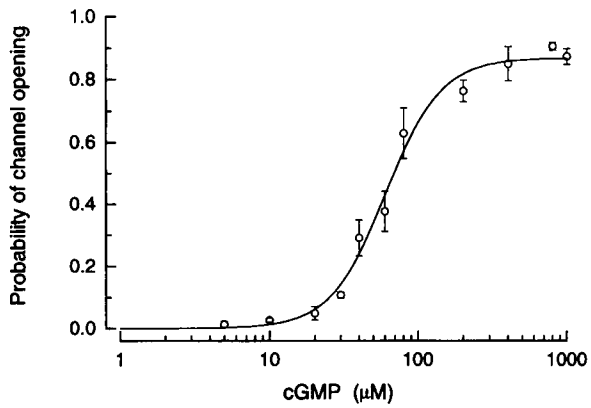

FIGURE 3 Dependence on cGMP concentration of probability of channel opening. Currents were measured at −50 mV. Data points are the mean (± SEM). The continuous line is the best fit to the data of text Eq. 6. The optimized fit yields the following values (± SD): $P_o^{max} = 0.87 \pm 0.03$, $K_m = 60.2 \pm 3.7$ μM, and $n = 2.33 \pm 0.32$.

each membrane patch, the values of i and N were determined as described above; knowing these values, we computed open channel probability for each mean current amplitude measured at various cGMP concentrations (Eq. 2). Fig. 3 illustrates the dependence of P_o on cGMP concentration for the average of 14 patches. Combining Eqs. 1 and 2 yields the following expression to describe the dependence of P_o on cGMP concentration

$$P_0 = \frac{P_0^{max}}{1 + \frac{K_m^n}{[cGMP]^n}} \quad (6)$$

where $P_o^{max} = I_m^{max}/iN$. This function provides an excellent description for the experimental data (Fig. 3). The optimum fit to data measured at −50 mV in 14 different patches yielded the following average values (± SD): $P_o^{max} = 0.87 \pm 0.03$, $K_m = 60.2 \pm 3.7$ μM, and $n = 2.33 \pm 0.32$.

Because the conductance of a single channel is ohmic (Haynes and Yau, 1990), the apparent unitary event conductance (γ) can be directly calculated from the value of i

$$\gamma = \frac{i}{V_m - V_{rev}} \quad (7)$$

where V_m is the membrane voltage and V_{rev} is the reversal potential, which was equal to 0 under the symmetric ionic solutions in our experiments. The calculated γ at −50 mV was 18.2 ± 4.6 pS (mean ± SD; $n = 14$).

Power spectral density distribution of membrane fluctuations

The power spectral density of the cGMP-dependent current noise is illustrated in Fig. 4. The experimental data were well described by the sum of two Lorentzian components

$$S(f) = \frac{S(0)_l}{1 + \left(\frac{f}{f_l}\right)^2} + \frac{S(0)_h}{1 + \left(\frac{f}{f_h}\right)^2} \quad (8)$$

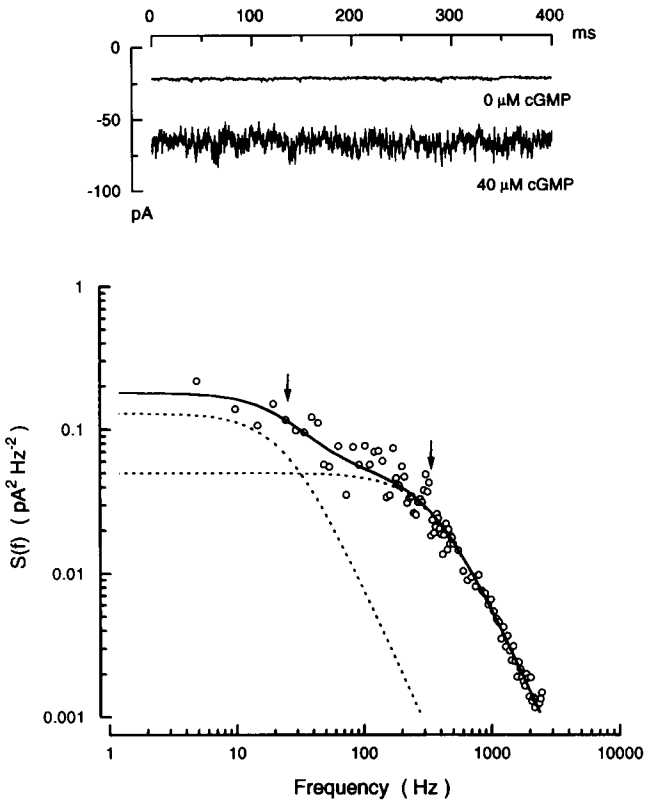

FIGURE 4 Difference power spectrum of the fluctuations in cGMP-dependent current. The upper panel illustrates representative single sweep recordings of macroscopic currents in the absence (*control, upper trace*) and in the presence of 40 μM cGMP (*lower trace*). Patch resistance in the absence of nucleotide was 2.5 GΩ. The lower panel shows the spectral density distribution function of the difference current measured with and without the nucleotide. The continuous line is the fit to the data of text Eq. 8 with $S(0)_l = 0.13$ pA² Hz⁻¹, $f_l = 25$ Hz; $S(0)_h = 0.05$ pA² Hz⁻¹; $f_h = 350$ Hz. The individual Lorentzian components are illustrated by the discontinuous lines. The arrows indicate the corner frequencies for each component (f_l and f_h).

where $S(0)_l$ and $S(0)_h$ are the zero-frequency asymptotes for the low- and high-frequency components, each characterized by half-power frequencies f_l and f_h. We analyzed the spectra of current noise in the presence of 40 μM cGMP in 10 patches. In these patches the mean amplitude of the cGMP-dependent current at −50 mV was −102.4 ± 84.8 pA (± SD). At this concentration, average values (± SD) for the corner frequencies were: $f_l = 26.2 \pm 17.9$ Hz, and $f_h = 318.0 \pm 57.9$ Hz.

The spectral density distribution can also be used to estimate the unitary current of the cGMP-gated channels. The variance of the current fluctuations is the integral over the positive frequencies of the spectral density distribution (DeFelice, 1981). Thus, for Eq. 8

$$\sigma^2 = \frac{\pi}{2}[S(0)_l f_l + S(0)_h f_h]. \quad (9)$$

In individual patches we determined I and σ^2 in the presence of 40 μM cGMP. At this concentration P_o is 0.29 (see Fig. 3). With these values we computed the value of i (Eq. 4). In 10 patches and at −50 mV (average ± SD), i was −0.86

± 0.21 pA and γ was 18.1 ± 4.3 pS. These values are essentially the same as those reported above and measured in a different set of membranes through the analysis of variance.

DISCUSSION

We have characterized the properties of cGMP-gated channels in cone photoreceptor outer segments through statistical analysis of current noise. A comparable study has not appeared before, although the cGMP-gated channels of rod photoreceptors have been analyzed using these techniques (Matthews, 1986). We undertook this investigation to determine whether cGMP-gated channels in cones are a homogeneous class of channels by comparing the functional properties of the channels inferred from their ensemble behavior with those measured directly in single-channel recordings. If a population of channels is not functionally homogeneous, macroscopic measurements reflect the features of the state most prevalently occupied, whereas single-channel recordings may be dominated by slow and large conductance states more readily recorded.

Probability of channel opening

Statistical analysis of current fluctuations is particularly useful in determining the probability and kinetics of channel openings. We have characterized the dependence of the probability of channel opening, P_o, on cGMP concentration. The maximum probability of opening at saturating cGMP concentrations is about 0.9. Single-channel recordings reveal nearly the same limiting value for P_o in other cones (Haynes and Yau, 1990), although single-channel data are limited because they depend on the rare experimental condition in which the outer segment membrane patch contains only one active channel and is explored at various cGMP concentrations (two patches in Haynes and Yau, 1990). Our result, however, appears to differ from findings in the cGMP-gated channel of rods. In studies both of macroscopic currents in the rod outer segment (Matthews, 1986) and on single-channel measurements (Matthews and Watanabe, 1988), Matthews has suggested that the limiting value for P_o at saturating cGMP concentration is between 0.3 and 0.4. These studies, however, were conducted in the presence of 0.2 mM calcium, a condition known to block current through the channels (review in Yau and Baylor, 1989), and which might have affected the probability of opening. Ildefonse and Bennet (1991) and Ildefonse et al. (1992) report that, in the absence of divalent cations, P_o^{max} in bovine rod channels "incorporated" in black-lipid membranes is nearly 1. Therefore, the value of P_o^{max} is likely to be similar in channels from rods and cones.

Channel kinetics

The spectral density distribution of the cGMP-dependent current fluctuations in cones in the presence of 40 μM cGMP is well fit by the sum of two Lorentzians, within our recording bandpass (≤2 kHz). In recent studies of current fluctuations in rods at higher bandpass (Torre et al., 1992) (≤4 kHz), a third high-frequency Lorentzian becomes apparent. Within the 2-kHz recording bandpass, however, the spectral density of cGMP-dependent current in rods is also fit by the sum of two Lorentzians. The corner frequencies of the Lorentzian components in cones are, on average, slower than those reported for rods, and this may be added to the list of detailed biophysical features that distinguish rod from cone channels (Picones and Korenbrot, 1992*a*). Within the same recording bandpass, previously reported average values for the low-frequency component in rods are (in Hz ± SD) 70 ± 5 (Bufo rods, Matthews, 1986), 91–143 in range (Bufo rods, Haynes et al., 1986), which compare with our value of 26.2 ± 17.9 in cones. The high-frequency component reported for rods is (in Hz ± SD) 1190 ± 76 (Bufo rods, Matthews, 1986), 667–1111 in range (Bufo rods, Haynes et al., 1986), which compare with our value of 318.0 ± 57.9 in cones. Two Lorentzian functions in the frequency domain identify two exponential processes in the time domain. In cone channels, therefore, the macroscopic behavior would be consistent with the existence of two exponentially distributed populations in the histogram of open state lifetimes. The slow time constant would be about 6.1 ms and the fast one 0.5 ms. The fast time constant measures the mean lifetime of the open state of the channel, whereas the slow one measures the mean lifetime of "bursts" of channel activity.

The records of cGMP-gated single-channel activity in cones show individual openings that are brief in duration with a "spiky appearance" (Haynes and Yau, 1990). The kinetics of channel opening we have inferred from analysis of fluctuations at 40 μM cGMP are similar in value to those measured in single-channel recordings in the presence of 4 μM cGMP. The mean channel open lifetime is 0.5 ms at −50 mV in our results, 0.5 ± 0.10 at −30 mV in the single channel results of Haynes and Yau (1990) and 0.42 ± 0.12 at −80 mV in the results of Watanabe and Murakami (1991). Burst mean duration is 6.1 ms in our analysis and 2.26 ± 0.56 in the analysis of Haynes and Yau (1990). Watanabe and Murakami (1991) did not report a slow time process. In cones, then, frequency analysis suggests that the cGMP-gated channels are functionally homogeneous with respect to their kinetics because their functional characteristics determined through either noise analysis or single-channel recordings are essentially the same.

The channel kinetics we measured at 40 μM cGMP are similar to those measured at 4 μM cGMP (Haynes and Yau, 1990; Watanabe and Murakami, 1991). Indeed, the cGMP-gated channels exhibit a very low level of activity even in the absence of nucleotide, and the kinetics of this activity are the same as those measured in the presence of cGMP (Picones and Korenbrot, 1992*b*). Thus it appears that varying cGMP concentration changes primarily the probability of channel opening, but not channel kinetics.

Single-channel conductance

Although the features of channel kinetics and probability of opening are similar when measured by either noise fluctua-

tions or unitary activity, the same is not true of the single-channel conductance. Haynes and Yau (1990) report the largest conductance they observed and analyzed was ~50 pS in amplitude, but they also observed lower conductance states. Watanabe and Murakami (1991), on the other hand, report observing only one conductance state, about 14 pS in amplitude. Analysis of current noise is based on the presumption that a) all channels are identical, b) channels open and close independently of each other in a stochastic manner, and c) channels exist only in either a closed state or a single open state (Neumcke, 1982). Therefore, if multiple open states exist, noise analysis is likely to reflect the features of the state most frequently occupied. In our results, two independent methods of analysis indicate that the most frequently occupied open state has a conductance value of about 18 pS. Moreover, this most prevalent open state strongly dominates the current fluctuations at all cGMP concentrations because the variance as a function of mean current is well described by a parabolic function, as expected from theory (Fig. 2 *B*, text Eq. 5).

Noise analysis can underestimate the single-channel conductance of the most prevalent state if Lorentzian components with high corner frequencies are not detected because of bandpass limitations in the experimental recordings. Our data are limited to 2 kHz, and Torre et al. (1992) report that cGMP-gated channels in rods, studied at 4 kHz bandpass, exhibit a Lorentzian component with a corner frequency at ~4 kHz that represents about one third of the energy in the power spectrum. If cone channels exhibit a comparable high-frequency component, our calculation of single-channel conductance would underestimate its true value by ~30%. The similarity between our single-channel conductance and that reported by Watanabe and Murakami (1991), and the difference with that reported by Haynes and Yau (1990), suggests that cGMP-gated channels in cones are not homogeneous with respect to unitary conductance. The channels can exist in open state conductances as large as 50 pS (Haynes and Yau, 1990), and this number may even underestimate the true conductance because of frequency limitations in the recordings (Torre et al., 1992). However, on average, this is not the most frequently occupied open state. At 40 μM cGMP, the most frequently occupied open state, has a conductance of 18–25 pS. This open state conductance may represent transitions from the closed state or even steps between levels of a single, multilevel channel.

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