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Single-Channel Study of the cGMP-Dependent Conductance of Retinal Rods from Incorporation of Native Vesicles into Planar Lipid Bilayers

Michèle Ildefonse and Nelly Bennett

Laboratoire de Biophysique Moléculaire et Cellulaire, Unité de Recherche Associée 520 du Centre National de la Recherche Scientifique, Centre d'Etudes Nucléaires de Grenoble, 85x, 38041 Grenoble cedex, France

Summary. Unitary currents through cGMP-dependent channels of retinal rods are observed following incorporation into planar lipid bilayers of native vesicles from purified rod outer segment membranes washed free of soluble and peripheral proteins. The influence of the concentration of cGMP, inhibitors (*cis*-diltiazem, tetracaine and Ag^+) and divalent cations (Ca^{2+}, Mg^{2+}, and Co^{2+}) on the conductance and open probability of the channel is described, as well as the voltage dependence of these effects. The cGMP dependence suggests the existence of four binding sites for cGMP and reveals that sequential binding of four cGMP molecules corresponds to the opening of four discrete conductance levels. Finally, we provide conclusive evidence that activated G-protein does not directly inactivate the cGMP-dependent channels of bovine retinal rods.

Key Words vision · photoreceptors · cGMP-dependent channel · bilayers

Introduction

Phototransduction in retinal rods is initiated by absorption of a photon by the photosensitive pigment rhodopsin; photoexcited rhodopsin catalyzes binding of GTP on a GTP-binding protein, which in its GTP-bound state activates a cGMP-phosphodiesterase. This results in reduction of the cytoplasmic concentration of cGMP and closing of cGMP-dependent cation channels of the plasma membrane. The sodium current entering the rod outer segment in the dark is reduced, inducing hyperpolarization of the membrane (*see* reviews by Pugh & Cobbs, 1986; Yau & Baylor, 1989).

The cGMP-dependent channels of the plasma membrane are regulated by direct binding of cGMP (Fesenko, Kolesnikov & Lyubarski, 1985; Zimmerman et al., 1985; Haynes, Kay & Yau, 1986; Zimmerman & Baylor, 1986; Matthews, 1987). Most studies of the cGMP-dependent channels carried out by the patch-clamp technique consist of measurements of macroscopic currents because of the high channel density in the plasma membrane. Two strategies have nevertheless allowed single channels to be recorded with this technique: (*a*) Reducing the cGMP concentration to 1–2 μM, in order to reduce the open probability of the channels to such an extent that single-channel events are observed even if several hundred channels are present under the patch pipette. Using this approach, a conductance of 24 pS was measured in salamander rods (Zimmerman & Baylor, 1986), 25 pS in toad rods (Haynes et al., 1986; Matthews, 1987) and 6 pS in rat rods (Quandt, Nicol & Schnetkamp, 1988). Zimmerman and Baylor (1986) also report the existence of a conductance sublevel of 7 pS. (*b*) Studying the inner segment, in which the density of cGMP-dependent channels is much lower than in the outer segment (Watanabe & Matthews, 1988). This approach has allowed an elaborate study of the kinetic properties of toad rod channels (Matthews & Watanabe, 1988).

Another method is to purify the channel protein and to incorporate it into planar lipid bilayers. This was first performed by Hanke, Cook and Kaupp (1988), who purified the cGMP-dependent channels from bovine rods. It has then recently been possible to clone and express the cDNA coding for the channel in *Xenopus* oocytes (Kaupp et al., 1989). A unitary conductance of 26 pS (Hanke et al., 1988) or 20 pS (Kaupp et al., 1989) with, in both cases, a sublevel of 8–10 pS is reported. The kinetic properties (Hanke et al., 1988; Kaupp et al., 1989) are slower than those measured in the experiments of Matthews and Watanabe (1988).

We have recently reported a study of cGMP-dependent channels from bovine rods carried out by incorporation of native vesicles from rod outer segment membranes into planar lipid bilayer (Bennett et al., 1989), a technique which was first used by Tanaka et al. (1987). Using this technique, we usually observe incorporation of no more than 3–4

channels, and incorporation of a single channel is relatively frequent. We report here a study of the cGMP dependence and influence of various possible effectors of the channel (inhibitors, divalent cations, G-protein) at the single-channel level.

Materials and Methods

Membrane Vesicles and G-Protein Preparation

Bovine rod outer segments were purified as described by Kühn (1984). The membranes were then extensively washed in room light with hypotonic buffer in order to remove all soluble proteins as well as the peripheral cGMP-phosphodiesterase (PDE); further hypotonic washes in the presence of GTP dissociate the G-protein from bleached rhodopsin (Kühn, 1984). After at least four additional hypotonic washes, the membranes were frozen in liquid nitrogen. The amount of contaminant PDE was estimated as less than 1% of the initial concentration (about 0.01% of the amount of rhodopsin) from activity measurements.

G-protein was purified from rod outer segments during the washing of the membranes (*see above*) as described by Baehr et al. (1982). The G-protein preparation (before activation) was kept for less than a week. It was permanently activated with $GTP_\gamma S$ as follows: an aliquot of the G-protein preparation was incubated for 15–30 min in an ice bath with washed membranes (bleached) at a rhodopsin/G-protein ratio of 0.5 to 1 in the presence of excess $GTP_\gamma S$ (guanosine-5'-0-(3-thiotriphosphate) in hypotonic buffer. The suspension was then centrifuged for 5 min at 400,000 $\times$ *g* in a Beckman TL100 ultracentrifuge. Activated G-protein with bound $GTP_\gamma S$ (G_α-$GTP_\gamma S$ + G_β) obtained in the supernatant (recovery and activation yield > 95%) was used within the day; its quality was tested by its ability to activate the PDE in the presence of rod membranes by comparison with trypsin activation (Bennett & Clerc, 1989).

Planar Bilayers and Vesicle Incorporation

Phosphatidylethanolamine (PE) and phosphatidylserine (PS) 70 : 30(wt/wt), purified from bovine brain (Avanti Polar Lipids, Birmingham, AL) were dissolved in decane (30 mg/ml) and spread across a hole 200 μm in diameter. Fusion of the rod membrane vesicles with the bilayer was performed in the presence of a sodium gradient (500 mM NaCl in the *cis* chamber, 200 or 100 mM NaCl in the *trans* chamber); divalent cations: 100 μM $MgCl_2$ and 100 μM free calcium (150 μM $CaCl_2$ + 50 μM EGTA) were usually present in the solution in order to increase the probability of fusion. The *cis* chamber was then perfused with a solution of required ionic composition and concentrations. Some experiments were carried out in the incorporation buffer (500 mM NaCl, 100 μM Ca^{2+} and Mg^{2+}). However, the effect of divalent cations on channel activity being more pronounced at lower ionic strength (*see* Results), whenever 100 mM NaCl solutions were used, the concentrations of divalent cations were reduced to 200 nM Ca^{2+} (150 μM $CaCl_2$ + 250 μM EGTA, from dissociation constants reported by Sillen & Martell, 1964) and contaminant Mg^{2+} (no $MgCl_2$ added). These solutions will be referred to as "low divalents." All solutions were buffered at pH 7.4 with 10 mM HEPES. Rod membrane vesicles were added to the *cis* chamber (30 to 100 μg/ml) together with 20–30 μM cGMP in order to allow immediate visualization of channel incorporation, which usually occurred after a delay of a few minutes to 1 hr. If no incorporation had occurred after 1 hr, the chamber was thoroughly cleaned for a new assay. The volume of membranes added was lower than 1% that of the *cis* chamber (1.5 ml). After channel(s) incorporation, the *cis* chamber was perfused once or several times while stirring in order to reduce the membrane and cGMP concentrations and to change the composition of the solution if required (one perfusion providing at least a 20-fold dilution). The absence of channel opening was checked before any further addition, demonstrating that the incorporated channel was indeed cGMP dependent. Washout of the chamber or addition of chemicals does not modify the position of the baseline by more than 0.1 pA; this allows unambiguous recognition of the closed state. All experiments were carried out in room light at 20–22°C.

Recording of Channel Activity and Analysis

Currents were recorded with a Bio-Logic RK-300 patch-clamp amplifier equipped with a 10-Ω feedback head stage. Bandwidth was 1 kHz or 300 Hz. Potential values were defined as *cis* chamber minus *trans* chamber voltages according to the physiological convention. Amplitude histograms were built with the Bio-Patch software (Bio-Logic) and fitted with Gaussian curves using a nonlinear regression method. Records of 1-min duration (for open probabilities) or of 20 to 30 sec (for amplitude measurements) were sampled at 2.5 kHz (1-kHz bandwidth) or 750 Hz (300-Hz bandwidth) and then digitally filtered at 100 Hz unless otherwise specified. The total open probability is defined as $P_o = 1 - P_c$, with P_c = proportion of the Gaussian curve corresponding to the closed state; the open probability of a given level or sublevel is the proportion of the Gaussian curve corresponding to this level. The Gaussian curve corresponding to the closed state is unambiguously determined from the position of the baseline current in the absence of cGMP (*see* Planar lipid Bilayer and Vesicle Incorporation).

Chemicals

cGMP, *cis*-diltiazem (98% D-enantiomere according to the purchaser) and tetracaine were purchased from SIGMA, $GTP_\gamma S$ from Boehringer Mannheim.

Results

cGMP Concentration Dependence of the Conductance and Open Probability of the Channel

It has been previously reported from patch-clamp studies (Fesenko et al., 1985; Haynes et al., 1986; Zimmerman & Baylor, 1986), from studies of the purified and reconstituted channel protein (Hanke et

al., 1988) or from measurements of ionic fluxes (Koch & Kaupp, 1985; Cook et al., 1986; Puckett & Goldin, 1986) that the open probability of the cGMP-dependent channel increases with cGMP concentration; the existence of at least one conductance sublevel (Haynes et al., 1986; Zimmerman & Baylor, 1986; Tanaka et al., 1987) and perhaps two (Hanke et al., 1988; Bennett et al., 1989) was described; it was suggested that these sublevels may be associated with channel molecules having only one or two cGMP bound. The results in Fig. 1*A* (current records and amplitude histograms) indeed demonstrate that at low cGMP concentration, the sublevels are more frequent than the maximal level, while the opposite is observed at higher concentrations. The open probability of sublevels and of maximal level from six experiments as a function of cGMP concentration is shown in Fig. 1*B*; partial results consistent with the data in Fig. 1*B* were obtained in 14 other experiments. When cGMP was replaced with the analog 8-bromo-cGMP (11 experiments), which is known to activate the channels more efficiently (Zimmerman et al., 1985), a similar behavior was observed, except that smaller levels are more frequent and distinct at low concentration compared to experiments with cGMP at the same concentration (Fig. 2); in three experiments where both cGMP and 8-bromo-cGMP dependence could be studied on the same channel, the EC_{50} for 8-bromo-cGMP was smaller by a factor 4–5 than that for cGMP.

In the previous reports cited above on the cGMP dependence of the open probability of the channels, Hill plots suggested cooperative binding of at least three cGMP molecules. In our experiments, Hill coefficients calculated from six experiments were all between 3.1 and 3.4 (*see* an example in the inset of Fig. 1*B*), which would be more in favor of the existence of at least four sites. We also found that amplitude histograms from many experiments are better fitted with 5 Gaussians (closed state and four open states) than with 3 (*see* Materials and Methods): an example is shown in Fig. 3*A*, in which a sublevel having half the maximal current amplitude is very clearly observed; sublevels corresponding to $\frac{1}{4}$ and $\frac{3}{4}$ of the current can also be noted in the record (the same record and histogram are shown at two different filtering conditions, indicating that sublevels are not filtering artifacts, *see* Discussion). A sublevel with half-maximal amplitude at 20 μM cGMP is also shown in Fig. 1*A*. Although sublevels are usually less clearly resolved at negative potentials (inward current), they are also occasionally unambiguously observed: a sublevel opening corresponding to $\frac{1}{4}$ of the maximum level is shown in Fig. 3*B*. Further support for the existence of four conductance levels will be given below (*see* Fig. 5, experiments with tetracaine). It can be noted from the records shown in Figs. 1–3 that each conductance level can be reached from, or decays to, either the closed state or a neighboring level. This suggests that cGMP can bind to both the closed and the open states: indeed if cGMP could only bind to the closed state, then each conductance level could only undergo direct transitions to and from the closed state; on the other hand, if cGMP could only bind to the open state, transitions from closed state to maximal level could only occur stepwise via all intermediate levels.

The curves drawn in Fig. 1*B* are theoretical curves calculating the total open probability and the open probability of sublevels and maximal level as a function of cGMP concentration, using the model described in the legend for Fig. 1, in which 4 molecules of cGMP sequentially bind to the channel (cooperative binding); it is assumed that binding of 1, 2 and 3 cGMP molecules induces sublevel openings while binding of the fourth cGMP molecule induces maximal opening. The best fit with the experimental data in Fig. 1*B* is obtained taking dissociation constants for cGMP binding of 80 μM for the first site and 15–20 μM for the three others. A higher value for the first constant (lower affinity) is necessary to account for the sigmoidal shape of the curves. However, the scheme used (legend of Fig. 1) is oversimplified, since each of the dissociation constants is in fact an apparent constant, including at least two components: the affinity for cGMP (of both closed and open states) and the equilibrium constant for the open/closed transition. Because of the low signal/noise ratio and of the small amplitude of the sublevel currents, we have not been able to measure the duration of open times for each sublevel; therefore, we do not have access to parameters concerning any of the open/closed transitions. Since we have not taken into account in the model the possible existence of closed states with cGMP bound, the real affinities for cGMP may be higher than the values given above. The interest of Fig. 1*B*, therefore, does not reside in the exact value of these constants, but in the fact that the proportion of the different sublevels is indeed consistent with a cooperative model, in which binding of each cGMP molecule is associated with opening of a distinct conductance level. The theoretical curves calculated from a model with four identical independent channels is shown for comparison in Fig. 1*C*: an EC_{50} of 15–20 μM is obtained with a K_d for cGMP of 75–100 μM, and the probability of four channels being open simultaneously (noted "maximal sublevel" on the figure) remains much lower than the probability of (one + two + three

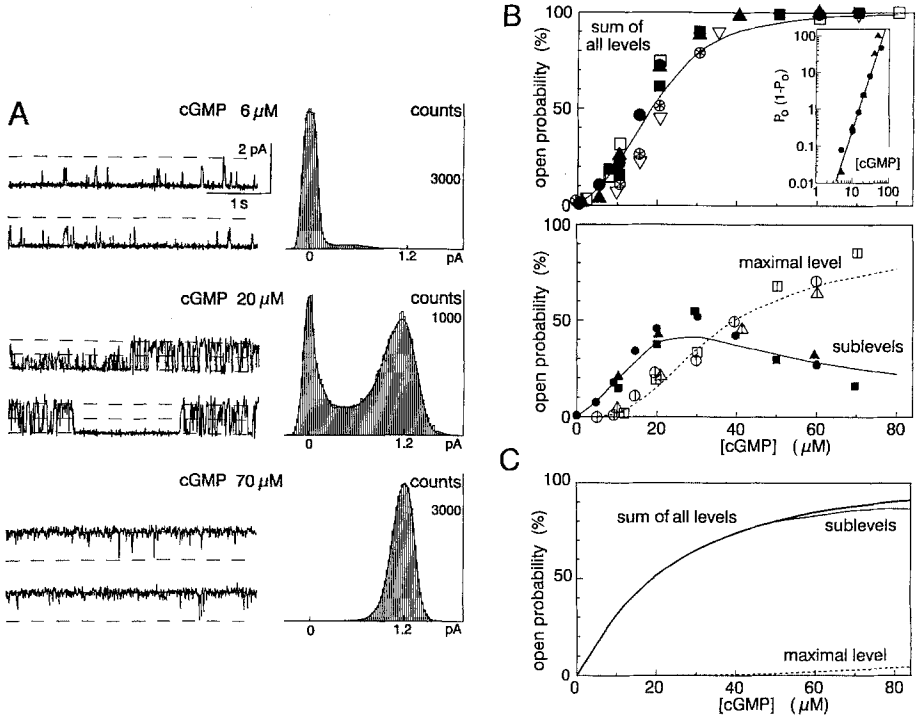


Fig. 1. cGMP dependence of the conductance and open probability of a single channel. (*A*) Current recordings and amplitude histograms from one experiment at different cGMP concentrations: NaCl: 500 mM *cis*/200 mM *trans*, 100 μM $CaCl_2$ and $MgCl_2$; $V = +40$ mV (outward current). Amplitude histograms were built from records of 1-min duration. (*B*) cGMP dependence of the open probability for the sum of all levels (upper frame, from amplitude histogram analysis of six different experiments) and for the maximal (●, ▲, ■) level and the sublevels (Ⓓ, ◬, ◫) (lower frame, from three of the six experiments above, same symbols). The experimental conditions for the different experiments were: NaCl 100 mM *cis*/*trans*, "low divalents" (■, ▲, ⊛), or Ca^{2+} *cis* 100 μM (□), $V = +60$ mV; NaCl 500 mM *cis*/100 mM *trans*, "low divalents" (●) or 100 μM $MgCl_2$ and $CaCl_2$ *cis*/*trans* (△), $V = +40$ mV. Notice that neither the ionic strength nor the presence of 100 μM divalent cations significantly affects the cGMP dependence. A Hill plot from two of the experiments (same symbols) is shown in inset in the upper frame. The slope is 3.2, suggesting the existence of four binding sites. The curves in the two frames correspond to theoretical curves calculating the proportions of the different components in a model with 4 molecules of ligand *L* sequentially binding to a protein *P* (cooperative binding):

$$P \underset{K1}{\rightleftharpoons} PL \underset{K2}{\rightleftharpoons} PL_2 \underset{K3}{\rightleftharpoons} PL_3 \underset{K4}{\rightleftharpoons} PL_4 .$$

The curve in the upper frame, which fits the sum of all conductance levels, is the proportion of $([PL] + [PL_2] + [PL_3] + [PL_4]) = 1 - [P]$. The two curves in the lower frame are the proportions of $([PL] + [PL_2] + [PL_3])$ (solid line, fitting the proportion of sublevels) and of $[PL_4]$ (dotted line, fitting the proportion of maximal level). The proportion of a component PL_i is given by $[PL_i]/[P\text{total}] = p \cdot [L]^i/K_i$, with $p = [P\text{free}]/[P\text{total}] = 1/(1 + [L]/K1 + [L]^2/K1 \cdot K2 + [L]^3/K1 \cdot K2 \cdot K3 + [L]^4/K1 \cdot K2 \cdot K3 \cdot K4)$. The concentration of free ligand is assumed to be equal to that of total ligand, the concentration of protein being negligible. The dissociation constants used for calculations are $K1 = 80$ μM, $K2 = K3 = K4 = 17.5$ μM. Most of the experimental points are contained between the two sets of curves calculated with $K1 = 80$ μM, $K2 = K3 = K4 = 15$ μM and with $K1 = 80$ μM, $K2 = K3 = K4 = 20$ μM. (*C*) Theoretical curves calculating the proportions of open levels in a model where 4 molecules of cGMP bind independently to four identical channels (K_d for cGMP = 100 μM), one open channel corresponding to the smallest current sublevel:

$$4\ (P \underset{K}{\rightleftharpoons} PL).$$

The curve representing the proportion of all open channels is noted "sum of all levels" (thick solid line); the proportion of (one channel open + two channels open simultaneously + three channels open simultaneously) is the thin solid line ("sublevels"), and that of four channels open simultaneously is the dotted line ("maximal level"). The probability of *k* channels being open simultaneously in a total of four is calculated from the equation:

$$P_o = C_4^k \cdot P_o^k (1 - P_o)^{(4-k)}$$

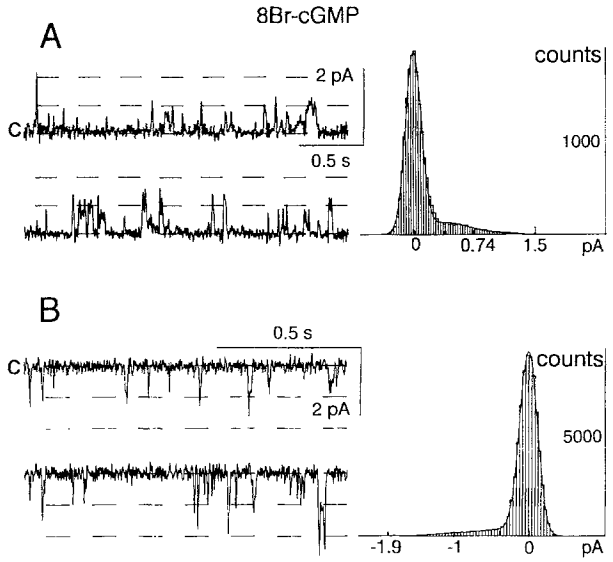


Fig. 2. Sublevel openings of single channels at low 8-bromo-cGMP concentration: outward current (*A*) and inward current (*B*). (*A*) Five μM 8-bromo-cGMP, NaCl 500 mM *cis*/200 mM *trans*, 100 μM Ca^{2+} and Mg^{2+}; $V = +40$ mV. (*B*) Three μM 8-bromo-cGMP, NaCl 100 mM *cis*/*trans*, "low divalents"; $V = -120$ mV. The maximal current level measured in the same experiment at saturating 8-bromo-cGMP concentration (1.5 pA in *A*; 1.9 pA in *B*) and the half-maximal level are indicated by a broken line

channels) being open simultaneously (noted "sublevels") in the concentration range studied.

Voltage Dependence of Conductance and Open Probability

The amplitude of the current in the experiment shown in Fig. 3*A* (at least 5.4 pA for a driving force of 140 mV) corresponds to a conductance of about 38 pS, a much higher value than previously reported. Similar high values (35–38 pS) were obtained in each of four experiments carried out at +140 mV. Indeed, we find that the current/voltage relation of single channels (maximal current level) is not linear: measurements from three experiments at low divalents concentrations (Fig. 4*A*) show a well-marked outward rectification.

We also observe that for a given cGMP concentration the open probability decreases with decreasing voltage: the equivalent gating charge ($z \sim 0.3$) measured from the plot in Fig. 4*B* is consistent with the value of $z \sim 0.5$ previously reported by Hanke et al. (1988). Thus, as also previously described by Karpen et al. (1988), Haynes et al. (1986) and Tanaka, Eccleston and Furman (1989), the EC_{50} for cGMP depends on voltage, with higher EC_{50} values as voltage decreases. In three experiments where the cGMP dependence was studied at two different voltages on the same channel, the experimental data at the lower potential could be fitted with the same constants except for the first one, which had to be still increased (lower affinity): a dissociation constant for the first binding of about 200 μM was thus obtained at -80 mV compared to 80 μM at $+80$ mV. This however does not necessarily imply that the binding of the first cGMP itself is voltage dependent, nor that the affinity of the first binding site is so low. One possibility, proposed by Karpen et al. (1988) is that the open/closed transitions (rather than cGMP binding) are voltage dependent: they indeed observed, from measurements of rate constants in experiments of voltage jump and flash photolysis of caged cGMP, that the rate constant for channel closure increases with hyperpolarization. Tanaka et al. (1989), on the other hand, suggest that the voltage dependence of the open/closed transition does not entirely account for the voltage-dependent shift of the $K_{0.5}$ for cGMP.

Inhibition of Channel Activity

cis-Diltiazem and Tetracaine: Modification of the Expression of Sublevels

L-*cis*-diltiazem has been described as an inhibitor of macroscopic currents (Stern, Kaupp & MacLeish, 1986; Quandt et al., 1988) and cGMP-dependent ionic fluxes (Koch & Kaupp, 1985; Schnetkamp, 1987; Pearce et al., 1988). Tetracaine is a local anesthetic which has been reported to inhibit cGMP-dependent ionic fluxes from rod membrane vesicles (Schnetkamp, 1987), but its effect on cGMP-activated currents has never been tested. In a previous report (Bennett et al., 1989), we noted that in the presence of diltiazem, the small conductance level was more frequently observed. The dependence of the open probability at different conductance levels (single-channel experiments) on the concentration of inhibitor at the cytoplasmic side is shown in Fig. 5*A* (diltiazem) and Fig. 5*B* (tetracaine); a similar dependence was observed in three other experiments for each inhibitor. At positive potentials (outward current), we reproducibly observe that before inducing total inhibition by increasing the proportion of the closed state, addition of diltiazem or tetracaine first reduces the open probability of maximal and intermediate levels, increasing at the same time the open probability of the smallest level. This effect is always very clear cut in the experiments with tetracaine: in the example shown (Fig. 5*C*) as well as in seven other experiments (including two records at 1 kHz), the amplitude of the smallest current level

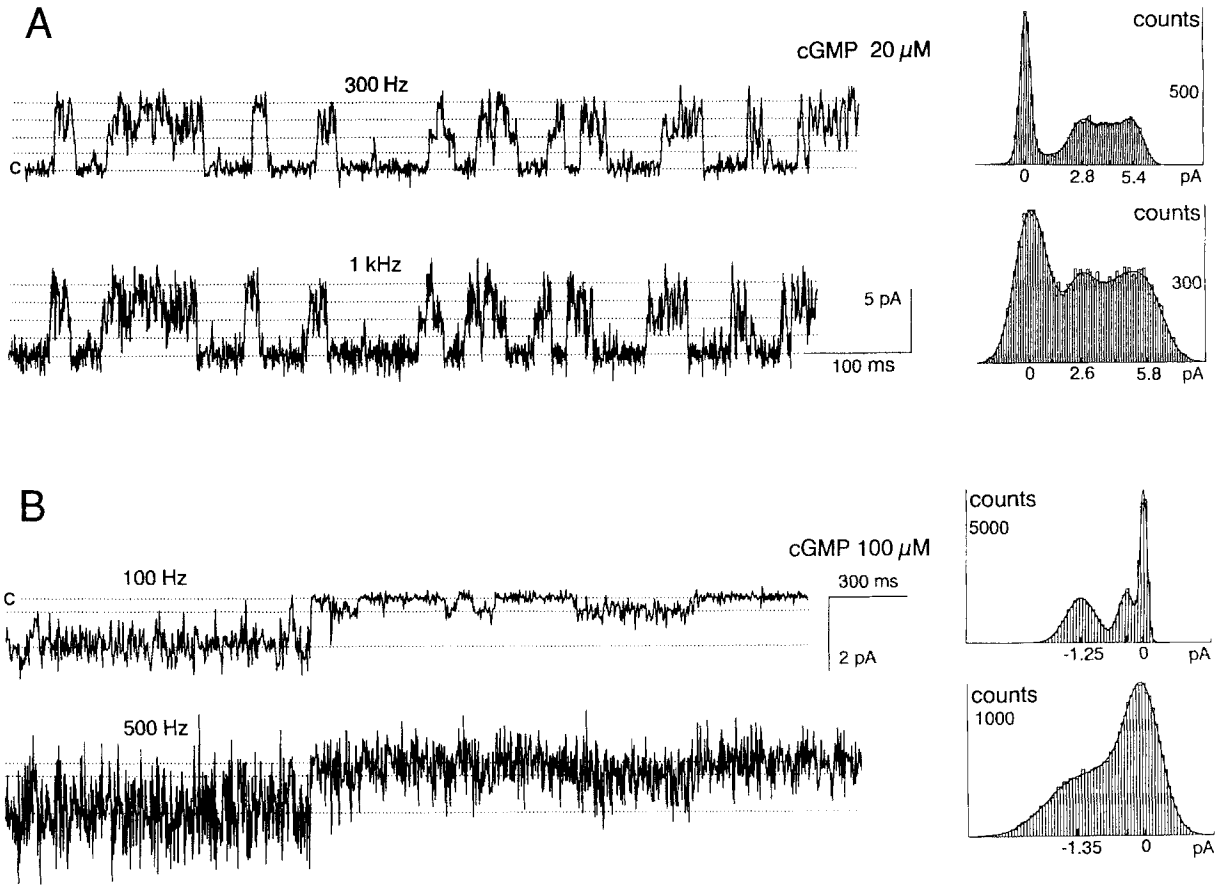


Fig. 3. Resolution of different sublevels at positive (*A*) and negative (*B*) potentials. (*A*) Example of single-channel recording showing four discrete opening levels at positive potential (outward current), filtered at 300 Hz (upper trace and histogram) or 1 kHz (lower trace and histogram). Histograms were fitted with 5 Gaussians, with two well-resolved current amplitudes at 2.8 (2.6) and 5.4 (5.8) pA. NaCl: 500 mM *cis*/100 mM *trans*, "low divalents"; $V = +100$ mV. (*B*) Expression of two different conductance levels at negative potential (inward current). Filtering at 100 Hz (upper trace and histogram) only slightly reduces the amplitude of the current compared to the same record filtered at 500 Hz (lower trace and histogram). The amplitude of the small level (0.34 pA) is close to $\frac{1}{4}$ that of the maximal level (1.25–1.35 pA). NaCl: 100 mM *cis*/*trans*, "low divalents"; $V = -100$ mV; 100 μM cGMP

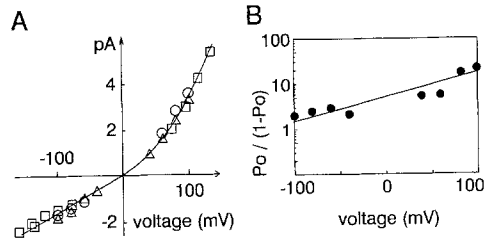


Fig. 4. Voltage dependence of the conductance (maximal level) and open probability of unitary currents. (*A*) Current/voltage relation showing outward rectification. NaCl 100 mM *cis*/*trans*, "low divalents", three different experiments. Bandwidth: △ and ○, 100 Hz; □, 1 kHz. (*B*) Increase of the open probability with increasing voltage (membrane depolarization). The equivalent valence charge (z) for opening the channel measured from this plot (according to the application by Hodgkin & Huxley (1952) of Boltzmann's equation: $P_o(1 - P_o) = \exp(-(w - zeV)/kT)$; *see* Hille, 1984*a*) is ~0.3, indicating a low voltage dependence. Conditions as in *A*

is clearly resolved and corresponds to $\frac{1}{4}$ of the maximal level. Raising the cGMP concentration after inhibition produces an increase of the total open probability, although 100% opening can no longer be reached, and the first sublevel remains more frequent than the other conductance levels with unchanged amplitude (Fig. 5*C*). Tetracaine does not modify the value of the conductances. On the other hand, diltiazem appears to slightly reduce these values as well as modifying the proportion of the different sublevels. Note that the EC_{50} of diltiazem in our experiments is somewhat higher than the values found in the literature (where total block is described from 10 to 50 μM), since we use a mixture mainly composed of the D form (*see* Materials and Methods).

The inhibitory effect of diltiazem and tetracaine is found to be more pronounced at positive voltages (one experiment with diltiazem, six with tetracaine). While in the absence of inhibitors, the open probabil-

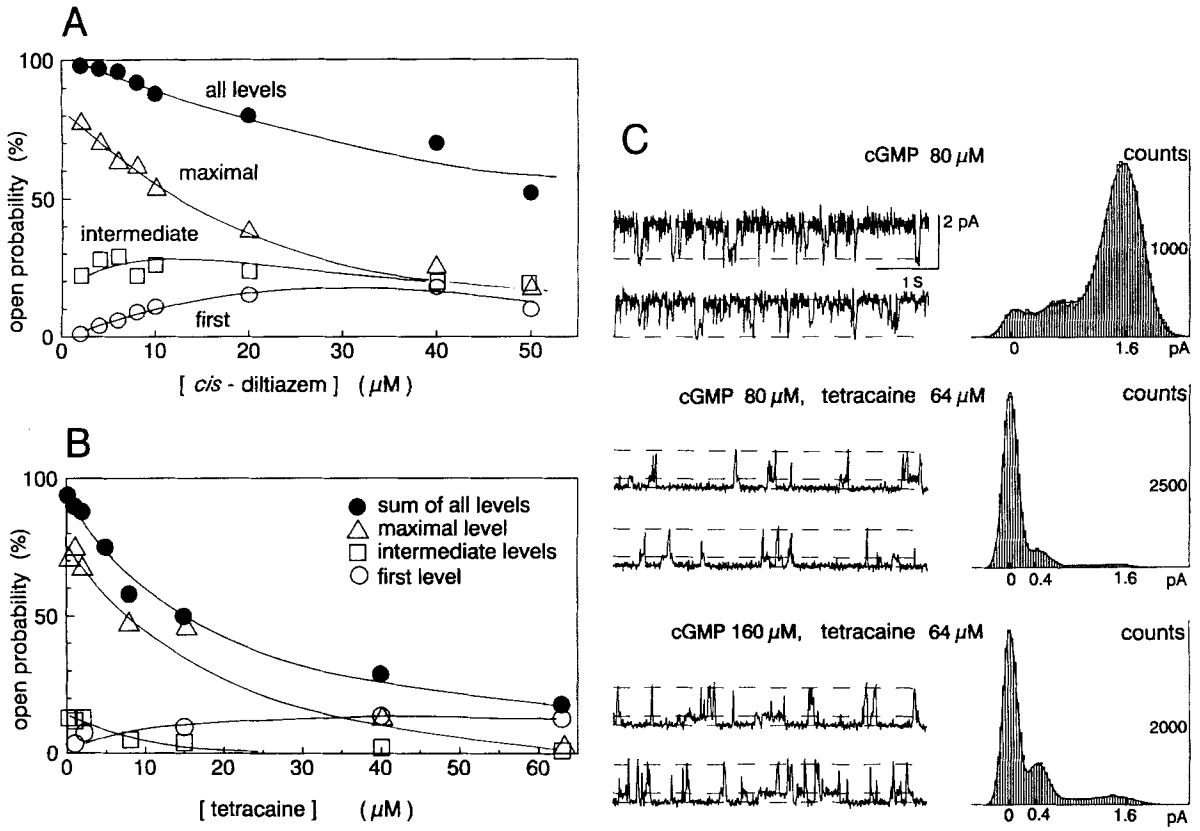


Fig. 5. Inhibition of single channels by tetracaine or *cis*-diltiazem. (*A*) Dependence of the open probability of different conductance levels on the concentration of *cis*-diltiazem (single experiment). The maximal and smallest current amplitudes at 50 μM diltiazem were 1.6 and 0.4 pA. The amplitude of the maximal current level before addition of diltiazem (1.9 pA) was slightly reduced with increasing diltiazem concentrations. Further increase of diltiazem concentration induces total inhibition of channel activity. Experimental conditions: NaCl 500 mM *cis*/100 mM *trans*, 100 μM Mg^{2+}, 100 μM Ca^{2+}, 80 μM cGMP, $V = +60$ mV. Diltiazem was prepared immediately before use. (*B*) Dependence of the open probability of different conductance levels on the concentration of tetracaine (single experiment). NaCl 100 mM *cis*/*trans*, 100 μM Mg^{2+}, 200 nM Ca^{2+}, 80 μM cGMP, $V = +80$ mV. (*C*) Current recordings and amplitude histograms before and after addition of 64 μM tetracaine (80 μM cGMP) and after a further addition of cGMP (160 μM final) (same experiment as in *B*)

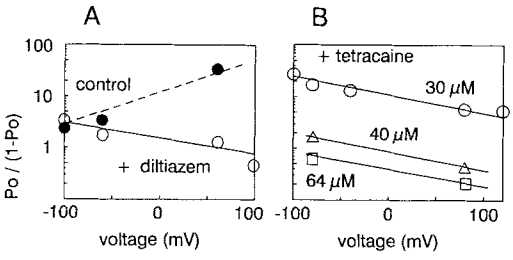


Fig. 6. Effect of diltiazem (*A*) and of tetracaine (*B*) on the voltage dependence of open probability. (*A*) Before (●) and after (○) addition of diltiazem (50 μM). NaCl 500 mM *cis*/100 mM *trans*, "low divalents", 50 μM cGMP. (*B*) After addition of tetracaine. NaCl 100 mM *cis*/*trans*, "low divalents". Two different experiments: ○, 200 μM cGMP: △ and □ (same experiment), 80 μM cGMP. Note the inversion of the voltage dependence by the inhibitors and the absence of inhibition by diltiazem at −100 mV. The value of z in the presence of either inhibitor is of the order of −0.2, compared to $z \sim 0.3$ in the control experiment, *A*, and Fig. 4*B*

ity of the channels at a given cGMP concentration increases as voltage increases (Fig. 4*B* and previous reports, *see above*), the reverse situation is observed in the presence of diltiazem (*see also* Quandt et al., 1988) and tetracaine: the open probability in the presence of diltiazem (Fig. 6*A*) or of tetracaine (Fig. 6*B*) decreases as voltage increases. No inhibition by 50 μM diltiazem is observed at −100 mV (Fig. 6*a*); tetracaine does, however, increase the duration of the closed state even at negative potentials, although higher concentrations are required than at positive potentials.

Local anesthetics have been shown to block Na^+channels in a voltage-dependent manner by

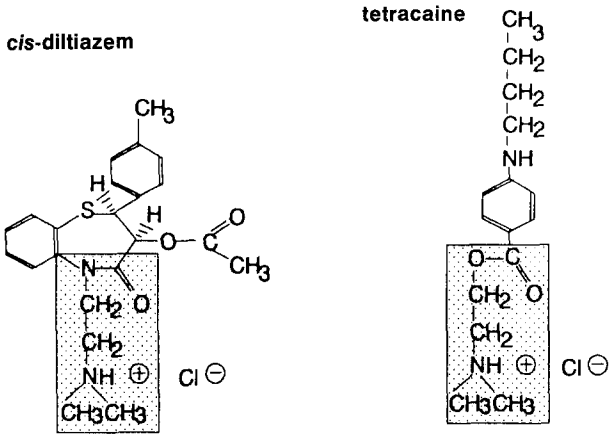


Fig. 7. Structure of *cis*-diltiazem and tetracaine

binding to hydrophobic sites in an internal vestibule (reviewed by Hille, 1984*b*): in the case of positively charged molecules, the voltage sensitivity suggests that the inhibitors follow the cation flux, entering the channel at positive voltage but not at negative voltage; alternatively, voltage-induced charge modifications of the channel protein could facilitate interaction with a positively charged inhibitor at positive voltage (negative charge increment at the cytoplasmic side); voltage-dependent inhibition of Na^+ channels by neutral local anesthetics has, however, also been described. The structure of diltiazem and tetracaine is shown in Fig. 7: the two molecules have hydrophobic parts, which could bind to internal or external hydrophobic sites of the channel, as well as a positively charged quaternary amine, which could also interact with the channel in a voltage-sensitive manner.

Silver

Silver ions (50–100 μM) were reported to induce channel opening from the external side of the plasma membrane of intact rods (Schnetkamp & Szerencsei, 1989). We find, however, that up to 200 μM $AgNO_3$ does not induce any channel activity when added to the *trans* chamber in the absence of cGMP (two experiments) and has no activating effect on the cGMP-induced activity. On the other hand, adding $AgNO_3$ to the *cis* chamber in the presence of cGMP drastically inhibits the channel, total inhibition being achieved at 1 to 2 μM, even in the presence of 100 mM Cl^- (nine experiments), indicating that the reaction with the protein is faster than AgCl precipitation. The block by silver is achieved by reduction of the open probability, the conductance remaining unchanged (Fig. 8), and is observed for both inward and outward currents.

Modifications of Conductance and Open Probability by Divalent Cations

The modifications induced by divalent cations are also strongly voltage dependent (reviewed by Yau & Baylor, 1989). We have limited our study to one positive potential (measurement of outward currents) and one negative potential (inward current) for each ion and each side of the chamber (*cis* = cytoplasmic side of the channel and *trans* = external side for plasma membrane).

Calcium and Magnesium

The effect of Mg^{2+} and Ca^{2+} in the *cis* chamber (cytoplasmic side) is shown in Fig. 9*A* and in the *trans* chamber (external side) in Fig. 9*B*. Each set of data was obtained from a single experiment in which only one channel was incorporated in the bilayer. Several other experiments (five for cytoplasmic and external calcium; nine for cytoplasmic magnesium; two for external magnesium), in which only a partial set of concentrations could be measured, or in which two channels were incorporated gave similar results. The lower calcium concentration used was chosen as 200 nM, which corresponds to the cytoplasmic concentration in the dark-adapted rod (Ratto et al., 1988; Korenbrot & Miller, 1989). The lower magnesium concentration (contaminant magnesium) was estimated as 10–20 μM.

Increasing the cytoplasmic calcium and magnesium concentration up to 1 mM (Fig. 9*A*) has no effect on the conductance nor on the open probability of the channel for the inward current. This result once more refutes the "calcium hypothesis" (Yoshikami & Hagins, 1973), according to which a light-induced increase in cytoplasmic calcium would have reduced the inward sodium current. When the current flows outwards, the open probability of the channel is little or not affected; its conductance, however, is notably reduced with increasing divalent concentrations.

Increasing the external concentration of calcium or magnesium (Fig. 9*B*) reduces both conductance and open probability of the channels, the inward current being more affected than the outward current. Examples of recordings are shown in Fig. 10. At the calcium and magnesium concentrations present in the extracellular medium (around 1.5 mM calcium and 0.5 mM magnesium), the conductance for the inward current is too small to be measured with

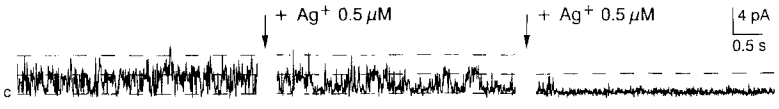


Fig. 8. Inhibition of channel activity by cytoplasmic Ag^+ (three channels incorporated). NaCl 100 mM *cis/trans*, "low divalents," 20 μM cGMP; $V = +80$ mV (outward current)

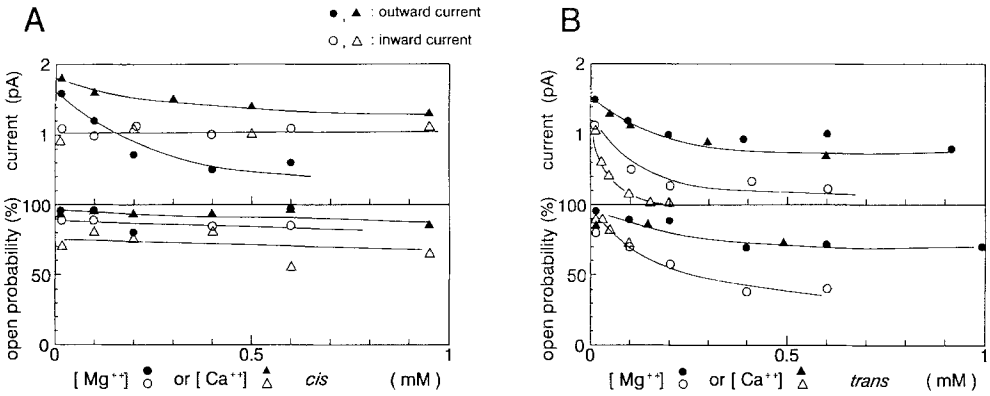


Fig. 9. Effects of cytoplasmic (*A*) and external (*B*) concentrations of Mg^{2+} and Ca^{2+} on unitary current amplitude and open probability for inward currents (△, ○) and outward currents (▲, ●). Each effect of Mg^{2+} (○, ●) and Ca^{2+} (△, ▲) was tested on a different channel. NaCl 100 mM *cis/trans*, "low divalents"; $V = -60$ mV (inward currents) or $+60$ mV (outward currents). cGMP concentration was 30 μM in all experiments. At $[Ca^{2+}]_{trans} > 100$ μM, the open probability of the channel for the inward current cannot be measured due to the reduction of the current amplitude to nonmeasurable values

our setup and the open probability of the channels is markedly lowered.

In a previous report (Bennett et al., 1989), we measured a cGMP-dependent sodium conductance (outward current) of 20–22 pS in the presence of 100 μM calcium and 100 μM magnesium (*cis* and *trans*), and the inward current was clearly measured. These experiments were, however, carried out at 500 mM NaCl in the *cis* chamber and 200 mM in the *trans* chamber (*see also* an example in this report, Fig. 1*A*). We indeed observed that the effect of divalent cations is reduced when ionic strength increases (*not shown*).

It must be noted that whether divalents are added to the *cis* or to the *trans* chamber, inhibition is more pronounced when the applied voltage is such that divalent cations can flow through the channel together with the sodium current, in agreement with previous reports suggesting the existence of a binding site inside the channel (Yau & Baylor, 1989).

In all these experiments, when the conductance is reduced, it happens in a progressive manner, clearly different from the effect of diltiazem and tetracaine, which reduce the proportion of maximal conductance level to the benefit of smaller conductance sublevels.

Cobalt

Addition of Co^{2+} on the external side of the channel (three experiments) reduces both the open probability and the channel conductance (Fig. 11), with a more pronounced effect on the inward current than on the outward current. This effect of Co^{2+}, which is known as an inhibitor of calcium currents (Tsien, 1983), has already been described by MacLeish, Schwartz and Tachibana (1984) and is very similar to that of Ca^{2+} and Mg^{2+}.

Addition of cobalt on the cytoplasmic side (*cis*) has an unexpected and spectacular effect: the activity of the channels in the presence of cobalt (EC_{50} for cobalt around 5–10 μM: eight experiments) is dramatically increased. In the experiment shown in Fig. 12 (outward current), in which two channels are present, the open probability in the control is very low and the current amplitude mainly corresponds to sublevels. At 40 μM $CoCl_2$, 80% simultaneous opening of the two channels (maximal level) occurs. The same potentiating effect is observed on the inward current. The cGMP dependence in the presence of 50 μM $CoCl_2$ (*not shown*) can be accounted for by a decrease of all the dissociation constants for cGMP by at least a factor 2 or 3, suggesting an

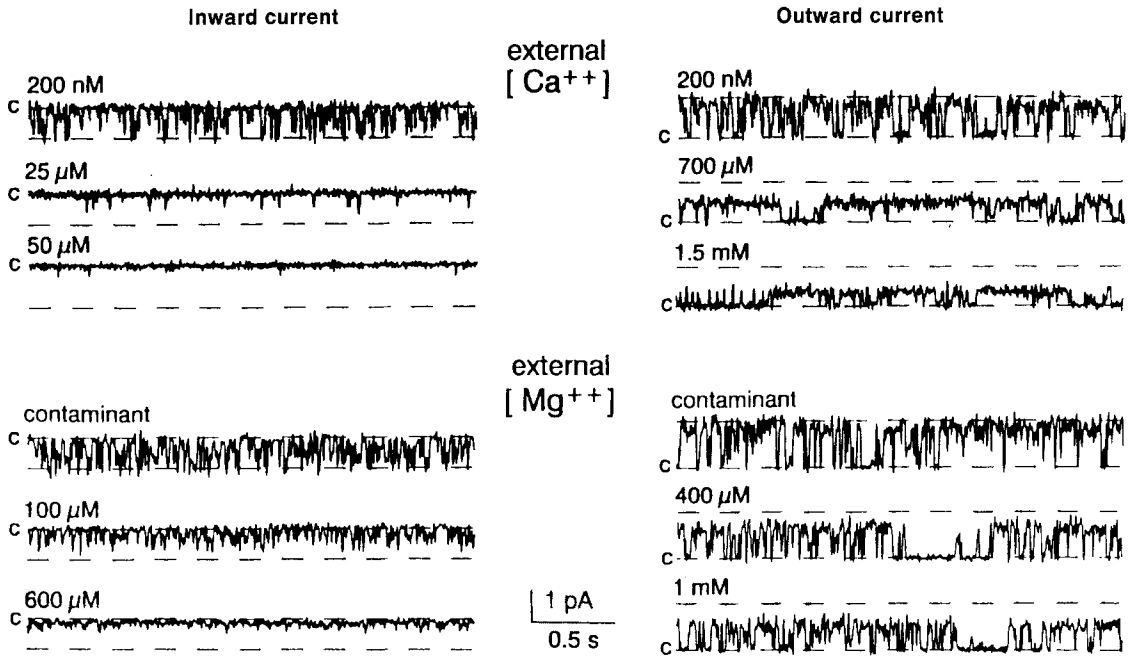


Fig. 10. Recordings of two experiments showing the effect of external calcium and magnesium on inward and outward unitary currents. NaCl 100 mM *cis/trans*, "low divalents," 20 μM cGMP (calcium addition) or 30 μM cGMP (magnesium addition); $V = -60$ mV (inward current) or $+60$ mV (outward current)

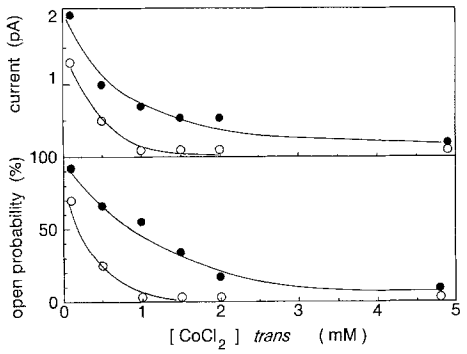


Fig. 11. Effects of external Co^{2+} on current amplitude and open probability of inward current (○) and outward current (●). NaCl 500 mM *cis*/100 mM *trans*, no addition of divalent (no EGTA); $V = +40$ and -100 mV

interaction with the binding sites or with cGMP itself. Similar concentrations of the closely related metals Ni^{2+} and Fe^{2+} produced the same potentiating effect; Mn^{2+}, which was tested because of its effect on guanylate cyclase activity, had no action at similar concentrations.

Absence of Direct Regulation by Activated G-Protein

Since Krapivinsky et al. (1989) reported direct inhibition of 8-bromo-cGMP activated currents in the presence of activated G-protein at the macroscopic level, we attempted to study this effect at the single-channel level. The channel was activated by addition of 8-bromo-cGMP, which is much more slowly hydrolyzed by cGMP-phosphodiesterase than cGMP (Barkdoll, Pugh & Sitaramayya, 1988). Purified G-protein from rod membranes preactivated with $GTP_\gamma S$ ($G_{GTP_\gamma S}$) was added to the *cis* chamber at concentrations ranging from 2 to 7 μM. The experiment was carried out on five different channels: for none of them did G-protein have the slightest inhibitory effect on the currents. An example is shown in Fig. 13*A*. In another experiment where 8-bromo-cGMP was replaced by cGMP (Fig. 13*B*), a progressive reduction of the open probability of the channels is observed, which is likely to be related to hydrolysis of cGMP following activation by G-protein of contaminant phosphodiesterase present in the membranes (note that the concentration of membranes in Fig. 13*B* is much higher than in Fig. 13*A*). In one other experiment with 8-bromo-cGMP, where the membrane concentration (and therefore also the concentration of contaminant phosphodiesterase) was not reduced by washout of the *cis* chamber, we observed a very slow reduction of the open probability as a function of time, consistent with the fact that 8-bromo-cGMP is also hydrolyzed, although much more slowly. We suggest that the inhibitory effect described by Krapivinsky et al. (1989) is in fact the hydrolysis of 8-bromo-cGMP by phosphodiesterase

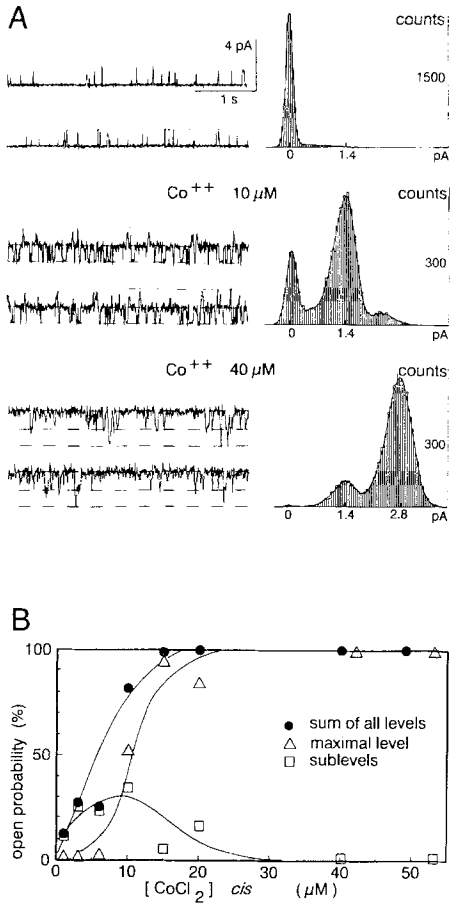


Fig. 12. Potentiating effect of cytoplasmic Co^{2+} on open probability of cGMP-dependent conductance (two channels incorporated). (*A*) Examples of current recordings and amplitude histograms. (*B*) Expression of different levels as a function of cobalt concentration. In this experiment, only the smaller sublevels were expressed before addition of Co^{2+} (*A*, upper trace); below 10 μM Co^{2+}, the smaller sublevels remained predominant; the maximal level became more frequent with increasing concentration of Co^{2+} at the same time as the open probability was increased (*B*). Note that the simultaneous opening of two channels which can be observed from 10 μM Co^{2+} (*A*, middle trace), is almost permanent at 40 μM Co^{2+} (*A*, lower trace). Broken lines indicate maximal opening of one and two channels. NaCl 500 mM *cis*/100 mM *trans* (no addition of divalent); $V = +40$ mV; 20 μM cGMP

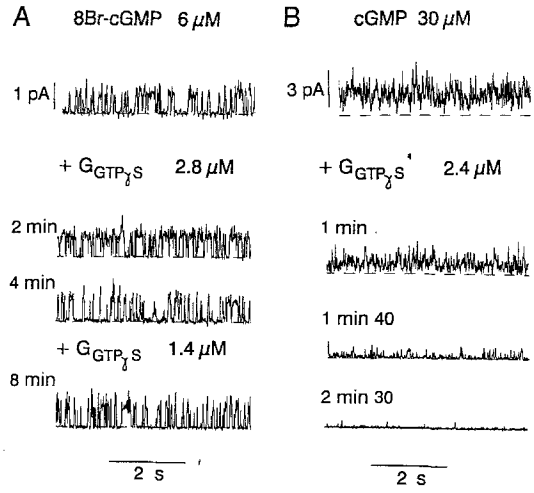


Fig. 13. Absence of direct regulation of cGMP-dependent channels by activated G-protein. (*A*) 8-bromo-cGMP activated current (single channel) before and after two successive additions of G-protein preactivated with $GTP_\gamma S$, $G_{GTP_\gamma S}$ (final $G_{GTP_\gamma S}$ concentration after second addition: 4.2 μM). The *cis* chamber was perfused twice after incorporation of the channel, so that the rhodopsin concentration was less than 8 nM (estimated contaminant PDE concentration: less than 1 pM, *see* Materials and Methods). NaCl 500 mM *cis*/200 mM *trans*, 100 μM Mg^{2+} and Ca^{2+}; $V = +40$ mV. (*B*) cGMP-activated current (at least three channels): in the presence of 3.3 μM of rhodopsin (no perfusion of the *cis* chamber after incorporation) a decrease of the activity was observed after addition of $G_{GTP_\gamma S}$, probably due to the hydrolysis of cGMP by $G_{GTP_\gamma S}$ activation of contaminant PDE (estimated concentration: less than 0.4 nM). PDE activity being about 400 cGMP/sec/PDE under the experimental conditions used (pH 7.4; 20°C; [cGMP] $\sim K_M/2$) (Bennett & Clerc, 1989), 30 μM cGMP are indeed expected to be hydrolyzed by 0.4 nM PDE in about 3–4 min. Same solutions as in *A*, $V = +20$ mV

present in the patch area. The amount of phosphodiesterase in their experiment is expected to be much larger than in ours since they use native rods; indeed, the membranes that we use are extensively washed during preparation in order to remove most peripheral proteins (*see* Materials and Methods). Recently, Ertel (1990) also concluded from patch-clamp experiments on macroscopic currents that activated transducin present in the patch area reduces the light-sensitive current, indirectly, by activation of phosphodiesterase.

Discussion

We report here a single-channel study of the cGMP-dependent cation conductance of rod outer segment membranes. The mechanism of known inhibitors (*cis*-diltiazem and tetracaine) is precised, and other tools which may be useful for future studies of the channel are described (Ag^+ and Co^{2+}). The direct inhibitory effect of activated G-protein reported by Krapivinsky et al. (1989) is not observed.

Effect of Divalent Cations

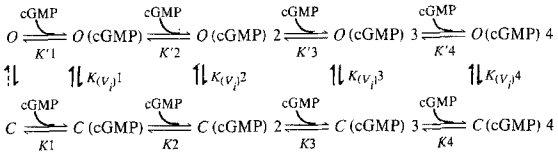
Our results on the effect of divalent cations on unitary currents confirm and complete previous results on macroscopic currents (Matthews, 1986; Stern et al., 1986; Yau, Haynes & Nakatani, 1986): briefly, no effect of cytoplasmic calcium and magnesium on the inward current and large reduction of both conductance and open probability of the

channels for the inward current by external calcium and magnesium, the conductance being too low to be measured at physiological divalent concentrations. This result is consistent with the value of the conductance (of the order of 0.1 pS) deduced from macroscopic current noise analysis (reviewed by Yau & Baylor, 1989). Very few and partial studies of the effect of divalent cations on unitary currents have been previously reported: Haynes et al. (1986) describe a flickering block by divalent cations in the cytoplasmic solution (175 μM Mg^{2+}); Matthews and Watanabe (1988), however, observed that flicker is not reduced in the presence of EDTA and EGTA. With our filtering conditions (*see below*), we would not be able to detect a fast flicker and cannot exclude that the reduction of the conductance by divalents could in fact be only apparent (although current noise analyses do suggest that the conductance is indeed very small under physiological conditions). It can be pointed out that in our experiments, the effects of calcium and magnesium are very similar, suggesting a low specificity of action; this is also confirmed by the similar effect of cobalt on the external side. The effect of cobalt on the cytoplasmic side appears on the contrary very specific and may involve cGMP binding sites. The dissymmetric action of divalent cations added in *cis* or *trans* with respect to the outward or inward current, respectively (stronger inhibition of inward current by *trans* divalent cations than of outward current by *cis* cations, Fig. 9), suggests that, in addition to a binding site inside the channel which would be accessible from both sides of the membrane by voltage-induced cation flux through the channel (Yau & Baylor, 1989), another binding site could be present at the external side. These sites are, however, distinct from the high affinity site (K_d: 1–10 nM) described by Tanaka and Furman (1990), which would be already fully saturated in the "low divalents" solution.

Sublevel Conductances and Model for Channel Activation

The cGMP- and the voltage-dependence of channel opening are compatible with a model in which at each voltage value corresponds a different charge repartition within the channel, which determines a set of closed states (with 0 to 4 cGMP bound), open states (with 0 to 4 cGMP bound) and rate constants for cGMP binding and open/closed transitions. Binding of one cGMP induces opening of the channel with a low conductance. If cGMP concentration is sufficient, binding of the first cGMP will be followed by cooperative binding of 3 other cGMP molecules, the conductance being increased stepwise at each binding by approximately the same value. Each conductance level can undergo transitions to/from the closed state and the neighboring levels, suggesting the existence of two pathways for cGMP binding (closed states- and open states-pathways). For a given voltage, V_i, the complete sequence can thus be represented by the scheme below:

Scheme (a)



The open state with no cGMP bound is theoretical, since no channel opening is observed in the absence of cGMP. In the model proposed by Karpen et al. (1988), only the fully liganded closed state with 3 cGMP molecules bound is in voltage-dependent equilibrium with a fully liganded open state. However, our results are consistent with the existence of open states of different conductance for each cGMP binding, the conductance corresponding to one cGMP bound being about $\frac{1}{4}$ that of the fully liganded channel. Stryer (1987) proposed an allosteric model with four binding sites, in which the channel is supposed to open at each binding step as in scheme (*a*); it is, however, different from scheme (*a*), since the existence of only two distinct conformations (open or closed) is postulated, independently of the number of bound cGMP, while we propose the existence of four distinct open conformations. In scheme (*a*), the different sublevels can be more or less apparent according to values of the equilibrium constants and the binding of cGMP ($K1$, $K'1$, etc.) is not necessarily, although it may be, voltage dependent. The existence of several distinct closed states could explain the existence of bursts, as well as the very long closed periods often observed even at high cGMP concentration (*see* an example at 20 μM cGMP in Fig. 1*A*), which have also been described by Matthews and Watanabe (1988). At least one subopen state has also recently been described in the catfish cone (Haynes & Yau, 1990); a model with five binding sites is proposed in which the large conductance corresponds to the fully liganded channel and the substate to a channel with one less cGMP bound. Although we have evidence for only four conductance sublevels, we cannot exclude that there may be five, since the precision of our measurements may not be sufficient to

detect a smaller level which would be even less frequent than our "first" level.

The existence of conductance sublevels raises the question of the physical structure of the channel. Since the 63-kDa protein has been shown to contain a single cGMP binding site (Kaupp et al., 1989), the channel may consist of a tetrameric structure forming a single pore, whose conductance is modified by cooperative binding of four cGMP molecules to the four units; alternatively, each 63-kDa unit may be itself a channel having a unitary conductance corresponding to the first sublevel, four identical channels being associated in a cooperative manner. According to the single-pore hypothesis, the effect of the inhibitors diltiazem and tetracaine could be speculatively interpreted in two ways: block of the closed-to-open transitions or inhibition of cGMP binding, which would both favor smaller conductance levels when inhibitor concentration increases; in the four-channels hypothesis, another plausible interpretation is the dissociation of the channels units, which would suppress the cooperativity of cGMP binding.

In our experiments, at 1–2 μM cGMP concentrations (which are used in patch-clamp experiments in order to observe single-channel openings), only the first or second sublevels would be observed. This is consistent with the conductance of 6 pS measured by Quandt et al. (1988) using rat rods. The value of 24–25 pS reported from experiments with salamander and toad rods (Haynes et al., 1986; Zimmerman & Baylor, 1986; Matthews & Watanabe, 1987) suggests that the mechanism, or at least the value of the dissociation constants, might be slightly different in lower vertebrates. The models proposed by Karpen et al. (1988) and Stryer (1987) would indeed explain how the channel conductance could be maximal at very low cGMP concentrations in salamander rods; although, the apparent affinity for cGMP is similar to the one that we measure. It is puzzling, however, that at least one conductance sublevel is observed in salamander rods (Zimmerman & Baylor, 1986).

Channel Conductance

The value of the maximal conductance level itself shows wide variations between previous reports. Values obtained from lower vertebrates are reasonably close: 24 pS at +75 mV, 25 pS at +60 mV and 20.5 pS (light-sensitive channel) or 24 pS (cGMP-activated channel) at negative potential according to Haynes et al. (1986), Zimmerman and Baylor (1986) and Matthews and Watanabe (1987), respectively. Results obtained with the bovine channel are significantly different: the conductance of the cGMP-gated channel expressed in *Xenopus* oocytes is estimated as 20 pS at +120 mV, while the slope of the *I-V* curve at $V_m = 0$ mV only corresponds to 12 pS. The slope at 0 mV of *I-V* curves obtained from single-channel currents with the purified channel yields a value of 27 pS (incorporation into bilayers, Hanke et al., 1988). From the *I-V* curve of Fig. 4, which presents a strong outward rectification, the value of the conductance is about 38 pS at +140 mV, 27 pS at +75 mV, 25 pS at +60 mV and 16 pS at negative potentials and at 0 mV. Rectification is also noted, although much less marked, in the previous data on single-channel conductance carried out with the purified protein by Hanke et al. (1988). The reason for these discrepancies is unclear. Although rectification has been suggested to be caused by divalent cations, the concentration of calcium in the experiments used for Fig. 4 (200 nM) is lower than that in the experiments of Hanke et al. (1988) (5–10 μM). In a previous report (Bennett et al., 1989), we also observed a strong rectification, although the values of the conductance were lower than in the present report, due to the different ionic conditions (*see* paragraph on the effect of calcium and magnesium). A possible explanation is that the purified channel protein may be slightly modified (indeed, its sensitivity to diltiazem is lost) and that the conductance of the lower vertebrate channel is different from that of the bovine channel.

Kinetics

Our experiments were recorded with a filtering frequency of either 300 Hz or 1 kHz. Because of a rather low signal/noise ratio, further filtering at 300 Hz (for 1-kHz bandwidth) or even 100 Hz with a digital Hanning filter (minimal duration measurable with less than 10% reduction of the signal amplitude: about 2 or 5 msec, respectively) was usually carried out before histogram analysis. Under these conditions, we do not have access to very fast kinetics. However, in all cases where analysis was possible at 1 kHz, the amplitudes of the current levels were not significantly reduced by filtering at 300 Hz (*see* an example in Fig. 3*A*: 20 μM cGMP, +140 mV) or even 100 Hz (Fig. 3*B*: 100 μM cGMP, −100 mV), indicating that the mean closed and open times (or at least mean bursts duration and mean closed times between bursts) under these conditions were certainly longer than 2 msec. Such slow kinetics have been reported by Hanke et al. (1988) with the purified bovine channel (mean open time > 5 msec for [cGMP] > 2 μM, +50 mV), and by Kaupp et al. (1989) from expression of the bovine channel in oocytes (mean open times: 1 msec and ≥ 10 msec, 5

μM cGMP, +120 mV). They can also be compared to the values reported by Haynes et al. (1986) from salamander rods (mean open time: 1 msec; mean burst duration: a few milliseconds at 2 μM cGMP and +60 mV). The values reported by Matthews and Watanabe (1988) at negative potential (inward current) are much shorter at low cGMP concentrations: mean burst duration of 0.7, 1.14, 1.75 and 3.4 msec at 5, 10, 20 and 50 μM cGMP, respectively (with a mean open time of 0.2–0.3 msec independent of cGMP concentration). They proposed that the duration of bursts may be related to different open conformations of the channel with a different number of bound cGMP. Although we do not know the duration of bursts at low cGMP concentrations, we nevertheless observe sublevel conductances at negative potentials with bovine membranes (Figs. 2*B* and 3*B*), again suggesting a possible difference between bovine and amphibian rods. It is also possible that incorporation of the channels into artificial bilayers slows down the kinetics. Another apparent difference is the maximal probability of channel opening: while we reproducibly observe $\geq$90% channel opening above 80 μM cGMP (26 experiments), above 30 μM cGMP in the presence of cobalt (8 experiments) or above 30 μM 8-bromo-cGMP (4 experiments), a maximal probability of 30 $\pm$ 5% is described by Matthews and Watanabe (1988) and predicted by the model of Tanaka et al. (1989). This difference may be accounted for by our filtering conditions which would indeed occult very short closed times, if they also exist for the bovine channel.

In conclusion, our results confirm and emphasize the fact that in the cell, all the conditions seem to be most unfavorable to the channel function: negative potential which favors the closed state, free cGMP concentration (of the order of 10 μM or less: Cobbs & Pughs, 1985; Yau & Nakatani, 1985) probably well below the dissociation constants, and finally concentrations of divalent cations in the external medium which reduce both the conductance and the open probability of the channels to minute values. The advantages for single photon detection, of having a large number of channels open with a low unit conductance rather than few channels with a large conductance are well discussed by Yau and Baylor (1989). A very low fractional activation (1% of the channels open in the dark) has been demonstrated by Nakatani and Yau (1988); it has been proposed (Stryer, 1987) to be optimal for allowing at the same time fastest responses and lowest energy expenses.

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