

Blockade of a Retinal cGMP-gated Channel by Polyamines

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ABSTRACT The cyclic nucleotide-gated (CNG) channel in retinal rods converts the light-regulated intracellular cGMP concentration to various levels of membrane potential. Blockade of the channel by cations such as Ca^{2+} and Mg^{2+} lowers its effective conductance. Consequently, the membrane potential has very low noise, which enables rods to detect light with extremely high sensitivity. Here, we report that three polyamines (putrescine, spermidine, and spermine), which exist in both the intracellular and extracellular media, also effectively block the CNG channel from both sides of the membrane. Among them, spermine has the greatest potency. Extracellular spermine blocks the channel as a permeant blocker, whereas intracellular spermine appears to block the channel in two conformations—one permeant, and the other non- (or much less) permeant. The membrane potential in rods is typically depolarized to approximately -40 mV in the dark. At this voltage, $K_{1/2}$ of the CNG channel for extracellular spermine is $3 \mu\text{M}$, which is 100–1,000-fold higher affinity than that of the NMDA receptor-channel for extracellular spermine. Blockade of the CNG channel by polyamines may play an important role in suppressing noise in the signal transduction system in rods.

KEY WORDS: cyclic nucleotide-gated channel • ion permeation • putrescine • spermidine • spermine

INTRODUCTION

Cyclic nucleotide-gated (CNG)¹ channels mediate visual signal transduction in vertebrate retinal rods (Yau and Baylor, 1989). The channel opens when intracellular cGMP concentration is elevated in darkness, and closes when cGMP concentration is reduced in light. An open CNG channel conducts Na^+ and K^+ almost equally well and consequently has a reversal potential near 0 mV (Werblin, 1975; Bader et al., 1979; Yau et al., 1981; Bastian and Fain, 1982; Woodruff et al., 1982; Capovilla et al., 1983; Hodgkin et al., 1984, 1985; Yau and Nakatani, 1984; Bododia and Detwiler, 1985; Baylor and Nunn, 1986). Activation of the channel by cGMP depolarizes the plasma membrane, whereas deactivation hyperpolarizes it. The CNG channel functions as a chemoelectrical signal converter: it converts the light-regulated intracellular cGMP concentration into various levels of membrane potential.

The CNG channel also passes Ca^{2+} and Mg^{2+} (Capovilla et al., 1983; Hodgkin et al., 1985; Torre et al., 1987; Nakatani and Yau, 1988). The Ca^{2+} influx through the CNG channel influences light adaptation, which is critical for the retinal rod's broad dynamic range of light sensitivity (Kaupp and Koch, 1992; Koutalos and Yau, 1993).

Permeation rates of Ca^{2+} and Mg^{2+} are considerably lower than those of Na^+ and K^+ . Therefore, Ca^{2+} and Mg^{2+} act as permeant blockers of the CNG channel (Haynes et al., 1986; Stern et al., 1987; Colamartino et al., 1991; Zimmerman and Baylor, 1992; Root and MacKinnon, 1993; Eismann et al., 1994; Park and MacKinnon, 1995). Blockade of the channel by divalent cations dramatically reduces the effective conductance of the channel. Consequently, membrane potential noise in rods is very low, which enables them to detect light with exceedingly high sensitivity (Yau and Baylor, 1989).

Like the CNG channel, inward-rectifier K^+ channels and some glutamate-gated channels are also blocked by divalent cations (Ault et al., 1980; Mayer et al., 1984; Nowak et al., 1984; Horie et al., 1987; Matsuda et al., 1987; Vandenberg, 1987). These two classes of channels were recently shown to be blocked by polycationic polyamines, ubiquitous intracellular constituents that also exist in extracellular media (Rock and Macdonald, 1992a,b; Araneda et al., 1993; Benveniste and Mayer, 1993; Ficker et al., 1994; Lopatin et al., 1994; Bowie and Mayer, 1995; Donevan and Rogawski, 1995; Igarashi and Williams, 1995; Fakler et al., 1995; Isa et al., 1995; Koh et al., 1995; Kamboj et al., 1995; Bähring et al., 1997; Williams, 1997). Polyamines block the pore of inward-rectifier K^+ channels from the intracellular side. They block some glutamate-gated channels from both the intracellular and the extracellular sides in a permeant manner. Motivated by these recent findings, we examined the effects of polyamines on a CNG channel. The channel was heterologously expressed in *Xenopus* oocytes by direct injection of RNA that encodes the α

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¹Abbreviations used in this paper: CNG, cyclic nucleotide-gated; I-V, current-voltage.

subunit of the bovine retinal cyclic nucleotide-gated channel (Kaupp et al., 1989). We found that three polyamines (spermine, spermidine, and putrescine) blocked the channel from both the intracellular and extracellular sides. The mechanisms by which polyamines block the CNG channel are complex.

METHODS

Molecular Biology and Oocyte Preparation

The cDNA of bovine rod cGMP-gated channel α subunit cloned into pGEM-HE plasmid was kindly provided by Dr. Steven Siegelbaum (Kaupp et al., 1989; Goulding et al., 1992; Liman et al., 1992). RNA was synthesized using T7 polymerase (Promega Corp.) from NheI-linearized cDNA. Oocytes harvested from *Xenopus laevis* (Xenopus One) were incubated in a solution containing (mM): 82.5 NaCl, 2.5 KCl, 1.0 MgCl₂, 5.0 HEPES, pH 7.6, and 2–4 mg/ml collagenase. The oocyte preparation was agitated using a platform shaker (80 rpm) for 60–90 min. It was then rinsed thoroughly and stored in a solution containing (mM) 96 NaCl, 2.5 KCl, 1.8 CaCl₂, 1.0 MgCl₂, 5 HEPES, pH 7.6, and 50 μ M gentamicin. Defolliculated oocytes were selected and injected with RNA at least 2 and 16 h after collagenase treatment, respectively. All oocytes were stored in an incubator at 18°C.

Patch Recording and Solutions

The CNG channel currents were recorded in either the inside-out or the outside-out configuration from *Xenopus* oocytes (injected with the CNG-channel cRNA) with an Axopatch 200B amplifier (Axon Instruments, Inc.). The recorded signal was filtered at 1 kHz and sampled at 5 kHz using an analogue-to-digital converter (DigiData 1200; Axon Instruments, Inc.) interfaced with a personal computer. pClamp6 software (Axon Instruments, Inc.) was used to control the amplifier and acquire the data. Macroscopic current–voltage curves were recorded as membrane voltage was linearly ramped (25–50 mV/s). For the inside-out configuration, the currents obtained in the absence of cGMP were used as templates for subsequent off-line background current corrections. For the outside-out configuration, currents at -80 mV in the presence of 20 mM extracellular Mg²⁺ were used to estimate the background currents. During background current corrections, the background currents were assumed to vary linearly as a function of membrane voltage between -80 and $+80$ mV.

Both the internal and external solutions contained (mM): 130 NaCl, 0.5 EDTA, and 5 HEPES, pH 7.6. To activate the channel, 1 mM (a near-saturating concentration) cGMP was included in the internal solution. Polyamine-containing internal and external solutions were prepared daily.

RESULTS

Blockade of the CNG Channel by Extracellular Polyamines

Fig. 1 A shows macroscopic current–voltage (I–V) relations of the CNG channel recorded in the outside-out configuration. Both sides of the membrane were exposed to 130 mM Na⁺ and 0.5 mM EDTA. To activate the channel, a near saturating concentration of cGMP (1 mM) was included in the pipette solution. In the absence of blocking ions, the I–V relation of the CNG channel was nearly linear, with very slight outward rec-

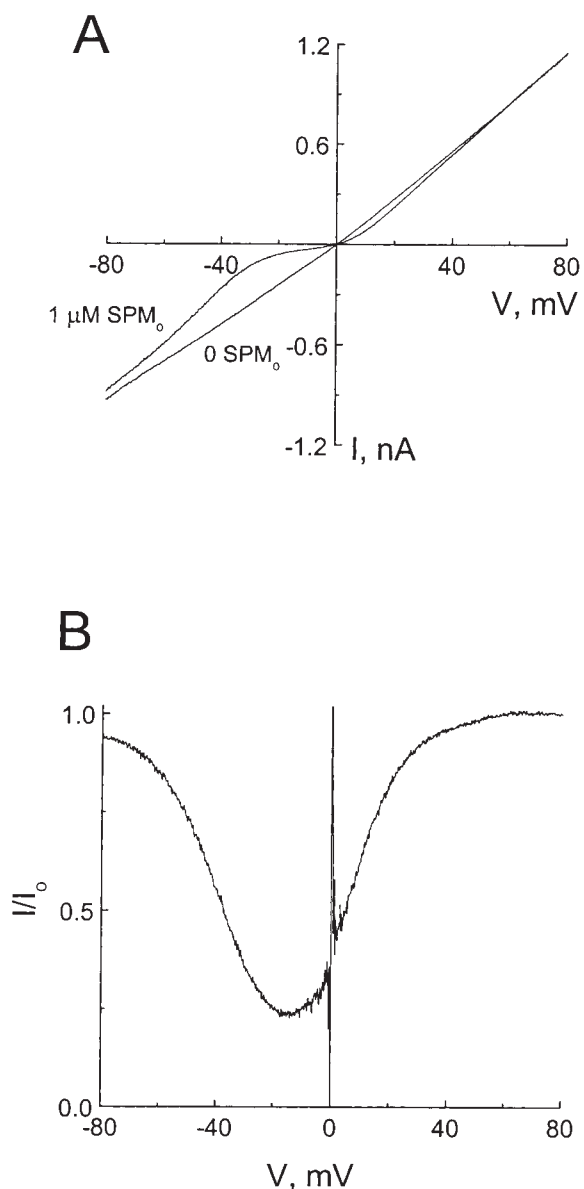


FIGURE 1. Blockade of the CNG channel by extracellular spermine. (A) Macroscopic current–voltage relations (I–V curves) of the CNG channel in the absence and presence of 1 μ M extracellular spermine. (B) The fraction of unblocked current (I/I_o), taken from A, is plotted against membrane voltage.

tification. We used the I–V relation in the presence of 1 μ M extracellular spermine to illustrate the basic properties of channel blockade. Between 50 and 80 mV, the I–V curves with and without spermine were superimposed, indicating that in this voltage range spermine had no effect. Below 50 mV, spermine blocked the channel and the extent of blockade increased when membrane voltage was reduced, as manifested by a gradual divergence of the two I–V curves. However, contrary to expectation for a typical ionic pore blocker, channel blockade by spermine was relieved (rather

than enhanced) at extreme negative membrane voltage, as manifested by a reconvergence of the I-V curves at negative voltages.

In Fig. 1 B, the fraction of unblocked current in the presence of 1 μM extracellular spermine (I/I_0) is plotted against membrane voltage. The curve has an inverted bell shape with maximal inhibition at -15 mV: channel blockade was enhanced when positive membrane voltage was lowered, and relieved again at large negative voltages. Extracellular spermine effectively caused the channel to conduct in a bidirectionally rectifying manner. This bimodal character of channel blockade as a function of voltage is commonly viewed as the hallmark of a permeant ionic pore blocker, and is explained as follows: hyperpolarization electrostatically enhances the binding of positively charged blockers into the pore; however, with sufficient hyperpolarization, the excessive transmembrane electrical force “drives” permeant blockers through the pore, which results in relief of blockade.

Fig. 2, A-C, shows CNG channel I-V relations in the absence and presence of various concentrations of ex-

tracellular putrescine, spermidine, and spermine, respectively. The fraction of unblocked currents is plotted against membrane voltage in D-F. All three polyamines exhibited qualitatively similar behaviors, predominantly blocking in a voltage-dependent manner at positive voltages and becoming permeant at negative voltages. The blocking phase of the curves for spermine and spermidine was similar but steeper than that for putrescine. Interestingly, steepness of the relief phases of the curve correlated qualitatively with the valence of the polyamine. (Putrescine, spermidine, and spermine carry up to two, three, and four positive charges, respectively.)

As shown in Fig. 2, current at negative voltages was progressively inhibited by increasing polyamine concentrations, and essentially vanished in the presence of 1 mM spermine. Therefore, although current enhancement at negative voltages resulted from polyamine permeation, Na^+ still carried nearly all the current.

Because the membrane potential in rods is depolarized to about -40 mV in darkness, we plotted the fraction of unblocked currents of the CNG channel at -40 mV against polyamine concentration and fitted the data

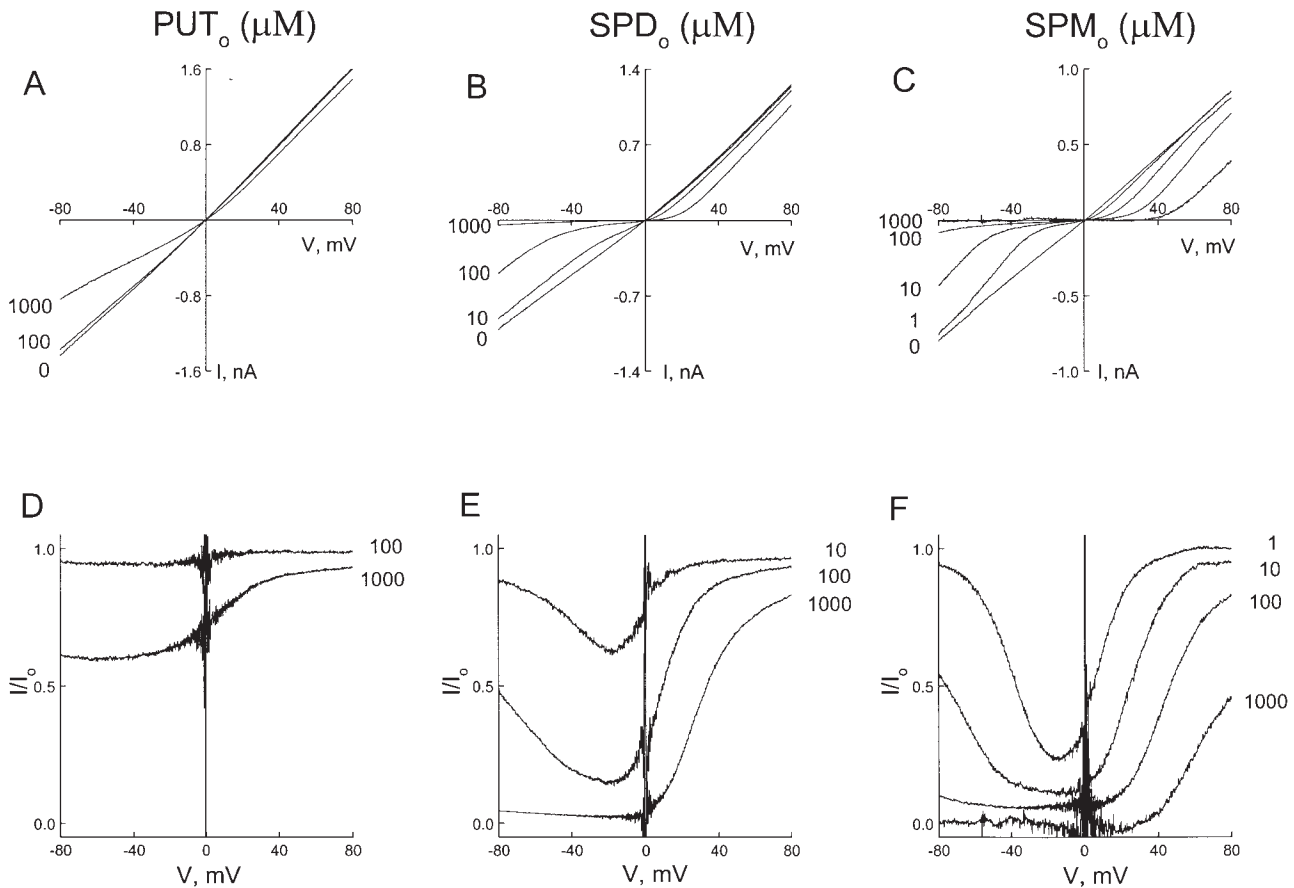


FIGURE 2. Comparison of CNG channel blockade by three extracellular polyamines. (A-C) Macroscopic I-V curves of the CNG channel in the absence and presence of various concentrations of extracellular putrescine (PUT), spermidine (SPD), and spermine (SPM). (D-F) The corresponding fraction of unblocked currents is plotted against membrane voltage.

with hyperbolic functions in Fig. 3. The least-squares estimates of $K_{1/2}$ for putrescine, spermidine, and spermine at -40 mV were 1.4 mM, 50 μ M, and 3.1 μ M, respectively.

Blockade of the CNG Channel by Intracellular Polyamines

Fig. 4 A shows macroscopic I-V relations recorded in the inside-out configuration, with 130 mM Na^+ and 0.5 mM EDTA on both sides of the membrane. To activate the channel, 1 mM cGMP was included in the bath solution. We used the I-V curve in the presence of 10 μ M intracellular spermine to illustrate the basic properties of channel blockade. At negative voltages, the shape of the I-V curve with intracellular spermine was very similar to that with extracellular spermine (compare Figs. 1 A and 4 A). Inhibition was stronger near zero than at negative voltages, consistent with spermine binding within the transmembrane electrical field. At positive voltages, the I-V curve was complex. If intracellular spermine, like extracellular spermine, simply acted as a typical permeant blocker, one would expect relief of blockade at positive voltages. That is, the I-V curves with and without spermine would converge at positive voltages. However, the I-V curves diverge dramatically at positive voltages, suggesting that intracellular spermine did not merely (or at all) act as a permeant ionic blocker.

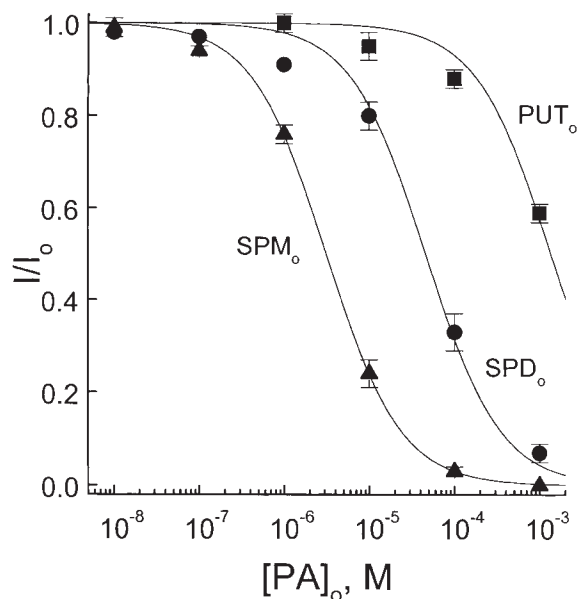


FIGURE 3. Comparative potency of the three extracellular polyamines. The fraction of unblocked currents at -40 mV is plotted against the concentrations of putrescine, spermidine, and spermine. The solid curves are least-squares fits of equations of the form $I/I_o = K_{1/2} / (K_{1/2} + [PA])$, where $K_{1/2}$ is the concentration at which a given polyamine blocks half the current through the CNG channel, and $[PA]$ is the concentration of the polyamine. The $K_{1/2}$ values for extracellular putrescine, spermidine, and spermine are 1.4 mM, 50 μ M, and 3.1 μ M, respectively.

The fraction of unblocked current in the presence of 10 μ M intracellular spermine is plotted against membrane voltage in Fig. 4 B. The curve displayed both a minimum and a maximum. Interestingly, the minima for both intracellular and extracellular spermine occur at about -15 mV (compare Figs. 1 B and 4 B).

Fig. 5, A-C, shows I-V relations for the CNG channel in the absence and presence of various concentrations of intracellular putrescine, spermidine, and spermine, respectively. The fraction of unblocked currents is plotted against membrane voltage in D-F. As shown in Fig. 5 F, the positive slope between the minimum and maximum

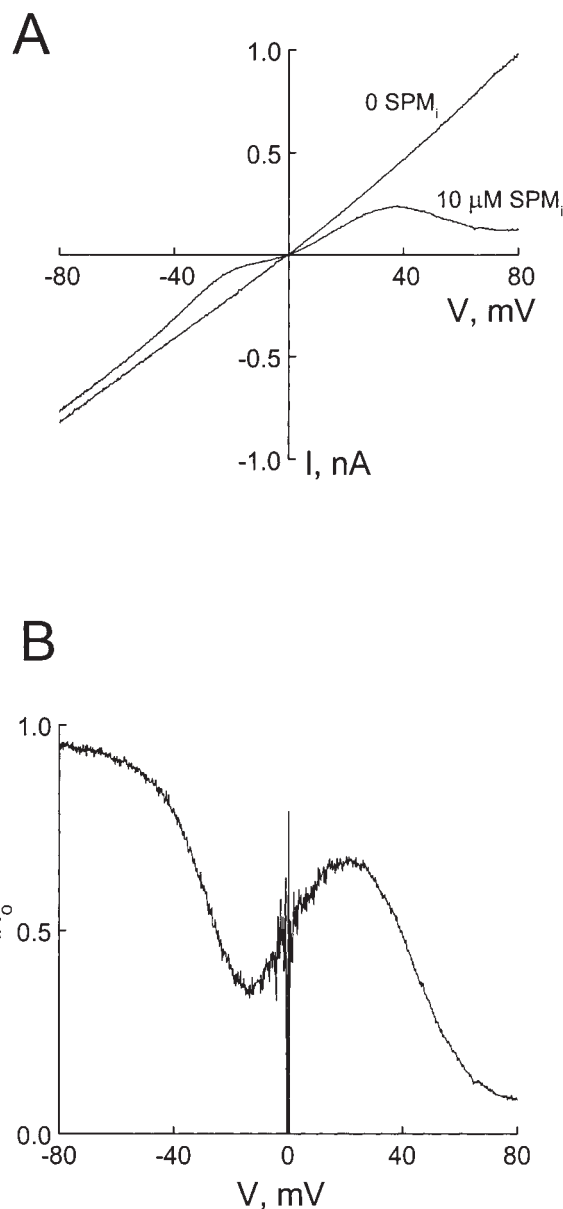


FIGURE 4. Blockade of the CNG channel by 10 μ M intracellular spermine. (A) Macroscopic I-V curves of the CNG channel in the absence and presence of spermine. (B) The fraction of unblocked current, taken from A, is plotted against membrane voltage.

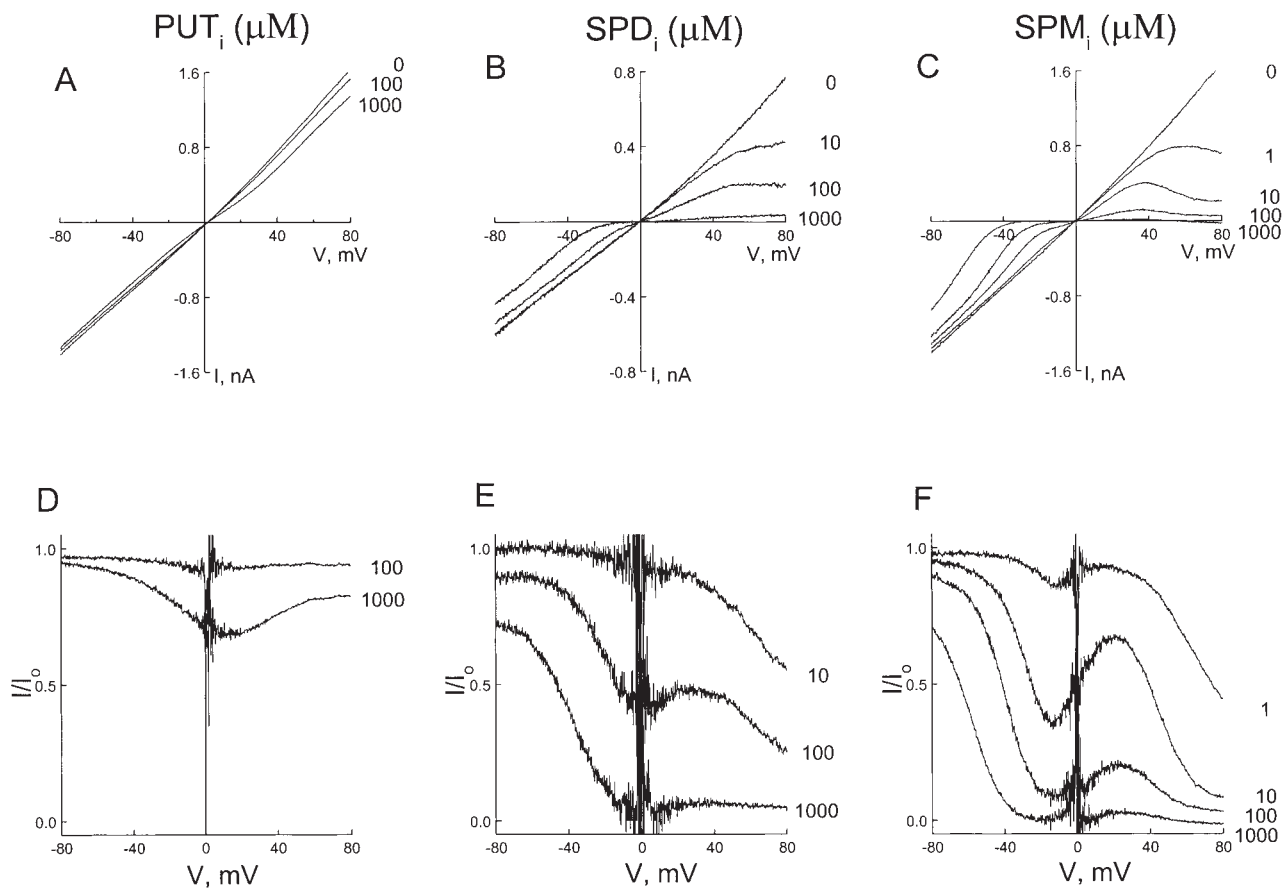


FIGURE 5. CNG channel blockade by three intracellular polyamines. (A–C) Macroscopic I–V curves of the CNG channel in the absence and the presence of various concentrations of intracellular putrescine, spermidine, and spermine. (D–F) The fraction of unblocked currents, taken from A–C, is plotted against membrane voltage.

varied with spermine concentration, and essentially disappeared when the concentration reached 1 mM. In the case of spermidine, the minimum and maximum were not as well defined as for spermine, almost fusing together to form a plateau (Fig. 5 E). For putrescine, the curve appeared to reach a plateau just below 80 mV.

In Fig. 6, the fraction of unblocked currents of the CNG channel at +40 mV is plotted against the concentration of three intracellular polyamines. The least-squares estimates of $K_{1/2}$ for putrescine, spermidine, and spermine were 3.0 mM, 80 μ M, and 6.7 μ M, respectively. These values are similar to those for extracellular polyamines at –40 mV (compare Figs. 3 and 6).

DISCUSSION

In the present study, we discovered that both intracellular and extracellular polyamines block the CNG channel in a voltage-dependent manner. Of the three polyamines tested, spermine, with the greatest number of ammonium and methylene groups, blocked the channel with the strongest voltage dependence and the

highest affinity. The value of $K_{1/2}$ was 7 μ M for intracellular spermine at +40 mV and 3 μ M for extracellular spermine at –40 mV. Because the higher affinity and stronger voltage dependence of spermine allow a more complete observation of its blocking behaviors within manageable ranges of concentration and membrane voltage, we will mainly discuss channel blockade by spermine.

As in some glutamate-gated channels (e.g., Bähring et al., 1997), extracellular spermine in the CNG channel acts like a typical permeant cationic pore blocker: reductions in positive membrane voltage enhances CNG channel blockade by spermine, whereas negative voltage relieved blockade (Figs. 1 and 2). Permeation of spermine through the CNG channel is physically possible because the estimated diameter of the CNG pore (~ 6 Å) exceeds that of spermine (~ 5 Å) (Goulding et al., 1993; Bähring et al., 1997). Compared with divalent cations, spermine (with up to four positive charges) appears to be more permeant over the same voltage range (e.g., compare Park and MacKinnon, 1995, with the present study).

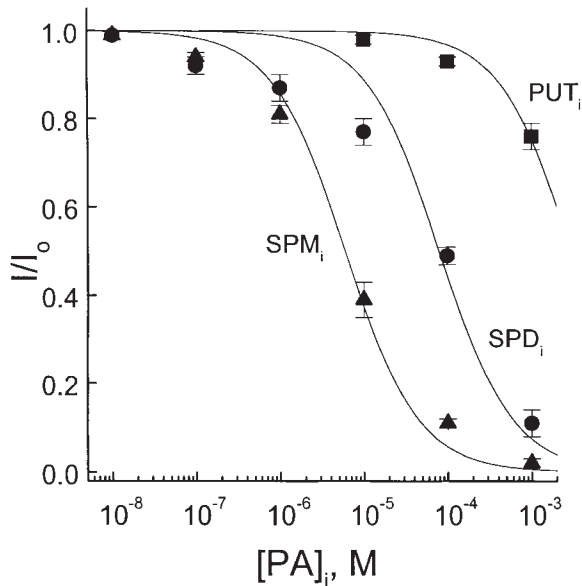


FIGURE 6. Comparative potency of the three intracellular polyamines. The fraction of unblocked currents at +40 mV is plotted against the concentrations of putrescine, spermidine, and spermine. The solid curves are least-squares fits of equations of the forms $I/I_0 = P^A K_{1/2} / (P^A K_{1/2} + [PA])$; see Fig. 3. The $K_{1/2}$ values for putrescine, spermidine, and spermine are 3.0 mM, 80 μ M, and 6.7 μ M, respectively.

CNG channel blockade by intracellular spermine is more complex. Unlike extracellular spermine, intracellular spermine does not simply behave as a permeant blocker. Nor does it act like a nonpermeant blocker as in inward-rectifier K^+ channels (Ficker et al., 1994; Lopatin et al., 1994; Fakler et al., 1995). As suggested in Fig. 7, the complex behavior of spermine can be explained by assuming that spermine adopts two conformations, one permeant and the other non- (or much less) permeant. For example, spermine may act as a permeant blocker when it binds in a linear conformation along the narrow region of the pore. It may also act as a nonpermeant blocker when it adopts a “curled” conformation(s) and jams itself in the wider internal region of the pore, similar to blockade of the sarcoplasmic reticulum K^+ channel by bis-quaternary ammonium compounds (Miller, 1982). The two conformations of spermine may be interconverted.

The noisy trace a in Fig. 7 B is from Fig. 4 B and shows how the fraction of unblocked current in the presence of 10 μ M intracellular spermine varies with membrane voltage. Fig. 7 B, b corresponds to the model in Fig. 7 A (see below and the figure legend for details), which proposes that intracellular spermine can bind to the channel in either a permeant (e.g., linear) or a nonpermeant (e.g., curled) conformation (external spermine concentration was set at zero). Fig. 7 B, c and d, were drawn by assuming that spermine

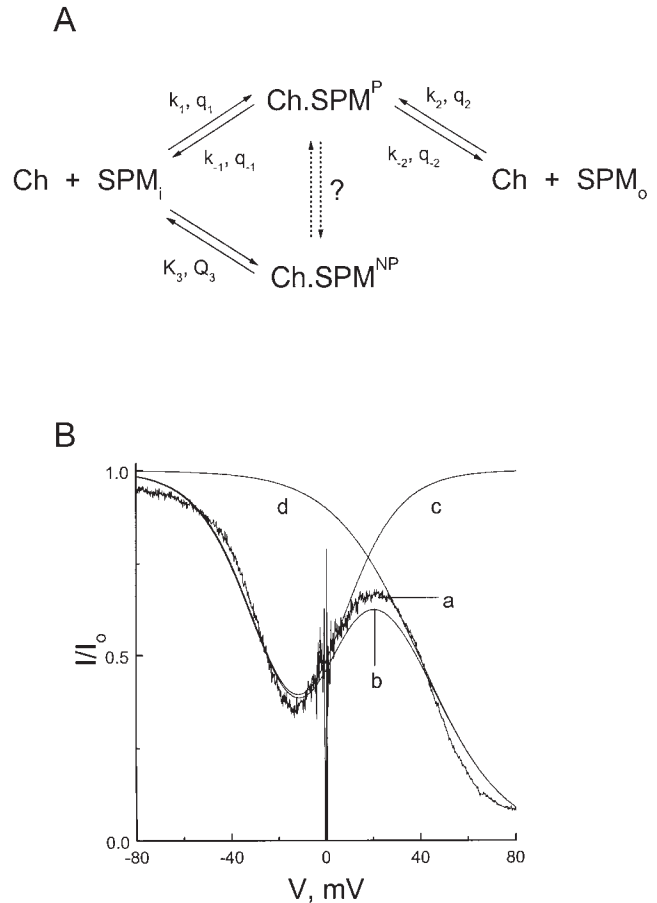


FIGURE 7. A kinetic model for the action of spermine. (A) Reaction scheme. Ch represents the CNG channel, SPM_i and SPM_o denote intra- and extracellular spermine, and $Ch.SPM^P$ and $Ch.SPM^{NP}$ denote the CNG channels blocked by spermine in the permeant and nonpermeant conformations, respectively. K_x and Q_x are, respectively, the equilibrium dissociation constant at 0 mV and the total number of equivalent charges moving across the electrical field (in either direction) for nonpermeant spermine binding. Rate constants at 0 mV (k_x or k_{-x}) and the number of equivalent charges traversing the electrical field (q_x or q_{-x}) for movements of the permeant conformation of spermine are also shown. (The number of equivalent charges is defined as the actual number of charges of the blocker multiplied by the fraction of the electrical field transversed by the blocker.) (B) Noisy trace a is the same as that in Fig. 4 B, obtained in the presence of 10 μ M of intracellular spermine. Curve b was drawn according to the scheme in A, with $k_1 = 1 * 10^6 M^{-1} s^{-1}$, $k_{-1} = 8 s^{-1}$, $k_2 = 1.3 * 10^6 M^{-1} s^{-1}$, $k_{-2} = 10.4 s^{-1}$, $K_3 = 333 \mu$ M, $q_1 = 2$, $q_{-1} = 0$, $q_2 = 2$, $q_{-2} = 0$, $Q_3 = 1.5$. (Any set of four rate constants in the given proportion will yield the same result.) Intracellular and extracellular spermine concentrations were set at 10 μ M and zero, respectively. Curves c and d were drawn using the same parameters, except that both k_1 and k_{-1} were zero for curve c and that K_3 was infinite for curve d.

acts only as either a permeant or a nonpermeant blocker. Our model interprets the first and second descending phases as reflecting blockade by spermine in the permeant and nonpermeant conformations, respectively. The intervening ascending phase reflects

spermine permeation. According to the model, we only observe such an interesting and complex blocking behavior when the pore has much higher affinity for the permeant than nonpermeant conformation of spermine. Fig. 7 B, b was drawn assuming that the pore has an ~ 40 -fold higher affinity for the permeant than nonpermeant conformation of spermine.

Fig. 7 A will also explain the action of extracellular spermine, provided one assumes that the binding state of the permeant form of spermine is the same regardless of the direction from which spermine enters the pore. In Fig. 8, we replotted the data from Figs. 2 F and 5 F. The theoretical curves superimposed on the data correspond to the model of Fig. 7 A, which allows extracellular spermine to act only as a permeant blocker, whereas intracellular spermine acts as a permeant as well as nonpermeant blocker. The model assumes that the binding of the permeant form of spermine is voltage independent, whereas the release of the permeant form of spermine into either the intracellular or the extracellular solution is voltage dependent. In the model, the spermine-binding site is located halfway through the transmembrane electrical field. (Although we have not exhausted all possibilities, the model appears to describe the data better if the voltage dependence mainly associates with the unbinding rather than the binding of spermine in the permeant form. The physical mechanism underlying this peculiar voltage dependence remains to be established.) Fig. 8 shows that this simple model qualitatively describes channel blockade by various concentrations of intracellular and extracellular spermine.

Many ion channels, such as inward-rectifier K^+ channels and some glutamate channels, are blocked by cations. The block of these channels is a critical feature that enables the channels to accomplish many important biological tasks. Initially, the block was attributed only to divalent cations (Ault et al., 1980; Mayer et al., 1984; Nowak et al., 1984; Horie et al., 1987; Matsuda et al., 1987; Vandenberg, 1987). Recently, polycationic polyamines were discovered to block inward-rectifier K^+ channels and some glutamate-gated channels (Rock and Macdonald, 1992a,b; Araneda et al., 1993; Benveniste and Mayer, 1993; Lopatin et al., 1994; Ficker et al., 1994; Bowie and Mayer, 1995; Donevan and Rogawski, 1995; Fakler et al., 1995; Igarashi and Williams, 1995; Isa et al., 1995; Kamboj et al., 1995; Koh et al., 1995; Bähring, 1997; Williams, 1997). We have shown here that polyamines also block the CNG channel. Spermine blocks the inward-rectifier K^+ channels as a nonpermeant blocker, whereas it blocks the glutamate-gated channels as a permeant blocker. In the CNG channel, spermine blocks the pore from the outside as a permeant blocker, qualitatively similar to that in the glutamate-gated channels. However, spermine blocks the CNG channel from the inside as a permeant as well as nonpermeant blocker, a

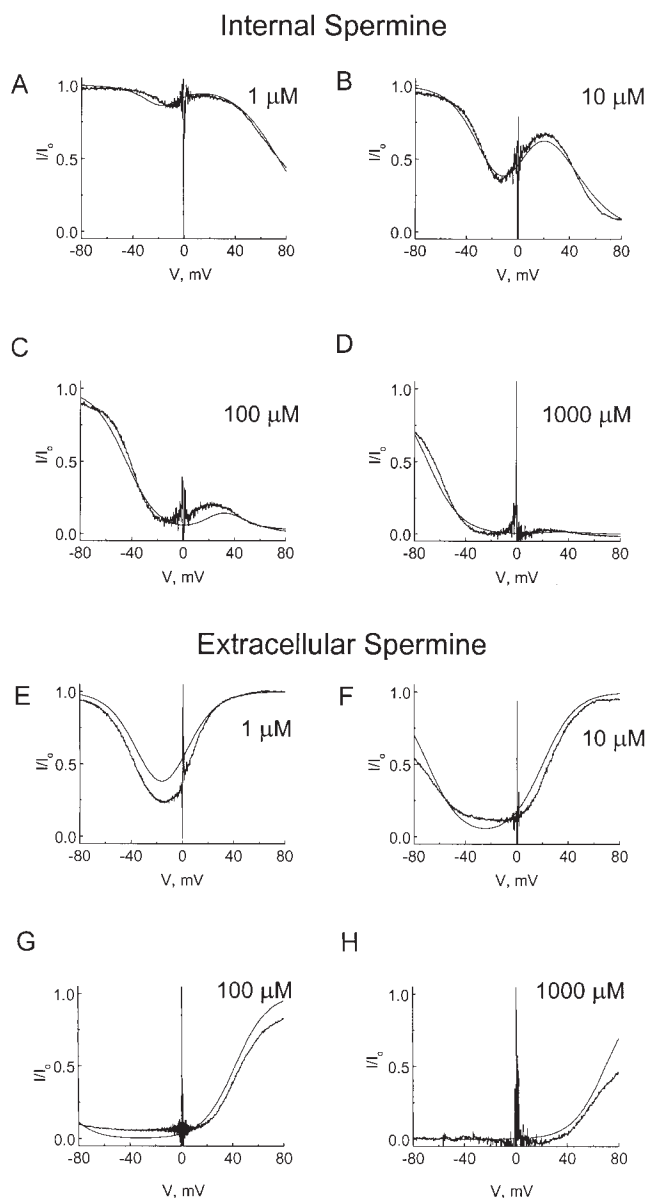


FIGURE 8. Modeling of channel blockade by various concentrations of intracellular and extracellular spermine. The experimental data (noisy traces) are the same as those in Figs. 2 F and 5 F. All smooth traces were computed according to the scheme in Fig. 7 A, using the same parameters as in Fig. 7 B and appropriate concentrations of intracellular and extracellular spermine. For example, in Fig. 8 A the model's intracellular spermine concentration was set at $1 \mu\text{M}$ and extracellular spermine concentration at zero.

combined behavior of spermine in the glutamate-gated channels and inward-rectifier K^+ channels. The emerging pattern is that channels blocked by (or conducting) divalent cations can also be blocked by polyamines. Polyamines appear to block selective (e.g., inward-rectifier) cation channels in a nonpermeant manner, but nonselective (e.g., glutamate- and cyclic nucleotide-gated) channels in a permeant manner.

The physiological significance of CNG channel blockade by polyamines remains to be determined. Polyamines are ubiquitous in cells. The concentrations at which intracellular polyamines block the CNG channel are well within the concentration range for polyamines found in many cell types (e.g., Bähring et al., 1997). Polyamines also exist in extracellular fluid (e.g., Williams, 1997). However, most studies on CNG channel conduction were carried out with defined artificial extracellular solutions free of polyamines. As shown here, 1 μM extracellular spermine dramatically inhibits the inward Na^+ current. This concentration is 100–1,000-fold lower

than that required to block *N*-methyl-D-arginine-gated channels (Rock and Macdonald, 1992a,b; Araneda et al., 1993; Benveniste and Mayer, 1995a,b; Igarashi and Williams, 1995; Chao et al., 1997). It will be important to establish to what extent CNG channels are affected by extracellular polyamines in their native environment. Polyamines likely block permeation not only of monovalent cations but also of divalent cations such as Ca^{2+} . Block of Ca^{2+} influx could have a major impact on the adaptation of visual transduction in vertebrate photoreceptors.

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