Rectification of Rabbit Cardiac Ryanodine Receptor Current by Endogenous Polyamines

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ABSTRACT The actions of three endogenous polyamines (spermine, spermidine, and putrescine) were defined on Ca^{2+} release channels (ryanodine receptors, RyRs) isolated from rabbit cardiac sarcoplasmic reticulum. The current-voltage relationship of the RyR channel was N-shaped in the presence of polyamine (1–5 mM). Polyamine blocked conduction near 0 mV, but the blockade was relieved at large potentials. Polyamines acted (blocked) from both sides of the channel. Polyamine efficacy was dependent on current direction and was inversely related to the ion selectivity of the RyR pore. This suggests that polyamine interacts with current-carrying ions in the permeation pathway. The apparent half-block concentration of spermine at 0 mV was <0.1 mM. The features of polyamine blockade suggest that the polyamines are permeable cationic blockers of the RyR channel. Further, the levels of polyamines found in muscle cells are sufficient to block single RyR channels and thus may alter the sarcoplasmic reticulum Ca^{2+} release process in situ.

INTRODUCTION

Polyamines are found in a variety of different cell types (Tabor and Tabor, 1984; Pegg 1986; Schuber, 1989). In nonmuscle cells, a rapid elevation in the concentration of polyamine is thought to be a potent signal that induces intracellular Ca^{2+} mobilization (Pegg and MacCann, 1982; Koenig et al., 1983; Iqbal and Koenig, 1985). It has also been suggested that polyamines act as second messengers that modulate β-adrenoreceptor-mediated membrane transport mechanisms (Koenig et al., 1983b, 1988). Over the last few years, it has been shown that the polyamines are important modulators of a variety of surface membrane ion channels (Lopatin et al., 1994; Ficker et al., 1994; Drouin and Hermann, 1994; Weiger and Hermann, 1994; Koh et al., 1995; Sunjeev et al., 1995). Thus, it has become apparent that polyamines can influence a wide range of cellular processes.

Muscle tissues contain significant levels of polyamine (Tabor and Tabor, 1964; Kaminska et al., 1982; Persson and Rosengren, 1983; Koenig et al., 1988). The impact of endogenous polyamines on muscle function has not yet been clearly defined. It is known that polyamines inhibit drug-induced Ca^{2+} release from skeletal muscle sarcoplasmic reticulum (SR) vesicles (Palade, 1987) and that polyamines specifically interact with the RyR protein (Zarka and Shoshan-Barmatz, 1992). Thus, it is reasonable to hypothesize that polyamines may alter the function of single RyR channels. In this study, we show that endogenous levels of polyamines can act as permeable cationic blockers of single RyR channels.

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METHODS

Purification of ryanodine receptor protein

Ventricular muscle from adult rabbit was collected and chopped into small pieces. The muscle was homogenized and then junctional SR membranes were isolated following the procedures of Holmberg and Williams (1990). The RyR-containing microsomes were solubilized and reconstituted in liposomes following the methods of Imagawa et al. (1989). The solubilized protein was stored at −70°C until use.

Solutions

The standard experimental solution contained 210 mM KCl, 10 μM $CaCl_2$ and 20 mM HEPES-KOH (pH 7.4). In some experiments, the 210 mM KCl was substituted for CsCl, $BaCl_2$, or other KCl concentrations. The pH values of polyamine stock solutions were adjusted to pH 7.4 using KOH (Sigma Chemical Co., St. Louis, MO). There was no pH change due to addition of polyamines to the experimental solutions. The free Ca^{2+} concentration was measured using a Ca^{2+} electrode. Lipids were obtained from Avanti Polar Lipids (Alabaster, AL). Because polyamines are known to bind to acidic or negatively charged phospholipids (Tadolini et al., 1985; Chung et al., 1985), charged lipids were not used. The experimental lipid mixture contained phosphatidyl-ethanolamine (PE; a neutral lipid) in decane. All other chemicals were of the highest available grade.

Single-channel recording

The RyR channels were incorporated into planar lipid bilayers. Bilayer membranes were formed by painting the lipid mixture across the hole in a Delrin partition separating two lucite chambers. All experiments were done under voltage-clamp conditions. Experimental solutions were connected to the amplifier via Ag/AgCl electrodes and KCl-agar bridges. Junction potentials were measured and the appropriate corrections were made.

Voltage changes and the RyR protein were applied to one side of the bilayer (*cis* side). The other side of the bilayer (*trans*) was grounded. The orientation of the RyR in the bilayer was established by the sidedness of ATP-, Ca^{2+}-, and Mg^{2+}-sensitivity of incorporated channels. The RyR channels were consistently oriented with the cytoplasmic side in the *cis* chamber. Single-channel currents were recorded using a custom-designed patch-clamp amplifier. The data were digitized at 10 KHz and stored on removable magneto-optical disk. Data were filtered at 4 KHz and then

analyzed using a Basic program (Shioya, 1990) and pClamp software (Axon Instruments, Burlingame, CA).

Data analysis

Single-channel current was measured as the difference between the means of the closed and open peaks in all-points amplitude histograms. Control current-voltage (I-V) relationships were fit by a linear regression using the least-squares method. The I-V relationships in the presence of polyamine were fit by eye using a polynomial fitting routine.

Chord conductances were derived from the I-V relationship using the expression,

$$g(Vm) = I(Vm)/(Vm - E_x) \quad (1)$$

where $g(Vm)$ is conductance, $I(Vm)$ is current at voltage (Vm), and E_x is the equilibrium potential for ion "x". The mechanism of polyamine blockade appeared analogous to blockade of K^+ channels by Na^+ as reported by Benzanilla and Armstrong (1972), French and Wells (1977) and French and Shoukimas (1985). In those studies, blockade was quantitated using I-V curves and chord conductance plots. Chord conductance plots were also generated here to, 1) clearly illustrate that channel conductance during polyamine block varies with voltage, and 2) demonstrate the similarities with the blocking mechanism outlined in those classical studies.

Conductance in the presence of blocking ion, $g_B(Vm)$, was fit to the following expression,

$$g_B(Vm) = g(\text{Limit})/\{1 + \exp(szF(Vm - E_{50})/RT)\} \quad (2)$$

where g(Limit) is the conductance obtained in control solutions, z is the valence of the blocking ion, E_{50} is the voltage where half-block occurs, and F, R, and T represent the Faraday constant, gas constant, and temperature (Kelvin), respectively. The s represents the apparent electrical distance from the membrane-solution interface to the blocking site (fraction of transmembrane voltage per unit charge).

The apparent affinities for polyamine block (K_B) were extrapolated from Hill-equation fits to percent-block (see below) data at several different membrane potentials. Assuming a one ion per channel reaction, the K_B at 0 mV, $K_B(0)$, was also estimated by,

$$K_B(0) = [\text{Blocker}] \exp(-szF(E_{50})/RT) \quad (3)$$

Percent block was determined from I-V relationships using the following expression.

$$\%\ \text{Block} = 100*(1 - I(Vm)_{\text{POLYAMINE}}/I(Vm)_{\text{CONTROL}}) \quad (4)$$

RESULTS

The cardiac SR Ca^{2+} release (RyR) channel is a Ca^{2+}-activated, large conductance channel that is modulated by ATP, Mg^{2+}, calmodulin, caffeine, phosphorylation, and ryanodine (Rousseau et al., 1986; Anderson et al., 1989; Rousseau and Meissner, 1989; Hain et al., 1995). Its large conductance and fast gating are consistent with its physiological function, which is to mediate a large Ca^{2+} flux on a millisecond time scale. The RyR channels from several tissues have been incorporated into planar lipid bilayers (Rousseau et al., 1986; Smith et al., 1988; Ashley, 1989; Hermann-Frank et al., 1991). Despite the successful efforts to clone and sequence rabbit cardiac RyR cDNA (Otsu et al., 1990; Nakai et al., 1990), the single-channel properties of this particular RyR channel have not yet been extensively explored.

In this study, single rabbit cardiac RyR channels were incorporated into planar lipid bilayers. Reconstitution of RyR proteins into liposomes reduced the possibility that other types of ion channels may be present in these experiments. It is possible, however, that accessory proteins that may be closely associated with the channel may still be present. Thus, efforts were made to positively identify the channel in the bilayer. In the presence of 10 μM Ca^{2+}, channel activity occurred spontaneously as illustrated in Fig. 1 (*left panels*). Channel gating was characterized by frequent, brief (ms), open events with few events lasting longer than 20 ms. The identity of the channel was established by its pharmacology and ion selectivity. The channels were activated by ATP, Ca^{2+}, and caffeine, and blocked by ryanodine, ruthenium red, and Mg^{2+}. The channels were also divalent selective (PBa/PK $\approx$ 6). These properties are consistent with the identification of this channel as the ryanodine receptor (Smith et al., 1988; Rousseau et al., 1986; Ashley, 1989; Hermann-Frank et al., 1991).

Action of spermine on single-RyR channels

After incorporation of a single-RyR channel into the bilayer, spermine (5 mM) was added to the cytoplasmic side of the channel. At pH 7.4, spermine will be protonated and have four positively charged moieties (Morris and Harada, 1980). Spermine significantly reduced unitary current amplitude (compared to control) in a voltage-dependent manner. At negative potentials (Fig. 1 *B*), where net current was inward (lumen $\rightarrow$ cytoplasm direction), spermine caused only small reductions in unitary current. At positive potentials (Fig. 1 *A*), where net current was outward (cytoplasm $\rightarrow$ lumen direction), spermine reduced unitary current to a much

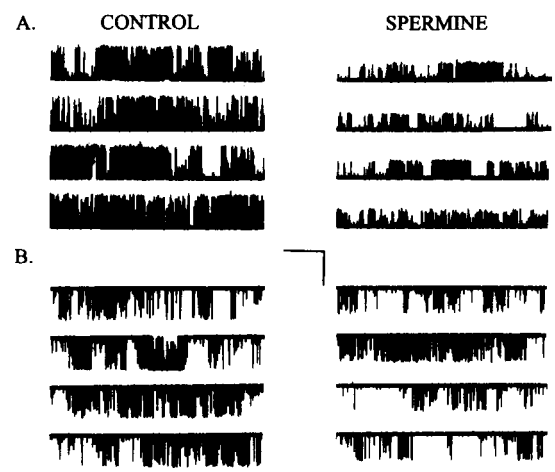

FIGURE 1 Single-channel recordings illustrating the action of spermine. Rabbit cardiac ryanodine receptor channels were incorporated into planar lipid bilayers in the presence of symmetrical 210 mM K^+ and 10 μM Ca^{2+}. Spermine (1 mM) was applied to only the cytoplasmic side of the channel (*cis*). Calibration bars represent 50 pA and 1000 ms. *A*. Records illustrating spermine action at +60 mV (holding potential, HP). Open events are upward deflections. *B*. Records illustrating spermine action at −60 mV (HP). Open events are downward deflections.

greater extent. This voltage-dependent action of spermine is illustrated in Fig. 2. In the absence of spermine, the I-V relationship (750 pS) was linear. In the presence of spermine, the I-V relationship was N-shaped. At membrane potential near 0 mV, spermine significantly attenuated the unitary current. Note that the block was apparently relieved at large membrane potentials.

Because the polyamines are polyvalent cations, it is possible that the polyamines may alter function through some sort of surface charge effect. Polyamine could alter the surface potential of the membrane or influence surface charges on the channel protein itself. If polyamine significantly altered membrane surface potential, then asymmetrically applied polyamine would be expected to shift the observed reversal potential. If polyamine added or screened surface charge on the channel protein, then asymmetrically applied polyamine would be expected to change the slope of the I-V relationship, but not the reversal potential. The N-shaped I-V relationship (Fig. 2), therefore, can not be easily explained by a simple surface-charge effect. Polyamine action at high salt concentrations and the dependence of polyamine efficacy on current direction (Fig. 4) also suggest that a surface charge effect cannot be the predominate underlying cause of the blockade. In work on other channel types, an N-shaped I-V relationship has been shown to be a principle feature of blockade induced by a permeable cationic channel blocker (Benzanilla and Armstrong, 1972; French and Wells, 1977). Permeable cationic blockers are thought to enter an open channel and attenuate current by interacting with current-carrying ions in the pore. The block is relieved at large membrane potentials because the cationic blocker can actually pass through the pore. Typically, current carried by the blocker is insignificant. A classical example of permeable cationic blockade is Na^+ block of neuronal K^+ channels (French and Shoukimas, 1985; Benzanilla and Armstrong, 1972; French and Wells, 1977).

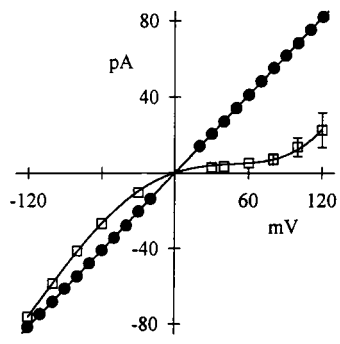

FIGURE 2 Spermine action on the unitary conductance of single ryanodine receptor channels. Solutions contained 210 mM and 10 μM $CaCl_2$ on both sides of channel. Spermine (5 mM) was applied to the cytoplasmic side of the channel. Current-voltage relationships before (*circle*), and after (*square*) polyamine application. Circles were fit by a linear regression (slope 750 pS). Squares were fit by eye. There was no measurable shift in the reversal potential. These data are representative of more than 20 similar experiments. The points represent means (± SE). In most cases, the symbol was larger than the standard error.

The hypothesis that the polyamines act as permeable cationic blockers of the RyR channel was tested here.

Sidedness and reversibility of spermine action

If spermine is a permeable cationic blocker, then it should act from both sides of the channel. To test this premise, I-V relationships were determined under the following conditions; 1) control, 2) *cis* only spermine, 3) symmetrical spermine (*cis* and *trans*), and 4) *trans* only spermine. In the absence of spermine (control), the unitary current (Fig. 3 *A*) and chord conductance (Fig. 3 *B*) were linear functions of membrane potential. Addition of spermine to either side of the channel attenuated current, and spermine action was readily reversible. These data indicate that the site of spermine action was accessible from both sides of the channel. Spermine action, however, was not symmetrical. This is best illustrated in the chord conductance plots (Fig. 3 *B*). The shape of the chord conductance plots depended on

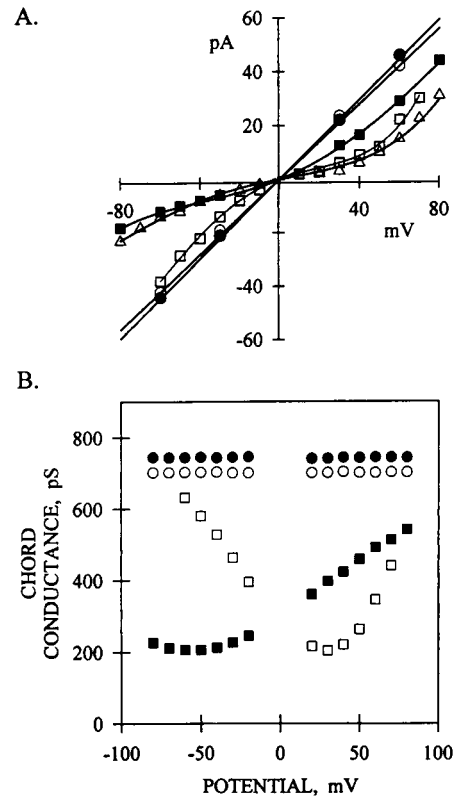

FIGURE 3 Sidedness and washout of spermine action on single ryanodine receptor channels. Solutions contained 210 mM and 10 μM $CaCl_2$ on both sides of channel. These data are representative of six single-channel experiments. The following experimental sequence was applied to each channel: control (*filled circle*), 1 mM *cis* only spermine (*open square*), 1 mM symmetrical spermine (*triangle*), washout of both chambers (*open circle*) then 1 mM *trans* only spermine (*filled square*). *A*. Representative I-V relationships under each experimental condition. There were no measurable shifts in reversal potential. Control and washout data were best fit by linear regressions (slopes 743 and 702 pS, respectively). *B*. Representative chord conductance plots. Chord conductance was calculated from I-V relationships using Eq. 1.

whether spermine was added to the *cis* or *trans* side of the channel. This could suggest that the site at which polyamine acts is asymmetrically positioned in the electrical field.

Spermine action depends on current direction

One way to show that a blocking cation can pass through the pore is to actually measure current carried by the blocking cation. This requires the use of large concentrations of blocker. Several attempts were made to measure polyamine current and/or permeability. At very high polyamine concentrations (20–30 mM), there was no measurable polyamine current and no discernable polyamine-dependent shift in reversal potential. Thus, if polyamine does enter and pass through the pore, then the $P_K/P_{SPERMINE}$ ratio must be quite large (i.e., polyamine poorly permeable).

To test if polyamine enters the pore and attenuates current by interacting with charge-carrying ions, polyamine action was defined with charge-carrying ions passing predominately in one direction or the other. To assure that conduction at any particular voltage was saturated, this set of experiments was done in the presence of a large concentration of current-carrying ion (1.4 M). Spermine was applied only to one side (*cis*) of the channel. The voltage-dependence of chord conductance at 210 mM and 1.4 M KCl are compared in Fig. 4 *A*. The general features of spermine blockade, peak block near 0 mV and relief of block at large potentials, were also present in high KCl. Polyamine efficacy, however, was less in the high KCl. Decreased efficacy would be predicted if polyamine and potassium were competing in the pore.

Unitary currents were determined at large negative (−50 → −120 mV) and positive (+50 → +120 mV) potentials with spermine on the cytoplasmic (*cis*) side of the channel. At such large potentials (>50 mV from reversal potential), the net current was essentially a unidirectional flux (Tu et al., 1994). The net current values at large potentials were transformed into percent block using Eq. 4. The percent block was plotted as a function of membrane potential (absolute value) in Fig. 4 *B*. When current was predominantly inward (lumen → cytoplasm direction), the percent block was near zero. When the current was predominately outward (cytoplasm → lumen direction), there was substantial block. Thus, spermine block depended on current direction suggesting that polyamine interacts with current-carrying ions in the permeation pathway.

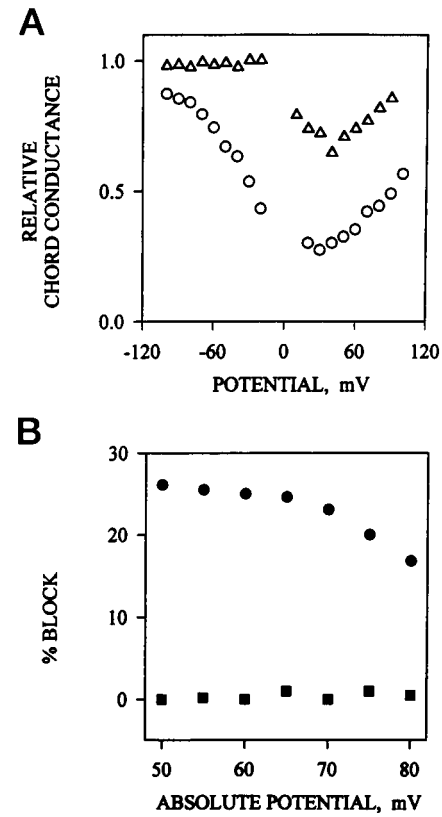

FIGURE 4 Polyamine block depended on current direction. Solutions contained 3 mM spermine (*cis* only) and either 210 or 1400 mM KCl. These data are representative of 11 single-channel experiments in 210 mM KCl and 4 experiments in 1400 mM KCl. The control (no spermine) conductances in each of the ionic conditions were 698 and 928 pS, respectively. *A*. Relative chord conductance at both salt concentrations (210 mM *circle*; 1400 mM *triangle*). Chord conductance was calculated from the current-voltage relationships using Eq. 1. Data were normalized by dividing chord conductance by control conductance. *B*. Dependence of spermine block on current direction. Membrane potential was plotted as absolute values. At large negative potentials (*square*), current-carrying ions moved predominately in the lumen → cytoplasm direction. At large positive potentials (*circle*), current-carrying ions moved predominately in the cytoplasm → lumen direction.

Spermine action depends on channel selectivity

If polyamine interacts with current-carrying ions in the pore, then polyamine action should be influenced by the ion selectivity of the pore. For example, the RyR channel is more selective for Ba^{2+} than for K^+ (Tinker et al., 1992). Because Ba^{2+} will compete with polyamine more effectively than K^+, polyamine efficacy should be less when Ba^{2+} is the primary charge-carrying ion. Thus, the selectivity of the pore and polyamine efficacy should be inversely related.

Spermine action in the presence of equal molar levels of Ba^{2+}, K^+, or Cs^+ was defined. The I-V relationships in symmetrical Cs^+ (Fig. 5 *A*) and symmetrical Ba^{2+} (Fig. 5 *B*) are shown. The N-shaped I-V relationship was evident in all the salts tested. The chord conductance plots illustrate the effectiveness of spermine in each salt. Spermine was least effective when Ba^{2+} was the current carrier and most effective when Cs^+ was present. The order of spermine efficacy was $Cs^+ > K^+ > Ba^{2+}$. The selectivity sequence of the RyR pore is $Ba^{2+} > K^+ > Cs^+$ (Tinker et al., 1992). Thus, spermine efficacy and the ion selectivity of the RyR pore were inversely related suggesting that polyamine and the charge-carrying ion compete in the pore.

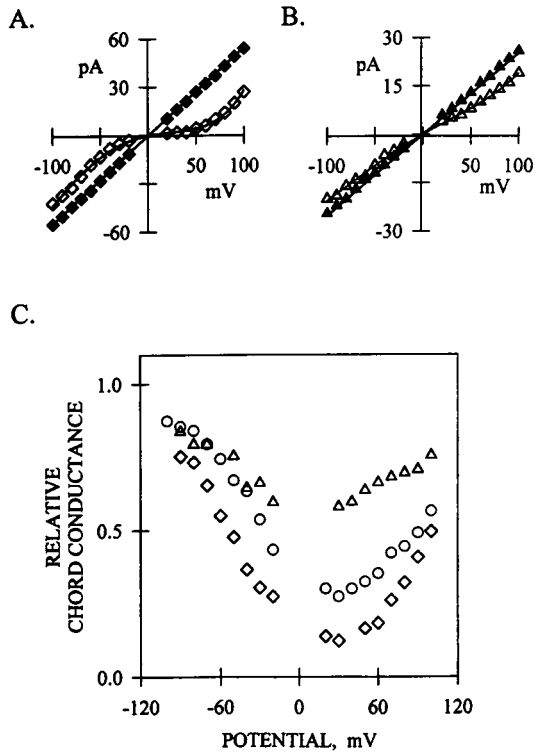

FIGURE 5 Polyamine blockade in presence of different charge carriers. Solutions contained 3 mM spermine in the *cis* chamber, and either symmetrical 210 mM CsCl (*diamond*), 210 mM KCl (*circles*) or 210 mM $BaCl_2$ (*triangle*). These data are representative of seven single-channel experiments. *A*. Current-voltage relationship in 210 mM CsCl (*square*). Data were collected under control conditions (no spermine; *filled*) and with spermine present (*open*). Control data were best fit by a linear regression (slope 546 pS). *B*. Current-voltage relationship in 210 mM $BaCl_2$ (*triangle*). Data were collected in control conditions (*filled*) and with spermine present (*open*). Control data were best fit by a linear regression (slope 252 pS). *C*. Chord conductance plots in the different charge carriers. Chord conductance was calculated from the current-voltage relationships using Eq. 1. Relative chord conductance was calculated by dividing chord conductance by conductance in the absence of spermine. The KCl points (*diamond*) are replotted from Fig. 5.

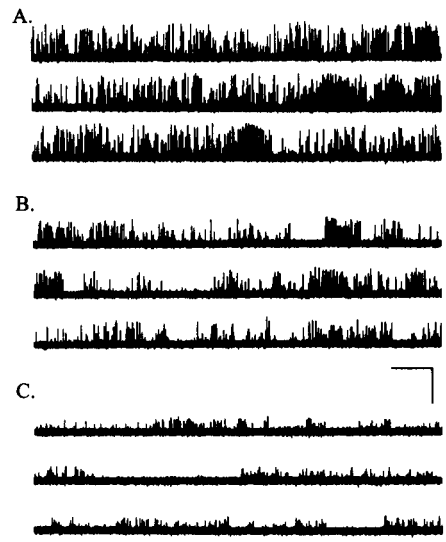

FIGURE 6 Single-channel recordings illustrating the concentration dependence of spermine action. Solutions contained symmetrical 210 mM K^+ and 10 μM Ca^{2+}. Increasing concentrations of spermine were applied to the cytoplasmic side of the channel (*cis*). Calibration bars represent 50 pA and 1000 ms. *A*. Records obtained in the absence of spermine. *B*. Records illustrating the action of 0.3 mM spermine. *C*. Records illustrating the action of 1.0 mM spermine.

Concentration dependence of spermine action

The degree of spermine blockade was concentration dependent. Single-channel records illustrating blockade at different spermine concentrations are shown in Fig. 6. As spermine concentration increased, unitary current decreased and became more and more difficult to resolve (signal to noise ratio decreased). Current-voltage relationships in the presence of different concentrations of spermine (*cis* only) are plotted in Fig. 7 *A*. Low concentrations of spermine (0.03 mM) induced little attenuation of current. At higher spermine concentrations, the characteristic N-shaped I-V relationship was more evident. The spermine concentration dependence of chord conductance is illustrated in Fig. 7 *B*. The degree of block increased with polyamine concentration. Percent block was plotted as a function of spermine concentration at each potential (Fig. 7 *C*, *inset*). To estimate the apparent half-block concentration (K_B), the Hill equation ($n = 1$) was fit to the percent block data. Apparent K_B was then plotted as a function of membrane potential (Fig. 7 *C*). Errors that may be associated with the individual fits of percent block at any one potential did not appear to effect the consistency of K_D values between potentials. The shape of the K_B vs. potential relationship was V-shaped with highest affinities occurring near 0 mV. Because it is thought that the potential across the SR in situ is near 0 mV (Somlyo et al., 1977), the data were extrapolated to estimate the apparent K_B at 0 mV. The apparent K_B at 0 mV was <0.1 mM.

Action of spermine on single-channel gating

If polyamines interact with charge carriers in the channel's pore, then a reduction in ion translocation (current amplitude) may not be the only functional consequence. It is possible that the presence of spermine in the pore may disrupt single-channel gating. The single-channel records shown in Fig. 8 illustrate channel gating in the absence (*left panel*) and presence of 3 mM spermine (*right panel*). In the presence of spermine, channel gating remained fast with few individual openings lasting more than 20 ms. Single-channel open probability (P_o), mean open time, and mean closed time were determined under each condition and summarized in Table 1. Gating experiments were performed under different salt and spermine concentrations. There were no discernable differences in P_o, mean open time, or mean closed time. There was no clear evidence that spermine blocks the pore for extended periods of time. Although open channel noise was generally larger in the presence of

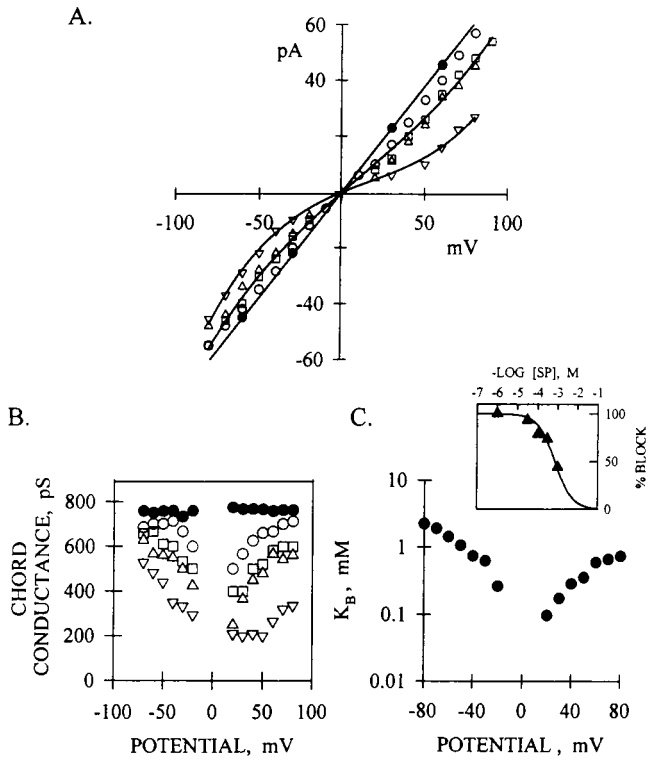

FIGURE 7 Blockade at different *cis* spermine concentrations. Solutions contained 210 mM and 10 μM $CaCl_2$ on both sides of channel. These data are representative of nine single-channel experiments. *A*. Current-voltage relationships collected in control conditions (*filled circle*) and in the presence of 0.03 mM *cis* spermine (*open circle*), 0.1 mM *cis* spermine (*square*), 0.3 mM *cis* spermine (*triangle*), and 1.0 mM *cis* spermine (*inverted triangle*). Control data were best fit by a linear regression (slopes 766). *B*. Concentration dependence of chord conductance. Chord conductance was calculated from the current-voltage relationships using Eq. 1. *C*. Measured values of the spermine half-block (K_B) concentration as a function of membrane potential. The K_B values were obtained from Hill fits to percent block versus spermine concentration plots. An example plot (at −80 mV) and fit are illustrated in the *inset*. The percent block was calculated from the current-voltage relationship using Eq. 4. Extrapolation to 0 mV (not shown) suggested a K_B(0 mV) of 43 μM.

spermine, pooling of data collected on five different channels indicated that the difference was not significant (5.64 ± 0.54 vs. 6.34 ± 0.67 pA, $p = 0.1024$ two-tailed *t*-test). An increase in open channel noise would suggest that spermine attenuates current by entering and leaving the pore very rapidly.

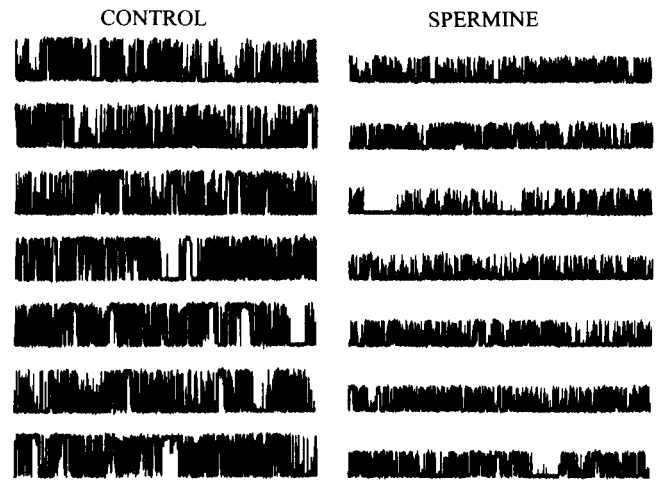

FIGURE 8 Single-channel recordings in the presence and absence of spermine. Solutions contained symmetrical 810 mM K^+ and 10 μM Ca^{2+}. Control (no spermine) records are on the left. Recordings from the same channel in the presence of 3 mM spermine (3 mM) are on the right. Calibration bars represent 50 pA and 1000 ms.

Action of other polyamines

In cells, the common endogenous polyamines are spermine, spermidine, and putrescine. These polyamines vary in size (202, 145, and 88 g/mole, respectively) and valence (+4, +3, and +2, respectively). Because size and valence are two key factors that influence permeability of ions through channels, it is possible that the different polyamines may alter channel function in very different ways. Thus, the actions of these three polyamines on the RyR channel were defined.

Relatively large concentrations of each polyamine (3 mM) were applied to the cytoplasmic (*cis*) side of the channel. The I-V relationships in control conditions and in the presence of the different polyamines are plotted in Fig. 9 *A*. The characteristic N-shaped I-V relationship was observed regardless of which polyamine was present. The blocks induced by the different polyamines are compared in the chord conductance plot (Fig. 9 *B*). Although the efficacies of the three polyamines were different (spermine > spermidine > putrescine), the general features of the blockade, peak block near 0 mV and relief of block at large potentials, were present. It appears that size and valence altered efficacy but did not significantly change the general characteristics of the blocking mechanism.

TABLE 1 Spermine action on channel gating

	Open probability (p_o)	Mean open time (MOT)	Mean closed time (MCT)
210 K^+ Control	12.9 ± 4.6 (n=8)	0.60 ± 0.35 (n=8)	15.3 ± 7.3 (n=8)
810 K^+ Control	17.2 ± 7.71 (n=4)	0.64 ± 0.27 (n=4)	8.2 ± 5.6 (n=4)
210 K^+ Spermine (1 mM)	10.5 ± 5.87 (n=8)	0.68 ± 0.47 (n=8)	13.6 ± 8.8 (n=8)
810 Spermine (3 mM)	16.2 ± 8.83 (n=4)	0.63 ± 0.28 (n=4)	10.6 ± 5.7 (n=4)

Data reported as means ± SD of several determinations (n) on different channels. There were no significant differences (two-tailed *t*-test) in P_o, MOT, or MCT.

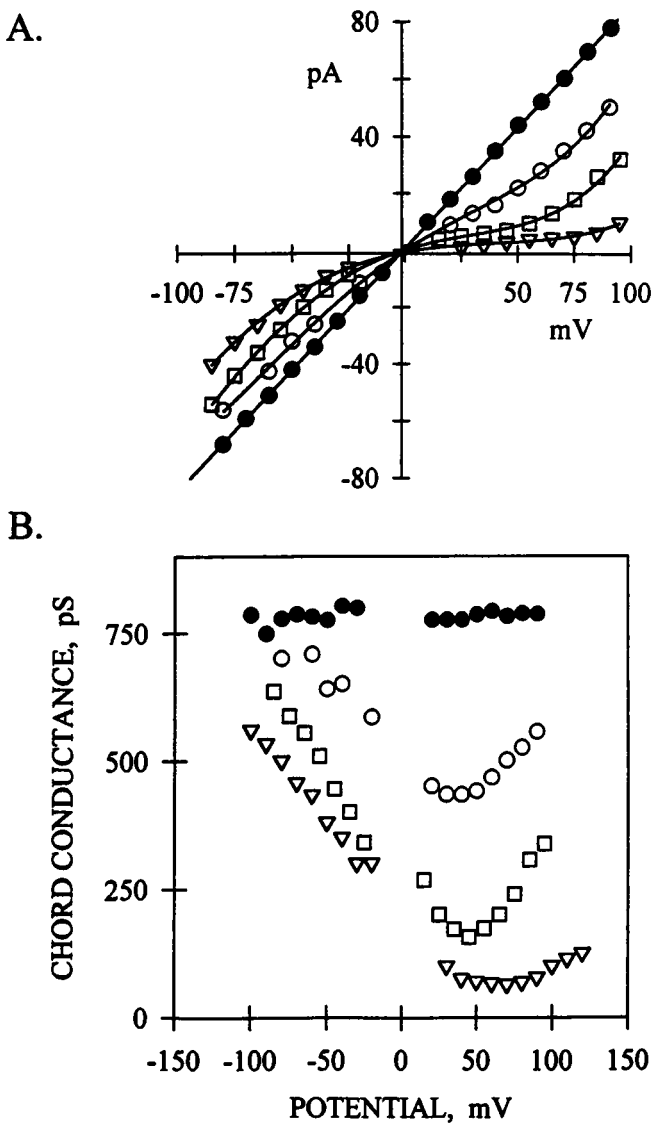

FIGURE 9 Action of three endogenous polyamines on single ryanodine receptor channels. Solutions contained 210 mM KCl and 10 μM $CaCl_2$ on both sides of channel. These data are representative of 13 single-channel experiments. *A*. Current-voltage relationships illustrating data were collected in control conditions (*filled circle*) and in the presence of 5 mM *cis* putrescine (*open circle*), 5 mM *cis* spermidine (*square*) or 5 mM *cis* spermine (*inverted triangle*). Control data were best fit by a linear regression (slope 795 pS). *B*. Action of the three polyamines on chord conductance. Chord conductance was calculated from the current-voltage relationships using Eq. 1.

Classically, cationic block has been analyzed following the strategy of Woodhull (1973). The Woodhull (1973) scheme has been applied, in a limited fashion, to blockade by permeable cationic blockers (French and Wells, 1977; French and Shoukimas, 1985). Here, a similar strategy was applied (over a limited voltage range) to estimate the apparent electrical distance of the spermine blocking site from one edge of the electrical field (see Methods). It was not assumed here that a particular electrical distance value is associated with a discrete binding site or physical position. Instead, these values were merely used as an index to compare the actions of the different polyamines. It appears that spermine, when applied to the cytoplasmic side of the channel, moves through 14.4 ± 0.6 (mean ± SD, n = 6) percent of the electrical field to reach its blocking site. Spermidine (15.9 ± 0.6%, n = 3) and putrescine (18.1 ± 0.5%, n = 3) appear to move through slightly more of the electrical field to reach their blocking sites. This suggests that the smaller, less-charged polyamines penetrate deeper into the RyR pore. Because the blocking mechanism was similar for all the polyamines tested, this analysis and the lack of sidedness (Fig. 3) suggest that the polyamines may simply occlude the pore instead of binding to a distinct site in the permeation pathway.

DISCUSSION

The RyR channel protein has an abundance of negatively charged amino acid residues (RyR isoelectric point 3.7). Thus, it is not surprising that polycationic metabolites, like polyamines, interact with the RyR protein. It has been suggested that the polyamine-RyR interaction is specific (Zarka and Shoshan-Barmatz, 1992; Palade, 1987). However, the mechanism and functional consequences of the polyamine-RyR interaction have not been explored. The data presented in this study suggest that the endogenous levels of polyamines present in cardiac muscle are sufficient to alter RyR function by acting as permeable cationic channel blockers.

Cationic block of ion channels

There are many well-documented examples of cationic blockade of ion channels. Typically, a cationic blocker is thought to act by entering the channel pore and obstructing normal ion passage. If the blocker enters the transmembrane electric field, then the blockade will be voltage dependent. Although most cationic blockers do not permeate through the channel, there is an interesting consequence when one does. If the blocker permeates, then the block will be relieved at large membrane potentials. This phenomenon has been previously termed "punch through." Relief occurs when the blocking cation translocates through the channel faster than the next blocking cation associates. Because the speed of blocking cation translocation through the channel increases with membrane potential, relief is usually observed only at large membrane potentials. Current carried by the primary current carrier dominates and thus the measured conduction will approach the unblocked value.

A classical example of a permeable cationic blockade is Na^+-block of neuronal K^+ channels (French and Shoukimas, 1985; Bezanilla and Armstrong, 1972; French and Wells, 1977). Some characteristic features of block by a permeable cationic blocker are an N-shaped I-V relationship, relief of block at large membrane potentials, and no measurable blocking cation current.

Polyamine-RyR interaction

The I-V relationship of RyR channels after exposure to polyamine was N-shaped. The block was relieved at large membrane potentials and there was no measurable polyamine current. This suggests that polyamine may act as a permeable cationic blocker of the RyR channel. Several lines of experimental evidence support this possibility. First, polyamine blocked from both sides of the channel and the block was readily reversible. Second, polyamine efficacy depended on current direction. The current direction results suggest that the site of polyamine action is in the RyR channel permeation pathway. Third, the apparent affinity of the RyR for polyamine (half-block concentration, K_B) depended on membrane potential. The voltage dependency of K_B indicates that the site of polyamine action lies within the electrical field. Fourth, polyamine efficacy was inversely related to the ion selectivity of the RyR pore. This suggests that polyamine and the primary charge-carrying ion interact in the permeation pathway. Fifth, the polyamine blockade was relieved at large membrane potentials, potentials which would tend to push the blocker further into the pore. The relief of block can be explained if polyamine actually passes through the RyR pore. The sidedness, reversibility, voltage dependence, current direction dependence, pore selectivity dependence, and the relief of blockade at large membrane potentials support the conclusion that polyamines act as permeable cationic blockers of the RyR channel. Thus, polyamine enters the pore and attenuates the current by interacting with current-carrying ions in the RyR pore.

The data presented here show that spermine attenuates unitary current through the ryanodine receptor channel without altering channel-gating properties. This could suggest that spermine enters and leaves the pore very rapidly and that the bandwidth of the measuring system is too narrow to adequately track individual blocking events. In this case, the actual degree of blockade would depend on the dwell time of spermine in the pore, and on the frequency response of the measuring system. As the dwell time of spermine in the pore shortens, the apparent attenuation of current would increase. Unfortunately, the inherent bandwidth limitations of the bilayer experiments limited exploring this possibility at corner frequencies faster than 2 kHz. Within the bandwidth limitations of our recording system, the possibility that spermine acts as a fast cationic blocker could not be clearly established.

In skeletal muscle SR vesicle preparation, Palade (1987) demonstrated that polyamine inhibited the Ca^{2+} release process. Zarka and Shoshan-Barmatz (1992) showed that polyamine increased the ryanodine-binding by about fivefold. Thus, polyamine blockade is one of the few cases where ryanodine binding does not correlate well with the activity of the channel. Most pharmacological agents that alter ryanodine binding and channel function in parallel actually bind allosterically to the channel protein. Our data suggest that polyamine does not bind to the channel protein, but instead polyamine enters the channel pore and physically impairs ion translocation. Thus, the basis of the disparity between binding and function may simply reflect the nature of the polyamine-channel interaction.

Physiological implication of polyamine blockade

Polyamines (spermine, spermidine, and putrescine) are endogenous constituents of muscle and exist in substantial concentrations ($\approx$0.8, $\approx$0.6, and $\approx$0.04 mM, respectively; Zarka and Shoshan-Barmatz, 1992). Thus, endogenous polyamines may alter RyR function. The affinity of the RyR for polyamine at 0 mV was estimated. At rest, the membrane potential of the sarcoplasmic reticulum (SR) is thought to be essentially clamped at 0 mV by the high resting K^+ permeability of the SR (Somlyo et al., 1977). The apparent affinity (K_B) at 0 mV was estimated in two ways. First, it was derived from chord conductance data using Eq. 3. These estimates suggested that K_B at 0 mV was $<$0.25 mM. Second, K_B at 0 mV was extrapolated from the voltage dependency of K_B plot (Fig. 7). The extrapolation indicated that K_B at 0 mV was $<$0.1 mM. Because there is more than 1 mM polyamine in muscle cells, endogenous polyamines can potentially alter RyR function. As polyamine appears to be permeable through the RyR channel, there may be substantial polyamine concentrations inside and outside the SR. Consequently, the endogenous polyamine may block inward and/or outward current through the channel. Thus, the possibility that polyamines alter RyR function in vivo should not be ignored.

It is interesting that the endogenous polyamines have been implicated in the Mg^{2+}-dependent inward rectification of the inward rectifying K^+ channel (Matsuda et al., 1987; Lopatin et al., 1994; Ficker et al., 1994). Although the blocking mechanism is clearly different, polyamine does block conduction through both channels. This might suggest that endogenous polyamines are ubiquitous modulators of ion channel function.

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