Ring of Negative Charge in BK Channels Facilitates Block by Intracellular Mg^{2+} and Polyamines through Electrostatics

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Intracellular Mg^{2+} and natural polyamines block outward currents in BK channels in a highly voltage-dependent manner. Here we investigate the contribution of the ring of eight negatively charged residues (4 x E321/E324) at the entrance to the inner vestibule of BK channels to this block. Channels with or without (E321N/E324N) the ring of negative charge were expressed in oocytes and unitary currents were recorded from inside-out patches over a range of intracellular Mg^{2+} and polyamine concentrations. Removing the ring of charge greatly decreased the block, increasing K_B^{ap} (0 mV) for Mg^{2+} block from 48.3 ± 3.0 to 143 ± 8 mM, and for spermine block from 8.0 ± 1.0 to 721 ± 9 mM (150 mM symmetrical KCl). Polyamines with fewer amine groups blocked less: putrescine < spermidine < spermine. An equation that combined an empirical Hill function for block together with a Boltzmann function for the voltage dependence of K_B^{ap} described the voltage and concentration dependence of the block for channels with and without the ring of charge. The Hill coefficients for these descriptions were <1 for both Mg^{2+} and spermine block, and were unchanged by removing the ring of charge. When KCl_i was increased from 150 mM to 3 M, the ring of charge no longer facilitated block, Mg^{2+} block was reduced, spermine block became negligible, and the Hill coefficients became ~1.0. BK channels in cell-attached oocyte patches displayed inward rectification, which was reduced for channels without the ring of charge. Taken together, these observations suggest that the ring of negative charge facilitates block through a preferential electrostatic attraction of Mg^{2+} and polyamine over K^+. This preferential attraction of multivalent blockers over monovalent K^+ would decrease the K^+ available at the inner vestibule to carry outward current in the presence of Mg^{2+} or polyamines, while increasing the concentration of blocker available to enter and block the conduction pathway.

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INTRODUCTION

Intracellular Mg^{2+} and the natural polyamines, spermine, spermidine, and putrescine are ubiquitously present in the cytoplasm of cells, blocking outward currents through many different ion channels giving rise to inward rectification (Nichols and Lopatin, 1997; Hille, 2001; Bichet et al., 2003; Lu, 2004). Mg^{2+} and polyamine block are major contributors to inward rectification of the Kir channel family (Matsuda et al., 1987; Vandenberg, 1987; Ficker et al., 1994; Lopatin et al., 1994; Nichols and Lopatin, 1997; Pegan et al., 2005), and polyamines also block Na^+ channels (Huang and Moczydlowski, 2001), gap junction channels (Musa and Veenstra, 2003; Musa et al., 2004), and cyclic nucleotide-gated channels (Lu and Ding, 1999). Mg^{2+} block also induces inward rectification in the TRPC5 transient receptor potential channels (Obukhov and Nowycky, 2005). Large-conductance Ca^{2+} and voltage-activated K^+ (BK) channels also demonstrate inward rectification when recording from cell-attached patches (Morales et al., 1996; Snetkov et al., 1996), and intracellular Mg^{2+} (Ferguson, 1991; Laver, 1992; Zhang et al., 1995; Morales et al., 1996), spermine, and spermidine reduce outward single-channel currents through BK channels in excised patches of membrane (Snetkov et al., 1996).

Rings of negative charge in the inner vestibule (D172) and cytoplasmic pore domain (Glu224 and Glu299) of Kir 2.1 channels are important for the strong inward rectification by intracellular Mg^{2+} and polyamines, acting as electrostatic attractors of the blocking agents (Lu and MacKinnon, 1994; Stanfield et al., 1994; Wible et al., 1994; Taglialatela et al., 1995; Yang et al., 1995; Minor et al., 1999; Lu et al., 1999; Kubo and Murata, 2001; Nishida and MacKinnon, 2002; Xie et al., 2002; Kuo et al., 2003; Kurata et al., 2004). The negatively charged residues in gap junction channels (Musa et al., 2004) and CNG channels (Guo and Lu, 2000) are also critical for polyamine block.

BK channels also have a ring of negative charge in the conduction pathway. By comparing the amino acid sequences of KcsA and MthK channels whose structures are known (Doyle et al., 1998; Jiang et al., 2002a,b) to that of BK channels, Brelidze et al. (2003) and Nimigean et al. (2003) found a ring of negative charge at the entrance to the inner vestibule of BK channels. This ring of eight negative charges arises from two charges per subunit (E321 and E324). Although it is known that this ring of negative charge doubles the amplitudes of

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outward unitary currents through BK channels by increasing the concentration of K^+_i at the entrance to the inner vestibule ~3.3-fold (Brelidze et al., 2003), the contribution of the ring of negative charge to Mg^{2+}_i and polyamine block in BK channels is unknown. In this paper we investigate this question. The term "block" will be used in this paper to refer to the reduction of current by Mg^{2+}_i and polyamines without implication as to specific mechanism, as several different mechanisms may contribute to block.

By comparing Mg^{2+}_i block in wild-type channels to block in mutated channels without the ring of negative charge (E321N/E324N), we find that the ring of negative charge greatly facilitates intracellular Mg^{2+} and polyamine block of BK channels. The ring of charge decreased K_B^{ap} (0 mV) for Mg^{2+} block threefold, from 143 to 48 mM in 150 mM symmetrical KCl, and decreased the K_B^{ap} (0 mV) for spermine block 90-fold, from 721 to 8 mM. The facilitating effect of the ring of negative charge on intracellular Mg^{2+} and polyamine block was removed with 3 M intracellular KCl. The above observations are consistent with the ring of negative charge facilitating block through a preferential electrostatic attraction of blocker over K^+ to the entrance to the intracellular vestibule. This preferential attraction would reduce outward K^+ current in two ways: by increasing the concentration of blocker available to enter the channel to block K^+ currents and by screening the ring of negative charge so that the negative charge is less effective at increasing the concentration of K^+ available to enter the vestibule. We also find that the inward rectification of BK channels on cell-attached patches (Morales et al., 1996; Snetkov et al., 1996) is greatly reduced by removing the ring of negative charge, indicating that the ring of charge contributes to apparent block under physiological conditions. Some of the results have appeared in abstract form (Zhang, Y., X. Niu, T.I. Brelidze, and K.L. Magleby. 2004. *Biophys. J.* 86:119a).

MATERIALS AND METHODS

Expression and Mutagenesis

The construct encoding the WT channels (mSlo1 in pcDNA3), initially cloned by Pallanck and Ganetzky (1994), was provided by Merck Research Laboratories with all 5′ noncoding sequence removed up to the second potential translation initiation site (1–940 base pairs were removed). Using the wild-type construct, the mutant constructs were generated by replacing E321, E324, or both E321 and E324 with asparagines, as described (Brelidze et al., 2003; Brelidze and Magleby, 2004). The cRNA was transcribed using the mMessage mMachine kit (Ambion). Recordings were made 2–5 d after injecting either WT or mutant cRNA into *Xenopus* laevis oocytes (0.5–2 ng per oocyte).

Solutions

The extracellular (pipette) solution contained 150 mM KCl, 5 mM TES (5 *N*-tris[hydroxymethyl]methyl-2-aminoethane-sulfonic acid), and 50 μM $GdCl_3$ to block endogenous mechanosensitive channels (Yang and Sachs, 1989) and was the same for all experiments. The intracellular solutions contained (unless indicated) 150 mM KCl, 5 mM TES, and 1 mM EGTA plus 1 mM HEDTA (1 *N*-(2-hydroxyethyl)ethylenediamine-*N*,*N*′,*N*′-triacetic acid) to buffer the Ca^{2+} to prevent possible Ca^{2+} block from contaminating Ca^{2+}. As shown in Brelidze and Magleby (2004), 1 mM EGTA, 1 mM HEDTA, and 50 μM $GdCl_3$ do not alter the unitary current amplitudes of BK channels (Brelidze and Magleby, 2004). The concentrations of the intracellular Mg^{2+} (added as $MgCl_2$) and K^+ (added as KCl) were increased for some of the experiments, when indicated. The required added Mg^{2+} for the indicated free Mg^{2+} was calculated with a custom buffer program. Both intracellular and extracellular solutions were adjusted to pH 7.0. The natural polyamines (spermine, spermidine, and putrescine) were purchased from Sigma-Aldrich and made to a 100 mM stock solution in 150 mM KCl, with pH adjusted to 7.0. The polyamines were then added before the experiments. The solutions bathing the intracellular side of the patch were changed with a micro chamber as previously described (Barrett et al., 1982). In a typical experiment, the effects of many different concentrations of intracellular Mg^{2+} could be examined over a range of voltages on a single patch because the effects of Mg^{2+} reversed as rapidly as the solution could be changed. Because it was difficult to totally wash out polyamines, the oocyte was changed for each new experiment after perfusion with polyamines.

Single-channel Recording and Data Analysis

Single-channel currents were recorded from BK channels using the inside-out configuration of the patch-clamp technique (Hamill et al., 1981), and in a few experiments, where indicated, currents were recorded from BK channels in cell-attached patches. Data were acquired with an Axopatch 200B amplifier (Axon Instruments), sampled at a 5-μs interval using a Digidata 1322A and PCLAMP9 (Axon Instruments) and low pass filtered at an effective frequency of 5–10 kHz. BK channels were identified by their large conductance and characteristic Ca^{2+} and voltage sensitivity. Single-channel (unitary) current amplitudes were measured in an unbiased manner by using all-point histograms of the current records (pClamp 9.0), with the single-channel current amplitudes indicated by the distance between the adjacent peaks of the histograms. Each of the plotted points in the figures represents the average of observations obtained from three or more patches, with the SEM indicated by error bars. The absence of visible error bars indicates that the SEM is less than the symbol size. The value and SEM for each fitted parameter were determined during the fitting of the mean data using SigmaPlot 2000. Experiments were performed at 21–23°C.

RESULTS

A Ring of Negative Charge Encircles the Entrance to the Inner Vestibule of BK Channels

Previous studies based on the crystal structure and sequence alignment with MthK have suggested that a ring of negative charge encircles the entrance to the inner vestibule of BK channels (Jiang et al., 2002a,b; Brelidze et al., 2003; Nimigean et al., 2003). Fig. 1 A presents a ribbon structure of two opposed subunits of MthK viewed from the side, and Fig. 1 B presents a view looking from the cytoplasm toward the channel for all four subunits (Jiang et al., 2002a,b). The negative charged residues E321 and E324 in BK channels are projected onto the ribbon structure as space filling molecules.

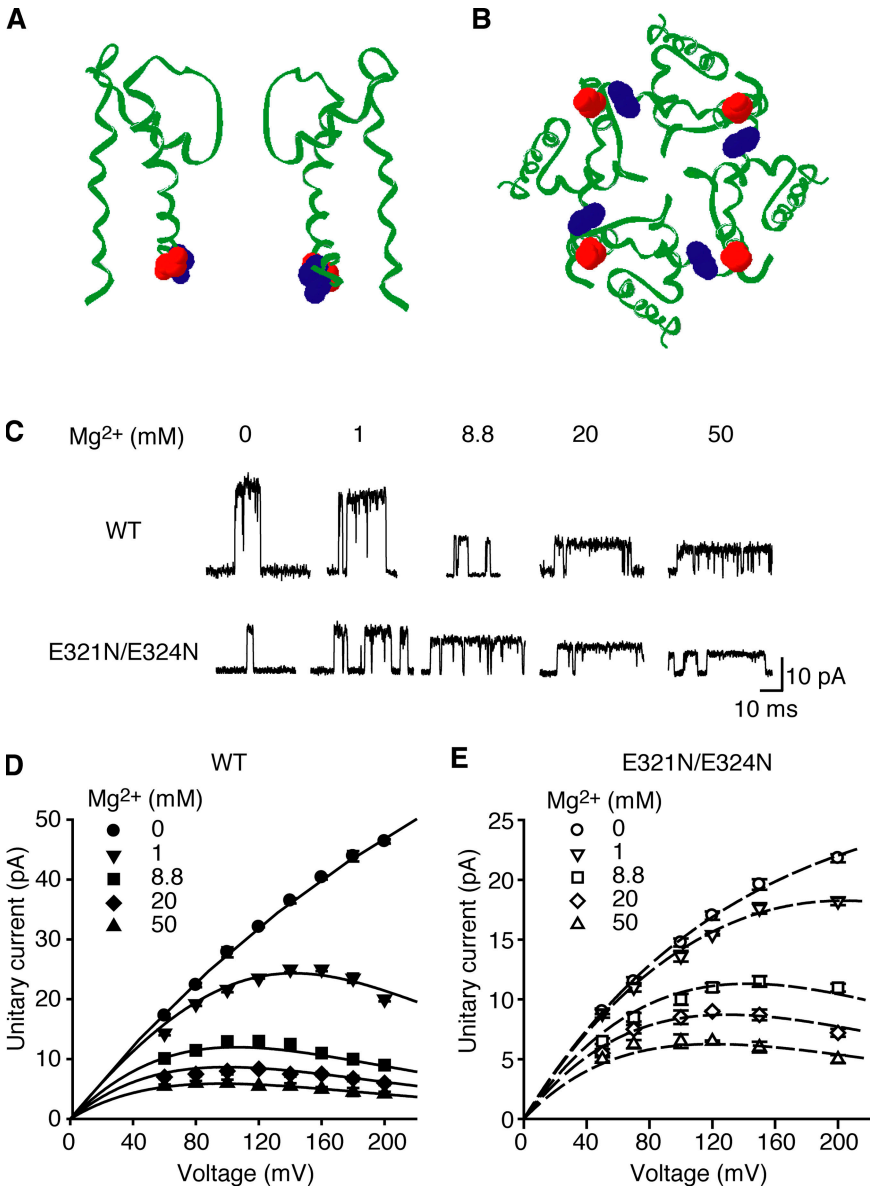

Figure 1. Intracellular Mg^{2+} blocks both WT and mutant (E321N/E324N) channels in a voltage- and concentration-dependent manner. A and B present ribbon structures of MthK for a side view of two opposed subunits and for a bottom view looking from the cytoplasm toward the channel for all four subunits, obtained with Swiss Protein Viewer and coordinates from Jiang et al. (2002a). The negatively charged residues E321 and E324 in BK channels are projected onto the ribbon structures as space filling structures (red and blue), which are substituted for E92 and L95 in MthK. Brelidze et al. (2003) substituted for R93 and E96 in their Fig. 1. It is unclear which substitution would be closer to the unknown structure of the BK channel. (C) Representative single-channel currents recorded from WT (top traces) and E321N/E324N (lower traces) BK channels expressed in oocytes with symmetrical 150 mM KCl and the indicated Mg^{2+}_i concentration. Membrane potential: +100 mV. Effective filtering: 5 kHz. Channel opening indicated by upward going currents. (D and E) Plots of outward unitary current amplitude versus voltage at the indicated Mg^{2+} from WT (filled symbols) and mutant (open symbols) channels, respectively. The intracellular solutions for all figures before adding the blocking ions or increasing intracellular KCl were (in mM) 150 KCl, 5 TES, 1 EGTA, 1 HEDTA, pH 7.0. The lines are descriptions with Eq. 5 for simultaneous fitting of the data in D for WT channels: $K_B^{ap}(0)$ for Mg^{2+}_i = 48.3 ± 3.0 mM, d = 0.25 ± 0.01, and n = 0.64 ± 0.01, and simultaneous fitting of the data in E for E321N/E324N channels: $K_B^{ap}(0)$ for Mg^{2+}_i = 143 ± 16.6 mM, d = 0.19 ± 0.01, and n = 0.61 ± 0.02.

The eight residues arising from the two negative charges per subunit (E321/E324) form a ring of negative charge that encircles the entrance to the inner vestibule.

Mg^{2+}_i Blocks WT and Mutant Channels (E321N/E324N) in a Concentration- and Voltage-dependent Manner

To investigate the contribution of the ring of negative charge (Fig. 1, A and B) to the block by intracellular Mg^{2+} (Mg^{2+}_i), single-channel (unitary) currents were recorded from WT channels and also from mutant BK channels in which the ring of eight negatively charged glutamates was replaced with neutral asparagines by making the double mutation E321N/E324N on each subunit. Fig. 1 C presents currents from single WT and E321N/E324N channels at a membrane potential of +100 mV with symmetrical 150 mM KCl. The unitary currents for WT channels in the absence of Mg^{2+}_i were 28.0 pA. Adding Mg^{2+}_i then decreased the currents. For example, 1 mM Mg^{2+}_i decreased the currents to 21.6 pA (23% reduction), and 20 mM Mg^{2+}_i decreased the currents to 8.9 pA (68% reduction).

For E321N/E324N channels, the unitary current in the absence of Mg^{2+}_i was 14.9 pA, about half that observed in WT channels (Fig. 1 C). The reduced current in E321N/E324N channels in the absence of blocker occurs because the negative ring of charge, which concentrates K^+ in the vestibule of the WT channels through an electrostatic mechanism, was removed (Brelidze et al., 2003; Nimigean et al., 2003). Adding 1 mM Mg^{2+}_i for E321N/E324N channels then decreased the unitary current to 13.4 pA (10% reduction), and 20 mM Mg^{2+}_i reduced the current to 6.31 pA (58% reduction).

To investigate the voltage dependence of the Mg^{2+} block, plots of unitary current amplitude versus

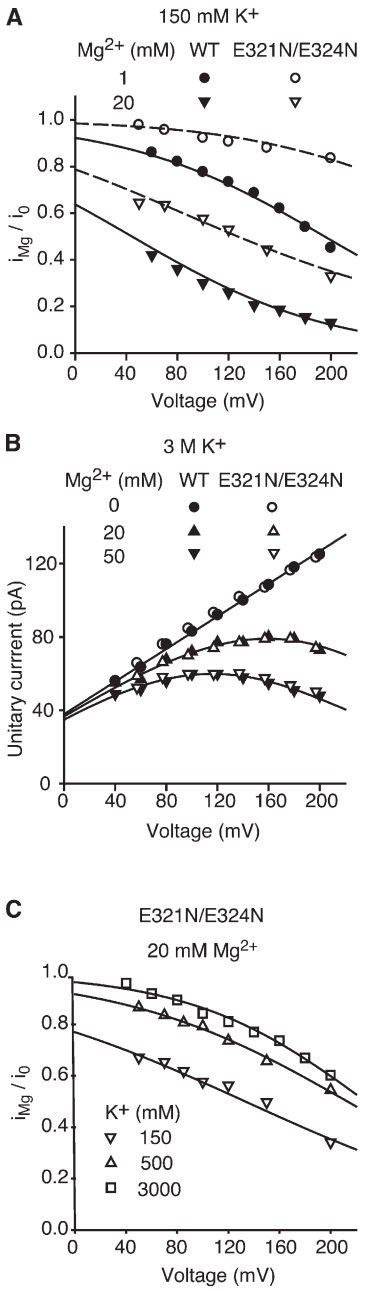

Figure 2. The ring of charge facilitates Mg^{2+}_i block through an electrostatic mechanism. (A) The ring of negative charge increases the Mg^{2+}_i block for 150 mM K^+_i at all examined voltages. Plots of the ratio of unitary current with and without Mg^{2+}_i (i_{Mg2+}/i_0) versus voltage from both WT (filled symbols) and E321N/E324N (open symbols) channels. The parameters for the lines calculated with Eq. 5 are the same as in Fig. 1. (B) The ring of negative charge no longer increases Mg^{2+}_i block when K^+_i is increased to 3 M. Plots of unitary current for WT channels (filled symbols) and mutant channels (open symbols) with intracellular KCl increased to 3 M. The WT symbols were shifted right by 5 mV so that they can be seen. The lines are fits from Eq. 5 with $K_B^{ap}(0)$ for Mg^{2+}_i = 561 ± 12 mM, d = 0.19 ± 0.01, and n = 1.01 ± 0.05 for both WT and E321N/E324N channels. (C) Increasing K^+_i decreases Mg^{2+} block. Plots of the ratio of unitary current from E321N/E324N channels with and without 20 mM Mg^{2+}_i (i_{Mg2+}/i_0) versus voltage for three different K^+_i. The parameters for the fits with 150 and 3 M K^+_i are given in Figs. 1 and 2, respectively. The parameters for 500 mM K^+_i are $K_B^{ap}(0)$ = 492 ± 32 mM, d = 0.19 ± 0.01, and n = 0.85 ± 0.04.

membrane potential were made for four different Mg^{2+}_i (Fig. 1, D and E). For all voltages, the reduction of the unitary currents became greater as Mg^{2+}_i was increased, and for each fixed Mg^{2+}_i, the reduction of unitary currents became greater as the membrane potential was made more positive. Thus, Mg^{2+}_i blocks in a concentration- and voltage-dependent manner for both WT and E321N/E324N channels. However, the fractional reduction of current by Mg^{2+}_i was greater for WT channels than for E321N/E324N channels. Consistent with previous studies on WT BK channels, Mg^{2+}_i reduced outward currents without increasing the apparent level of open channel noise (Ferguson, 1991; Zhang et al., 1995), and this was also observed to be the case for E321N/E324N channels (Fig. 1 C).

The Ring of Negative Charge Facilitates Mg^{2+}_i Block through an Electrostatic Mechanism

The greater fractional reduction of current by Mg^{2+}_i for WT channels suggests that the ring of negative charge facilitates Mg^{2+}_i block. To examine this facilitated block, the ratios of the unitary current amplitudes with 1 mM and 20 mM Mg^{2+}_i to those without Mg^{2+}_i for both WT (Fig. 1 D) and E321N/E324N channels (Fig. 1 E) were plotted against voltage in Fig. 2 A. A greater Mg^{2+}_i block was observed for WT channels at all voltages. If the greater Mg^{2+}_i block in WT channels arises because the negative ring of charge increases the concentration of Mg^{2+} in the vestibule through an electrostatic mechanism, then the Mg^{2+}_i block of WT and mutant channels should be the same with very high K^+_i, which would screen the ring of charge (Brelidze et al., 2003). Fig. 2 B plots the single-channel current amplitudes for WT and E321N/E324N channels vs. voltage with 3 M KCl for 0, 20, and 50 mM Mg^{2+}_i. With 3 M K^+_i, the unitary currents were greatly increased to 128 pA at +200 mV for both channel types. This large increase arises from the increased K^+ available to carry current and also from the increased driving force due to the increased concentration gradient of K^+ (Brelidze et al., 2003).

In contrast to the data obtained with 150 mM K^+_i (Fig. 1 and Fig. 2 A), with 3 M K^+_i, there were no differences in unitary currents between WT (filled symbols) and E321N/E324N channels (open symbols) with or without Mg^{2+}_i (Fig. 2 B). Thus, high K^+_i essentially removes any differences between WT and E321N/E324N channels in terms of Mg^{2+} block and single channel currents. Since the difference between these channels is the ring of negative charge, then high K^+_i must act by removing or swamping out any electrostatic effects of the ring of charge on attracting Mg^{2+} and K^+ to the entrance of the inner vestibule. These observations are

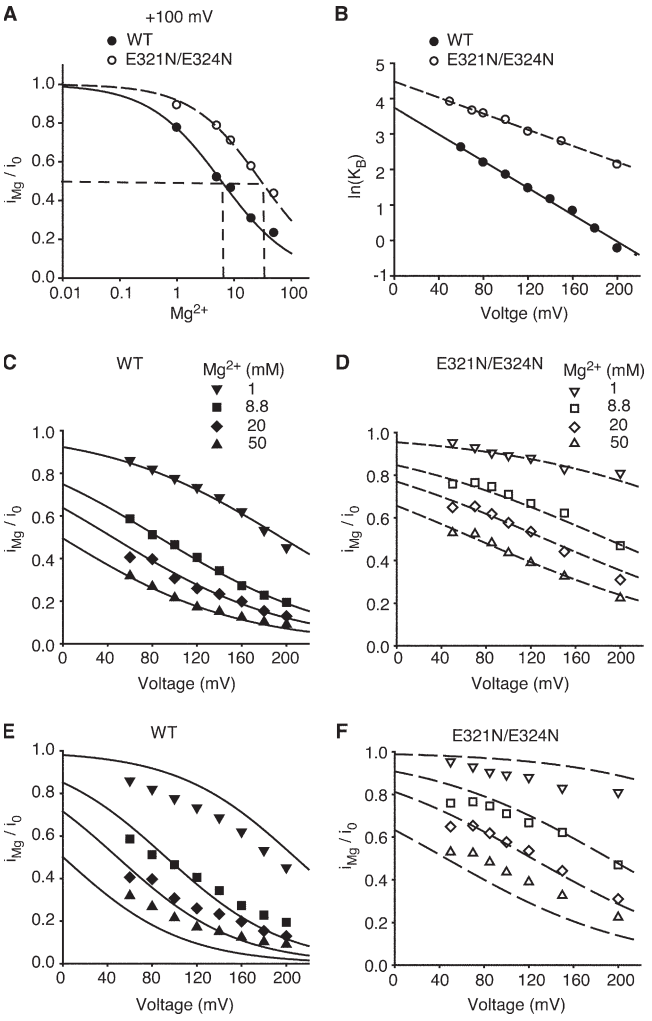

Figure 3. The ring of negative charge decreases both the apparent K_B and increases the voltage dependence of Mg^{2+}_i block. (A) Removing the ring of charge increases the K_B^{ap} for Mg^{2+}_i block. Plots of ratio of current amplitude at +100 mV with and without Mg^{2+}_i (i_{Mg2+}/i_0) over a range of Mg^{2+}_i in WT (filled circles) and E321N/E324N (open circles) channels. The lines are fits with the Hill equation (Eq. 1): WT channels, K_B^{ap} (+100 mV) for Mg^{2+}_i = 6.4 mM, n = 0.64 (continuous lines); E321N/E324N channels, K_B^{ap} (+100 mV) = 33.8 mM, n = 0.65 (dashed lines). (B) Removing the ring of charge increases K_B^{ap}. Semilogarithmic plots of K_B^{ap} against voltage for both WT and E321N/E324N channels. The lines are the fits with Eqs. 2 and 3 with projected K_B^{ap} (0 mV) for Mg^{2+}_i block of 42.3 mM for WT and 89.1 mM for E321N/E324N. (C and D) The Woodhull equation with added (negative) cooperativity accounts for Mg^{2+}_i block in both WT and E321N/E324N channels. Plots of i_{Mg2+}/i_0 over a range of voltages at the indicated Mg^{2+}_i for WT (filled symbols) and E321N/E324N (open symbols) channels. The continuous lines are from simultaneously fitting the WT data in C and the dashed lines are from simultaneously fitting the E321N/E324N data in D. The parameters for the fitting are given in Fig. 1. (E and F) Same as C and D, except that n in Eq. 5 was set to 1.0 so that there was no cooperativity. The Woodhull equation without negative cooperativity could not simultaneously describe the data obtained over a range of concentrations of Mg^{2+}_i.

consistent with an electrostatic mechanism for the effect of the ring of charge on enhancing Mg^{2+}_i block (see Discussion).

A Possible Secondary Site of Mg^{2+} Action

If the ring of negative charge were the sole site of action of Mg^{2+}_i block, then removing the ring of charge, either through mutation or by screening the ring of charge with high K^+_i, would be expected to remove all of the Mg^{2+}_i block. The observation of residual Mg^{2+} block when the ring of charge is removed by either mutation (E321N/E324N) (Fig. 1 E), by 3 M KCl (Fig. 2 B), or by both mutation and 3 M KCl (Fig. 2 B) suggests, but does not establish (see Discussion), that there may be a secondary site of Mg^{2+} action in addition to the ring of negative charge. The pronounced voltage dependence of the residual block suggests that the additional blocking site is located in the electric field of the membrane or that access to this site by Mg^{2+} involves the coupled movement of other ions thorough the electric field (Armstrong, 1971; Hille and Schwarz, 1978; Thompson and Begenisich, 2003, 2005; Gomez-Lagunas et al., 2003; Shin and Lu, 2005).

Ferguson (1991) found that Mg^{2+} block in WT channels was consistent with apparent competition between K^+ and Mg^{2+}, where K^+ inhibits the action of Mg^{2+}. If the ring of charge were the only site of this apparent competition, then removing the ring of charge should remove the ability of increased K^+_i to alleviate the Mg^{2+}_i block. To test this possibility, the effect of changing the concentration of K^+_i on Mg^{2+}_i block was investigated for E321N/E324N channels. Fig. 2 C plots the ratio of i_{Mg}/i_0 vs. voltage for 20 mM Mg^{2+}_i with three different K^+_i of 150, 500, and 3000 mM KCl. Increasing K^+_i progressively decreased Mg^{2+}_i block over the range of examined voltages. This observation of apparent competition between K^+_i and Mg^{2+}_i for channels without the ring of charge is consistent with a possible secondary site of action of Mg^{2+} other than the ring of charge, but other interpretations cannot be ruled out (see Discussion).

The Ring of Negative Charge Decreases K_B^{ap} for Mg^{2+}_i Block

To gain quantitative insight into the facilitating action of the ring of charge on Mg^{2+}_i block, the concentration of blocker in the bulk intracellular solution for 50% reduction of currents for Mg^{2+}_i block of WT and E321N/E324N channels, K_B^{ap}, was estimated at different voltages. These are apparent K_B's because the estimated concentration is that in the bulk solution rather than at the site of action. A typical experiment is shown in Fig. 3 A, which plots the ratio of the unitary current amplitudes with and without Mg^{2+}_i for WT and E321N/E324N channels over a range of Mg^{2+}_i. Data were fit with the following empirical Hill function (Lopes et al., 2000; Hille, 2001; Park et al., 2003),

$$i_{(Mg)} / i_{(0)} = 1/(1+(Mg_i^{2+} / K_B^{ap})^n), \quad (1)$$

where K_B^{ap} is the concentration of the blocker in the bulk intracellular solution required to achieve 50% reduction in current, and n is the Hill coefficient that describes the steepness of the curve. At +100 mV, K_B^{ap} for the WT channels was 6.4 mM, whereas K_B^{ap} for the E321N/E324N channels was 33.8 mM. This indicates that the ring of charge facilitates Mg^{2+}_i block approximately fivefold at +100 mV, presumably by preferentially increasing the effective concentration of the divalent Mg^{2+}_i at the entrance to the inner vestibule a greater amount than it increases the effective concentration of the monovalent K^+_i required to carry the single-channel currents (see Discussion).

The Hill coefficients for the Mg^{2+}_i block were 0.65 ± 0.01 for WT channels and 0.63 ± 0.02 for E321N/E324N channels. Hill coefficients <1 indicate that the block did not obey a single-binding site isotherm, such that the degree of block increases at a slower rate than expected for the increase in the concentration of the blocker in the bulk solution (Park et al., 2003). Hill coefficients <1 will be referred to as apparent negative cooperativity (Wyman and Gill, 1990), without implication as to possible mechanisms, which will be considered in a later section and in the Discussion. The observation that the Hill coefficients were the same for WT and E321N/E324N channels (notice the similar slopes in Fig. 3 A) indicates that a ring of negative charge is not required for apparent negative cooperativity of Mg^{2+} block.

K_B^{ap} for Mg^{2+}_i Block Decreases Exponentially with Depolarization

The K_B^{ap}'s in Fig. 3 A were determined for a fixed voltage of +100 mV. To investigate the effect of voltage on K_B^{ap}, plots like those in Fig. 3 A were made for data collected over a range of voltage, and then the K_B^{ap}'s determined from each figure were plotted semilogarithmically against voltage in Fig. 3 B. The data were well described by straight lines, such that

(WT channel)

$$K_B^{ap}(V) = (42.3\ \text{mM})\exp(-0.0189\ V) \quad (2)$$

(E321N/E324N)

$$K_B^{ap}(V) = (89.1\ \text{mM})\exp(-0.0127\ V), \quad (3)$$

where V is the holding (membrane) potential in millivolts, $K_B^{ap}(V)$ is the concentration of Mg^{2+} in the bulk intracellular solution required to reduce the unitary currents to 50% as a function of voltage, and 89.1 ± 3.4 and 42.3 ±1.6 mM are the concentrations of Mg^{2+}_i, $K_B^{ap}(0)$, required in the bulk solution to reduce the currents to 50% at 0 mV. $K_B^{ap}(0)$ is given by the projected intercept of the fitted lines in Fig. 3 B with the abscissa. Whether experimental data would remain linear on the semilogarithmic plot over the range of the projection is not known, but determining $K_B^{ap}(0)$ provides a means for a simple quantitative description of the data through Eqs. 2 and 3. The $K_B^{ap}(V)$ decreased with increasing voltage for both channel types, with WT channels having a greater voltage dependence for Mg^{2+}_i block than E321N/E324N channels (Fig. 3 B). At 0 mV, 2.1-fold greater Mg^{2+}_i (89.1 vs. 42.3 mM) in the bulk solution would be required for 50% reduction of unitary currents in E321N/E324N channels compared with WT channels, whereas at +200 mV, 13.9-fold greater Mg^{2+}_i (8.5 vs. 0.61 mM) would be required in the bulk solution to block E321N/E324N channels than WT channels. In addition, E321N/E324N channels also had a decreased voltage dependence for block, with a slope of −0.0189 natural log units/mV for WT channels compared with −0.0127 natural log units/mV for E321N/E324N channels.

Simultaneous Description of the Concentration and Voltage Dependence of Mg^{2+} Block

The Boltzmann function describes how an applied electric field can change the concentration of an ion at a fractional distance d through an electric field (Woodhull, 1973; Hille, 2001; Nimigean and Miller, 2002). If the ion is a blocker and the concentrations are those that give 50% block, then

$$K_B^{ap}(V) = K_B^{ap}(0)\exp(-zdVF/RT), \quad (4)$$

where z is the valence of the ion, and $F/RT = 1/25.4$ mV at 22°C (Woodhull, 1973; Hille, 2001). For K^+ channels with their single-file multi-ion pores (Neyton and Miller, 1988; Doyle et al. 1998), the apparent voltage sensitivity of a blocking ion also includes the voltage dependence of the permeant K^+ ions whose movement is coupled to the blocking event (Armstrong, 1971; Hille and Schwarz, 1978; Thompson and Begenisich, 2001, 2005; Gomez-Lagunas et al., 2003; Shin and Lu, 2005). Consequently, d in Eq. 4 is the effective average fractional distance moved by the various charges on the blockers and the coupled K^+ ions through the electric field for a blocking event. The exponential relationship between $K_B^{ap}(V)$ and voltage in Fig. 3 B is of the same form as Eq. 4, indicating, but not establishing, that the experimentally observed responses described by Eqs. 2 and 3 are consistent with the implied mechanism in Eq. 4. Combining Eq. 1, which describes block at a fixed voltage over a range of Mg^{2+}, with Eq. 4, which describes the change in K_B^{ap} with voltage, gives the empirical Eq. 5.

$$i_{Mg} / i_0 = 1/(1+(Mg_i^{2+} / K_B^{ap}(0)\exp(-zdVF/RT))^n) \quad (5)$$

To examine whether Eq. 5 could account for Mg^{2+}_i block over a range of voltage, the data in Fig. 1 D were replotted in Fig. 3 C as the ratio of the unitary current

TABLE I

Parameters for Mg^{2+} and Polyamine Block from Eq. 5

	WT				E321N/E324N			
Blocker	$K_B^{ap}(0)$	d	zd	n	$K_B^{ap}(0)$	d	zd	n
				150 mM K^+_i				
Mg^{2+}	48.3 ± 3	0.25 ± 0.01	0.50	0.64 ± 0.01	143 ± 16.6	0.19 ± 0.01	0.38	0.61 ± 0.02
Putrescine	180 ± 17	0.33 ± 0.01	0.66	0.85 ± 0.04				
Spermidine	38.4 ± 3	0.25 ± 0.03	0.75	0.75 ± 0.04				
Spermine	8.0 ± 1.0	0.19 ± 0.01	0.76	0.54 ± 0.02	721 ± 9	0.19 ± 0.02	0.56	0.53 ± 0.07
				3 M K^+_i				
Mg^{2+}	561 ± 12	0.19 ± 0.01	0.38	1.01 ± 0.05	561 ± 12	0.19 ± 0.01	0.38	1.01 ± 0.05

K_B^{ap} is the apparent affinity for the blocker projected to 0 mV; d is the average fractional distance moved through the electrical field by the blocker and any coupled K^+ ion; z is the valence of the blocker, with Mg^{2+} = 2, putrescine = 2, spermidine = 3, and spermine = 4; zd, gives a measure of the voltage dependence; n is the Hill coefficient. Since the charge on the polyamines is distributed and K^+ may be moving with blocker, d and zd are relative rather than giving direct physical distances.

amplitudes in the presence of Mg^{2+}_i to the amplitudes in the absence of Mg^{2+}_i for WT channels. All of the data in Fig. 3 C were then fitted simultaneously with Eq. 5 to obtain the best single estimate of the parameters. The Mg^{2+}_i block over a range of both Mg^{2+}_i and voltage was well described for WT channels (continuous lines) with $K_B^{ap}(0)$ = 48.3 ± 3.0 mM, d = 0.25 ± 0.01, and n = 0.64 ± 0.01. The same analysis was also performed for E321N/E324N channels starting with the data in Fig. 1 E. Eq. 5 also described the Mg^{2+} block for E321N/E324N channels, with $K_B^{ap}(0)$ = 143 ± 16.6 mM, d = 0.19 ± 0.01, and n = 0.61 ± 0.02 (Fig. 3 D, dashed lines). The parameters fitted with Eq. 5 are presented in Table I to facilitate comparison.

Fixing the Hill coefficient in Eq. 5 to 1.0 transforms the empirical Eq. 5 to the classical Woodhull model for voltage-dependent block at a single site (Woodhull, 1973). With n set to 1.0, Eq. 5 no longer simultaneously described Mg^{2+} block over a range of Mg^{2+}_i and voltage for WT channels (Fig. 3 E), as observed previously (Ferguson, 1991), and this was also the case for channels without the ring of charge (Fig. 3 F). Thus, an observation of $n < 1$ in Eq. 5 indicates that the titration curve for Mg^{2+} block of BK channels, both with and without the ring of charge, is broader than would be expected for a simple one-site binding isotherm.

To gain insight into possible mechanisms for the apparent negative cooperativity, we determined the value of n in Eq. 5 in the presence of 3 M KCl_i, which would be expected to reduce any surface potential effects generated by the ring of charge to negligible levels (see Discussion). The continuous lines in Fig. 2 B show that Eq. 5 can simultaneously describe the effects of Mg^{2+}_i and voltage on block, with $K_B^{ap}(0)$ for Mg^{2+}_i block = 561 ± 12 mM, d = 0.19 ± 0.01, and n = 1.01 ± 0.05. These same values apply to Mg^{2+} block of both WT and E321N/E324N channels, which responded essentially identically with 3 M K^+_i. This increase of $K_B^{ap}(0)$ for Mg^{2+}_i block, from 48.3 mM (WT) and 143 mM (E321N/E324N) in 150 mM KCl to 561 mM for both channel types with 3 M KCl indicates a greatly decreased Mg^{2+} block in the presence of high K^+_i for both types of channels. With 150 mM KCl, the value of d was 0.25 ± 0.01 in WT channels and 0.19 ± 0.01 in E321N/E324N channels, indicating that removing the ring of charge leads to a small decrease in the apparent voltage dependence of Mg^{2+}_i block. Adding 3M KCl did not change the voltage dependence of the block of E321N/E324N channels, which remained at d = 0.19, but reduced the voltage dependence of the block for WT channels to match that of E321N/E324N channels.

The observation that the Hill coefficient became ~1 for both channel types after applying 3 M intracellular KCl suggests that the apparent negative cooperativity for Mg^{2+} block observed with 150 mM K^+_i for both channel types may require electrostatic or competitive factors (see Discussion), but that these factors do not necessarily have to be associated with the ring of charge, because the apparent negative cooperativity ($n \sim 0.6$) with 150 mM K^+_i was essentially unchanged after removing the ring of negative charge.

Natural Polyamine Block of BK Channels Is Voltage and Concentration Dependent

The following sections of this paper will examine the contribution of the ring of negative charge to polyamine block. The natural polyamines spermine, spermidine, and putrescine consist of carbon chains with interposed amine groups (Fig. 4 E). The pKa's for the amine groups range from 8.1 to 10.9, so that at pH 7.0, each amine group is normally protonated, carrying a positive charge. Snetkov et al. (1996) previously found for examined voltages up to +80 mV that spermine and spermidine reduced outward unitary currents of WT BK channels in smooth muscle cells and that putrescine had little effect.

To extend the studies of Snetkov et al. (1996), polyamine block of WT and also E321N/E324N BK channels was examined over a wide range of voltages for

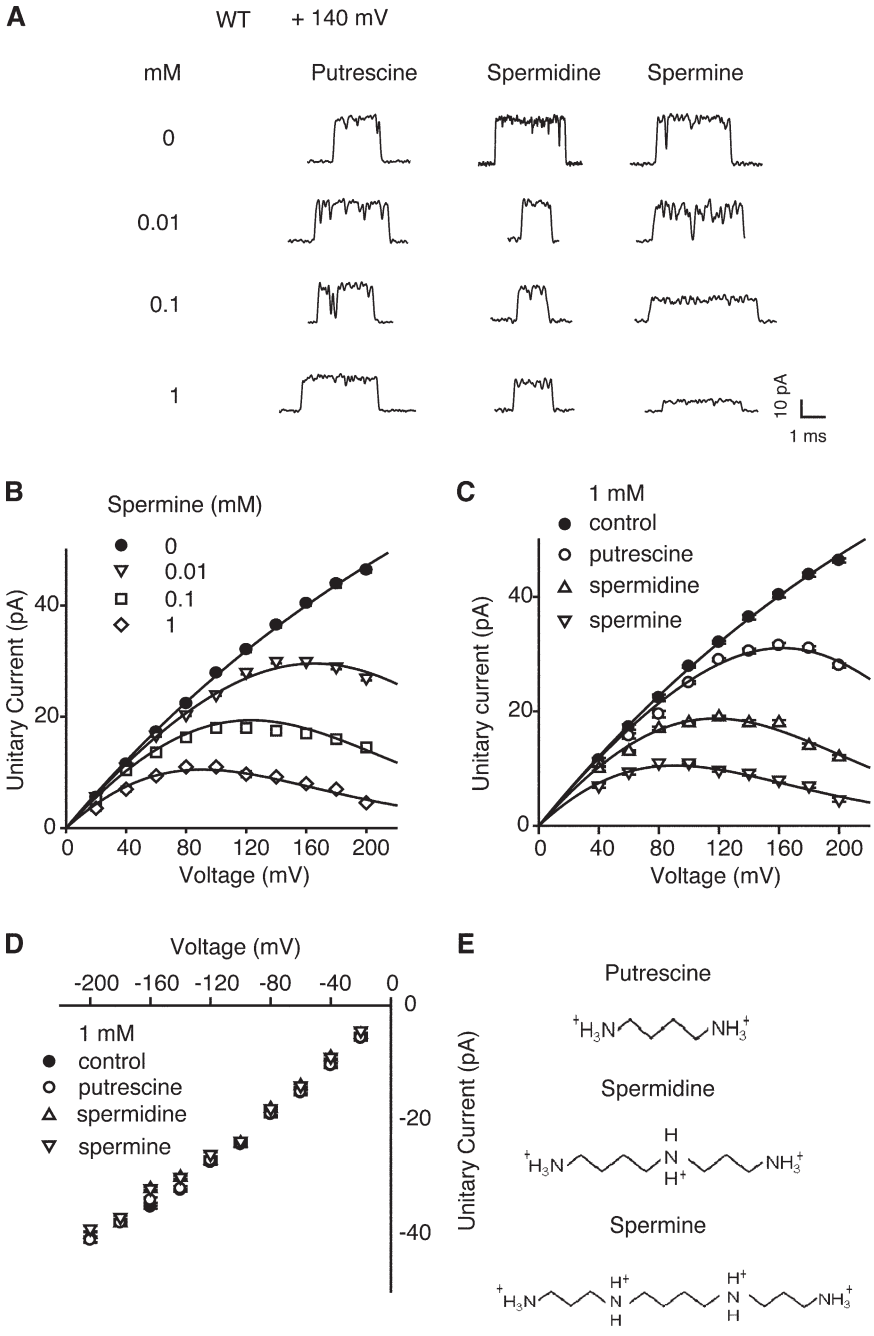

Figure 4. Natural polyamines block WT BK channels in a concentration- and voltage-dependent manner, with the blocking sequence: spermine > spermidine > putrescine. (A) Representative single-channel currents at +140 mV with the different polyamines at the indicated concentrations. (B) Concentration and voltage dependence of spermine block. Plots of unitary current versus voltage at the indicated concentrations of spermine. The lines are simultaneous fits with Eq. 5 with $K_B^{ap}(0)$ = 8.0 ± 1.0 mM, d = 0.19 ± 0.01, n = 0.54 ± 0.02. (C) Comparison of block by spermine, spermidine, and putrescine. Plots of unitary current versus voltage for the indicated natural polyamines at 1 mM. The fitted parameters for each polyamine were determined by simultaneously fitting data obtained over a range of concentrations for each polyamine, as in B. Only the data at 1 mM are presented. The data for spermine are from B. The lines are separate fits with Eq. 5 for spermidine: $K_B^{ap}(0)$ = 38.4 ± 3.3 mM, d = 0.25 ± 0.03, n = 0.75 ± 0.04, and for putrescine: $K_B^{ap}(0)$ = 180 ± 17 mM, d = 0.33 ± 0.01, n = 0.85 ± 0.04. (D) Natural polyamines at 1 mM do not block inward currents through BK channels. Plots of inward unitary currents vs. voltage with and without 1 mM polyamines. (E) Structures of the natural polyamines.

intracellular polyamines ranging from 10 to 1000 μM. Fig. 4 A presents currents from single WT BK channels with symmetrical 150 mM K^+ at +140 mV with and without each of the natural polyamines. Increasing the concentration of each polyamine led to a decrease in the unitary current amplitudes. The block was least for putrescine and greatest for spermine. The concentration and voltage dependence of the spermine block are shown in Fig. 4 B, where unitary current amplitude is plotted against voltage for three different concentrations of blocker and for no blocker. At each voltage, increasing the concentration of spermine increased the block, and for each concentration, increasing depolarization also increased the block. The continuous lines in Fig. 4 B show that Eq. 5 could simultaneously describe the voltage and concentration dependence of spermine block in WT channels, with $K_B^{ap}(0)$ = 8.0 ± 1.04 mM, d = 0.19 ± 0.01, and n = 0.54 ± 0.02.

To compare the block by spermine with that for other natural polyamines, the same types of experiments were performed for putrescine and spermidine. Fig. 4 (C and D) plots outward (C) and inward (D) unitary current amplitudes versus voltage for the three natural polyamines applied at a concentration of 1 mM. The natural polyamines

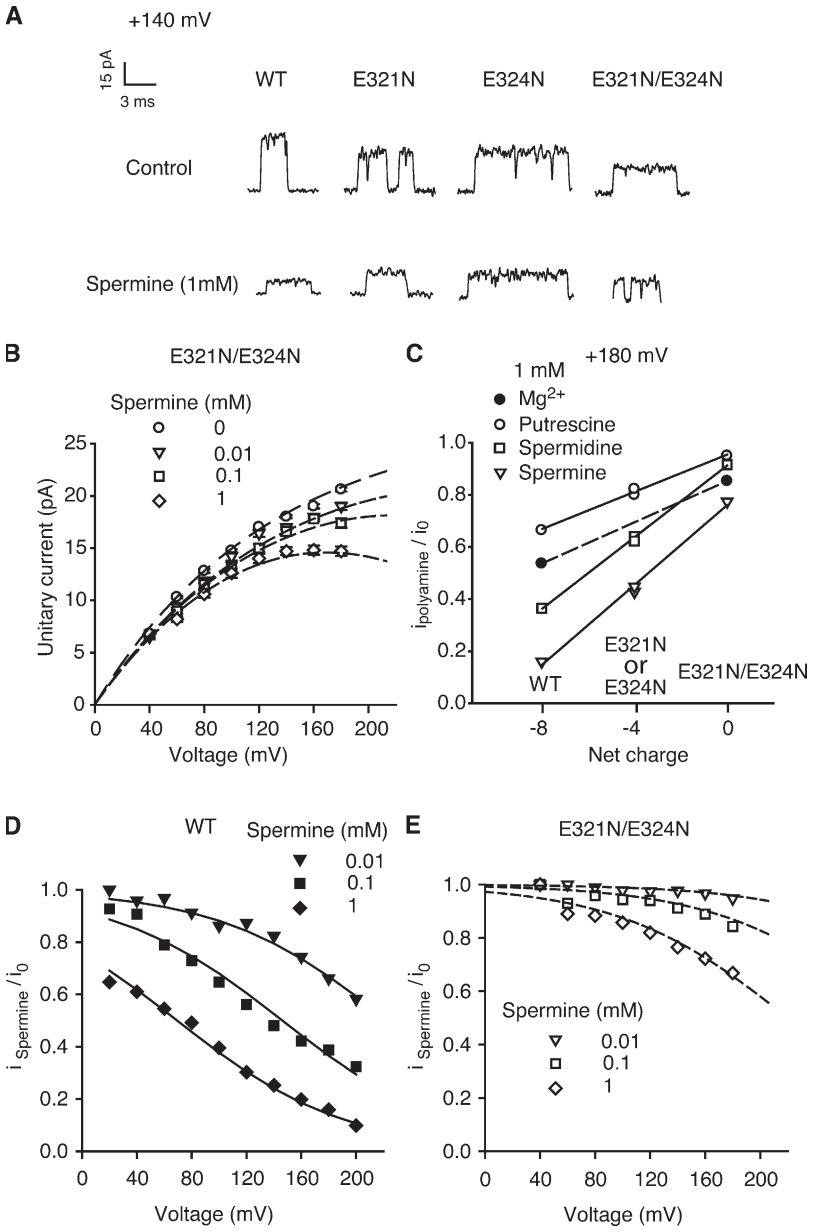

Figure 5. The ring of negative charge is a major site contributing to polyamine block, with block decreasing as the net charge in the ring of charge is decreased. (A) Representative single-channel currents with and without 1 mM spermine at +140 mV from channels with different net charge in the ring of charge: WT (−8), E321N (−4), E324N (−4), and E321N/E324N (0). The net charge in the ring of charge also changes the unitary current amplitude (Brelidze et al., 2003) so block must be judged by the ratio of unitary currents in the lower panel to those in the upper panel. (B) Removing the ring of negative charge greatly decreases spermine block. (Compare to Fig. 4 B). Plots of unitary currents versus voltage for E321N/E324N channels for the indicated spermine concentrations. The lines are simultaneous fits from Eq. 5 with $K_B^{ap}(0) = 721 \pm 9$ mM, $d = 0.19 \pm 0.02$, $n = 0.53 \pm 0.07$. (C) The degree of natural polyamine block is proportional to the net charge of the ring of charge. Plots of the ratio of unitary current with and without 1 mM spermine versus the net charge on the ring of charge for putrescine, spermidine, and spermine. For comparison, the ratio of unitary currents with and without 1 mM Mg^{2+}_i is also shown (filled circles). The overlapping points for a net charge of −4 indicate that E321 and E324 have equivalent effects on the block. (D and E) The Woodhull equation with added (negative) cooperativity accounts for spermine block in both WT and E321N/E324N channels. Plots of $i_{spermine}/i_0$ over a range of voltage at the indicated spermine for WT (filled symbols) and E321N/E324N (open symbols) channels. The continuous lines are from simultaneously fitting the WT data in D, and the dashed lines are from simultaneously fitting the E321N/E324N data in E. The parameters for the fitting are given in Fig. 4 B and Fig. 5 B.

had no obvious effect on inward unitary current through BK channels (Fig. 4 D), but all three reduced outward unitary current in a voltage-dependent manner (Fig. 4 C). These observations suggest that voltage drives the positively charged polyamines and/or any coupled K^+ ions into the channel, as is the case for polyamine block in Kir channels (Lu, 1994; Pearson and Nichols, 1998; Shin and Lu, 2005), for voltage-dependent block of Na^+ in KscA channels (Nimigean and Miller, 2002), for TEA block in delayed rectifier K^+ channels, BK channels, and Shaker channels (Armstrong 1966; Blatz and Magleby, 1984; Thompson and Begenisich, 2005), and Mg^{2+}_i block in BK channels (see previous sections).

The effectiveness of the voltage-dependent block of BK channels by polyamines followed the sequence spermine > spermidine > putrescine (Fig. 4 C), indicating that different natural polyamines have different blocking effects on BK channels. This difference is evident in the parameters for Eq. 5 describing the block (continuous lines in Fig. 4 C): putrescine, $K_B^{ap}(0) = 180 \pm 17.2$ mM, $d = 0.33 \pm 0.01$, $n = 0.85 \pm 0.04$; spermidine, $K_B^{ap}(0) = 38.4 \pm 3.35$ mM, $d = 0.25 \pm 0.03$, $n = 0.75 \pm 0.04$; spermine, $K_B^{ap}(0) = 8.0 \pm 1.04$ mM, $d = 0.19 \pm 0.01$, $n = 0.54 \pm 0.02$. The change in $K_B^{ap}(0)$ from 180 to 38.4 to 8 mM, for putrescine, spermidine, and spermine, respectively, indicates more effective block as the polyamines get longer and/or have an increased number of positive charges. Snetkov et al. (1996) found the same sequence of blocking ability, but in their experiments they observed no block for putrescine because they only examined lower voltages before the block would have become apparent.

The Ring of Charge Greatly Facilitates Block by Natural Polyamines

To investigate the contribution of the ring of negative charge to polyamine block, the net negative charge in the ring of charge was reduced from eight to four (E321N or E324N) or to zero charge (E321N/E324N). Fig. 5 A presents single-channel currents from a WT channel (net charge: −8), an E321N channel (net charge: −4), an E324N channel (net charge: −4), and an E321N/E324N channel (net charge: 0) with and without 1 mM spermine at +140 mV. In the absence of blocker, progressively removing charge in the ring of charge progressively reduced the unitary current amplitudes because less K^+ was concentrated in the inner vestibule by the ring of charge (Brelidze et al., 2003; Nimigean et al., 2003). The addition of 1 mM spermine to channels with different levels of charge in the ring of negative charge then reduced the unitary current amplitudes further, with the greatest reduction in amplitude occurring for channels with the greatest amount of negative charge (compare upper to lower current records for each channel type in Fig. 5 A). The unitary current amplitudes from E321N and E324N channels were similar, consistent with the observations from Brelidze et al. (2003), that these charges are equivalent in their effects on conductance.

Fig. 5 B plots the voltage and concentration dependence of spermine block for channels with no charge in the ring of charge (E321N/E324N). Comparing this figure to the pronounced spermine block observed in WT channels with their ring of charge (Fig. 4 B) indicates that spermine block was greatly reduced without the ring of charge. This is more clearly shown in Figs. 5 (D and E), where the ratio of the unitary current amplitudes in the presence and absence of three different concentrations of spermine are plotted against voltage for the WT and E321N/E324N channels, respectively. Simultaneously fitting the data over the range of concentrations and voltages with Eq. 5 for E321N/E324N channels gave: $K_B^{ap}(0) = 721 \pm 9$ mM, $d = 0.19 \pm 0.02$, and $n = 0.53 \pm 0.07$. A comparison of these values to those obtained for WT channels (Fig. 4 B; Fig. 6 D; Table I) indicates that the ring of charge decreases the $K_B^{ap}(0)$ for spermine block >90-fold.

Although spermine block was greatly reduced in the absence of the ring of charge, spermine still reduced the unitary current amplitude at the higher voltages and concentrations (Fig. 5 E), indicating that the ring of charge, while greatly facilitating spermine block, is not essential for all block by spermine. This suggests that spermine can still enter the inner vestibule in the absence of the ring of charge. The voltage dependence of this block suggests that movement of either the blocker or coupled K^+ ions within the electric field of the membrane is involved.

To quantify the contribution of the number of negative charges in the ring of charge on polyamine block,

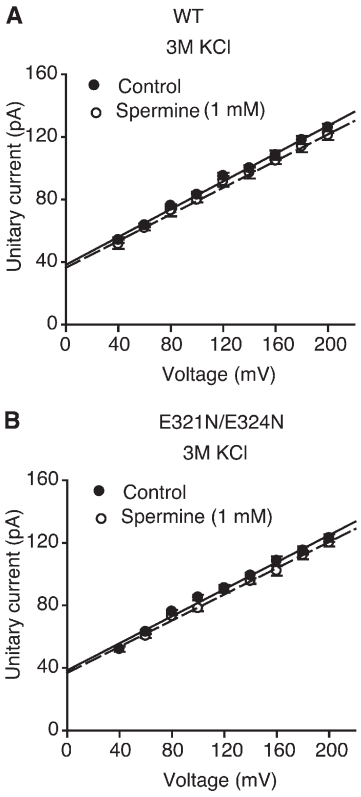

Figure 6. The ring of negative charge facilitates polyamine block through an electrostatic mechanism. (A and B) Plots of unitary current versus voltage with and without 1 mM spermine in the presence of 3 M intracellular KCl for WT and E321N/E324N channels. With 3 M K^+_i, the ring of charge has no effect on either spermine block or on the unitary current amplitude.

the ratio of the unitary current amplitude with 1 mM putrescine, spermidine, or spermine to the amplitude without polyamine for data obtained at +180 mV is plotted against net charge on the ring of charge in Fig. 5 C. For all three polyamines, the block increased as the net negative charge in the ring of charge increased (continuous lines through open symbols). For comparison, data are also plotted for 1 mM Mg^{2+}_i (dashed line through filled symbols). The ring of charge facilitates spermine (net charge +4) and spermidine (net charge +3) block more than putrescine (net charge +2) and Mg^{2+}_i (net charge +2) block.

The Ring of Negative Charge Facilitates Block by Natural Polyamines through an Electrostatic Mechanism

If polyamine block involves an electrostatic attraction of polyamines to blocking sites, then 3 M KCl should reduce polyamine block by screening the attracting charges and also by displacing polyamines directly at the blocking sites by mass action. To explore these possibilities, single-channel currents were recorded from both WT and E321N/E324N channels with 3 M intracellular KCl. (The pipette solution remained at 150 mM KCl.) With 3 M KCl, spermine no longer blocked WT channels

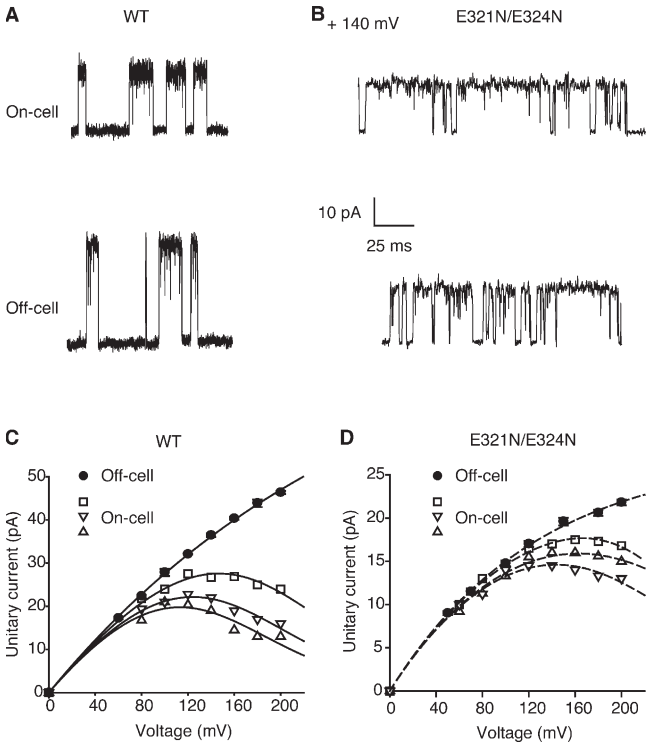

Figure 7. The ring of negative charge increases the inward rectification of BK channels for cell-attached patches of membrane. (A and B) Representative single-channel currents recorded from WT and E321N/E324N channels for on-cell recording from *Xenopus* oocytes (top traces) and after excising the patch (bottom traces). Membrane potential: +140 mV. The oocytes were bathed in 150 mM KCl and 5 mM TES (pH 7) before forming a patch so that the membrane potential would be close to zero. (C and D) Plots of unitary current versus voltage from cell-attached (open symbols) and excised patches (filled symbols) from WT and E321N/E324N channels, respectively. Excised patch data are the same as in Fig. 1 (D and E). Three representative on-cell recordings, each from a different patch, are shown for WT and E321N/E324N channels. Note the variability in response between on-cell patches from different oocytes. The lines have no theoretical meaning. Filtering was 10 kHz in A and 5 kHz in B.

(Fig. 6 A, compare with Fig. 4 B). Three molar KCl also abolished the remaining polyamine block observed in E321N/E324N channels with the ring of charge neutralized (compare Fig. 6 B with Fig. 5 B). The relief of the spermine block by 3 M KCl would be consistent with a number of mechanisms for spermine block: electrostatic attraction of spermine to the entrance of the inner vestibule by the ring of negative charge for WT channels, electrostatic attraction of spermine to other possible sites of action for both WT and E321N/E324N channels, and competitive displacement of spermine from its sites of action by the high K^+_i for WT and E321N/E324N channels.

The Ring of Negative Charge Increases the Inward Rectification of BK Channels for On-Cell Recording

Snetkov et al. (1996) and Morales et al. (1996) have observed inward rectification for recording from WT BK channels in on-cell patches on smooth muscle. Oocytes, like smooth muscle and other cells, would be expected to contain intracellular Mg^{2+}_i (1–3 mM) and free natural polyamines (~0.02–0.2 mM) (Igarashi and Kashiwagi, 2000; Hille, 2001). Consequently, it would be expected that unitary currents recorded from BK channels in on-cell patches of membrane would display inward rectification from block by intracellular Mg^{2+}_i and polyamines, and that removing the ring of negative charge would decrease the block. To test this possibility, single-channel currents were recorded from on-cell patches on oocytes bathed in pipette solution containing 150 mM KCl (see Materials and Methods), so that the intracellular membrane potential would be held close to zero. Fig. 7 A shows representative unitary currents (+140 mV) from a WT channel, first for on-cell recording and then several minutes after excising the patch for off-cell recordings. Similar data are presented for an E321N/E324N channel in Fig. 7 B. In both cases, the unitary current amplitudes increased after excising the patch, but the ~60% increase for WT channels was considerably greater than the ~10% increase for E321N/E324N channels.

Both WT and E321N/E324N channels displayed inward rectification at positive membrane potentials for on-cell recording (Fig. 7, C and D), but the rectification was considerably greater for WT channels. In contrast to excised patches where the blocking response was consistent from channel to channel (filled circles, average of four or more channels with the error bars typically less than the symbol diameters), the apparent blocking response for on-cell patches was highly variable, as indicated by the three representative on-cell i/V curves for both channel types, indicating that there may be different concentrations of blockers in different oocytes. Whereas the data in Fig. 7 do not indicate to what extent the observed inward rectification for on-cell patches is from Mg^{2+}_i, polyamines, Na^+, and other possible blockers, the data do show that the ring of negative charge doubles the magnitude of the outward unitary currents (notice the different scale on the ordinates) and also greatly facilitates the block of outward currents for on-cell BK channels, just as it does for BK channels in excised patches.

DISCUSSION

This study investigated the role of the ring of negative charge located at the intracellular entrance to the inner vestibule of BK channels on block of the channels by intracellular Mg^{2+}_i and natural polyamines. Single-channel (unitary) current amplitudes were examined to distinguish effects on conductance from those on kinetics. We found that the ring of negative charge increased Mg^{2+}_i block threefold and spermine block 90-fold, as measured by the change in apparent K_B's

projected to 0 mV. When intracellular KCl was increased from 150 mM to 3 M, the ring of negative charge no longer facilitated block. As discussed below, these observations are consistent with the ring of charge facilitating block through an electrostatic mechanism.

A Ring of Eight Negative Charges Encircles the Entrance to the Inner Vestibule of BK Channels

The residues E321 and E324 on each subunit that form the ring of negative charge are likely to be located at the entrance to the inner vestibule. This proposal is supported by the sequence alignment of BK channels with the crystal structure of MthK, a large conductance Ca^{2+}-modulated bacterial K^+ channel, where the residues in MthK equivalent to E321 and E324 are located at the entrance to the inner vestibule (Jiang et al., 2002b; Brelidze et al., 2003; Nimigean et al., 2003), as indicated in Fig. 1 (A and B). Further support that E321 is located near the entrance into the inner vestibule comes from the study of Nimigean et al. (2003), where replacing a neutral amino acid in KcsA channels with a negative residue at a position equivalent to E321 in BK channels increased the conductance in KcsA, just as negative charge at this residue increases the conductance in BK channels. Both E321 and E324 in BK channels are likely to face the conduction pathway, as Kurata et al. (2004) have found that charged residues that do not face the pore have no influence on spermine block in Kir channels. If E321 and E324 in BK channels are on an α helix, then they would be separated by one turn, suggesting that both residues could face the entrance to the inner vestibule. Supporting this possibility, we found that E321 and E324 contribute equally to facilitating the block of BK channels by polyamines (Fig. 5 C). This finding is in agreement with those of Brelidze et al. (2003) that E321 and E324 contribute equally to increasing the conductance of BK channels.

Attraction of Cations by the Ring of Negative Charge

The ring of negative charge would attract cations and repel anions through electrostatics. With 150 mM KCl, the surface potential generated by the fixed charge in the ring of charge would decrease with a Debye length of ~7.8 Å (Hille, 2001, pp. 342 and 546), in the same range as the estimated radius for the entrance to the inner vestibule of BK channels (Brelidze and Magleby, 2005). Hence, the ring of charge would attract a cloud of excess cations that would increase the concentration of cations at (and within) the entrance to the inner vestibule. The ability of negative charge to concentrate cations and deplete anions through surface potential effects can be considerable (Hille et al., 1975; Jordan, 1987; Hille, 2001, Fig. 17.6 and p. 653; Park et al., 2003). Consistent with this concentrating effect, Brelidze et al. (2003) found that the ring of negative charge increased the effective concentration of K^+ at the entrance to the inner vestibule approximately threefold, doubling the magnitude of outward single-channel currents.

In addition to attracting K^+, the ring of negative charge would also attract the Mg^{2+} and polyamine blockers because of their positive charge. Because the attractive force for multivalent blockers would be greater than for monovalent K^+ (Hille, 2001, p. 653), the electrostatic attraction would be expected to favor the accumulation of Mg^2_i and polyamines at the ring of charge over the accumulation of K^+. Such a differential accumulation of blockers could reduce outward movement of K^+ through the channel by at least two mechanisms: (1) indirectly, by decreasing the effective concentration of K^+ available to enter the inner vestibule to carry current, and (2) directly, by increasing the concentration of blockers available to enter the inner vestibule to slow or prevent the passage of K^+. Such a dual action of block has been proposed previously for spermine action at Kir2.1 channels (Xie et al., 2002). Mechanisms for the indirect block of BK channels will be considered first, followed by mechanisms for direct block.

Indirect Block by Mg^{2+} and Polyamines

As mentioned above, blockers added to the intracellular bulk solution would be attracted to the ring of charge, where they would screen the ring of charge and may also bind to and neutralize the ring of charge. For concentrations of blockers in the tens of millimolar range, the blockers would also increase the ionic strength of the solution, as the contribution of an ion to the ionic strength goes as the square of the valence of the ion. Such increases in ionic strength would further screen the ring of charge. In addition, since the blockers are multivalent, they would be attracted more strongly to the ring of charge than K^+, displacing K^+. Thus, for all the above reasons, blockers would act to decrease the cloud of excess K^+ that is attracted to the inner vestibule by the ring of negative charge. This blocker-induced decrease of the excess K^+ would reduce the outward single-channel current amplitudes, giving rise to indirect block.

The question arises as to whether other fixed charges in addition to the ring of charge might contribute to indirect block. For example, do the charged head groups of the lipid membranes surrounding the BK channels also attract Mg^{2+} and polyamines to the inner vestibule? This possibility seems unlikely because the charge on the lipid head groups does not alter intracellular Ba^{2+} block of BK channels, indicating that the conduction pore of the BK channel is electrostatically isolated from the lipid head groups by a distance >20 Å (Park et al., 2003). Charge located at this distance would not be expected to alter the concentration of either K^+ or the blockers at the entrance to the inner vestibule. Another possibility is that there might be negative charges on the channel protein in the vicinity of the ring of charge

that also concentrate ions at the entrance to the inner vestibule. Without knowing the crystal structure of BK channels, it is not possible to rule this out. It is also possible that the Asn residues used to replace the Glu residues in the E321N/E324N mutation could add some partial negative potential, so that the ring of charge is not entirely removed in the mutated channel.

Direct Block by Mg^{2+} and Polyamines

In the above section it was discussed that the ring of charge would attract blockers, reducing the concentration of K^+ at the entrance to the inner vestibule through screening of the ring of charge. A further consequence of this attraction is that the local concentration of the blockers themselves would be increased at the entrance to the inner vestibule. This increased blocker concentration could further reduce currents by direct action at possible blocking sites within the conduction pathway. One possible site for direct block would be at the focus of the pore helixes deep in the vestibule that is occupied by K^+ in the frozen crystal structure of KcsA channels (Doyle et al., 1998; Roux and MacKinnon, 1999; Zhou and MacKinnon, 2004), but this pore helix site may involve only weak interactions (Chatelain et al., 2005). A second possible site for direct block would be at the inner entrance to the selectivity filter where Ba^{2+} has been shown to reside in frozen crystal structures of KcsA (Jiang and MacKinnon, 2000). Mg^{2+} or polyamine at either of these two sites within the inner vestibule could directly interfere with the movement of K^+ from the bulk solution into the selectivity filter, reducing currents and blocking the channel. Because the ring of charge would increase the local concentration of the divalent blockers in preference to monovalent K^+, the fractional reduction of currents by blocker should be greater in the presence of the ring of charge than in its absence, as was observed. The observation that Mg^{2+} and polyamine block was not accompanied by either increases in open single-channel noise or the presence of discrete subconductance blocking levels indicates that Mg^{2+} and the natural polyamines reside at their blocking sites for durations less than can be resolved with the frequency response of the recordings (typically 5 kHz). Thus, Mg^{2+} and natural polyamines are fast blockers and do not bind tightly within the conduction pathway.

Electrostatic Action of the Ring of Negative Charge

It was suggested in the above sections that the ring of negative charge increases Mg^{2+} and polyamine block through differential attraction of blocker over K^+ to the entrance to the inner vestibule. Such an electrostatic mechanism is consistent with our observation that the ring of charge no longer facilitated block when intracellular KCl was increased 20-fold, from 150 mM to 3 M (Fig. 2 B and Fig. 6). 3 M KCl would be expected to negate the enhancing effects of the ring of charge on block for three reasons. (1) At 3 M KCl, the fractional increase in the local concentration of K^+ induced by the ring of charge would be considerably less than that at 150 mM KCl in the bulk solution because the electrostatic surface potential is effectively screened at high ionic strength. (2) Increasing KCl 20-fold would increase the ionic strength of the solution ~20-fold, decreasing the Debye length to 22% of its original value, from 7.8 to 1.7 Å, (see Hille, 2001, p. 342). With such a short Debye length, the cloud of excess K^+ and blockers attracted by the ring of charge would extend only marginally into the inner vestibule, having little effect on the local concentration of these ions at the entrance to the inner vestibule. (3) Increasing K^+_i 20-fold would facilitate the displacement of blockers from their sites of action. For these three reasons, 3 M KCl would be expected to negate the electrostatic effects of the ring of charge on facilitating the action of the blockers, as was observed (Fig. 2 B and Fig. 6).

The observations of residual Mg^{2+} block after removing the ring of negative charge (Fig. 1 E and Fig. 2 A) and also of residual Mg^{2+} block in 3 M KCl that was identical for both WT and mutant (E321N/E324N) channels (Fig. 2 B) suggests, but does not establish, that there may be another site of Mg^{2+} action in addition to the Glu's in the ring of negative charge, perhaps in the inner vestibule. An argument against another site is that the Asn's used to replace the Glu's in the mutant channel might still have an electrostatic action at the ring of charge because of their weak polar charge. Although we cannot exclude this possibility, it seems unlikely that the Asn's would be a major contributor to the residual block in the mutant channel. Because 3 M KCl was sufficient to totally mask the effects of the large charge difference between WT and mutated channels on Mg^{2+} block, as indicated by identical Mg^{2+} block for WT and E321N/E324N channels in 3 M KCl (Fig. 2 B), then it would also be expected that the same 3 M KCl would be sufficient to also mask any weak polar charge from the Asn's used to replace the Glu's. Yet, there was still appreciable Mg^{2+} block in the mutant channels in 3 M KCl (Fig. 2 B), consistent with an additional site of action for Mg^{2+} other than at the Asn's used to replace the Glu's at the ring of charge.

Apparent Negative Cooperativity

The empirical Eq. 5, which combined the voltage dependence of the K_B^{AP} for block (Fig. 3 B; Eqs. 2–4) with an empirical Hill function, described Mg^{2+} and polyamine block over a wide range of blocker concentrations and voltage with Hill coefficients, n, of ~0.62 for Mg^{2+} block and ~0.53 for spermine block, indicating apparent negative cooperativity (Figs. 1–5; Table I). For both Mg^{2+} and polyamine block, removing the ring of charge reduced block without changing the value of n. Hence, the ring of charge is not directly required for apparent

negative cooperativity. Such apparent negative cooperativity indicates deviation from a simple Langmuir isotherm. This deviation could arise from several possible mechanisms. Increasing the concentration of the blockers to carry out the titration of the blocking site(s) would increase the ionic strength of the solution, which would screen the ring of charge. For example, adding 50 mM $MgCl_2$ (the highest concentration of blocker used) to 150 mM KCl would double the ionic strength, from 0.15 to 0.3 M, which would decrease the Debye length from 7.8 to 5.5 Å (see Hille, 2001, p. 342). This decrease in Debye length would decrease the cloud of excess cations at the entrance to the inner vestibule with increasing $Mg^{2+}{}_i$, which could contribute to values of $n < 1$ (Ravindran et al., 1991; Latorre et al., 1992; Park et al., 2003). However, changes in ionic strength cannot be the mechanism for the apparent negative cooperativity associated with spermine block, as spermine gave substantial block at concentrations ≤1 mM, where any changes in ionic strength would be negligible. Apparent negative cooperativity might also arise if block occurs at multiple sites, with blocker at one site inhibiting the access or action of blocker at other sites (Wyman and Gill, 1990). Such sites might include the focus of the pore helices and the Ba^{2+} site at the entrance to the selectivity filter (Jiang and MacKinnon, 2000), where Mg^{2+} might weakly bind. 3 M KCl changed the value of n in Eq. 5 to ~1, perhaps by effectively removing one or more sites of action for Mg^{2+}. The actual basis of the apparent negative cooperativity must await a full theoretical treatment of the conductance and selectivity of the channel.

Like $Mg^{2+}{}_i$, $Sr^{2+}{}_i$ also blocks BK channels with negative cooperativity (Sugihara, 1998). To describe the block, Sugihara (1998) added cooperativity by raising the blocker concentration to a power before the blocker was concentrated by the electrical field of the membrane. In our formulation, the cooperativity was added after the concentrating effect of the voltage drop in the vestibule on the blocker. The formulation of the block used by Sugihara (1998) and our Eq. 5 gave an equivalent description of our data (not depicted).

Voltage-dependent Block by $Mg^{2+}{}_i$ and Polyamines

A characteristic feature of intracellular Mg^{2+} and polyamine block in BK channels (Figs. 1 and 4) is that the block becomes stronger with depolarization (Ferguson, 1991; Morales et al., 1996; Snetkov et al., 1996), leading to inward rectification, similar to block of other channels by various cations (Armstrong, 1966; Woodhull, 1973; Lu and MacKinnon, 1994; Nichols and Lopatin, 1997; Hille, 2001; Voets et al., 2003; Brelidze and Magleby, 2004). For K^+ channels with their single-file multi-ion pores (Neyton and Miller, 1988; Doyle et al. 1998), the voltage sensitivity of a blocking ion also includes the voltage dependence of the permeant K^+ ions whose movement is coupled to the blocking event (Armstrong, 1971; Hille and Schwarz, 1978; Thompson and Begenisich, 2001, 2005; Gomez-Lagunas et al., 2003; Shin and Lu, 2005). Consequently, d in Eq. 4 is the effective average fractional distance moved by the charge on the blocker together with any coupled K^+ ions through the electric field for a blocking event. For polyamines, movement of multiple charges different distances in the field may contribute. A measure of the relative voltage dependence of the block is given by zd, which takes into account the valence of the blocker. The estimated values of d for Mg^2, putrescine, spermidine, and spermine block were 0.25, 0.33, 0.25, and 0.19, respectively, and the values of zd were 0.50, 0.66, 0.75, and 0.76, respectively, indicating that the voltage dependence of the block was greatest for spermine and least for Mg^{2+}. If the intracellular gating ring of BK channels (Jiang et al., 2002b; Niu et al., 2004) extends the intracellular conduction pathway as proposed in Kir channels (Nishida et al., 2002; Kuo et al., 2003), then the extended intracellular pathway might also contribute to d in some manner.

Structural data from MthK (Jiang et al., 2002b) and electrophysiological data from BK channels showing that large quaternary ammonium ions (Li and Aldrich, 2004) and sugar molecules (Brelidze and Magleby, 2005) can enter the inner vestibule, suggest that BK channels have large diameter inner vestibules. On this basis and from electrostatic calculations for MthK (Jiang et al., 2002b), it would be expected that the fractional voltage drop in the inner vestibule of BK channels in the absence of large space–occupying molecules would be small (<20%), with the majority of the voltage drop occurring across the selectivity filter, as is the case for *Shaker* and Kir channels (Kurata et al., 2004; Shin and Lu, 2005; Thompson and Begenisich, 2005). For Mg^{2+} block of NMDA receptors (Ruppersberg et al., 1993), TEA_i block of Shaker K^+ channels (Thompson and Begenisich, 2003, 2005), and polyamine block of Kir2.1 channels (Spassova and Lu, 1998; Guo and Lu 2000, 2002, 2003; Lu, 2004; Shin and Lu, 2005), the voltage dependence of block may arise in large part from the movement of permeant ions through the electric field in the selectivity filter in front of the blocker. If the blockers move into the selectivity filter, then this would also contribute to the voltage dependence of the block. For Kir 6.2 channels there is disagreement as to what extent polyamines enter and bind to sites within the selectivity filter (Pearson and Nichols, 1998; Kurata et al., 2004; Guo and Lu, 2000, 2002, 2003; John et al., 2004; Shin and Lu, 2005). For BK channels it is not known whether Mg^{2+} and the natural polyamines enter the selectivity filter.

Schematic Diagram of $Mg^{2+}{}_i$ and Polyamine Block in BK channels

Fig. 8 summarizes possible blocking actions of Mg^{2+} and polyamines for BK channels using the open channel

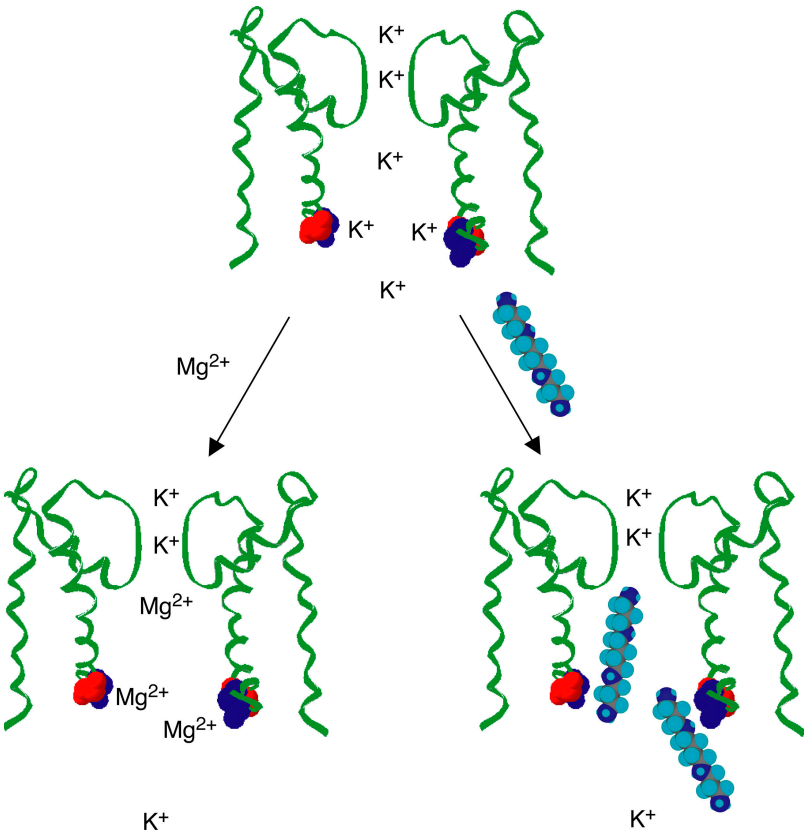

Figure 8. Schematic diagram of a possible mechanism for intracellular Mg^{2+} and polyamine block in BK channels. The ring of negative charge attracts Mg^{2+} and polyamines, which then displaces K^+ from the entrance to the inner vestibule. Mg^{2+} and polyamine also enter further into the inner vestibule, attracted by the dipole formed by the pore helixes, where they can displace K^+ both physically and electrostatically from the inner vestibule and also from the entrance to the selectivity filter.

structure of MthK (Doyle et al., 1998; MacKinnon et al., 1998; Lu et al., 2001; Jiang et al., 2002a,b), with the residues forming the ring of negative charge in BK channels projected onto MthK. Although Fig. 8 will be used for discussion, it most likely represents an oversimplification, as the large cytoplasmic gating ring of the channel is not shown (Jiang et al., 2002a). The observation that replacing the RCK2 domains of the gating ring of BK channels with the related RCK2 domains of Slo3 channels reduces the conductance ~15% suggests that the gating ring may also contribute to the conduction pathway (Moss and Magleby, 2001). Focusing on the ring of charge, in the absence of blocker ions, the ring of charge attracts K^+ to the entrance of the inner vestibule, increasing the effective localized concentration of K^+ to ~500 mM when there is 150 mM K^+ in the bulk intracellular solution (top drawing) (Brelidze et al., 2003). Mg^{2+} and polyamines, when present, would be attracted to the ring of charge, where they would screen the ring of charge (bottom drawings). The screening would then decrease the attractive force on K^+, leading to a decrease in the local concentration of K^+ available to enter the vestibule, which would decrease the outward currents. Mg^{2+} and polyamines attracted by the ring of charge would also enter into the inner vestibule where they would physically and electrostatically displace K^+ from the vestibule. Mg^{2+} and polyamines may also compete with K^+ at the site where K^+_i enters the selectivity filter and/or at sites deeper in the selectivity filter, directly preventing the outward movement of K^+.

In WT BK channels with their ring of negative charge, the K_B^{ap} (0 mV) for spermine block was 8.0 mM, whereas the K_B^{ap} for Mg^{2+}_i block was 42 mM, indicating that spermine blocks BK channels more effectively than Mg^{2+}_i. The length of spermine (~16 Å) would allow one end of spermine to be stabilized by the ring of charge when the other end is positioned at the entrance to the selectivity filter. This stabilization of spermine could increase the dwell-time of spermine in the vestibule, allowing greater block at lower concentrations, as suggested for Kir channels (Lopatin et al., 1995; Guo and Lu, 2003; Xie et al., 2003; Yeh et al., 2005; Kurata et al., 2006). Our observation that neutralization of the ring of negative charge increased the K_B^{ap} (0 mV) for spermine block 90-fold compared with a threefold increase for Mg^{2+}_i block, lends support to the notion that spermine is stabilized in the vestibule by the ring of charge. In addition, compared with the small size of Mg^{2+}, the large physical size of spermine together with the four positive charges on spermine would be more effective both physically and electrostatically than Mg^{2+} in excluding K^+ from the vestibule.

The actions of the ring of charge depicted in Fig. 8 would also apply to BK channels in surface cell membranes on intact cells. Recordings from on-cell patches

for BK channels expressed in *Xenopus* oocytes showed inward rectification similar to that observed for excised patches with either ~1 mM Mg^{2+}_i, ~0.1 mM spermine, or ~1 mM spermidine (Figs. 1, 4, and 7). Removing the ring of charge for on-cell recordings then reduced the single channel current to about half and also decreased the inward rectification, similar to the decrease observed in excised patches of membrane (Figs. 1, 5, and 7). Whereas these observations do not identify the intracellular substances in oocytes responsible for the blocking, they do indicate that the ring of negative charge still doubles the single-channel current and also facilitates block when BK channels are exposed to all of the components of cytoplasm.

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