

Interactions of external K^+ and internal blockers in a weak inward-rectifier K^+ channel

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We investigated the effects of changing extracellular K^+ concentrations on block of the weak inward-rectifier K^+ channel Kir1.1b (ROMK2) by the three intracellular cations Mg^{2+} , Na^+ , and TEA^+ . Single-channel currents were monitored in inside-out patches made from *Xenopus laevis* oocytes expressing the channels. With 110 mM K^+ in the inside (cytoplasmic) solution and 11 mM K^+ in the outside (extracellular) solution, these three cations blocked K^+ currents with a range of apparent affinities ($K_i(0) = 1.6$ mM for Mg^{2+} , 160 mM for Na^+ , and 1.8 mM for TEA^+) but with similar voltage dependence ($z\delta = 0.58$ for Mg^{2+} , 0.71 for Na^+ , and 0.61 for TEA^+) despite having different valences. When external K^+ was increased to 110 mM, the apparent affinity of all three blockers was decreased approximately threefold with no significant change in the voltage dependence of block. The possibility that the transmembrane cavity is the site of block was explored by making mutations at the N152 residue, a position previously shown to affect rectification in Kir channels. N152D increased the affinity for block by Mg^{2+} but not for Na^+ or TEA^+ . In contrast, the N152Y mutation increased the affinity for block by TEA^+ but not for Na^+ or Mg^{2+} . Replacing the C terminus of the channel with that of the strong inward-rectifier Kir2.1 increased the affinity of block by Mg^{2+} but had a small effect on that by Na^+ . TEA^+ block was enhanced and had a larger voltage dependence. We used an eight-state kinetic model to simulate these results. The effects of voltage and external K^+ could be explained by a model in which the blockers occupy a site, presumably in the transmembrane cavity, at a position that is largely unaffected by changes in the electric field. The effects of voltage and extracellular K^+ are explained by shifts in the occupancy of sites within the selectivity filter by K^+ ions.

INTRODUCTION

Inward-rectifier K^+ channels are named for their ability to pass inward currents more easily than outward currents. This characteristic reflects voltage-dependent block of the channels by intracellular cations, including Mg^{2+} (Matsuda et al., 1987; Vandenberg, 1987; Lu and MacKinnon, 1994; Nichols et al., 1994; Taglialatela et al., 1994) and polyamines (Ficker et al., 1994; Lopatin et al., 1994; Fakler et al., 1995). In the “strong” inward rectifiers (Kir2 and Kir3 families), outward currents are almost entirely eliminated at voltages very positive to the K^+ equilibrium potential. In the “weak” rectifiers (Kir1 and Kir6 families), outward currents are observed even at large positive membrane potentials, but their conductance is reduced relative to that of inward currents.

Another defining property of inward rectification is its dependence on extracellular K^+ . Increasing external K^+ (K_o^+) relieves block by internal cations similarly to membrane hyperpolarization, such that block is better correlated with the electrochemical potential difference for K^+ than with voltage per se. This feature was reported long ago (Hagiwara et al., 1976), but its basis has not been precisely defined. Although it has similarities to a competition between permeant and blocking

cations for a binding site, it is unclear how an external permeant ion can directly compete with an internal blocker. In voltage-gated K^+ channels, acceleration of inward K^+ currents displaced quaternary ammonium ions from their internal blocking sites through an apparent knock-off mechanism (Armstrong, 1971). The effects of external K^+ on inward rectifiers are different in that the affinity of block by internal cations decreases with increasing K_o^+ even when K^+ is driven out of the cell by the electrochemical activity gradient.

Recent work has supported the idea that voltage dependence of block in Kir2.1 arises in large part from a movement of K^+ across the transmembrane electric field concomitant with the blocking event (Spassova and Lu, 1998; Guo et al., 2003; Xu et al., 2009). This suggests that K_o^+ might affect internal block by altering the number and/or location of permeant ions within the electric field. However, the analysis of block of the strong rectifiers by polyamines is complicated by the large apparent valence of block (Ficker et al., 1994; Lopatin et al., 1994, 1995; Fakler et al., 1995; Xu et al., 2009), uncertainty over the precise position of the

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Abbreviation used in this paper: WT, wild type.

blocking particle (Guo et al., 2003; Kurata et al., 2006), and the possible presence of more than one blocking site (Ishihara and Ehara, 2004; Shin et al., 2005; Liu et al., 2012). We therefore chose to analyze the comparatively simple system of the weak inward-rectifier Kir1.1. Here, interactions with polyamines are less important than those with Mg^{2+} ; block by Mg^{2+} has a modest effective valence, but the affinity remains dependent on external K^+ (Lu and MacKinnon, 1994; Nichols et al., 1994; Yang et al., 2010).

We find that effects of K_o^+ on internal block are quite general and are similar for monovalent as well as divalent cations that interact with the intracellular aspect of the channel. Using mathematical simulations, we show that they can be largely accounted for by changes in the occupancy of the selectivity filter by K^+ ions.

MATERIALS AND METHODS

Expression of Kir1.1 in *Xenopus laevis* oocytes

Oocytes were harvested from *Xenopus* according to the guidelines and with approval of the Institutional Animal Care and Use Committee of Weill Cornell Medical College. The animals were anesthetized through immersion in 1 liter of tap water containing 1.9 g L^{-1} tricaine methanesulphonate and HEPES, adjusted to pH 7.4, for 5–10 min. Once the animals were anesthetized, a small incision was made in the abdomen and part of the ovary was removed. The oocytes were then dissociated through incubation in OR2 solution (mM: 82.5 NaCl, 2.5 KCl, 1 $MgCl_2$, 1 Na_2HPO_4 , and 5 HEPES, pH 7.4) supplemented with 2 mg ml^{-1} collagenase type II (Worthington) and 2 mg ml^{-1} hyaluronidase type II (Sigma-Aldrich) for 1 h.

pSport plasmids containing wild-type (WT) or mutant Kir1.1b (ROMK2) were linearized with NotI restriction enzyme, and cRNAs were transcribed with T7 RNA polymerase using mMESSAGE mMACHINE T7 kit (Ambion). cRNA pellets were dissolved in nuclease-free water and stored in $-70^\circ C$ before use. The oocytes were injected with 10 ng RNA and incubated overnight in L-15 solution supplemented with HEPES, pH 7.4, 63 mg L^{-1} penicillin, and 145 mg L^{-1} streptomycin in $18^\circ C$. All chemicals were from Sigma-Aldrich unless otherwise noted.

The C13 chimera in which the cytoplasmic C-terminal tail of Kir1.1 was replaced by that of Kir2.1 was described previously (Choe et al., 2000). Site-directed mutations were constructed using the QuikChange kit (Agilent Technologies) according to the manufacturer's instructions. Sequences were confirmed by the BioResource Center of Cornell University.

Patch clamp

Immediately before patch-clamp measurements, the vitelline membranes of the oocytes were mechanically removed in a hypertonic solution containing 200 mM sucrose. Patch-clamp pipettes were prepared from hematocrit capillary glass (VWR Scientific) using a vertical puller (Kopf Instruments). They had resistances of 2–8 M Ω when filled with 110 mM KCl. Measurements were made in excised inside-out patches. Pipette and bath solutions contained Cl^- salts of K^+ and Na^+ as indicated, plus 5 mM HEPES buffered to pH 7.4. Bath solutions contained, in addition, 0.5 mM EGTA. Currents were recorded with an EPC-7 patch-clamp amplifier (HEKA), digitized with a Digidata 1332A interface (Axon Instruments). Data were filtered at 1 kHz and analyzed with pCLAMP 9 software (Axon Instruments).

Data analysis

In most cases, the kinetics of block were too rapid to allow us to resolve individual blocking events. We therefore assessed block as a decrease in single-channel currents (i), measured from selected recording intervals containing one to three active channels using all-points histograms. Histograms were fit with Gaussian functions, and the current amplitude was defined as the distance between adjacent peaks.

The effects of internal blocking ions were analyzed according to the equation:

$$i(B) = i(0) / (1 + [B] / K_i), \quad (1)$$

$$\text{with } K_i(V) = K_i(0) \bullet \exp(-z\delta FV / RT), \quad (2)$$

where $[B]$ is the blocker concentration, z is the blocker valence, V is the transmembrane voltage, and δ is the effective fraction of the electric field at the blocking site. $K_i(0)$ and $z\delta$ were estimated from linear regression analysis of a linearized form of Eqs. 1 and 2:

$$\ln(i(0) / i(B) - 1) = \ln([B] / K_i(0)) + z\delta FV / RT. \quad (3)$$

In some cases, the blocking kinetics were sufficiently slow to resolve individual events. Here, open probability (P_o) was measured instead of the single-channel current, using pCLAMP 9 software. P_o was plotted as a function of voltage and analyzed using the appropriate forms of Eqs. 1–3 above.

Kinetic modeling

A kinetic model for permeation was based on the premise that the selectivity filter contains four potential binding sites for K^+ (S1–S4) and is occupied simultaneously by two K^+ ions that move in concert between positions S1,S3 and S2,S4 (Bernèche and Roux, 2001; Morais-Cabral et al., 2001) (see Fig. 6 A). For simplicity, and to minimize the number of and kinetic parameters for the fits, we did not consider states in which the filter is occupied by more than or fewer than two ions. Ions entering the filter from the outside or inside interact with an external binding site (S0) and a site within the transmembrane cavity (Scav), respectively. The scheme is based on that proposed by Kutluay et al. (2005). Ions at adjacent positions can interact with each other through a repulsive energy term. When external Na^+ was present, simulated currents were decreased by a factor

$$1 / [1 + [Na^+]_o / (K_{Na}(0) \bullet \exp(\delta FV / RT))]$$

to account for Na^+ block, using parameters for $K_{Na}(0) = 272$ mM and $\delta = 0.15$ obtained previously (Yang et al., 2012).

The model was evaluated using Matlab 7.8.0 as described previously (Yang et al., 2012). Rate constants were calculated based on the values set for binding energies, energy barriers, and electrical distances. The model was fitted to the appropriate experimental data using the *lsqcurvefit* function in Matlab.

The basic strategy behind the modeling was to first make simplifying assumptions, reducing the number of free parameters. For example, in case 1 we assumed no repulsion between ions in S4 and Scav and no voltage drop between the cytoplasm and Scav. We then fit the reduced model to an entire set of current-voltage relationships measured at different external K^+ in the presence and absence of monovalent and divalent internal blocking cations. To access as much parameter space as possible, we varied the different starting conditions and used values giving the best fit from at least 100 trials.

Supplemental online material

The supplemental material includes tables summarizing measured blocking parameters for different intracellular ions and channel constructs, and fitting parameters for the kinetic models with different assumptions. It also includes figures showing fits of the model under two scenarios described in the text. The supplemental material is available at <http://www.jgp.org/cgi/content/full/jgp.201210835/DC1>.

RESULTS

Currents through Kir1.1 channels with 110 mM K^+ on the inside and 11 mM on the outside of inside-out patches are shown in Fig. 1. In the absence of an internal blocker, the i - V relationship is nearly linear over a large voltage range and reverses close to -60 mV, consistent with K^+ selectivity. When blocking cations were added to the inner solution at the concentrations shown, inward single-channel currents were not significantly changed, but outward single-channel currents were reduced by increasing fractions as the voltage became more positive. There was no consistent effect of any of the blockers on the open probability of the WT channels, although we cannot rule out modest changes; in

some cases, recordings at a given voltage were too short to reliably measure P_o . As changes in unitary currents appear to represent the primary blocking events for the ions studied here, we focus on these effects in Figs. 1–3.

To assess the voltage dependence quantitatively, the plots were transformed according to Eq. 3. Despite the different affinities of block for the three ions, the apparent valences ($z\delta$) of the blocking reactions, estimated from the slopes of the lines in Fig. 1 C, were similar, corresponding to equivalent charges moving across the electric field of ~ 0.6 – 0.7 . Similar values for Mg^{2+} block of Kir1.1 channels were reported previously (Lu and MacKinnon, 1994; Nichols et al., 1994; Yang et al., 2010). The finding that a monovalent blocker (e.g., Na^+) and a divalent blocker (e.g., Mg^{2+}) have the same apparent valence could be explained if Na^+ senses a larger fraction of the transmembrane voltage at its blocking site. Alternatively, the two blockers could bind to the same site if the voltage dependence arises not from the binding per se but from the displacement of permeant ions (K^+) across the electric field.

We next investigated the effects of changing $[K^+]_o$ on block by these internal cations. Increasing $[K^+]_o$

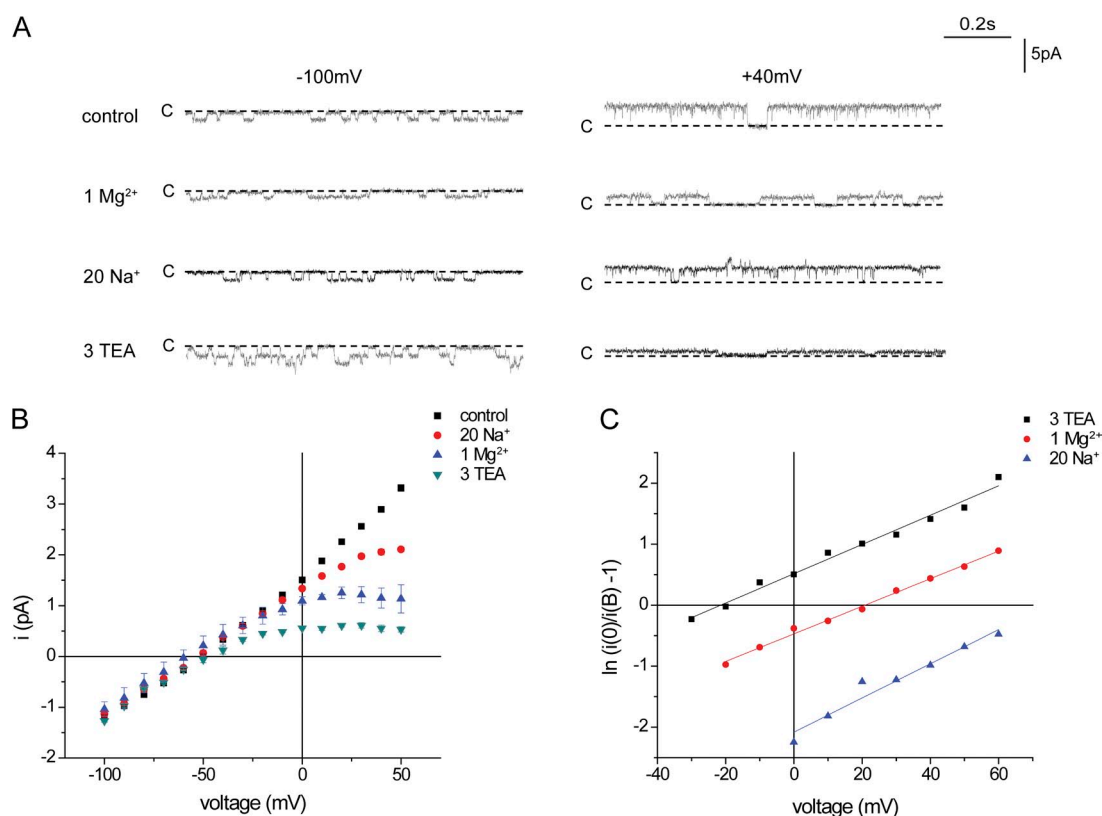


Figure 1. Mg^{2+} , Na^+ , and TEA^+ block Kir1.1 channels from the inside with similar voltage dependence. Currents through Kir1.1 channels were measured in inside-out patches with 11 mM K^+ plus 99 mM Na^+ in the extracellular (pipette) solution and 110 mM K^+ in the cytoplasmic (bath) solution, with or without 1 mM Mg^{2+} , 3 mM TEA^+ , or 20 mM Na^+ . (A) Typical current traces at voltages of -100 mV and $+40$ mV. Dashed lines show the closed state of the channel. (B) i - V relationships. Data represent means \pm SEM for 3–10 different patches. (C) Analysis of voltage dependence. Fractional block is linearized and fit to Eq. 3. Linear regression gives estimates of $K_d(0) = 1.6$ (Mg^{2+}), 1.8 (TEA^+), and 160 (Na^+) mM, and $z\delta = 0.58$ (Mg^{2+}), 0.61 (TEA^+), and 0.72 (Na^+).

decreased the affinity for Mg^{2+} (Fig. 2 A) as was reported previously (Lu and MacKinnon, 1994; Yang et al., 2010). With 110 mM K_o^+ , the $K_i(0)$ for Mg^{2+} block increased from 1.6 to 4.3 mM. The change was statistically significant (Table S1). The value of $z\delta$ was not substantially or significantly different. Similar results were obtained with Na^+ and TEA^+ as blockers. In all three cases, $K_i(0)$ increased about threefold for a 10-fold increase in $[\text{K}_o^+]$. The effect on block by TEA^+ is similar to that reported previously (Spasova and Lu, 1998). This indicates that the shift in affinity with external K^+ is quite general and does not depend on the nature or the valence of the blocker.

We also assessed the ability of internal K^+ to modulate block. Fig. 3 illustrates the effect of lowering $[\text{K}^+]_{\text{in}}$ from 110 to 25 mM on block by internal Mg^{2+} . The $K_i(0)$ for Mg^{2+} decreased from 2.38 to 0.92 mM, whereas the $z\delta$ value fell slightly from 0.68 to 0.61. The increased apparent affinity is consistent with a competitive interaction between K^+ and Mg^{2+} . With this assumption and the equation $K_i^{\text{app}} = K_i (1 + [\text{K}^+]_{\text{in}}/K_{\text{K}^+})$, we estimate an affinity K_{K^+} of the blocking site for K^+ of 30 mM. Because channels exhibited rapid rundown with low $[\text{K}^+]_{\text{in}}$, we did not extend these results further.

The similarity of the modulation of block by Mg^{2+} , Na^+ , and TEA^+ by K_o^+ suggests that, despite the differences in size and charge, the three internal cations may block at or near the same place in the channel. The most likely site is the transmembrane cavity. Consistent with this location, the apparent affinity of block by Mg^{2+} increases when negative charge is added to this part of the pore (Lu and MacKinnon, 1994; Taglialatela et al., 1995; Yang et al., 1995). Furthermore, x-ray crystallography

showed the cavity of KcsA channels to be a binding site for quaternary ammonium-like compounds similar to TEA^+ (Zhou et al., 2001). To explore this further, we first used a mutant of Kir1.1 in which the asparagine at position 152 was changed to aspartate (Fig. 4). As was found previously (Lu and MacKinnon, 1994; Taglialatela et al., 1995; Yang et al., 1995), this mutation increased the apparent affinity of the internal blocking site for Mg^{2+} , without significantly affecting the voltage dependence of block (Fig. 4 A and Table S1). In contrast, the $K_i(0)$ for Na^+ was virtually identical for WT and N152D channels (Fig. 4 B). With TEA^+ as a blocker, $K_i(0)$ was slightly increased by the mutation. In both cases, the apparent valence was again unchanged.

We next substituted a tyrosine at the same position. This mutation did not alter block by either Mg^{2+} or Na^+ (Table S1). However, the affinity for TEA^+ was strongly increased; the N152Y mutant was sensitive to 50 μM TEA^+ . In this case, the block was observed as a decrease in open P_o rather than in single-channel conductance, because the blocked state was sufficiently long-lived to be resolved within the bandwidth of the single-channel recordings (Fig. 5). The high frequency of blocking events allowed measurements of P_o even in short recordings. As shown in Fig. 5 (B and C), P_o decreased with depolarization in the presence of the blocker, whereas the single-channel currents were unchanged. We therefore analyzed the affinity and voltage dependence of the block in terms of P_o rather than in single-channel current, substituting P_o for i in Eq. 3. Analysis of the voltage dependence revealed an apparent valence of 0.8, similar to that assessed as a decrease in single-channel current for the WT channel. As with the WT, the $K_i(0)$

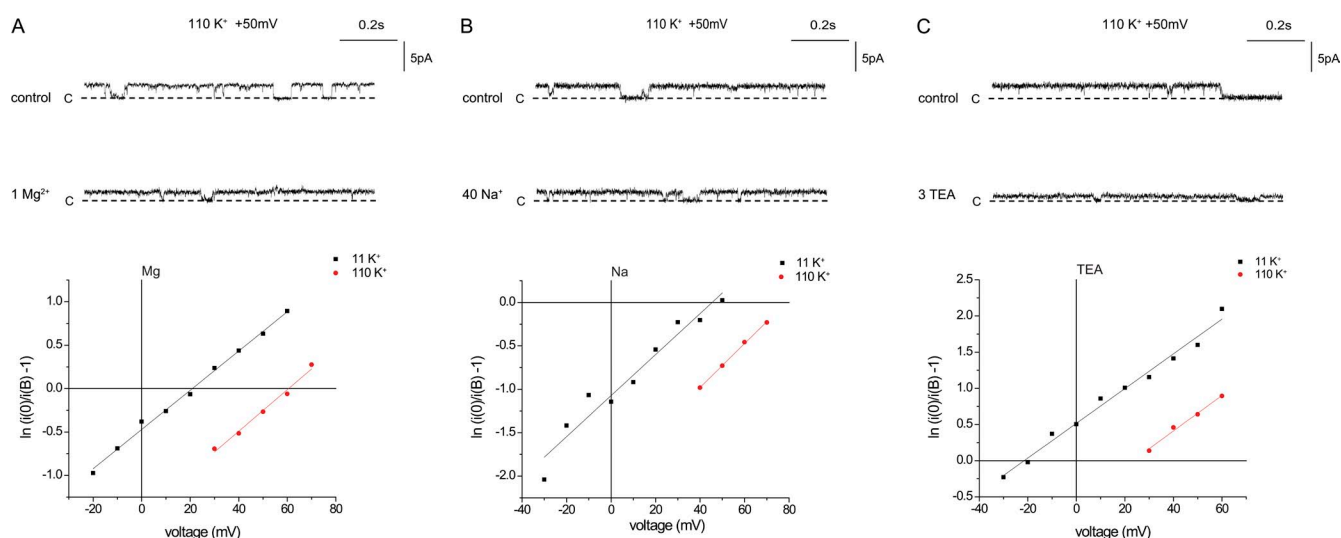


Figure 2. Blocking affinity is decreased by external K^+ . Block was analyzed according to Eq. 3 at extracellular K^+ concentrations of 11 and 110 mM for (A) 1 mM Mg^{2+} , (B) 40 mM Na^+ , and (C) 3 mM TEA^+ . Typical current traces at two voltages with and without blockers are shown at the top of each panel. Values of $K_i(0)$ increased from 1.6 to 4.2 mM (Mg^{2+}), 117 to 293 mM (Na^+), and 1.8 to 5.3 mM (TEA^+). No significant changes in $z\delta$ were observed for any of the blockers.

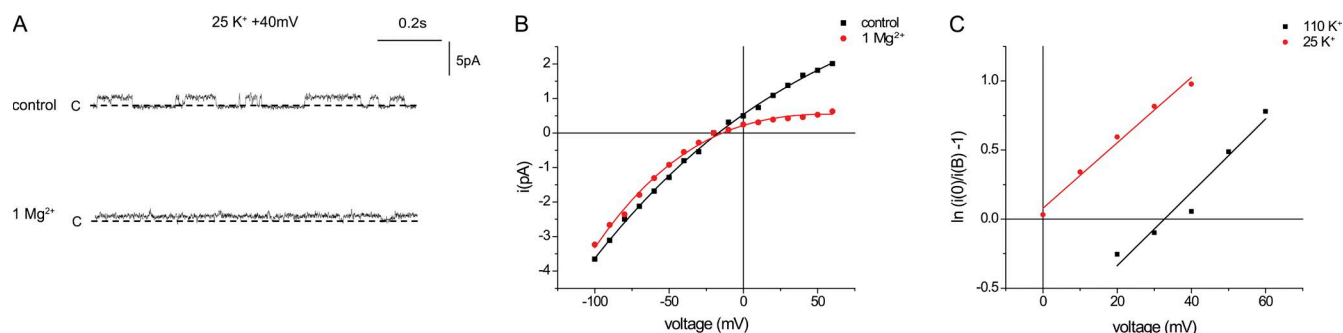


Figure 3. Blocking affinity of Mg^{2+} is decreased by internal K^+ . Voltage-dependent reduction in single-channel current was analyzed as in Fig. 1, with 25 or 110 mM K^+ in the cytoplasmic (bath) solution. The extracellular (pipette) solution contained 11 mM K^+ and zero Na^+ . (A) Current traces at +40 mV with 25 mM $[\text{K}^+]_{\text{in}}$ with and without 1 mM Mg^{2+} . (B) i - V relationships for the conditions in A. (C) Analysis of voltage dependence. Fractional block is linearized and fit to Eq. 3. Linear regression gives estimates of $K_i(0) = 2.38$ and $z\delta = 0.68$ (110 mM $[\text{K}^+]_{\text{in}}$), and $K_i(0) = 0.92$ and $z\delta = 0.61$ (25 mM $[\text{K}^+]_{\text{in}}$).

increased by about threefold when K_o^+ was increased from 11 to 110 mM (Fig. 5 D). The simplest interpretation of these results is that TEA^+ binds within the transmembrane cavity and that the affinity of the site is increased by the N152Y mutation, possibly because of cation- π electron interactions between TEA^+ and the aromatic rings of the tyrosines (Ahern et al., 2006).

Finally, we investigated the effects of exchanging the C-terminal domain of Kir1.1 for that of the strong rectifier Kir2.1. The resulting chimera is a strongly rectifying channel under physiological conditions or in the presence of polyamines on the cytoplasmic side of the membrane (Taglialatela et al., 1995; Yang et al., 1995), possibly caused by a different distribution of charges in the cytoplasmic pore (Robertson et al., 2008). However, in excised patches in the absence of polyamines, outward

currents can be readily measured. Consistent with previous reports (Taglialatela et al., 1995; Yang et al., 1995), we observed an increase (approximately fivefold) in the affinity of Mg^{2+} block, with a small increase in the voltage dependence (Fig. 6 A). The effect of the same mutation on Na^+ block was more modest, with $K_i(0)$ decreasing from 117 to 77 mM and the voltage dependence remaining constant (Fig. 6 B). The effects on TEA^+ block were more complex. Concentrations as low as 10 μM induced reductions in single-channel currents. This inhibition had a $K_i(0)$ of $\sim 50 \mu\text{M}$, lower than in WT, but a similar $z\delta$ value of 0.75. However, at this concentration, we also observed individual blocking and unblocking events reducing P_o . The $K_i(0)$ for this effect was also close to 50 μM , but the voltage dependence was steeper, with a $z\delta$ value of 1.6 (Fig. 6 C).

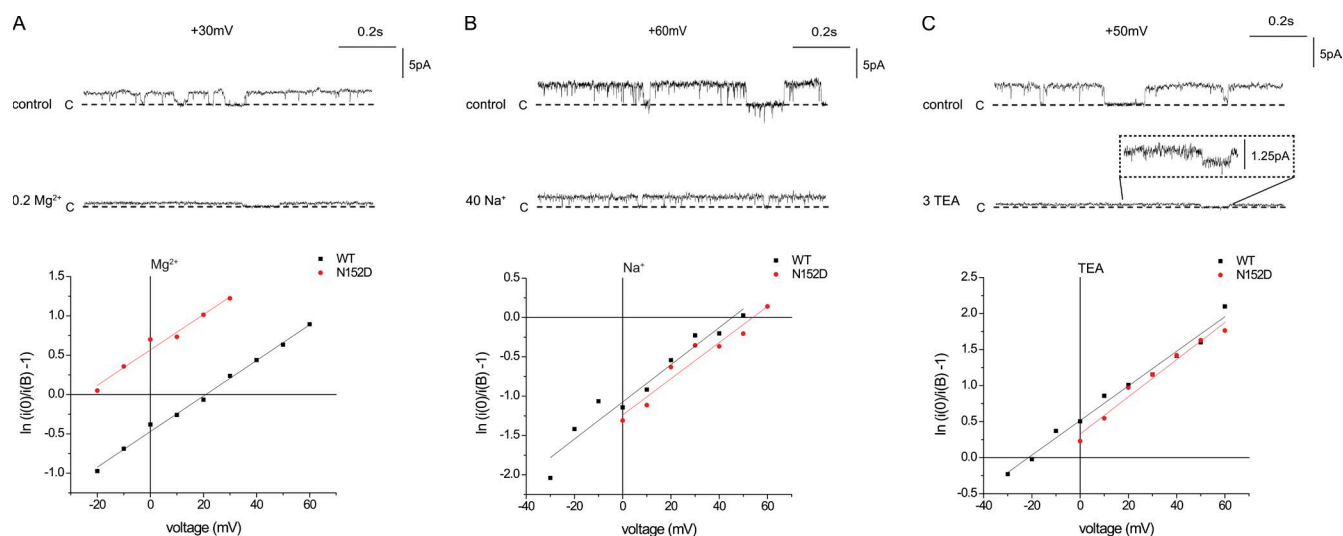


Figure 4. Adding negative charge to the transmembrane cavity increases affinity for Mg^{2+} but not for Na^+ or TEA^+ . Block was analyzed in WT and N152D channels according to Eq. 3 at 11 mM of extracellular K^+ for (A) 0.2 mM Mg^{2+} , (B) 40 mM Na^+ , and (C) 3 mM TEA^+ . Typical current traces at two voltages with and without blockers are shown at the top of each panel. A transition in the presence of TEA^+ is magnified in the inset. Values of $K_i(0)$ decreased from 0.81 to 0.11 mM (Mg^{2+}), and increased from 117 to 138 mM (Na^+) and 1.8 to 2.2 mM (TEA^+). No significant changes in $z\delta$ were observed.

These results suggest the possibility of two independent effects of TEA on this channel. In light of this added complexity, we did not pursue these interactions further.

Modeling the results

We next asked if these results were compatible with a standard kinetic model for K^+ permeation through these channels. We used a model with six permeant ion-binding sites (Fig. 7A). Four of these (S1–S4) are in the selectivity filter itself; the number of states is constrained such that at least two of the sites are occupied at all times. The other two sites represent binding to the external mouth of the pore (S0) and to the transmembrane cavity (Scav). We assume that the blocking sites for internal ions are at or near the Scav site, such that the cavity cannot be simultaneously occupied by both a permeant ion and a blocking ion. In general, voltage dependence of block by internal cations in such a model can arise from three sources. (1) An intrinsic voltage dependence will result from the blockers' crossing part of the electric field to reach their bindings site(s)

in the cavity. (2) An additional voltage dependence may come from the displacement of K^+ ions across the electric field subsequent to blocker binding, particularly if blocking ions repel K^+ ions in the selectivity filter. (3) Finally, changing the voltage can reduce the occupancy of a blocking site by permeant ions, increasing the availability of the site for binding of a blocker. In this case, the voltage dependence arises from the movement of K^+ ions into the selectivity filter, leaving the potential blocking site unoccupied.

In modeling the experimental findings, we examined three different scenarios. (1) In the first case, we assumed that the cavity site is completely outside the electric field. Furthermore, we assumed no direct interaction between blocking and permeant cations other than competition for Scav. An optimal fit of the data for Mg^{2+} is shown in Fig. S1. Kinetic parameters are listed in Table S2. Even with these simplifying assumptions, we can account for the basic voltage dependence of block at high $[K^+]_o$, which in this case arises from the increase in the fraction of empty sites as the membrane is depolarized

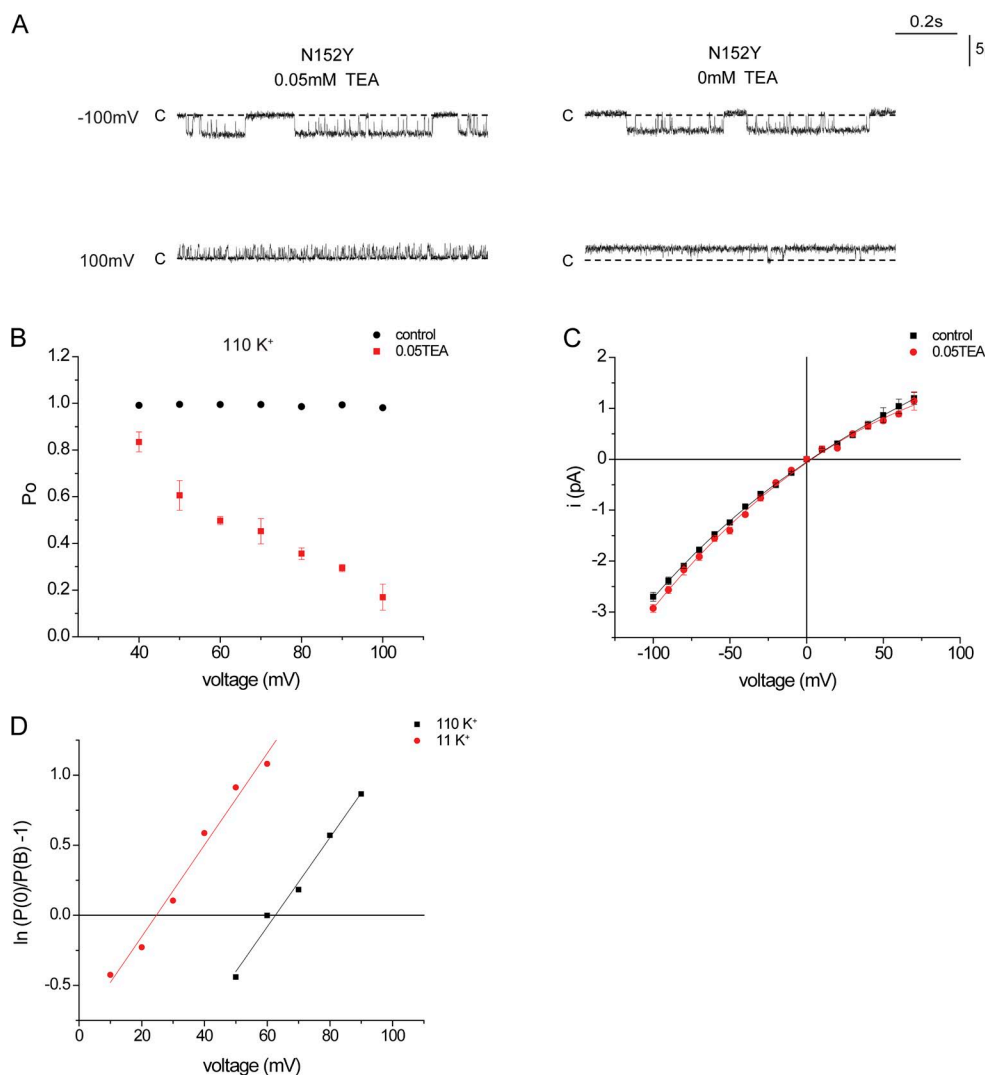


Figure 5. Adding an aromatic residue to the transmembrane cavity increases affinity for TEA⁺. (A) Typical currents through Kir1.1 N152Y channels in inside-out patches with 110 mM K⁺ on the outside and 110 mM K⁺ with and without 0.05 mM TEA⁺ on the inside of the patch. Currents are shown at -100 and +100 mV. (B) Analysis of P_o in the absence and presence of TEA⁺ (two to five measurements per point). (C) i - V relationships with and without TEA⁺. (D) Voltage dependence analyzed using Eq. 3 with P_o substituted for i for $[K^+]_o = 110$ and 11 mM. Values of $K_i(0)$ were 0.37 mM ($[K^+]_o = 110$ mM) and 0.11 mM ($[K^+]_o = 11$ mM). Values of $z\delta$ were 0.82 ($[K^+]_o = 110$ mM) and 0.84 ($[K^+]_o = 11$ mM).

as the other mechanisms are assumed not to exist. The increased availability of sites is shown as the black curve in Fig. S1 F. However, the predicted voltage dependence is somewhat less than that measured at low $[K^+]_o$, and somewhat larger than that measured at high $[K^+]_o$ (Fig. S1, A–C). Furthermore, the fraction of blocked current at positive V_m is virtually the same for all $[K^+]_o$, contrary to the data (Fig. S1 E). It therefore fails to recapitulate one of the primary observations that we are trying to understand.

(2) In a second formulation, we again assumed that the cavity site is outside the field but added a repulsion energy between ions in Scav and in S4. The repulsion was assumed to be proportional to charge, with twice the energy for Mg^{2+} relative to K^+ . This means that the cavity site either has a high affinity (S4 empty) or low affinity (S4 occupied) for the blocking ions. Here, voltage dependence can, in principle, arise from both a change in the number of Scav sites available and from a shift in the occupancy of the selectivity filter by K^+ . Under these conditions, the fits to the data were reasonably good over the entire range of $[K^+]_o$ (Fig. 7, B–D). Kinetic parameters are listed in Table S3. This model also accounts for the “crossover” effect in which outward current is decreased when $[K^+]_o$ is reduced, despite an increase in driving force (Fig. 7 D, inset). Furthermore, the fractional block of outward currents was clearly shifted in response to changes in $[K^+]_o$ (Fig. 8 A). The shift was greater between 110 and 11 mM than between 11 and 1 mM, consistent with experimental results (Yang et al., 2010). The simulation gave somewhat less block at negative voltages and low $[K^+]_o$ than was measured. Monovalent block could also be reasonably well

explained with this model (Fig. 8 C). Finally, the simulation described reasonably well the decrease in apparent $K_i(0)$ for internal Mg^{2+} when $[K^+]_{in}$ was reduced from 110 to 25 mM (Fig. 8 B), without adjustment of any parameters.

The basis for the voltage dependence with these parameters is different from those in scenario 1. The dependence of the fraction of open Scav sites on voltage again contributes. However, in the presence of Mg^{2+} , the fraction of channels with K^+ in sites S1 and S3 increases, whereas that with K^+ in sites S2 and S4 decreases with voltage (Fig. 8 D). Thus, the apparent valence of the block is caused in large part by an outward shift of K^+ within the selectivity filter. The effect of $[K^+]_o$ arises from an increase in occupancy of S0 as $[K^+]_o$ increases, pushing the ions in the selectivity filter in the opposite direction, into the S2/S4 configuration.

(3) Although the model shown in Figs. 7 and 8 gave a fairly good account of the data, the assumption that the cavity blocking site is completely outside the electric field may not be realistic. We therefore examined a third scenario in which an intrinsic voltage dependence of blocker occupancy of Scav was permitted. The fraction of the electric field sensed by a K^+ ion in Scav ($\delta_{K,cav}$) was fixed at 0.07. This value is somewhat arbitrary but is consistent with previous models of KcsA and inward-rectifier K^+ channels (Kutluay et al., 2005; Edvinsson et al., 2011; Yang et al., 2012). The blockers were assumed to bind to the same site. The best fit for the Mg^{2+} data are shown in Fig. S2. Kinetic parameters are listed in Table S4. The voltage and K_o^+ dependence were described slightly better than in case 2 (Fig. S2 E). The basis for the voltage dependence of block was even more

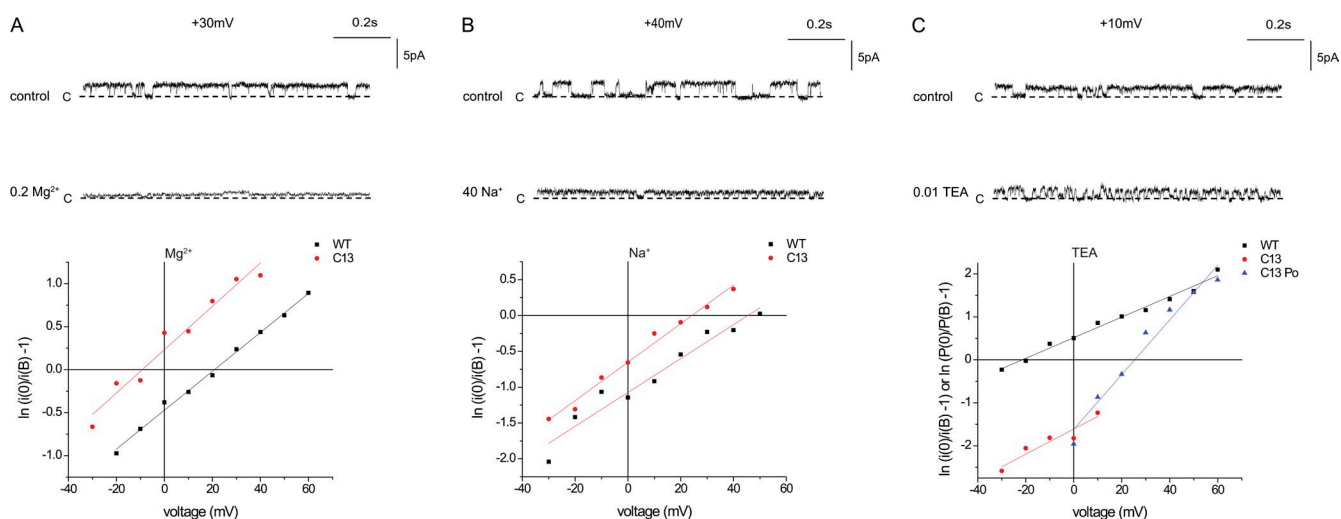


Figure 6. Switching the C terminus of Kir1.1 to that of Kir2.1 (C13 chimera) increases the affinity of block by Mg^{2+} and TEA^+ . Typical current traces at two voltages with and without blockers are shown at the top of each panel. Block was analyzed according to Eq. 3 at extracellular K^+ concentrations of 11 mM for (A) 0.2 mM Mg^{2+} , (B) 40 mM Na^+ , and (C) 0.01 mM TEA^+ . Values of $K_i(0)$ decreased from 0.82 to 0.16 mM (Mg^{2+}), 117 to 77 mM (Na^+), and 1.8 to 0.05 mM (TEA^+). No significant differences in $z\delta$ were observed for Na^+ , or Mg^{2+} , or TEA^+ measured from changes in i , but $z\delta$ for TEA^+ block measured as P_o was 1.63.

complex in this scenario. Depolarization increased the intrinsic binding of the blocker, increased the availability of open cavity sites, and shifted K^+ occupancy of the selectivity filter toward S1/S3 (Fig. S2 F). Thus, although the assumption of binding of the blockers within the electric field is not necessary to describe the data, a small intrinsic voltage dependence of blocker interaction is also compatible with the observations.

DISCUSSION

Physiological significance of block in weak rectifiers

Kir1.1 is a so-called “weak” inward rectifier; blocks by both Mg^{2+} and polyamines have low affinities compared with “strong” rectifiers such as Kir2.1 (Taglialatela et al., 1995; Yang et al., 1995). Physiological concentrations of intracellular polyamines appear to have little effect on these channels. However, millimolar concentrations of intracellular Mg^{2+} decrease but do not abolish outward currents through Kir1.1 (Lu and MacKinnon, 1994;

Nichols et al., 1994; Yang et al., 2010). This effect on the channels could play a role in preventing urinary K losses when K intake is low, as reduced luminal K^+ concentrations observed under these circumstances would increase the affinity for Mg^{2+} (Yang et al., 2010). In addition, this interaction could help explain the difficulty in maintaining plasma K^+ concentrations in patients with hypomagnesemia (Huang and Kuo, 2007). Thus, channel block by internal cations, especially Mg^{2+} , has potential physiological and pathophysiological relevance.

In strong inward rectifiers, intracellular polyamines are thought to be the dominant intracellular blockers under physiological conditions (Lopatin et al., 1995). Block by these compounds is more complex because of their multiple positive charges, block at more than one site (Ishihara and Ehara, 2004; Shin et al., 2005; Liu et al., 2012), and large apparent valences ($z\delta$ values) of block up to values of 4 (Xu et al., 2009). In particular, the work of Lu and colleagues (Spassova and Lu, 1998; Guo et al., 2003; Xu et al., 2009) suggests that the pronounced voltage dependence of the strong rectifiers

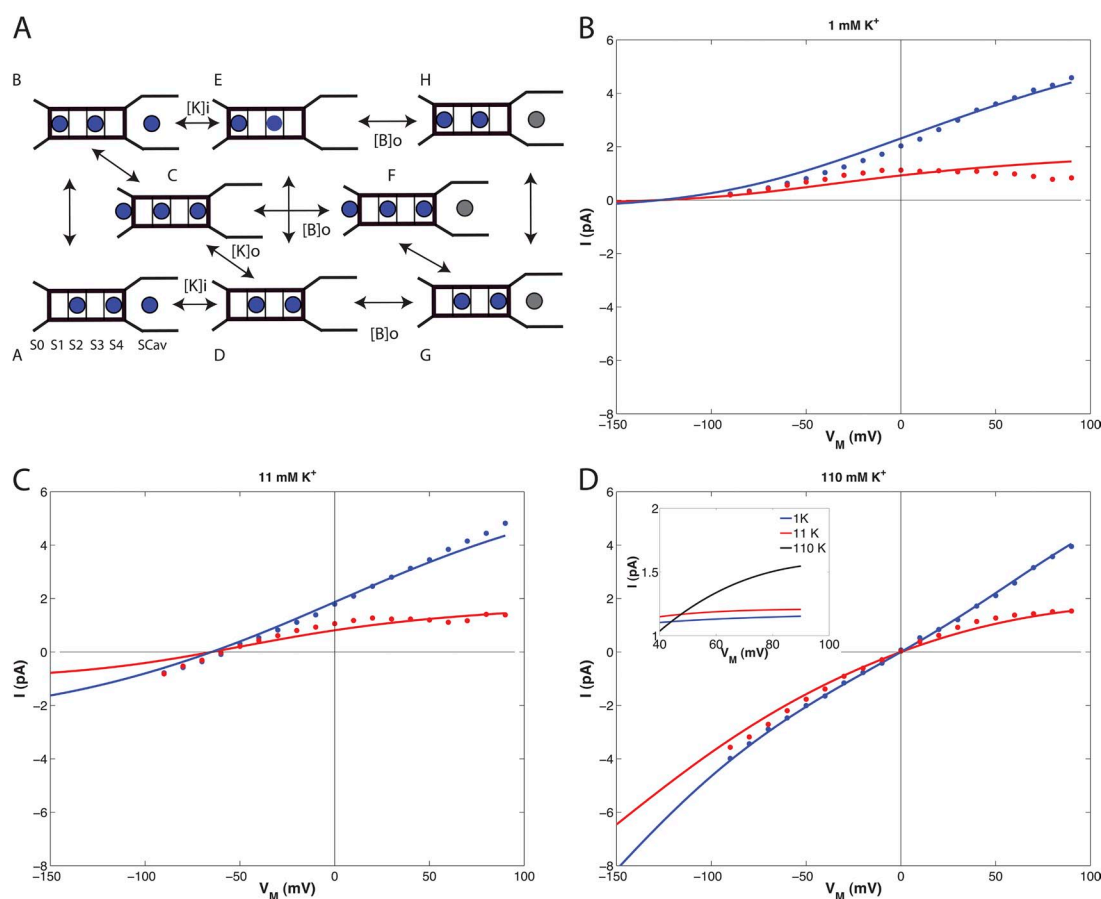


Figure 7. Results for block of Kir1.1 by Mg^{2+} are simulated with a kinetic model for permeation, with blocker binding to the transmembrane cavity. The model is shown in A. In this case, we assume no intrinsic voltage dependence of blocker binding and an electrostatic repulsion of 4.6 RT between ions in the cavity and those in S4. Currents in the presence (red) and absence (blue) of Mg^{2+} with $[K^+]_o = 1$ mM (B), 11 mM (C), and 110 mM (D). Symbols show measured values. Solid lines show predicted currents from the best-fit model parameters. (Inset) Crossover of currents, indicating decreased outward current with increased driving force caused by changes in $[K^+]_o$, predicted from the model.

entails the single-file movement of K^+ ions by the blockers from the cavity as well as the cytoplasmic domain through the electric field, pushed by the piston-like action of polyamines with “gaskets” preventing the permeant ions from leaking back into the cytoplasm. In contrast, the smaller voltage dependence of the weak rectifier Kir1.1 can be explained by the displacement of K^+ ions within the selectivity filter.

Shift of apparent affinity by K_o^+ applies to many intracellular blockers

We compared the effects of three different intracellular blockers of Kir channels: Mg^{2+} , Na^+ , and TEA^+ . These have a range of apparent affinities, with $K_i(0)$ values for Na^+ around 100 mM and those for Mg^{2+} and TEA^+ in the low millimolar range. However, they have two properties in common. First, they block in a voltage-dependent manner with similar apparent valences. Second, the apparent affinities of all three were affected similarly by changing $[K^+]_o$; reducing $[K^+]_o$ from 110

to 11 mM decreased $K_i(0)$ by about threefold in each case. This occurs without a measurable change in voltage dependence.

Sites of block

The similar $z\delta$ values could indicate that the monovalent cations block at a site deeper into the pore than does Mg^{2+} , such that the lower z is compensated by a larger δ . Alternatively, all the blockers could bind to the same site if the movement of K^+ ions in the pore accounts for most of the voltage dependence. We think the latter interpretation is a more parsimonious and more attractive explanation. Experimental evidence supports the binding of both Mg^{2+} and TEA^+ in the transmembrane cavity. Previous findings, confirmed here, showed that a negative charge in this part of the pore (N152D) increases the affinity for Mg^{2+} block (Lu and MacKinnon, 1994; Taglialatela et al., 1995; Yang et al., 1995). By the same reasoning, adding an aromatic side chain to the same amino acid (N152Y) increases the affinity for TEA^+ ,

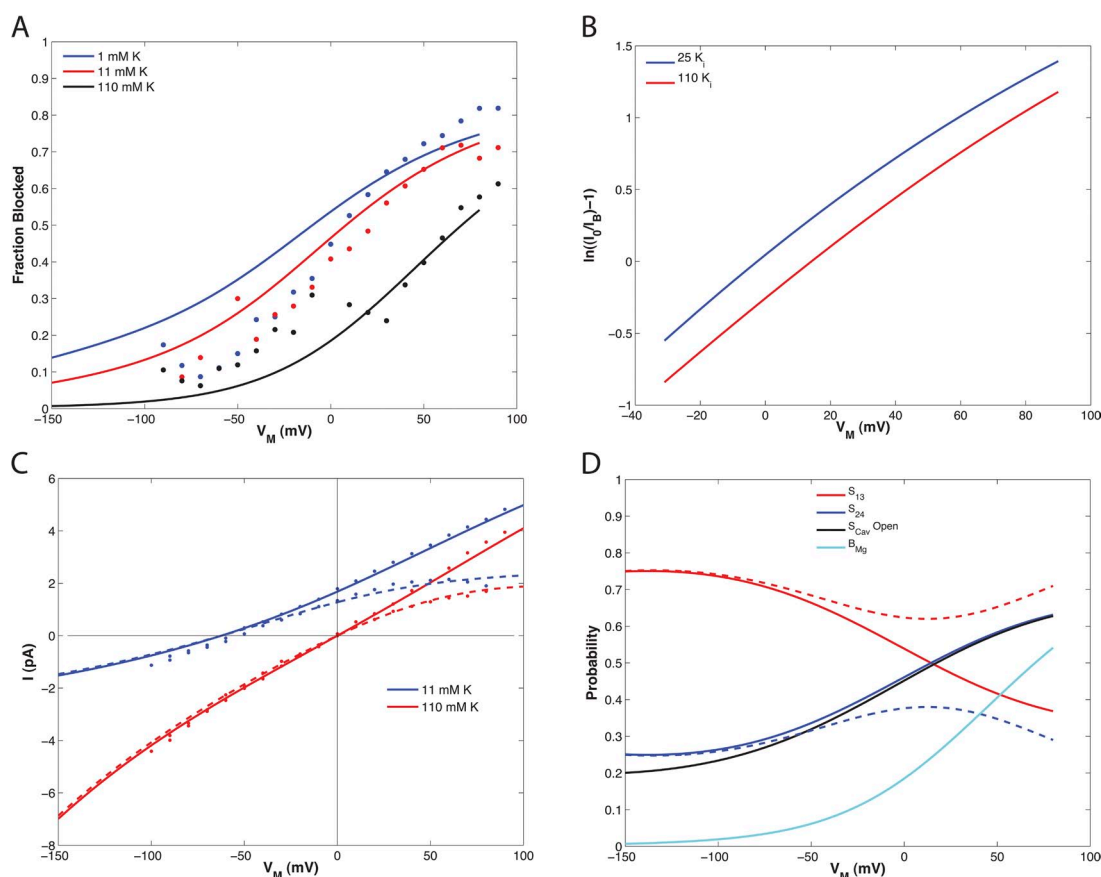


Figure 8. Results for block of Kir1.1 by Mg^{2+} and Na^+ are simulated with a kinetic model for permeation, with blocker binding to the transmembrane cavity. (A) Fractional block by Mg^{2+} at different voltages and values of $[K^+]_o$. (B) Predicted voltage dependence of Mg^{2+} block at 110 and 25 mM $[K^+]_{in}$. (C) Currents with $[K^+]_o = 110$ mM (red) or 11 mM (blue) and 110 mM of internal K^+ with (dashed lines) and without (solid lines) 40 mM Na^+ . Symbols represent data points. Solid lines show predictions of model parameters listed in Table S2. (D) Predicted occupancies of K^+ ions in the S1/S3 positions (red) and S2/S4 positions (blue) in the absence (solid lines) and presence (dashed lines) of 1 mM Mg^{2+} . The black line shows the fraction of unoccupied cavity sites in the absence of Mg^{2+} . The green line indicates the fraction of blocked channels.

possibly through cation- π electron interactions, suggesting that this monovalent cation also binds close to this position. In this case, not only was the affinity for TEA⁺ increased, but the kinetics of block were slowed such that individual blocking events could be resolved. X-ray crystallography provides independent experimental support for the interaction of a quaternary ammonium analogue within the cavity of the KcsA channel (Zhou et al., 2001). Thus, the simplest interpretation of the findings is that both divalent and monovalent blockers bind to the same site; that this site is within the cavity close to residue N152; and that to reach this site from the cytoplasm, the ions do not cross a substantial part of the transmembrane electric field. The similar voltage dependence for Na⁺ and TEA⁺ inhibition is most compatible with Na⁺ block within the transmembrane cavity rather than within the selectivity filter, as proposed for KcsA channels (Thompson et al., 2009). In that channel, the apparent valence for internal block by Na⁺ (>0.4; Thompson et al., 2009) is larger than that for TEA⁺ (0.16; Kutluay et al., 2005).

This raises the question of why the N152D mutation did not alter block by Na⁺ or TEA⁺. One explanation for this finding is that the blockers must compete with K⁺ for this site. This competition was shown directly for the case of Mg²⁺ (Fig. 3). The more negative electrostatic potential would increase the binding affinity for K⁺ to a similar extent as for Na⁺ and TEA⁺. The effect on Mg²⁺ binding would be larger because of the second positive charge, giving Mg²⁺ but not Na⁺ or TEA⁺ a competitive advantage over K⁺ for occupancy of the site.

The C13 chimera, with the cytoplasmic C terminus from Kir2.1 attached to Kir1.1, also has a higher affinity for block Mg²⁺ as well as for polyamines (Taglialatela et al., 1995; Yang et al., 1995). We confirmed that finding but again did not see an increase in affinity for Na⁺ block. This may also be explained by the difference in valence. We showed previously that the static charge distribution on the cytoplasmic domain of Kir2.1 is better suited to binding divalent (and polyvalent) cations than that of Kir1.1 (Robertson et al., 2008). The accumulation of Mg²⁺ in the cytoplasmic pore will both increase the availability of Mg²⁺ to enter the cavity and decrease the concentration of K⁺ available to the cavity. This effect would be greatly diminished with a monovalent blocker such as Na⁺.

The increased affinity of the C13 chimera for TEA⁺ was more surprising. Previous results showed that Kir2.1 has a particularly high sensitivity to block by TEA⁺ relative to other quaternary ammonium ions (Spassova and Lu, 1999), but the structural basis for this phenomenon was not explored. It appears that this strong interaction requires the cytoplasmic domain, but exactly how and where it comes about is not clear. It is theoretically possible that in the chimera, TEA⁺ can enter the selectivity filter, binding with a greater intrinsic voltage dependence

and/or further displacing K⁺ ions in the filter. However, this seems unlikely because at least in KcsA channels, TEA⁺ entry into the selectivity filter is energetically highly unfavorable (Kutluay et al., 2005). In addition, the increased valence was not observed for Na⁺ block. Alternatively, the block may involve first binding to the cytoplasmic pore followed by translocation to the cavity site, with the associated single-file movement of K⁺ ions through the extended pore, as suggested for polyamine block of Kir2.1 (Xu et al., 2009). These findings indicate that the chimera is behaving more like a strong rectifier with respect to its interactions with TEA⁺, and its behavior cannot be fully described by the simple formalisms developed here for the weak rectifiers.

Mechanism of voltage and K⁺ interactions

As shown by the modeling exercises, the voltage dependence of block can be explained in several ways. The classical mechanism (Woodhull, 1973) entails the blocking ion's crossing part of the electric field to reach its site. This premise accounts naturally for the shape of the *i*-*V* curves in the presence of the blockers and the ability to fit the data with *z* δ values that are independent of voltage over a substantial range. However, this interpretation does not explain the similar values of *z* δ for monovalent and divalent cations, despite circumstantial evidence that the blockers bind to similar sites. Furthermore, it does not easily account for the dependence of the apparent *K_i* value on external K⁺, a hallmark of inward-rectifier K⁺ channels that extends even to the weak rectifier Kir1.1.

A second type of mechanism explains the effect of voltage through changes in the number of unoccupied sites in the pore that are available to the blockers. Increasing the outward current either by depolarization of the membrane voltage or decreases in external K⁺ can, under some conditions, lead to an increased probability of the cavity site's being empty, even when this site is outside the electric field (Figs. 8 C, S1 F, and S2 F). In a sense, this is a reversal of the classical knock-off mechanism in which current coming into the cell displaces the blocker (Armstrong, 1971). This idea explains the lack of dependence of the apparent valence of block on the actual charge on the blocker, but by itself fails to describe the dependence of block on external K⁺ or the crossover of currents at large positive voltages.

The simplest model that accounts for these phenomena is one in which the blockers bind to the same site, which is mostly outside the transmembrane electric field but which interacts electrostatically with ions in the S4 site of the selectivity filter. The idea that the cavity site is mostly outside the electric field, at least for an open pore, is consistent with an analysis of TEA⁺ block of KcsA channels (Kutluay et al., 2005). This implies that only a small voltage drop is required to move ions through

the cytoplasmic pore, at least of Kir1.1, when the channel is open. The idea of repulsion between ions in the cavity and the S4 position of the selectivity filter is also physically reasonable; a similar value of repulsion energy was assumed in a previous study of KcsA (Kutluay et al., 2005).

Under these conditions, most of the voltage dependence arises from blocker-dependent shifts in the positions of K^+ in the selectivity filter, with S1/S3 occupancy being favored over S2/S4 at depolarized potentials. A similar conclusion was reached based on a simpler permeation model by Spassova and Lu (1998). This scenario accounts naturally for the effects of $[K^+]_o$; increases in $[K^+]_o$ will favor S2/S4 occupancy, directly opposing the binding of the blocker.

The model presents a simple picture of permeation and block, and the parameters derived from it do not constitute a unique description of these events. It can provide a reasonably good simulation of the data even with different basic assumptions about the binding sites, indicating that the parameters are not highly constrained. We nevertheless believe that the model does give some insight into the nature of the dependence of internal blockers on transmembrane voltage and external K^+ . The main conclusion is that it is possible to explain these interactions with a minimal scheme in which pairs of K^+ ions within the selectivity filter move outward across the electric field when blockers bind to Scav from the inside and inward across the field when K^+ binds to S0 from the outside. Although the reality is likely to be much more complex, this basic mechanism seems to be sufficient to explain the properties of block in this weakly inwardly rectifying K^+ channel.

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