

Electrostatics in the Cytoplasmic Pore Produce Intrinsic Inward Rectification in Kir2.1 Channels

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Inward rectifier K⁺ channels are important in regulating membrane excitability in many cell types. The physiological functions of these channels are related to their unique inward rectification, which has been attributed to voltage-dependent block. Here, we show that inward rectification can also be induced by neutral and positively charged residues at site 224 in the internal vestibule of tetrameric Kir2.1 channels. The order of extent of inward rectification is E224K mutant > E224G mutant > wild type in the absence of internal blockers. Mutating the glycines at the equivalent sites to lysines also rendered weak inward rectifier Kir1.1 channels more inwardly rectifying. Also, conjugating positively charged methanethiosulfonate to the cysteines at site 224 induced strong inward rectification, whereas negatively charged methanethiosulfonate alleviated inward rectification in the E224C mutant. These results suggest that charges at site 224 may control inward rectification in the Kir2.1 channel. In a D172N mutant, spermine interacting with E224 and E299 induced channel inhibition during depolarization but did not occlude the pore, further suggesting that a mechanism other than channel block is involved in the inward rectification of the Kir2.1 channel. In this and our previous studies we showed that the M2 bundle crossing and selectivity filter were not involved in the inward rectification induced by spermine interacting with E224 and E299. We propose that neutral and positively charged residues at site 224 increase a local energy barrier, which reduces K⁺ efflux more than K⁺ influx, thereby producing inward rectification.

INTRODUCTION

Inward rectifier K⁺ channels (Kir) are expressed in various excitable cells, including heart muscles (Sakmann and Trube, 1984), neurons (Constanti and Galvan, 1983), pancreatic β -cells (Cook and Hales, 1984), and endothelial cells (Silver and DeCoursey, 1990). These channels play an important role in maintaining the resting membrane potential near the K⁺ equilibrium potential (E_K), thereby regulating membrane excitability (Hille, 2001). Kir channels efficiently conduct inward currents when potential is negative to E_K but permit much smaller currents at depolarization (Hille, 2001). The mechanism underlying this inward rectification is prevalently attributed to the voltage (V_m)-dependent block of outward K⁺ currents by internal Mg²⁺ and polyamines interacting with D172, E224, and E299 sites (Fig. 1) (Matsuda et al., 1987; Vandenberg, 1987; Ficker et al., 1994; Lopatin et al., 1994; Lu and MacKinnon, 1994; Yang et al., 1995; Kubo and Murata, 2001). The occluding site of spermine interacting with D172 is believed to be located in the water cavity or the selectivity filter (Kurata et al., 2004). Recently, we have shown that the interaction of spermine with D172 prevents 2-trimethylammonioethylmethane thiosulfonate (MTSET) from accessing the substituted cysteines at sites 164 and 168, indicating that the spermine blocking site is near the site 164 (Fig. 1) (Chang et al., 2003).

The mechanism underlying the inward rectification induced by polyamines interacting with E224 and E299 has also been investigated. It has been proposed that E224 and E299 facilitate the entry and exit of polyamines to and from the final pore-plugging site located deeper in the pore and that internal blockers bind to E224 and E299 without occluding the pore (Kubo and Murata, 2001; John et al., 2004). Furthermore, Xie et al. (2002) have shown that screening the negative charges at the internal vestibule reduces conductance and contributes to a shallow V_m -dependent inhibition of the Kir2.1 channel. Recently, they suggest that E224 and E299 may allosterically regulate a fast gate located at the bundle crossing or below in the Kir2.1 channel (Xie et al., 2004). It remains unclear whether the binding of internal polyamines to E224 and E299 or screening charges at sites 224 and 299 can directly contribute to inward rectification.

Our previous study has shown that spermine, interacting with E224 and E299, inhibits K⁺ efflux, yet MTSET remains accessible to the cysteines at site 164 (Fig. 1) in Q164C/D172N mutants at depolarization, suggesting that spermine does not occlude the channel at a position internal to Q164 with the D172N mutation (Chang et al., 2003). Also, we show that spermine does

Abbreviations used in this paper: Kir, inward rectifier K⁺; E_K , K⁺ reversal potential; MTSES, 2-sulfonatoethylmethane thiosulfonate; MTSET, 2-trimethylammonioethylmethane thiosulfonate.

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not affect the exit of Ba^{2+} from its binding site, T141 (Alagem et al., 2001), which is located at the internal end of the selectivity filter (Fig. 1), and thus we suggest that the spermine-occluding site is not external to T141 in the D172C mutant (Chang et al., 2003). Therefore, in the absence of D172, spermine interacting with the cytoplasmic pore inhibits the Kir2.1 channel either by blocking at a position between 141 and 164 or by other mechanisms.

Although the E224G mutant shows milder inward rectification in cell-attached patches than the wild type (Yang et al., 1995; Kubo and Murata, 2001), the former is more inwardly rectifying than the latter in inside-out patches perfused with polyamine- and Mg^{2+} -free solutions (Xie et al., 2004). In this study we examined the effects of charges at site 224 on the inward rectification of the Kir2.1 channel. We found strong inward rectification in E224C mutants treated with the positively charged MTSET and in E224K mutants in the absence of internal blockers. Also, spermine interacting with E224 and E299 did not occlude the pore in the presence of the D172N mutation. We propose that the negative charges at site 224 decrease a local energy barrier for positively charged ions and molecules such as K^+ and polyamines so that their entry and exit rates to and from the pores are high. Decreasing negative charges at the internal vestibule (e.g., the E224G and E224K mutants and spermine binding to E224 and E299) via an increase in electrostatic potential and/or a constriction in permeation pathway raises the local energy barrier, which decreases K^+ efflux more efficiently than K^+ influx.

MATERIALS AND METHODS

Channel Mutagenesis and Expression

Mutations were constructed by using PCR and checked by sequencing. Cysteine mutants were generated in MTS-insensitive channels, IRK1J (C54V, C76V, C89I, C101L, C149F, and C169V) (Lu et al., 1999a). The cRNA was obtained by *in vitro* transcription (mMessage mMachine, Ambion) and injected in *Xenopus* oocytes, which were used 1–3 d after cRNA injection. *Xenopus* oocytes were isolated by partial ovariectomy from frogs anaesthetized with 0.1% tricaine (3-aminobenzoic acid ethyl ester). The incision was sutured and the animal was monitored during the recovery period before it was returned to its tank. Following the final oocyte collection, frogs were anaesthetized as described above and killed by decapitation. All surgical and anesthetic procedures were reviewed and approved by the Academia Sinica Institutional Animal Care and Utilization Committee. Oocytes were maintained at 18°C in Barth's solution containing (in mM) NaCl (88), KCl (1), NaHCO_3 (2.4), $\text{Ca}_2\text{N}_2\text{O}_6$ (0.3), CaCl_2 (0.41), MgSO_4 (0.82), HEPES (15), pH 7.6, with gentamicin (20 $\mu\text{g}/\text{ml}$).

Electrophysiology

Currents were recorded at room temperature (23°–25°C) using the giant patch clamp technique (Hamill et al., 1981; Hilgemann, 1995) with an Axopatch 200A amplifier (Axon Instruments). The resistance of electrodes ranged from 0.15 to 0.25 M Ω when filled

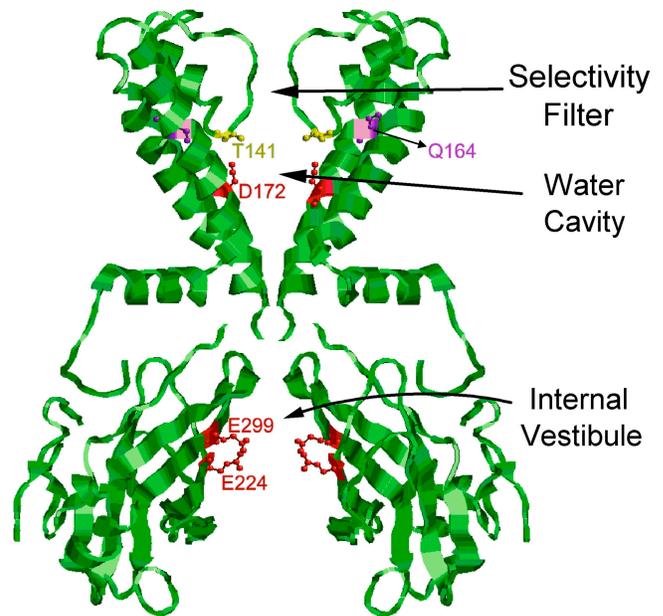


Figure 1. Homology model of Kir2.1 structure. The model was constructed based on a sequence alignment with the structure of a KirBac1.1 channel (Kuo et al., 2003). Two of the four subunits of a Kir2.1 channel are shown. Residues involved in spermine and Ba^{2+} bindings are shown in ball-and-chain models.

with a 100 mM $[\text{K}^+]$ solution. The external $[\text{K}^+]$ ($[\text{K}^+]_o$) and internal $[\text{K}^+]$ ($[\text{K}^+]_i$) (20–300 mM) solutions contained (in mM) KCl + KOH (2–282), EDTA (5), K_2HPO_4 (8), and KH_2PO_4 (2), pH 7.4. Sucrose (160 mM) was added to the 20 mM $[\text{K}^+]$ solution. In selectivity experiments, the $[\text{K}^+]_i$ solution contained (in mM) KNO_3 (100), EDTA (5), and HEPES (5), at pH 7.4 (titrated with HNO_3). For internal NH_4^+ and Tl^+ solutions, KNO_3 was replaced by NH_4NO_3 and TlNO_3 , respectively. 2-sulfonatoethylmethane thiosulfonate (MTSES) and MTSET (Toronto Research Chemicals) were stored at -20°C and dissolved immediately before application. Rundown of channel activity was delayed by treating inside-out patches with 25 μM 1- α -phosphatidylinositol-4,5-bisphosphate (Sigma-Aldrich) (Huang et al., 1998; Shieh et al., 1998). The command V_m and data acquisition functions were processed using a Pentium-based personal computer, a DigiData board, and pClamp6 software (Axon Instruments). Averaged data were presented as mean \pm SEM. Student's independent *t* test was used to assess the statistical significance.

RESULTS

Charges at Site 224 Control Inward Rectification in Tetrameric Kir2.1 Channels

To examine the effect of charges at residue 224 on both inward and outward K^+ conductance, we measured macroscopic currents from inside-out patches perfused with a symmetrical 100 mM $[\text{K}^+]$ solution free of polyamines and Mg^{2+} . In the absence of internal blockers, outward currents through the wild-type Kir2.1 channel were observed but showed slightly time-dependent decay (Fig. 2 A). At more hyperpolarizing V_m the I-V relationship was superlinear (Fig. 2 B). Outward

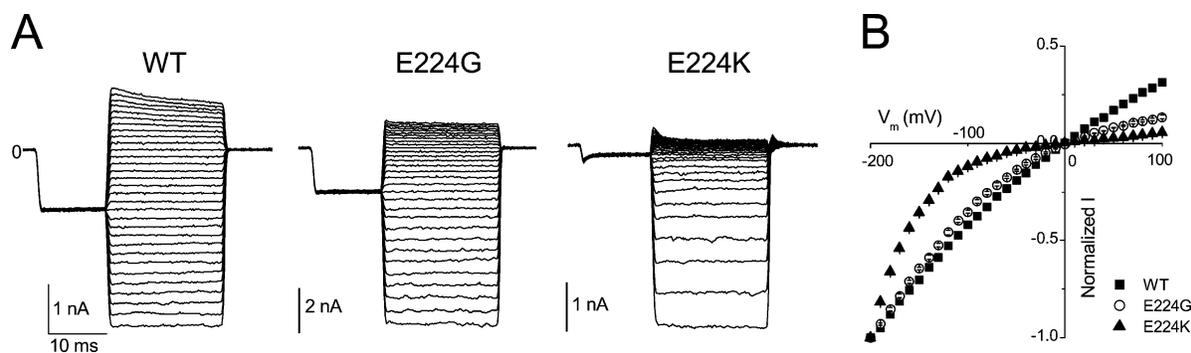


Figure 2. Neutral and positive residues at site 224 induce inward rectification in Kir2.1 channel. (A) Macroscopic currents recorded from inside-out patches containing the wild type, E224G, and E224K mutants in 100 mM symmetrical $[K^+]$. Patches were held at 0 mV, prepulsed to -80 mV for 12 ms and then stepped to V_m ranging from -200 to $+100$ mV for 20 ms. Capacitive and leak currents were corrected by subtracting the currents recorded after complete channel rundown from those measured during channel activities. (B) I-V relationships of the wild type, E224G, and E224K mutants. Currents were normalized to that at -200 mV. $n = 5-7$.

currents through the E224G mutant did not decay (Fig. 2 A) yet the instantaneous currents were more inwardly rectifying than the wild type (Fig. 2 B). Also, the super-linear I-V relationship was more obvious in the E224G mutant than the wild type. When residues 224 were mutated into positively charged lysines, outward currents no longer could be observed even after extensive wash-out in the control solution (Fig. 2 A). Also, inward rectification was so strong that inward currents could only be recorded at very hyperpolarizing V_m in the E224K mutant (Fig. 2, A and B). These results demonstrate that the more positively charged residue 224 is the stronger the inward rectification is in the absence of internal blockers.

Spermine Interacting with E224 and E299 Does Not Occlude the Pore with D172N Mutation

Our previous study suggests that spermine, interacting with E224 and E299, inhibits K^+ efflux by blocking at a position between 141 and 164 or by other mechanisms (Chang et al., 2003). To test the former possibility we examined whether the inhibition of T141C/D172N mutants by spermine can prevent MTSET from accessing the substituted cysteines at site 141. To avoid trapping the reagent in the pore following brief channel openings, membrane patches were held at $+40$ mV and stepped to -140 mV for a short period (5 ms) at 0.2 Hz to monitor channel activities. Using such a protocol, the open probability in the presence of spermine is 0.001, low enough to avoid trapping the reagent (Phillips et al., 2003). In the control, outward currents were observed and MTSET progressively inhibited the T141C/D172N mutant (Fig. 3 A). Spermine inhibited the outward current recorded at the holding potential (Fig. 3 B), presumably due to channel inhibition effected by spermine binding to E224 and E299 (Yang et al., 1995; Kubo and Murata, 2001). However, MTSET remained capable of irreversibly inhibiting the T141C/

D172N mutant, indicating that spermine does not block the pore internal to the site 141.

To further exclude the possibility that MTSET may be trapped inside the channel pore after brief openings at -140 mV, we next examined the effect of MTSET on the T141C/D172N mutant treated with spermine and continuously held at $+40$ mV without pulsing. Fig. 3 C shows that before MTSET treatment the current was stable for 50 s. The patch was exposed to MTSET for 100 s at $+40$ mV and then washout. Subsequently, short pulses to -140 mV revealed that the current was completely inhibited and could not be reversed by washout. Same results were observed in two other patches. These results confirm that MTSET accessing to the T141C/D172N mutant shown in Fig. 3 B is not due to trapped MTSET.

It has been suggested that the diffusion of MTSET through the nonpore pathway is unlikely (Xiao et al., 2003). To further exclude this possibility we next determined whether MTSET could access site 141 in T141C mutants (D172 is available) blocked by spermine. Fig. 3 D shows that MTSET could modify the T141C mutant in the absence of spermine (left panel; rate = 24.2 ± 4.9 $M^{-1}s^{-1}$, $n = 3$), but it could no longer do so when the channel was first blocked by spermine (right panel; same results were obtained in other five patches). These results suggest that spermine interacting with E224 and E299 can inhibit K^+ efflux through a mechanism other than occluding the pore.

The rates of MTSET modification were faster ($P = 0.04$) in spermine (84.9 ± 14.8 $M^{-1}s^{-1}$, $n = 5$) than those in the control (37.6 ± 8.7 $M^{-1}s^{-1}$, $n = 4$). This finding raises one question. Does spermine interacting with E224 and E299 increase rather than decrease the diffusion rates of MTSET and K^+ through the internal vestibule, bundle crossing, and water cavity? MTSET is a large molecule (~ 5.8 Å), and its reaction to cysteines in proteins is highly affected by steric fit, V_m , and [MT-

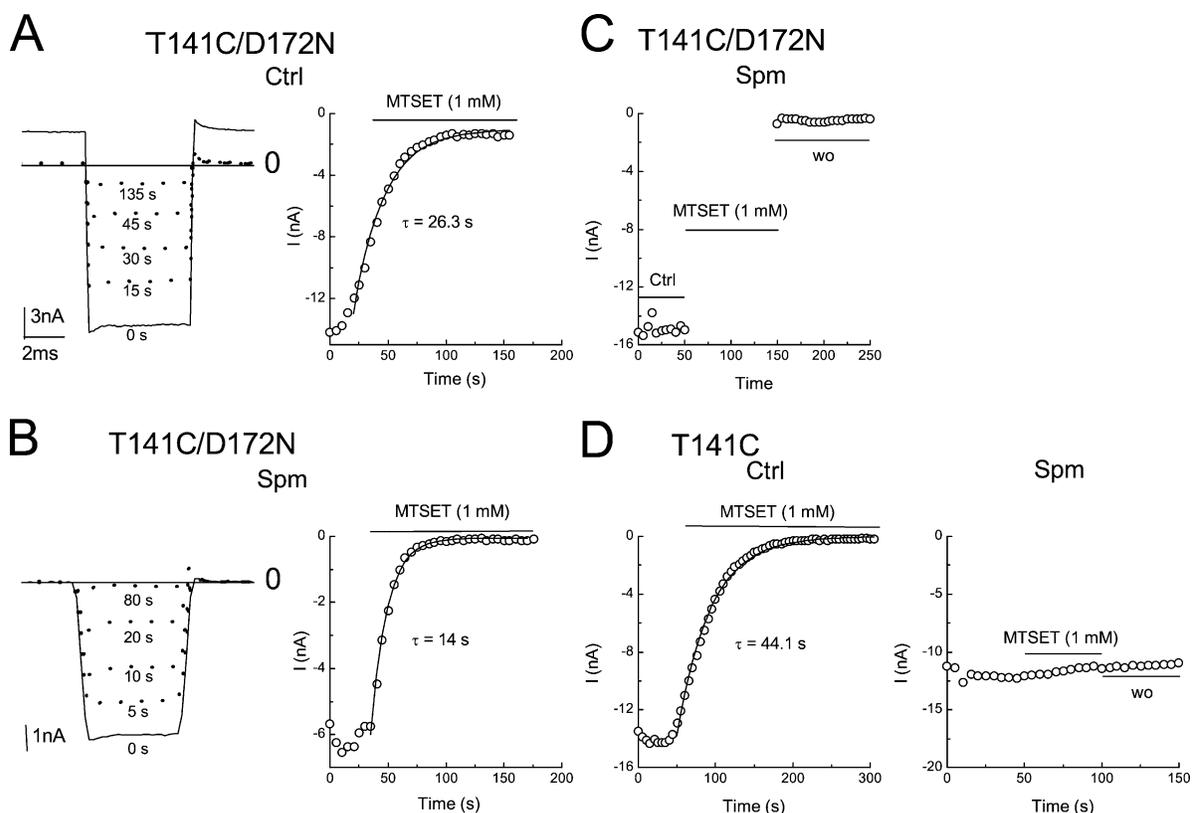


Figure 3. MTSET remains accessible to T141C when spermine inhibits K^+ efflux in the T141C/D172N mutant. Current traces in the T141C/D172N mutant exposed to the control (A) and 100 μ M spermine (B) solution. Current was recorded at -140 mV from a holding potential of $+40$ mV before (solid line) and after (dotted line) MTSET treatment (1 mM). The number of seconds on the bottom of each dotted line indicates the time after MTSET treatment. The time courses of MTSET modification in the control and spermine are shown in the right panels. The horizontal lines indicate the zero current levels. (C) In the presence of 100 μ M spermine, currents were monitored for 50 s before MTSET treatment. MTSET was applied for 100 s as the patch was held at $+40$ mV without pulsing. Thereafter, MTSET was washed out and currents were recorded with brief hyperpolarizing pulse to -140 mV. (D) Time courses of MTSET reaction in T141C mutants in control and spermine as indicated.

SET]. Therefore, MTSET modification rate (much slower than the diffusion limit) cannot be indicative of K^+ diffusion through the pore. To examine whether K^+ diffusion through the internal vestibule and bundle crossing is affected by spermine interacting with E224 and E299, we next measured the rate of Ag^+ modification in D172N/I176C mutants. In the absence of spermine, internal Ag^+ (1.5 nM) inhibited currents through the D172N/I176C mutant with an averaged time constant of 3.3 ± 0.2 s (rate = 2.0×10^8 s $^{-1}$ M $^{-1}$, $n = 3$). In the presence of spermine, 1.5 nM Ag^+ inhibited the D172N/I176C mutant with an averaged time constant of 26.9 ± 2.5 s (rate = 2.5×10^7 s $^{-1}$ M $^{-1}$, $n = 3$). By assuming that the local accessibility of Ag^+ to I176C is not decreased by spermine (spermine increases the MTSET modification rate by 1.5-fold in the I176C/D172N mutant [Chang et al., 2003], implying that the accessibility of the side chain may be increased), the Ag^+ modification experiments suggest that the diffusion of internal Ag^+ and also likely K^+ across the internal vestibule and/or bundle crossing is decreased by

spermine interacting with E224 and E299. The exact mechanism underlying the increasing effect of spermine on the MTSET modification rate in the T141C/D172N mutant is unclear. It is possible that spermine interacting with E224 and E299 may allosterically induce conformational changes in the water cavity such that the side chain of I141C is more accessible to MTSET than the control. Also, spermine may shift the electrical profile within the Kir2.1 channel pore toward a more positive potential (Xie et al., 2002). These effects may increase the first-order rate constant of MTSET modification by a factor more than the reduced local effective [MTSET]. Thus, the apparent second-order reaction rate is increased by spermine interacting with E224 and E299.

Positively Charged Residues at Site 224 Induce Inward Rectification by a Mechanism Different from Channel Block
What is the mechanism underlying the strong inward rectification of the E224K mutant recorded from inside-out patches perfused with the polyamine- and Mg^{2+} -free

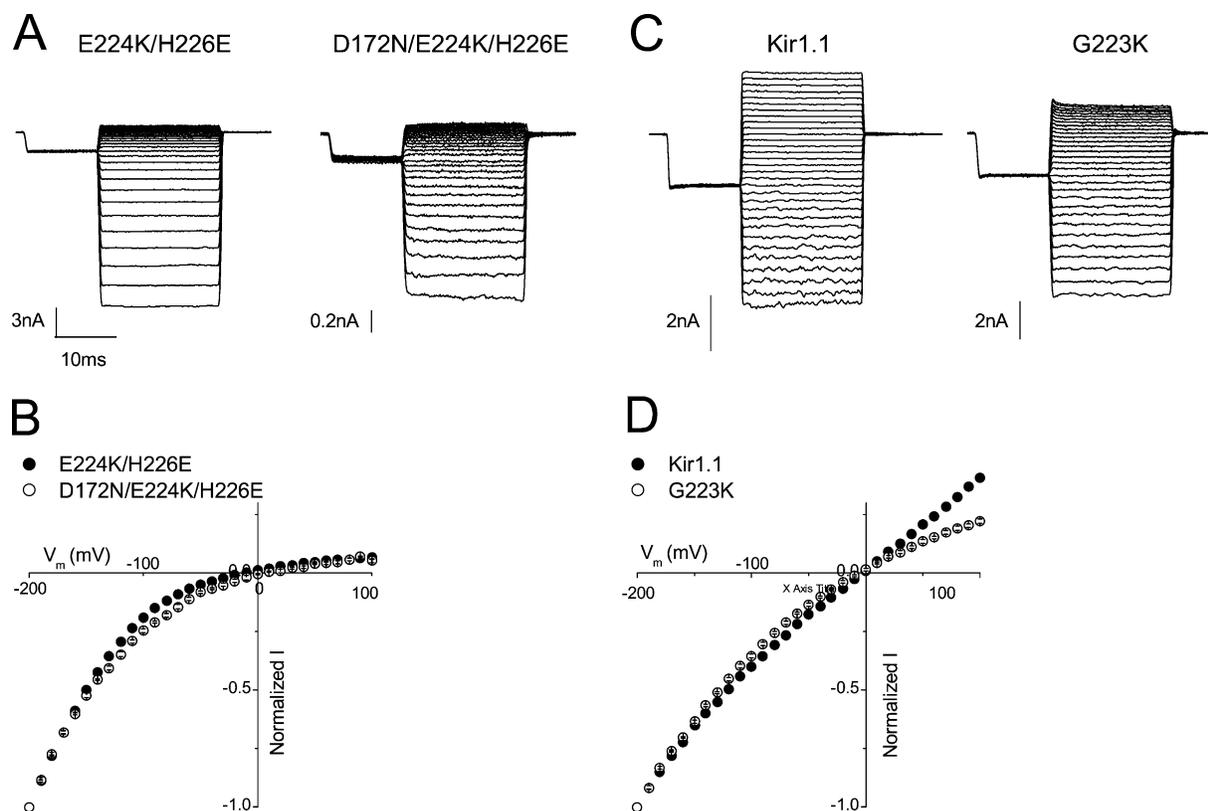


Figure 4. Inward rectification with E224K mutation is not due to channel block. (A and C) Macroscopic currents recorded from inside-out patches containing the indicated channel. (B and D) I-V relationship of the indicated channel. $n = 3-6$.

control solution? It has been suggested that residual polyamines can induce inward rectification in the Kir2.1 channel (Guo and Lu, 2000). Next, we examined the effects of an additional D172N mutation, which should decrease the affinity of residual blockers on the inward rectification of the E224K mutant. Because the expression level of the D172N/E224K mutant was too low to be assayed, we constructed an additional mutation H226E, which increased the expression of the E224K mutant and maintained strong inward rectification (Fig. 4 A). The rescuing effect of the H226E mutation on the conductance of an E224Q mutant has been shown (Xie et al., 2004). Neither the E224K/H226E nor D172N/E224K/H226E mutant demonstrated prominent outward currents (Fig. 4, A and B). The D172N/E224K/H226E mutant showed slightly larger currents at V_m ranging from -140 to -60 mV than those in the E224K/H226E mutant (Fig. 4 B). In summary, the additional D172N mutation does not alleviate the inward rectification in the E224K/H226E mutant, indicating that the inward rectification of the E224K/H226E mutant is not due to residual block.

To further explore the residual block possibility, we examined the mutation of glycines at the corresponding sites to lysines (G223K) in Kir1.1 channels (Ho et al., 1993), which show low sensitivity to internal Mg^{2+}

and polyamine block (Lopatin et al., 1994; Lu and MacKinnon, 1994). Fig. 4 C shows currents through the wild-type Kir1.1 channel and G223K mutant. The I-V relationships demonstrate that the Kir1.1 channel became more inwardly rectifying with the G223K mutation (Fig. 4 D). These results further support that introducing positive charges to the internal vestibule of Kir channels can induce inward rectification through a mechanism distinct from channel block.

To further examine the effects of charges on inward rectification in the Kir2.1 channel we next studied the effects of conjugating positively charged MTSET to the cysteines at site 224 on the inward rectification. Fig. 5 A shows the macroscopic currents recorded in the E224C mutant before and after MTSET treatment. Macroscopic currents through the E224C mutant in the control were similar to those in the E224G mutant. After the E224C mutant was treated with MTSET, outward currents were completely inhibited. Inward currents were also dramatically reduced but some residual currents remained observable. MTSET inhibited outward but not inward currents through the pseudo wild type, IRK1J (Lu et al., 1999a), the effect was completely reversible by washout (Fig. 5 B). On the other hand, the inwardly rectifying effect of MTSET on the E224C mutant could not be reversed by washout (Fig. 5 A). In ad-

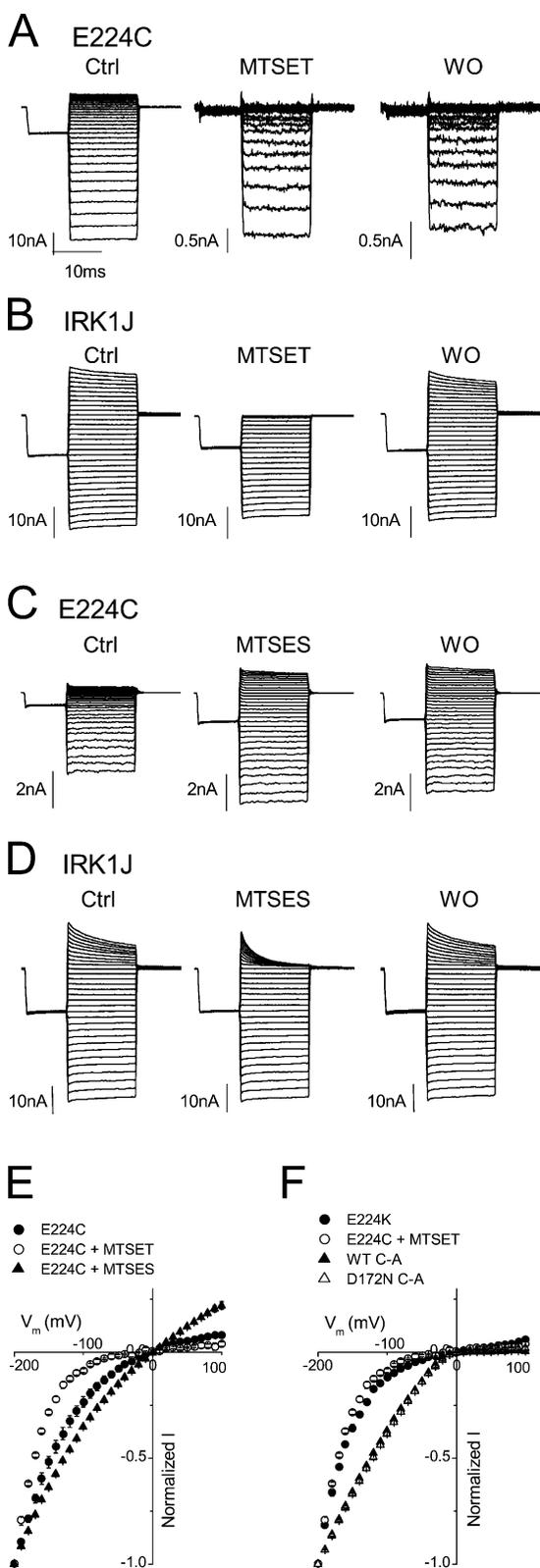


Figure 5. Charges at residues 224 control inward rectification of the Kir2.1 channel. Currents through the E224C mutant before and after treatment with MTSET (A) or MTSES (C) and after washout were recorded from the same patch. Traces of the IRK1J channel before and after MTSET (B) or MTSES (D) treatment

dition to placing positive charges at site 224 by conjugating to cysteines, MTSET also reduced the dimension of aqueous pores (Lu et al., 1999b). It has also been shown that the extent of rectification depends on the size of residue 224 in the Kir2.1 channel (E224Q > E224A > E224G) (Xie et al., 2004). Therefore, the inwardly rectifying effect of MTSET on the E224C mutant may not be exclusively attributed to a charge effect. To further investigate the role of charges at site 224 on inward rectification we next examined the effects of a negatively charged reagent, MTSES, on the E224C mutant. Fig. 5 C shows that MTSES irreversibly increased both inward and outward currents. Note that MTSES, similar in physical size to MTSET, did not reduce current and thus did not seem to obstruct the aqueous pore. Previously, we have shown that pretreatment with MTSES could only partially protect the E224C mutant from subsequent MTSET modification, suggesting that not all four cysteines are modified by MTSES (Chang et al., 2005). MTSES increased both inward and outward currents through the E224C mutant (Fig. 5 C), suggesting that the electrostatic effect may dominate over the steric effect of MTSES bound to the partially modified E224C mutant. Fig. 5 D shows that MTSES slightly reduced outward currents and accelerated time-dependent delay at strong depolarization in the IRK1J channel. These effects were reversible by washout. The normalized instantaneous I-V relationships show that MTSET induced strong inward rectification whereas the negatively charged MTSES alleviated inward rectification in the E224C mutant (Fig. 5 E). The I-V relationship of the E224C mutant treated with MTSET was the same as that in the E224K mutant (Fig. 5 F). Also, the inward rectification is stronger in the E224K mutant than the wild type and D172N mutant in cell-attached modes (Fig. 5 F), indicating that a mechanism different from channel block is involved in the inward rectification of the E224K mutant. Taken together, these results suggest that charges at residue 224 play a crucial role in controlling inward rectification in the Kir2.1 channel.

Effects of Neutral and Positive Charges at Site 224 on Selectivity

Fixed charges near the entrances of many channel types have been shown to affect channel gating and permeation (Chandler et al., 1965; Hille et al., 1975; Dani, 1986; Green et al., 1987; Imoto et al., 1988; Kell and DeFelice, 1988; MacKinnon et al., 1989; Xie et al., 2002; Brelidze et al., 2003; Nimigeon et al., 2003). The effects of charges at site 224 on inward rectification are consistent with electrostatic effects. However, the selec-

and after washout. (E and F) The I-V relationships of the indicated channel and treatment. $n = 3-5$.

tivity filter (Lu et al., 2001; Proks et al., 2001), the M2 bundle crossing (around the site 180) near the cytoplasmic end of the pore (Enkvetchakul et al., 2000; Loussouarn et al., 2000, 2001; Sadjja et al., 2001; Yi et al., 2001; Xie et al., 2004), and G loop of the internal vestibule (Pegan et al., 2005) have also been proposed for channel gating in the Kir family. The selectivity of the pore may be changed if the mutation of E224 allosterically alters the conformation of the selectivity filter or affects the energetics of ion movement in the narrow filter (Ding and Horn, 2002). Next, we determined the ion selectivity of the wild type, E224G, E224K, and E224K/H226E mutants. Fig. 6 shows the I-V relationships of the indicated channel exposed to internal solution containing 100 mM $[K^+]$, $[NH_4^+]$, and $[Tl^+]$, respectively, in 100 mM external $[K^+]$. Estimated from the reversal potential changes, the relative permeability of $K^+ : Tl^+ : NH_4^+$ was $1 : 2.02 \pm 0.02 : 0.14 \pm 0.02$ in the wild type ($n = 5$), $1 : 1.94 \pm 0.11 : 0.16 \pm 0.02$ in the E224G mutant ($n = 5$), and $1 : 2.07 \pm 0.21 : 0.41 \pm 0.16$ in the E224K/H226E mutant ($n = 5-10$). Because the strong inward rectification of the E224K mutant started at negative V_m and outward currents were not obvious, the reversal potentials cannot be properly estimated. Thus, the relative permeability was not determined in the E224K mutant. The permeability ratio of K^+ to NH_4^+ was significantly reduced ($P < 0.0001$) in the E224K/H226E as compared with the wild type, suggesting that the E224K mutation may alter the selectivity filter through long-range effects. However, the change in the selectivity is probably subtle since the relative permeability of K^+ to Tl^+ was not affected in the E224K/H226E mutant and the selectivity was not affected in the E224G mutant. Also, the exit rates of Ba^{2+} from the pore to the external side at +40 mV were about the same for the wild type (101.3 ± 5.9 ms, $n = 3$) and E224K/H226E mutant (82.2 ± 4.5 ms, $n = 4$). In addition, we have previously shown that spermine does not affect Ba^{2+} exit from D172C mutants, indicating that the channel inhibition induced by spermine binding to E224 and E299 in the D172C mutant is unlikely to occur through an occlusion at the selectivity filter (Chang et al., 2003). Together, these results suggest that the E224K mutation and spermine interacting with E224 and E299 do not produce a major conformational change in the selectivity filter.

Bundle Crossing Is Not Involved in the Inward Rectification Induced by Spermine Interacting with E224 and E299

Fig. 3 and our previous study (Chang et al., 2003) together show that MTSET can still access cysteines at sites 141, 164, 169, 172, and 176 in T141C/D172N, Q164C/D172N, V169C/D172N, D172C, and I176C/D172N mutants at V_m (+40 mV) where outward currents are completely inhibited by spermine interacting

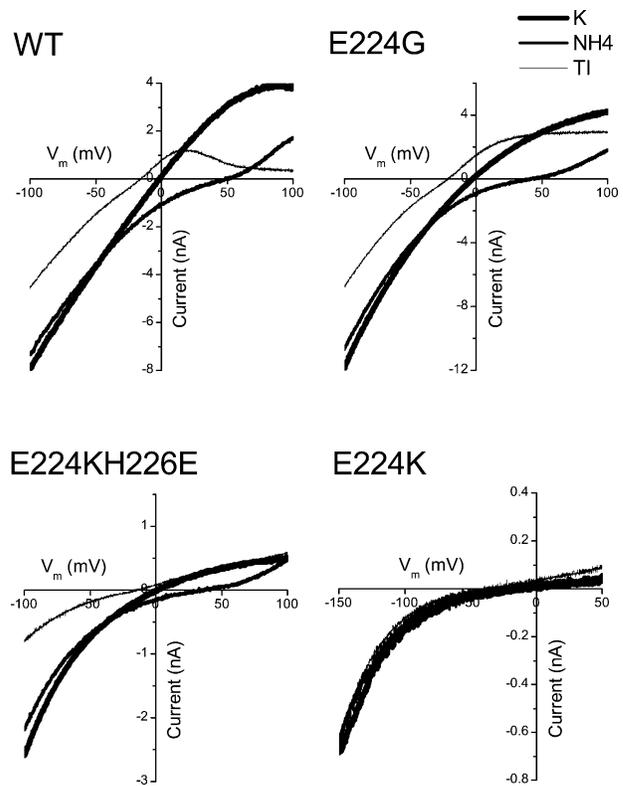


Figure 6. Effects of neutral and positive charges at site 224 on selectivity. I-V relationships recorded from the inside-out patches of the indicated channel exposed to 100 mM internal $[Tl^+]$, $[K^+]$, and $[NH_4^+]$. I-V records were obtained by voltage ramps from -200 to $+100$ mV over 1.4 s. $n = 3-8$.

with E224 and E299. We therefore consider that the gating of the bundle crossing is unlikely to be involved in the inward rectification induced by spermine interacting with E224 and E299 in the absence of D172. We next examined whether the E224K mutation allosterically closes the M2 bundle crossing. Cysteine mutations at sites located external (164, 172, and 176) and internal (184) to M180 (Fig. 7 A) were individually constructed with the E224K mutation. Cysteines at these sites were chosen because they have been shown to be accessible to MTS reagents and thus are probably exposed to the aqueous pore (Lu et al., 1999a; Chang et al., 2003; Xiao et al., 2003).

Fig. 7 B shows current traces and the corresponding time courses of MTSET modifications in the I176C/E224K mutant held at +40. In comparison to the I176C and I176C/E224K/H226E mutants, MTSET modification rates were much slower in the I176C/E224K mutant (Fig. 7 C). On the other hand, MTSET reaction rates were faster in the A184C/E224K and A184C/E224K/H226E mutants than the A184C mutant (Fig. 7 C). These results suggest that the E224K mutation may induce conformational changes around the bundle crossing. Since MTSET rates were compared in differ-

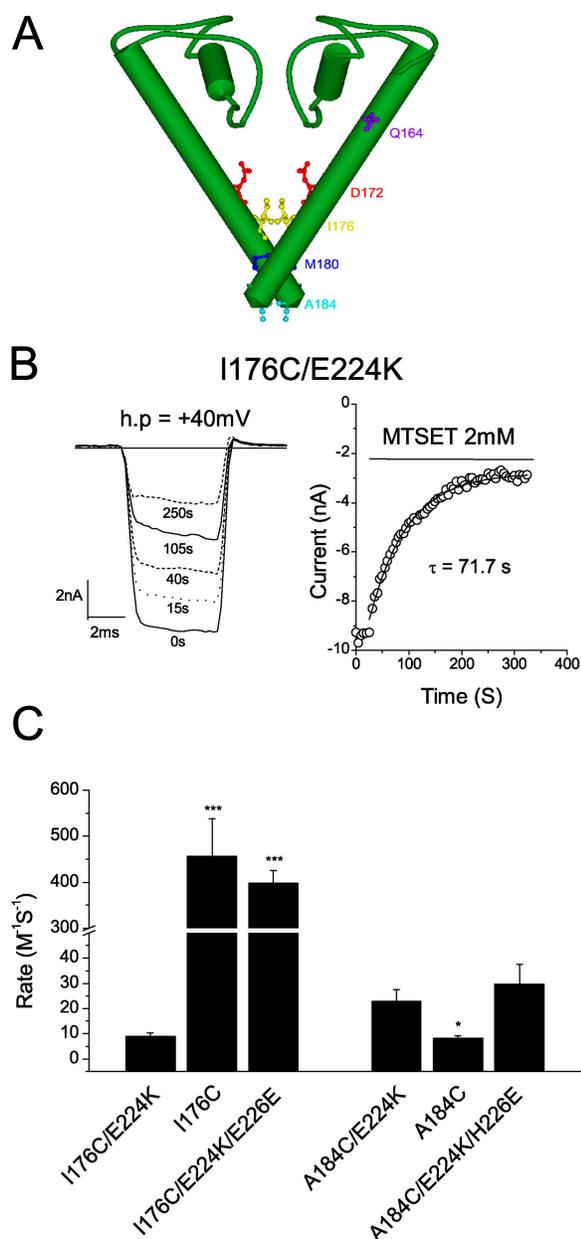


Figure 7. Effects of E224K mutation on MTSET modification to cysteine mutants external and internal to the bundle crossing. (A) A homology model of Kir2.1 structure showing the positions for cysteine mutations. (B) Current traces recorded at -200 mV (5 ms) from the indicated holding potential (pulse frequency 0.2 Hz) in the absence (solid line) and presence (dotted line) of MTSET from the I176C/E224K mutant. (C) MTSET modification rates for various mutants at the indicated holding potential. Currents were measured at the end of the test pulse. $n = 3-6$. *, $P < 0.05$; ***, $P < 0.005$.

ent mutants we cannot conclude whether the conformational changes induced by the E224K mutation result in a V_m -dependent gating involved in the bundle crossing or if they simply change the accessibilities of side chains at sites 176 and 184 to MTSET. Because Q164C/E224K and D172C/E224K did not result in

functional expression we could not examine the effect of E224K mutation on MTSET accessibility to other cysteine mutants located externally to M180.

Next, we further determined whether a V_m -dependent channel closure (e.g., the selectivity filter closure) allosterically controlled by charges at site 224 is involved in the inward rectification induced by spermine interacting with E224 and E299 and the E224K mutant. We examined the effects of $[K^+]_o$ on the I-V relationships of the D172N mutant treated with spermine and the E224K mutant. For a V_m -dependent channel closure, we would expect the gating for inward rectification to be dependent on V_m but not $[K^+]_o$. However, if inward rectification is due to a raised electrostatic potential then the rectification will be dependent on the direction of ion flows and K^+-K^+ interaction in the selectivity filter, which are sensitive to both V_m and $[K^+]_o$ (see DISCUSSION and Fig. 10). Fig. 8 A shows the relative currents (the instantaneous current in 100 μ M spermine divided by that in control) of the wild type and D172N mutant measured in three different $[K^+]_o$. Increases in $[K^+]_o$ shifted the relative currents of both wild type and D172N channels to more positive V_m . Also, the relative currents of the D172N mutant showed steeper V_m dependence at V_m close to E_K but the V_m dependence was less steep compared with the wild-type channel (steeper due to spermine block) (Xie et al., 2002). These results are consistent with asymmetrical effects of local electrostatics on ion permeation through the Kir2.1 channel (see Fig. 10 for discussion), although we cannot rule out that $[K^+]_o$ may also allosterically regulate a V_m -dependent closure of the channel. Detailed analysis will require a quantitative model, similar to that proposed by Xie et al. (2002), that describes the effects of local electrostatics and maybe also K^+-K^+ interaction in the selectivity filter (to account for the steep V_m dependence) on the rectification induced by spermine interacting with E224 and E299. A qualitative model is described in DISCUSSION.

Fig. 8 B shows the I-V and chord conductance (g -V) relationships of the E224K mutant in 100 and 300 mM $[K^+]_o$ recorded from two different patches. Because g -V relationships did not show saturation we could not determine whether $[K^+]_o$ shifts the g -V curves. Therefore, we cannot conclude whether channel closure is involved in the inward rectification of the E224K mutant. However, our data support that the bundle crossing and the selectivity filter are not involved in the inward rectification induced by spermine interacting with E224 and E299, which is functionally important.

High $[K^+]_i$ Increases Conductance but Cannot Completely Correct Inward Rectification in E224K/H226E Mutant

Several studies have suggested that negative charges located in the pore mouths of ion channels may control

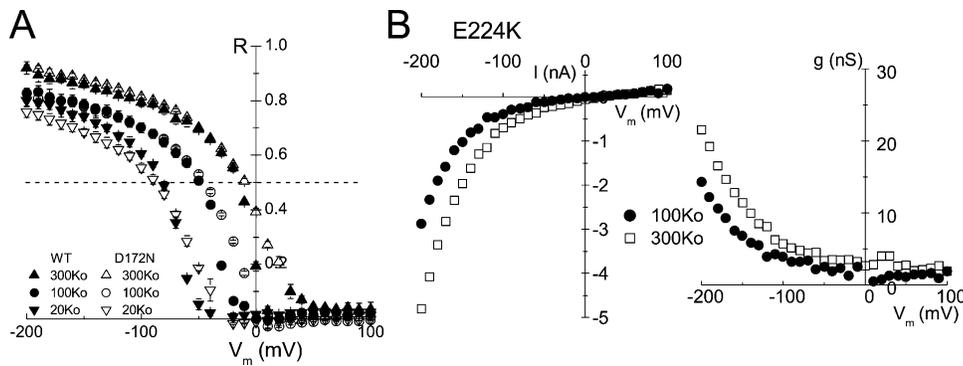


Figure 8. Effects of $[K^+]_o$ on the inward rectification induced by spermine in the D172N mutant and the E224K mutant. (A) Relative currents (R) were measured at the indicated $[K^+]_o$ in 100 mM $[K^+]_i$ with 100 μ M spermine as the inducer of inward rectification. The horizontal line indicates $R = 0.5$. $n = 2-3$. (B) I-V and g-V relationships of the E224K mutant in the indicated $[K^+]_o$.

ion permeation through ion-concentrating and surface charge effects (Chandler et al., 1965; Hille et al., 1975; Dani, 1986; Green et al., 1987; Imoto et al., 1988; Kell and DeFelice, 1988; MacKinnon et al., 1989; Chung et al., 2002; Xie et al., 2002; Brelidze et al., 2003; Nimigean et al., 2003). We next examined the effects of high $[K^+]_i$ on K^+ permeation and inward rectification in the E224K/H226E mutant. Elevating $[K^+]_i$ from 20 to 300 mM increased both inward and outward currents (Fig. 9 A). Also, the degree of inward rectification decreased when $[K^+]_i$ was increased from 20 to 100 mM. Further increasing $[K^+]_i$ to 300 mM did not reduce the extent of inward rectification (Fig. 9 B). These results suggest that in addition to a reduced ion-concentrating effect, another mechanism is involved in the inward rectification of the E224K/H226E mutant. For example, decreasing negative charges in the internal vestibule may also raise local electrostatic potential and produce an asymmetry in free energy barriers controlling ion permeation, thereby giving rise to inward rectification.

DISCUSSION

Local Electrostatics at Site 224 Controls Inward Rectification in Kir2.1 Channels

The mechanism for the inward rectification of the Kir2.1 channel has been attributed to voltage-dependent block. In this study, we show that inward rectification can also be induced by neutral or positive charges at residue 224 in the internal vestibule of tetrameric Kir2.1 channels. We provide several lines of evidence supporting that the inward rectification of the E224G and E224K mutants is due to the electrostatics around the site 224 instead of residual polyamines or Mg^{2+} block. Furthermore, we demonstrate that the M2 bundle crossing, G loop, or the selectivity filter is not completely closed at V_m where K^+ efflux is inhibited by spermine interacting with E224 and E299. Although it remains to be determined whether a V_m -dependent closure of the pore is involved in the inward rectification of the E224K mutation, our results suggest that the

charges at residues 224 may control inward rectification through changing the local electrostatic potential and/or regulating the opening size of the internal vestibule and/or bundle crossing in the Kir2.1 channel.

It has been shown previously that the H226E mutation can correct the inward rectification of the E224Q mutant (Xie et al., 2004). Our preliminary data also show that the H226E mutation can correct the inward rectification of the E224G mutant. However, the inward rectification remains strong in the E224K/H226E mutant. These results together suggest that negative charges at sites 226 can compensate for the electrostatic potential raised by neutral mutation at site 224 but they are not as effective as the negative charges at site 224 in lowering the electrostatic potential at the internal vestibule. The contribution of a charge to local electrostatics in the pore is dependent on the specific location of an amino acid and its surroundings. Further investigations are required to determine the relative contribution of charges to the electrostatics in the internal vestibule.

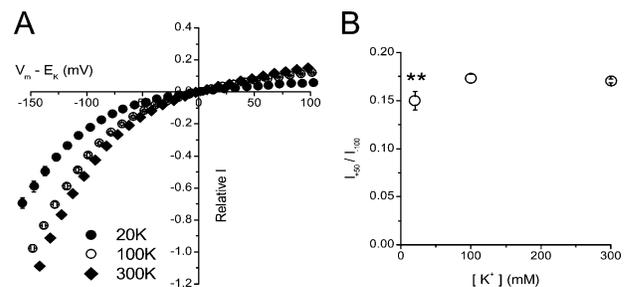


Figure 9. High $[K^+]_i$ increases conductance but cannot correct inward rectification in E224K/H226E mutant. (A) I vs. $(V_m - E_K)$ relationships of the E224K/H226E mutant in the presence of 100 mM external $[K^+]_o$ and various $[K^+]_i$. Currents through the E224K/H226E mutant exposed to 20 mM or 300 mM $[K^+]_i$ were always recorded with currents in 100 mM $[K^+]_i$ in the same patch. Currents were normalized to that in 100 mM $[K^+]_i$ at $V_m - E_K = -150$ mV in the same patch and then averaged. (B) Effects of $[K^+]_i$ on inward rectification. The extent of inward rectification is estimated by the ratio of current at $V_m - E_K = +50$ mV to that at -100 mV. $n = 5-11$. **, $P < 0.01$.

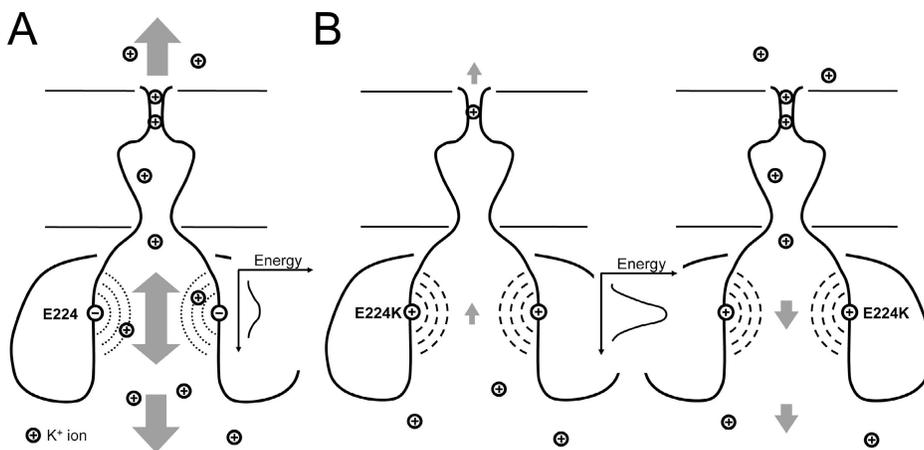


Figure 10. Schematic model for the effects of charges at site 224 on inward rectification. (A) Negative charges at site 224 guide internal K^+ entry to the internal pore and lower local electrostatics, thereby facilitating K^+ influx and efflux. (B, left) Positive charges at site 224 fail to guide internal K^+ entry, raise local energy barrier, and reduce K^+ - K^+ interaction in the selectivity filter. As a result, K^+ efflux is inhibited. (B, right) External K^+ entry and K^+ - K^+ interaction are not affected by K^+ influx. The high energy barrier at the internal vestibule limits K^+ influx. Arrow denotes K^+ fluxes. The larger the arrow is the greater the flux is.

Hypothesis for Inward Rectification Induced by Charges at Site 224

Charged residues at the pore mouth can guide the permeant ions from the bulk solution into the channel vestibule (MacKinnon et al., 1989) and serve as an energy barrier (Miloshevsky and Jordan, 2004). Also, negative charges located in the pore mouths of many channel types may control conductance and gating through ion-accumulating and surface charge effects (Chandler et al., 1965; Hille et al., 1975; Dani, 1986; Green et al., 1987; Imoto et al., 1988; Kell and DeFelice, 1988; MacKinnon et al., 1989; Chung et al., 2002; Xie et al., 2002; Brelidze et al., 2003; Nimigeon et al., 2003). Because high $[K^+]_i$ increases K^+ conductance without efficiently correcting inward rectification in the E224K/H226E mutant, we propose that in addition to ion-concentrating effect the local electrostatics at site 224 also serves as an energy barrier for K^+ conductance in the Kir2.1 channel. It is also possible that spermine interacting with E224 and E299 as well as an increase in local electrostatic potential may induce conformational changes resulting in a narrowing in the internal vestibule and/or bundle crossing and thereby reduce K^+ permeation. Both possibilities are consistent with the finding that spermine decreased Ag^+ modification in the D172N/I176C mutant. Fig. 10 illustrates how an increase in local electrostatic potential in the internal vestibule can produce inward rectification. A similar concept can be applied to reduced K^+ permeation induced by narrowing the internal vestibule and/or bundle crossing. Fig. 10 A shows that negative charges at residue 224 may steer K^+ ions into the pore mouth and decrease the energy barrier for K^+ to transport between the internal vestibule and the water cavity. On the other hand, neutral and positive charges at site 224 are not efficient in attracting internal K^+ ions and they raise the local electrostatic potential, thereby increasing the energy barrier (Fig. 10 B). The experiments of Ag^+ modification suggest that K^+ should still be able to

transport from the internal space to the water cavity, although with a reduced rate, when the local electrostatic potential is high in the internal vestibule. We further propose that K^+ - K^+ interaction in the selectivity is also affected. For example, when the rate of K^+ entering the pore is reduced, the selectivity filter is seldom occupied by two K^+ ions, resulting in a dramatic decrease in K^+ exit (Fig. 10 B, left) (Morais-Cabral et al., 2001). On the other hand, external K^+ entering the selectivity filter is affected to a smaller extent by the electrostatics at site 224 (Fig. 10 B, right). Therefore, a K^+ ion can still efficiently exit the selectivity filter into the water cavity through K^+ - K^+ interaction. According to the structure of the KcsA channel (Doyle et al., 1998), the concentration of one K^+ ion in the water cavity is ~ 1 M. This high K^+ gradient energetically forces the K^+ ion in the water cavity to pass through the high energy barrier at the internal vestibule. However, at V_m negative but close to E_K , K^+ - K^+ interaction in the selectivity filter is less efficient and thus K^+ influx is small. When V_m becomes more hyperpolarized, the high electrical energy in the selectivity will facilitate K^+ entering the water cavity, thereby increasing the chance of K^+ ions overcoming the high energy barrier. These processes may account for the steep V_m dependence of the relative currents of the D172N mutant at V_m close to E_K (Fig. 8 A). In summary, the raised electrostatic potential reduces both the entry and exit rates to larger degrees for K^+ efflux than influx and thereby induces inward rectification.

Functional Implication

E224 and E299 have been shown to be involved in the inward rectification of the Kir2.1 channel (Yang et al., 1995; Kubo and Murata, 2001). It has been proposed that these two residues serve as intermediate binding sites facilitating the transport of internal blockers between the internal space and the final pore-blocking site located deeper in the pore (Lee et al., 1999; Kubo

and Murata, 2001). It has also been demonstrated that polyamines screening the charges of E224 and E299 reduce single-channel amplitude at negative V_m (Xie et al., 2002). However, it is unknown whether polyamines interacting with E224 and E299 can directly contribute to inward rectification. Also, it is not clear whether the charge-screening effect of polyamines directly contributes to inward rectification, as the effect on inward rectification may be obscured by V_m -dependent block. Fig. 3 shows that spermine induces inward rectification but does not occlude the pore with the D172N mutation. Assuming that the spermine-induced inward rectification in the T141C/D172N mutant is due to a direct interaction of spermine with E224 and E299, we suggest that Mg^{2+} and polyamines may control the extents of inward rectification by screening charges (neutralization of the negative charges will induce intermediate inward rectification similar to the E224G mutant) or by adding positive charges (strong inward rectification similar to the E224K mutant) in the internal vestibule.

Conclusion

In this study we describe inward rectification modulated by the charges and local electrostatics at site 224 in the internal vestibule of the Kir2.1 channel. This mechanism is distinct from the prevalent channel block. The M2 bundle crossing, G loop, or the selectivity filter is not completely closed in the inward rectification induced by spermine interacting with E224 and E299. We propose that the neutral and positive charges at site 224 increase the local energy barrier, which reduces K^+ entry and exit rates to a larger degree for K^+ efflux than influx, thereby resulting in inward rectification.

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