Molecular Basis of Inward Rectification: Polyamine Interaction Sites Located by Combined Channel and Ligand Mutagenesis

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ABSTRACT Polyamines cause inward rectification of (Kir) K^+ channels, but the mechanism is controversial. We employed scanning mutagenesis of Kir6.2, and a structural series of blocking diamines, to combinatorially examine the role of both channel and blocker charges. We find that introduced glutamates at any pore-facing residue in the inner cavity, up to and including the entrance to the selectivity filter, can confer strong rectification. As these negative charges are moved higher (toward the selectivity filter), or lower (toward the cytoplasm), they preferentially enhance the potency of block by shorter, or longer, diamines, respectively. $MTSEA^+$ modification of engineered cysteines in the inner cavity reduces rectification, but modification below the inner cavity slows spermine entry and exit, without changing steady-state rectification. The data provide a coherent explanation of classical strong rectification as the result of polyamine block in the inner cavity and selectivity filter.

KEY WORDS: inward rectifier • spermine • diamine • rectification • selectivity filter

INTRODUCTION

Sir Bernard Katz discovered inward rectification of potassium channels over 50 yr ago (Katz, 1949). The defining features of classical strong inward rectification, very steep voltage dependence and strict dependence on external $[K^+]$ (Hagiwara and Takahashi, 1974; Hagiwara and Yoshii, 1979), underlie multiple physiological phenomena, including stable electrical activity in the heart and neuronal cells, potassium siphoning in glia, and blood $[K^+]$ control of vascular tone (Newman, 1993; von Beckerath et al., 1996; Lopatin and Nichols, 2001). The realization, almost 10 yr ago, that naturally occurring polyamines (spermine, spermidine, and putrescine) are responsible for strong rectification immediately led us to propose that these agents induce rectification by "long pore plugging," in which polyamines enter deeply into the single filing region of the channel in competition with K^+ ions (Nichols and Lopatin, 1997). Such a mechanism could intuitively underlie the steepness of the voltage dependence of pore block, particularly if K^+ ions were obligatorily displaced through the pore, and hence through the membrane field, in the blocking process (Ruppersberg et al., 1994; Pearson and Nichols, 1998; Guo et al., 2003). Furthermore, this mechanism could give rise to a strict dependence on external $[K^+]$ if polyamines reached sites that were directly sensitive to binding of K^+ ions from the outside of the membrane (Park and Miller, 1992; Lopatin and Nichols, 1996).

Molecular modeling of the Kir pore, using the crystal structures of MthK and KcsA as templates, has recently led us to propose that strong rectification may result from polyamine interaction within the selectivity filter itself (Dibb et al., 2003; Rose and Nichols, 2003). We propose that negative charges in the inner cavity, which control the strength of rectification (Nichols and Lopatin, 1997), act by reducing the energetic barrier to entry into the selectivity filter, and that excess charge movement (i.e., greater than the charge on the blocking polyamine) is then due to the obligatory displacement of K^+ ions from inner cavity and selectivity filter sites to the outside of the membrane (Rose and Nichols, 2003). Guo and colleagues (Guo and Lu, 2003; Guo et al., 2003) have recently proposed a quite different location of polyamine block during strong rectification.

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Considering diamine and monoamine block of Kir2.1 in detail, they proposed that the single amine of monoamines and one amine of diamines actually interacts with E224/E299 in the cytoplasmic vestibule, the remainder of the molecule protruding into the inner cavity toward D172 (Guo et al., 2003). It is not intuitively clear why such a "tail-first" binding of monoamines would occur, since block is voltage dependent, and the "head" amine might be expected to drag the "tail" into the field. Nevertheless, Guo and colleagues (Guo and Lu, 2003; Guo et al., 2003) raise two lines of argument in support of this arrangement. First, such an arrangement would place the head amine of long diamines in the vicinity of residue D172, thereby accounting for an observed energetic coupling between D172 and 1,9 diamino-nonane (DA9), but lack of coupling to the hydrophobic end of the monoamine alkyl chain in MA9. Second, this arrangement would move increasing numbers of K^+ ions through the pore from a column of ions extending from the selectivity filter through the inner cavity into the cytoplasmic pore. This implicitly requires that K^+ ions move in single file through the inner cavity and are obligatorily moved forward as the alkylamine enters the bottom of the pore. However, there is as yet no structural evidence for any ordering or restriction of ion movement in this region, and such ordering might be unlikely given the expected width (>10 Å) of the cavity (Doyle et al., 1998; Kuo et al., 2003). Instead, our proposed model directly predicts that the increase in K^+ ion movement with chain length arises from the progressive movement of longer alkylamines into the selectivity filter itself (Rose and Nichols, 2003).

In the present experiments, we have systematically examined the interaction of multiple diamines with charges placed throughout the Kir6.2 channel pore. By extensively scanning the parameter space, we show that introduction of negative charges at sites throughout the inner cavity, even immediately beneath the "GY/FG" sequence of the selectivity filter, can confer strong rectification. The energetic coupling between diamines of different chain length and the position of the introduced negative charge tends to increase for shorter diamines as the charge is moved closer to the selectivity filter, and for longer diamines as the charge is moved lower down, toward the cytoplasm. We further introduced positive charge by MTSEA$^+$ modification of cysteines in the inner cavity and the cytoplasmic pore. Modification of residues at the bottom of the inner cavity or below reduces spermine entry and exit rates but does not significantly change steady-state rectification. By contrast, modification of residues at deeper sites (i.e., closer to the selectivity filter) reduces, or abolishes, strong rectification. The data provide no evidence for a binding site involving the cytoplasmic pore, nor for specific interactions between polyamine/diamine charges and negative charges

in the inner cavity. Instead, they implicate the selectivity filter as the location of long-pore plugging during strong rectification (Lopatin et al., 1995).

MATERIALS AND METHODS

Expression of KATP Channels in COSm6 Cells and Xenopus Oocytes

Experimental methods are described in detail in previous publications (Loussouarn et al., 2000; Schulze et al., 2003). Point mutations were prepared by overlap extension at the junctions of relevant residues by sequential PCR as described. Dimers of Kir6.2 subunits were generated with a six glycine residue linker between the NH$_2$ terminus of a Kir6.2[N160D, C166S] subunit and the COOH terminus of another Kir6.2 subunit [N160D, C166S]. Importantly, the C166S mutation is not required for Kir6.2 to exhibit strong rectification and is included in some of our experiments, only to prevent nonspecific effects of MTSEA modification. An earlier study (Loussouarn et al., 2000) demonstrated Cd^{2+} accessibility of residue C166, and so the C166S mutation ensures that effects of MTSEA modification can be attributed specifically to introduced cysteine residues.

COSm6 cells were transfected with pCMV6b-Kir6.2 (with mutations as described), pECE-SUR1, and pGreenLantern (GIBCO BRL), as previously described (Loussouarn et al., 2000, 2001; Phillips et al., 2003). Patch-clamp experiments were made at room temperature, in a chamber that allowed the solution bathing the exposed surface of the isolated patch to be changed rapidly. Data were normally filtered at 0.5–2 kHz, and signals were digitized at 5 kHz and stored directly on a computer hard drive using Clampex software (Axon Instruments, Inc.). The standard pipette (extracellular) and bath (cytoplasmic) solution used in these experiments had the following composition: 140 mM KCl, 1 mM K-EGTA, 1 mM K-EDTA, 4 mM K$_2$HPO$_4$, pH 7 (Guo and Lu, 2000). All polyamines and diamines were purchased from Fluka AG. MTSEA (Toronto Research Chemicals) was dissolved in the standard recording solution on the day of experiments, to make a 10 mM stock, which was stored on ice. Further dilutions to 1 mM or 100 μM were used for channel modification, and unreacted excess MTSEA was washed off before recording. Off-line analysis was performed using Fetchan, pSTAT (Axon Instruments, Inc.), and Microsoft Excel programs. Wherever possible, data are presented as mean ± SEM. Microsoft Solver was used to fit data by least-square algorithm.

For oocyte expression, constructs were subcloned into the pBF expression vector. Capped cRNAs were synthesized in vitro using SP6 polymerase (Promega) and stored in stock solutions at $-70°$C. *Xenopus* oocytes were surgically removed from adult females and manually dissected. About 50 nl of a solution containing cRNA specific for SUR1 and Kir6.2 subunits was injected into Dumont stage VI oocytes. Oocytes were treated with collagenase type II (0.5 mg/ml; Sigma-Aldrich) and incubated at 19°C for 1–3 d and defolliculated before use. Giant patch recordings in inside-out configuration under voltage-clamp conditions were made at room temperature 3–7 d after cRNA injection. Pipettes were made from thick-walled borosilicate glass, had resistances of 0.2–0.4 MΩ (tip diameter of 20–30 μm), and were filled with (in mM, pH adjusted to 7.2 with KOH) 120 KCl, 10 HEPES, and 1.8 CaCl$_2$. Currents were recorded with an EPC9 amplifier (HEKA Electronics) and sampled at 1 kHz with analogue filter set to 3 kHz. Solutions were applied to the cytoplasmic side of excised patches via a multibarrel pipette and had the following composition in mM (Kint): 100 KCl, 10 HEPES, 2 K$_2$ EGTA (total K concentration was 120 mM, pH adjusted to 7.2 with KOH). Data

Figure 1. The inner cavity of Kir channels. (A) Selected sequence alignment of the pore (P) M2 region and cytoplasmic vestibule-lining region of Kir2.1, Kir6.2, and KirBac1.1. Identical residues in all three sequences are in red. Residues in bold were mutated to glutamates in this study. (B) Structure of Kirbac1.1, illustrating the results of scanning glutamate mutagenesis of Kir6.2 (see data in Fig. 2). Side chains of residues equivalent to those that induce strong spermine rectification, when glutamate is introduced, are indicated in red. Residues equivalent to those that do not affect rectification when glutamate is introduced are indicated in green. Residues are numbered according to Kirbac1.1 (and Kir6.2 in parentheses). Residue 151 (equivalent to Q173 in Kir6.2) is colored orange to indicate the intermediate rectification that is induced when mutated to glutamate.

analysis was done on a Macintosh G4 computer using IGOR (WaveMetrics) and Excel 2001 (Microsoft).

RESULTS

Scanning Mutagenesis Reveals Extent of Possible Locations of the "Rectification Controller"

Wild-type (WT) Kir6.2 contains no negative charges in M2 and encodes ATP-sensitive K^+ channels with only very weak rectification. However, introduction of either glutamate or aspartate at residue 160 (equivalent to the "rectification controller" residue 172 in the classic strong inward rectifier Kir2.1) results in channels that rectify essentially as strongly as Kir2.1 (Shyng et al.,

1997). The equivalent residue is aspartate in Kir2.1, 2.3, 2.4, and Kir3.1, asparigine in Kir1, Kir2.2, Kir3.2, 3.3, 3.4, Kir5, and Kir6, but is glutamate in strong rectifying Kir4.2, and in Kir5.1 and Kir7.1. We have employed the Kir6.2 channel in the present study for several technical reasons (Loussouarn et al., 2000). Most importantly, this channel is very stable in excised patches (unlike Kir2.1) and permits ATP inhibition to be used for accurate determination of channel-specific currents. The WT Kir 6.2 background provides the advantage of not rectifying, therefore allowing the introduction of negative charge to be directly correlated with the appearance of rectification.

To systematically examine the role of M2 negative charge in controlling rectification, we introduced glutamate residues throughout the M2 helix of Kir6.2 (Fig. 1). We initially assessed the sensitivity to spermine in inside-out patches using ramp protocols (Fig. 2 A). At certain positions, the introduced negative charge causes strong spermine block, but is without effect at other positions (Fig. 2 A). Sites where glutamate substitution results in strong rectification correspond exactly with previously identified pore-lining residues in Kir6.2 (Loussouarn et al., 2000). It is clear from this analysis that each M2 mutation has an essentially all-or-nothing effect (Fig. 1 B and Fig. 2); strong rectification is induced by introduction of negative charge at all pore-facing residues in the inner cavity, and no rectification is induced at residues facing away from the pore. The strength of spermine-induced rectification only begins to substantially change from this all-or-nothing nature when negative charges are introduced at position 172 located in the linker region immediately below the M2 helix "bundle crossing" that defines the cytoplasmic end of the inner cavity (Fig. 1 B).

In Fig. 1 B, we have indicated the likely location of introduced mutations on the structure of KirBac1.1, an archeabacterial Kir homologue (Kuo et al., 2003). Residues that are predicted to face the pore generate very similar spermine sensitivity, from residue 168, located at the M2 helix "bundle crossing" all the way to residue 156 beneath the selectivity filter. Strikingly, the introduction of a glutamate at two additional residues (V129 or T130), in the P-loop itself, also generates high spermine sensitivity (Fig. 2). There is some variability in both the steepness and potency of block that is induced by the different mutations (Fig. 2), but the clear implication of this initial scan is that strong rectification can be induced by introduction of negative charge at essentially any pore-facing residue in the inner cavity (Fig. 2 C). It is intriguing that we were able to detect currents from the G156E mutant of Kir6.2, as this position corresponds to the "glycine hinge" proposed to underlie activation gating in many cation channels (Jiang et al., 2002). Also, glutamates that do not face the pore are well tolerated
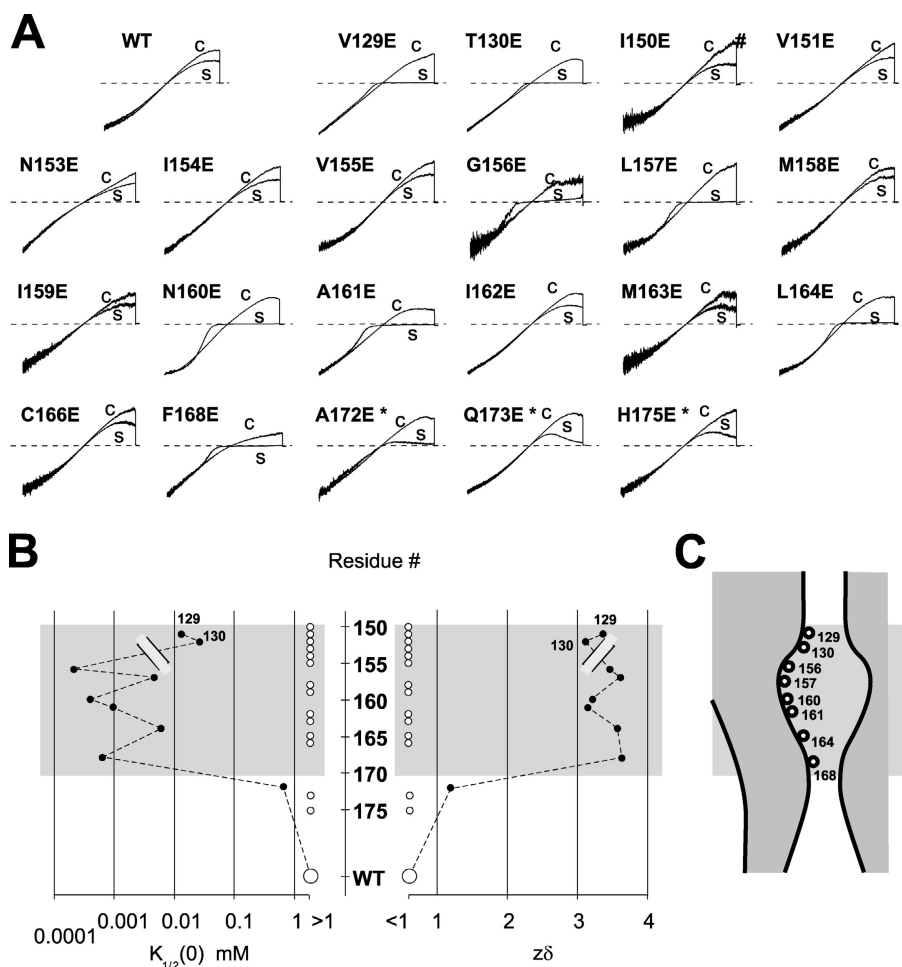
FIGURE 2. All-or-nothing nature of glutamate-induced rectification in the inner cavity. (A) Currents in response to a voltage ramp from −100 to +100 mV are shown from inside-out membrane patches excised from *Xenopus* oocytes in the presence and absence of 100 μM spermine. Data from WT and mutant channels, as indicated, are normalized to current at −100 mV. (B) $K_{1/2}(0)$ and $z\delta$ for spermine block of WT and mutant channels, obtained from I–V relationships as in A (current in spermine/control current, fitted with single Boltzmann functions). White circles denote WT and mutant channels with $z\delta < 1$ and $K_{1/2}(0) > 1$ mM. (C) Cartoon illustrating approximate physical location of the residues in the inner cavity and selectivity filter that induce strong rectification.

and generate functional channels in almost every case, but have no influence on spermine block.

Introduction of Negative Charge in the Inner Cavity Enhances Diamine Block

In Kir2.1 channels, linear (1,n)-diaminoalkanes (DAn) are potent blockers (Pearson and Nichols, 1998). As alkyl chain length increases, both potency and voltage dependence of block increase, and there is similar potency and voltage dependence for block by polyamines and diamines of equivalent molecular length (Pearson and Nichols, 1998; Guo and Lu, 2003; Xie et al., 2003).

We examined diamine block of WT Kir6.2 and of mutants with glutamate residues introduced throughout the inner cavity (V129E, L157E, N160E, L164E, and F168E) (Figs. 3 and 4). The first important observation is that WT channels, which lack any M2 negative charge, are quite sensitive to longer diamines, even though they are essentially insensitive to spermine (Fig. 4 A). DA12 blocks WT channels with $z\delta \sim 1.6$ and $K_{1/2}(0) \sim 200$ μM. This suggests that while introduction of an M2 negative charge increases potency and voltage dependence of block by both diamines and sper-

mine, it has a proportionally much greater effect on spermine block than on DA12. One interpretation of this finding is that the internal charges of spermine actually limit its blocking ability (versus DA12) in the absence of M2 charges, and that hydrophobic interactions between long diamines and pore-lining residues underlie the stronger interaction of DA12.

Nevertheless, the introduction of negative charges at all five of the positions examined in detail does substantially increase both potency and voltage dependence of all diamines (Figs. 3 and 4). In each case, the data are generally well fit by single Boltzmann functions (Fig. 3, B and C; Fig. 4) with a similar small offset (Fig. 5 C), with the single exception of F168E (Fig. 4 A). In this case, block by short diamines was very weak, requiring considerably larger offsets to fit the data (Fig. 4 A), a point considered further below.

As diamine length increases, the potency of block increases, in both mutant and WT channels (Fig. 5 B). This behavior is qualitatively similar to that observed in Kir2.1 and Kir2.1[D172N] mutant channels (Pearson and Nichols, 1998; Guo et al., 2003; Xie et al., 2003). Unlike Kir2.1, the valence of block ($z\delta$) does not sys-
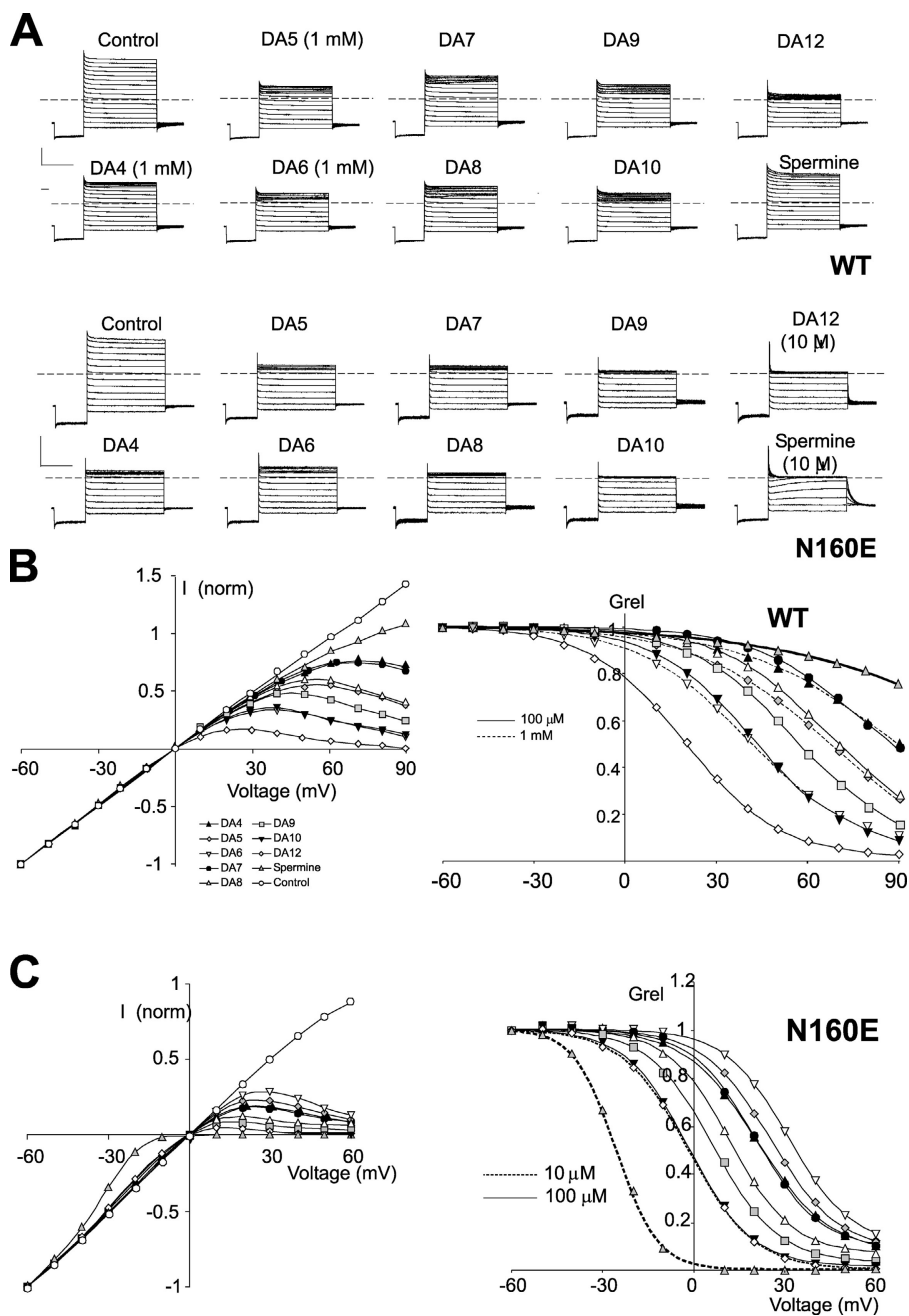
FIGURE 3. Potency of diamine block of Kir6.2 increases with chain length. (A) Representative currents from voltage step protocols illustrating currents in the absence of any diamine or polyamine (Control), and rectification induced by spermine and diamines of different alkyl chain length in WT and N160E mutant channels. In each case, the scale bar represents 200 ms and 3 nA. Diamines and polyamines were applied at 100 μM, unless otherwise indicated. (B and C) Representative steady-state current–voltage relationships (left), and relative conductance (Grel)–voltage relationships (right). Grel–V curves were fit with single Boltzmann functions, with an offset for nonblocked current (see Fig. 5).

tematically increase with diamine length. Quantitatively, the limiting voltage dependence of block in Kir6.2 mutants ($z\delta$ = 2–3, 4 for spermine; Fig. 5 A) is also lower than that observed in Kir2.1 channels (limiting $z\delta \sim$ 4 [Xie et al., 2003] to 5 [Guo et al., 2003]), but is still higher than can be accounted for by movement of the blocker itself into the field.

Energetic Mutant Cycle Analysis Reveals Complex Interactions of Diamines with Introduced Negative Charges

Intriguingly, Guo and colleagues (Guo and Lu, 2003; Guo et al., 2003) demonstrated a maximum energetic

contribution of the negatively charged aspartate at residue 172 in Kir2.1 for block by DA9, with an increasingly lower energetic contribution as chain length either increased or decreased from 9, leading them to propose a specific interaction between one of the amines (specifically the head amine) in DA9 and the charge at residue 172.

We have performed similar thermodynamic mutant cycle analyses using diamines of varying length to characterize interactions with introduced acidic residues in Kir6.2. We computed the energetic coupling coefficient (Hidalgo and MacKinnon, 1995), using $K_{1/2}$ (0 mV) of the WT and a mutant channel for a control di-
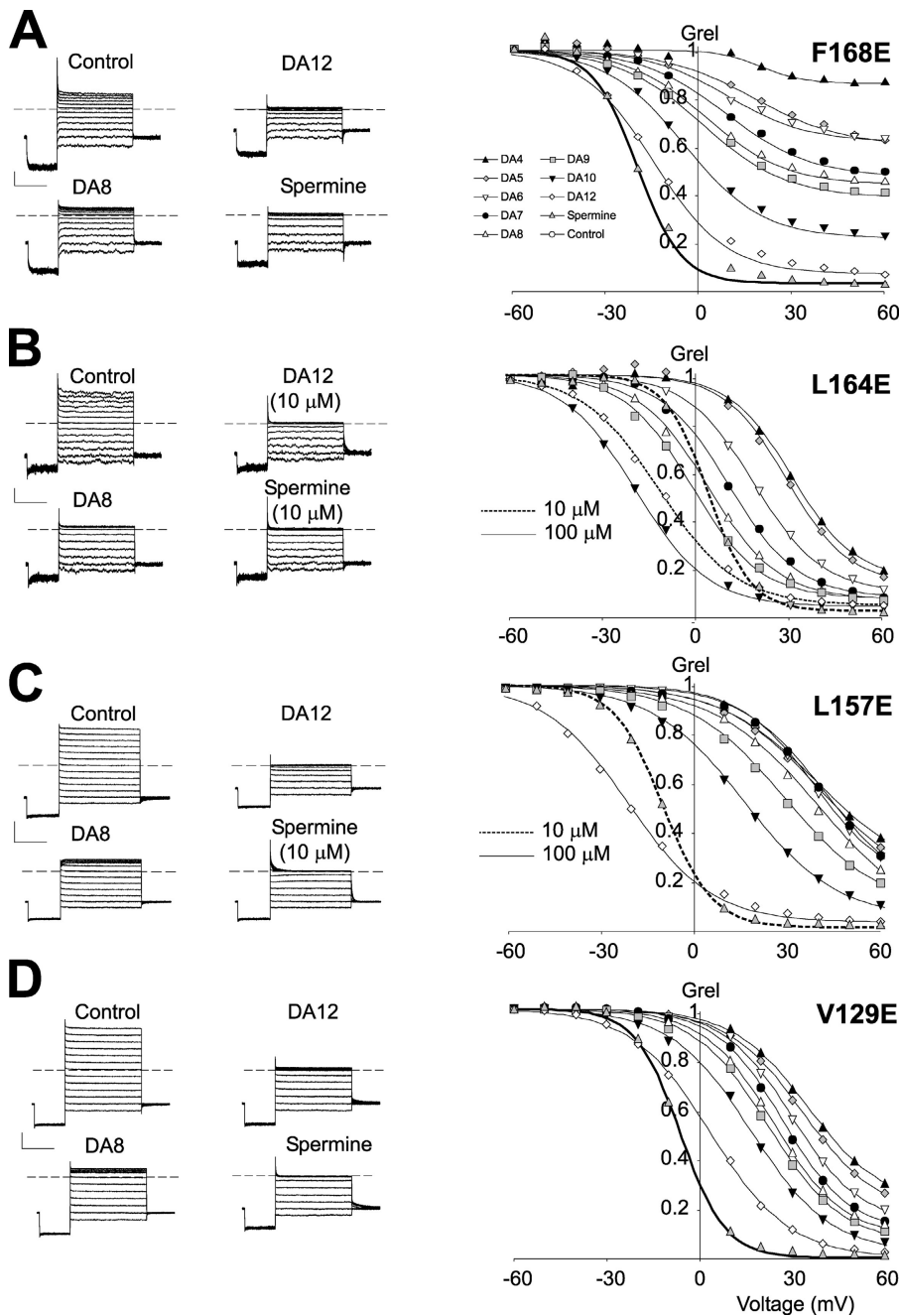
FIGURE 4. Potency of diamine block depends on glutamate location. (A–D) Left, representative currents from voltage step protocols illustrating currents in the absence of diamine/polyamine (Control), and rectification induced by spermine and diamines of different alkyl chain length in glutamate mutant channels (left). Scale bar represents 200 ms and 0.5 nA (A and B) or 2 nA (C and D). Diamines and polyamines were applied at 100 μM, unless otherwise indicated. Right, relative conductance (Grel)–voltage relationships, obtained as in Fig. 3, were fit with single Boltzmann functions, with an offset for nonblocked current (see Fig. 5).

amine (DA4 in Fig. 5) and for a given test diamine (DAn) ($[^{mt}K_{1/2}^{DA4} \times {}^{wt}K_{1/2}^{DAn}]/[^{wt}K_{1/2}^{DA4} \times {}^{mt}K_{1/2}^{DAn}]$). The results are presented in terms of the free energy difference between the interaction of DA4 with a particular channel residue and the interaction of a test DAn with that same residue, and we refer to Ω as the interaction energy, which can be positive or negative, implying that the test diamine interacts with a given residue more ($\Omega > 1$) or less ($\Omega < 1$) strongly than the "control" diamine (DA4).

Since we can get comparable induction of strong rectification by negative charge at residues through-

out the inner cavity, we can examine the energetic coupling between diamines and charges throughout the cavity. This allows us to systematically test the two contrasting hypotheses that (1) the location of diamine block is fixed with the tail at the bottom of the cavity, and the head extending upwards (Guo and Lu, 2003), or (2) the location of diamine block is fixed with the head at or in the selectivity filter and the tail extending backward into the inner cavity (Dibb et al., 2003; Rose and Nichols, 2003). In the first case, we would expect to see increased coupling to diamines of longer length as the charge moves upwards,
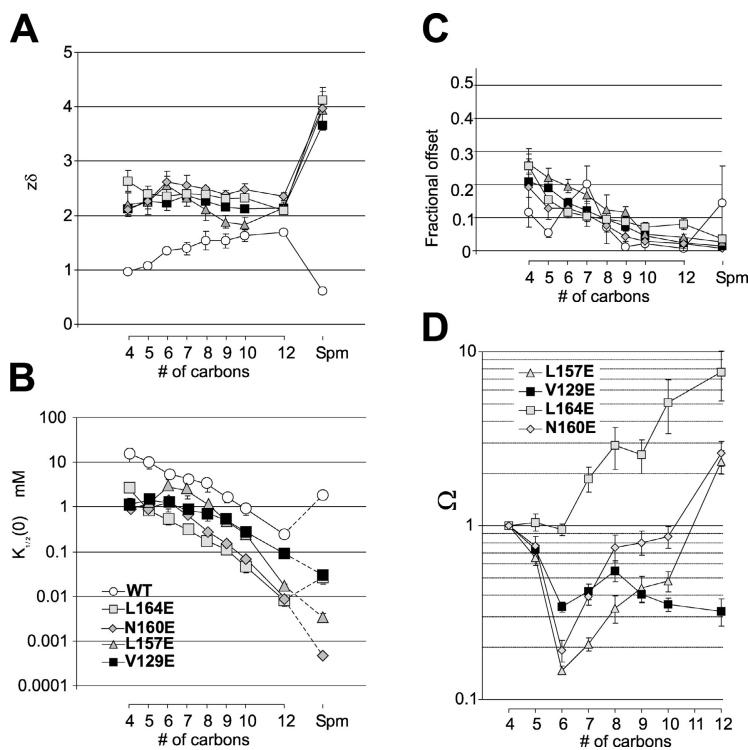
FIGURE 5. Energetic coupling of diamines with introduced glutamates, depends on location. (A–C) Averaged (mean ± SEM, $n = 4$–8) values of (A) zδ, (B) K$_{1/2}$(0), and (C) unblocked offset, obtained from fitted Grel–voltage relationships from experiments like those shown in Figs. 3 and 4 for WT and mutant channels, as indicated. For this analysis, blockade of glutamate mutants was examined using diamine concentrations of 10 or 100 μM, and a spermine concentration of 10 or 100 μM. Blockade of WT Kir6.2 channels was examined using diamine concentrations of 100 μM (for DA7 and longer diamines) or 1 mM (for DA6 and shorter diamines, and spermine). (D) Thermodynamic cycle analysis of energetic coupling between diamines of different length and introduced glutamates, relative to the glutamate-less WT channels, using K$_{1/2}$(0) values from B. Shown are Ω values computed for different length DAn compounds with respect to DA4 (see text).

whereas the opposite result is expected (i.e., decreased energetic coupling to diamines of longer length as the charge is moved upwards) in the second case. Fig. 5 D shows the interaction energy for each diamine with each of the tested residues in the inner cavity. While we do not observe any monotonic function of chain length for intermediate locations (L157E and N160E; c.f. Guo and Lu, 2003), there are very clearly opposing trends in the two most separated residues (V129E and L164E); i.e., in the V129E mutant, energetic coupling grows weaker with progressively longer diamines, whereas the L164E mutant exhibited considerably stronger coupling with progressively longer diamines. A simple interpretation of these findings is that the head amine binds at or in the selectivity filter and the tail amine occupies the inner cavity. For some of the mutants characterized here, we also observed a "well" in the energetic coupling at DA6 (Fig. 5 D). This feature is especially prominent in the L157E and N160E channel mutants, although at present we are unsure of the significance of this observation. We did not perform the analysis on F168E, because this mutant exhibited significant intrinsic rectification properties (Fig. 4 A, control) with a very large offset in the G–V curves for short diamines (Fig. 4 A) that complicates the analysis of blocking potency. However, while this mutant clearly stabilizes block by spermine and long diamines, the effect on block by short diamines is minimal, again most consistent with the second hypothesis.

Potency of Spermine Block Can Depend on Net Charge in the Inner Cavity

While examining the consequences of MTSEA$^+$ modification of channels with introduced cysteines (Loussouarn et al., 2001), we observed that modification of residues in the vicinity of residue 160 led to substantial reduction of spermine block. At some residues in the inner cavity, modification of cysteines in a homotetrameric channel reduces single channel conductance to such an extent that macroscopic currents cannot be reliably recorded (Phillips et al., 2003). To circumvent this problem, we have introduced cysteines to the inner cavity of only one half of a dimeric channel formed from two Kir6.2[N160D, C166S] subunits linked end-to-end with a hexaglycine linker (see MATERIALS AND METHODS), and characterized spermine block before and after MTSEA$^+$ modification of the two cysteines that are present in the resulting channels.

At five positions tested in detail, the potency, voltage dependence, and kinetics of spermine block are essentially unaltered by introduction of cysteines into the rear half of the dimer construct (Figs. 6 and 7). After complete modification with MTSEA$^+$, the current was reduced to a variable extent in each case (129C, 43%; 157C, 24 ± 2%, $n = 3$; 164C, 53 ± 1%, $n = 3$; 169C, 50 ± 2%, $n = 3$; 212C, 59 ± 1%, $n = 4$). Although current reduction after MTSEA$^+$ application suggests that cysteine modification has occurred, we cannot be sure whether both cysteines in the channel have reacted, or whether reaction with one cysteine somehow inhibits
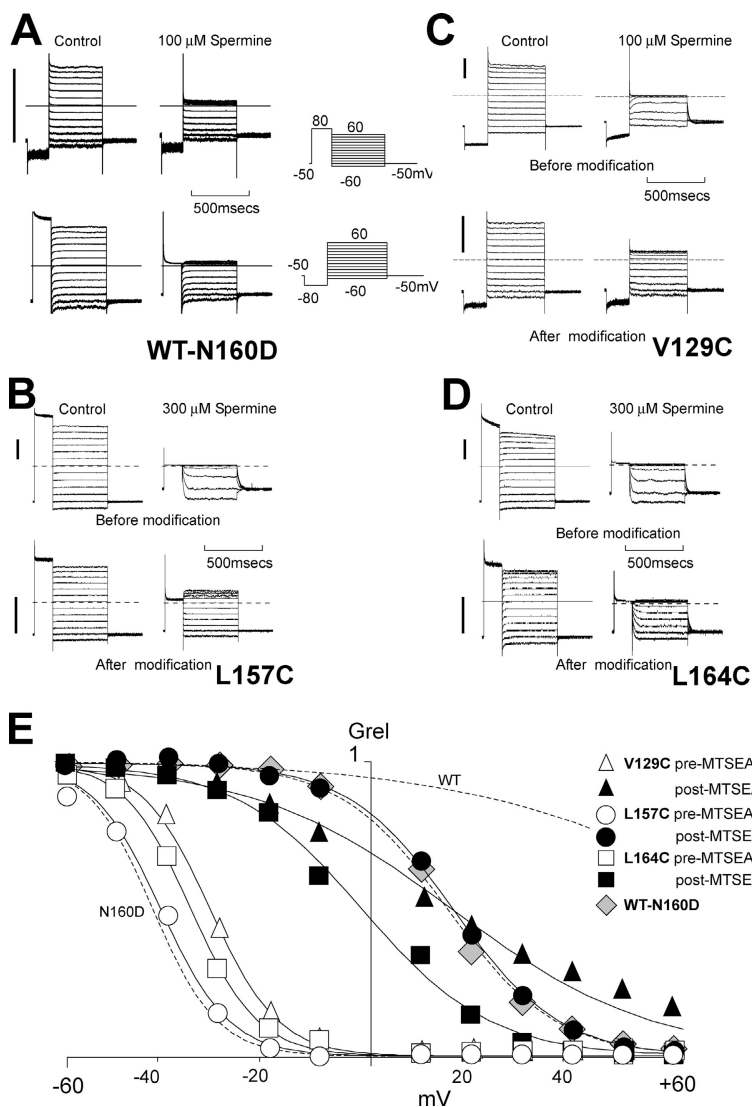
FIGURE 6. In situ introduction of positive charge to the inner cavity decreases spermine-dependent rectification. (A) Representative currents illustrating rectification induced by spermine in WT-N160D dimer channels. Two voltage protocols are indicated. (B) Representative currents illustrating rectification induced by spermine in WT-N160D, and in V129C, L157C, and L164C mutant channels (mutation in second half of N160D-N160D dimer background), before and after complete modification by MTSEA[+] (modification not shown). Voltage protocols as in A. Current scale indicates 0.5 nA in each case. (C) Steady-state current in spermine relative to control (Grel) plotted versus voltage, fitted with Boltzmann functions (no offset) as indicated.

MTSEA[+] modification at a second site in the channel. Given that each subunit in the dimer contains a negatively charged aspartate residue at position 160, complete modification with MTSEA[+] at two cysteine residues would result in a net charge of -2 in the inner cavity, whereas modification of a single cysteine would result in a net charge of -3. Modification with MTSEA[+] substantially altered the rectification properties in each case. While the effects are most likely due to electrostatic interactions between the positively charged ethylamine groups and the blocking polyamine, we cannot yet rule out steric effects of the MTSEA modification. Most importantly, modification altered rectification in very different ways depending on the modification site. As described below, at locations above N160D (L157C and V129C), modification by MTSEA[+] greatly decreases spermine sensitivity, generating steady-state rectification intermediate between that of WT and N160D channels (Fig. 6, B and C).

Mixed expression of WT and N160D mutant Kir6.2 subunits generates multiple channels with different spermine sensitivities, depending on the number of incorporated N160D subunits (Shyng and Nichols, 1997). To examine the rectification properties of a channel with only two negative charges in the inner cavity, we also engineered a dimeric construct consisting of an N160D subunit linked at the COOH terminus to the NH$_2$ terminus of a WT subunit by a hexaglycine linker. Rectification of the WT-N160D channel was intermediate between WT and tetrameric N160D (Fig. 6, A and E), and very similar to that of the MTSEA[+]-modified L157C (Fig. 6, B and E), implying that the block characteristics depend essentially on the net charge in that region. At residue V129C, the change in K$_{1/2}$ was similar to that for L157C, but in addition, the voltage dependence was substantially reduced. At residue L164C, one turn of the M2 helix below N160D, there was less reduction of block potency, and much less effect on block kinetics.
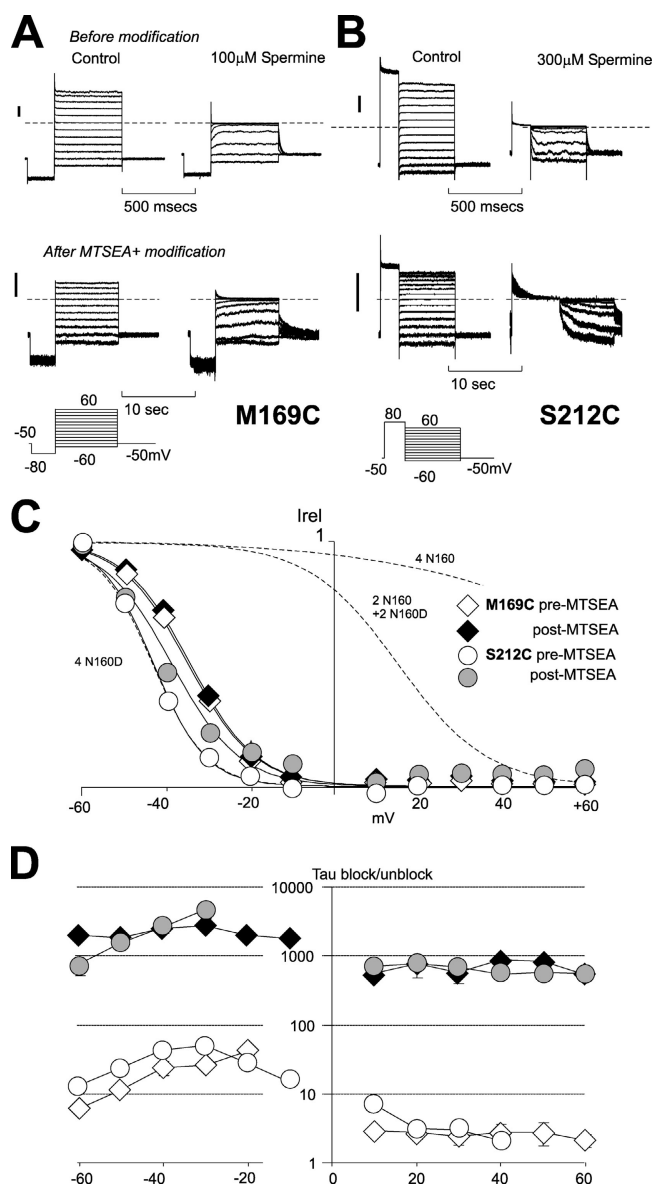
FIGURE 7. In situ introduction of positive charge at the bottom of, or below, the inner cavity slows spermine block kinetics. (A and B) Representative currents illustrating rectification induced by spermine in (A) M169C and (B) S212C mutant channels (mutation in second half of N160D-N160D dimer background), before and after complete modification by MTSEA⁺ (modification not shown). Current scale indicates 0.2 nA. Note changes of current and time scales, post-modification. (C) Steady-state current in spermine relative to control (Grel) plotted versus voltage, fitted with Boltzmann functions as indicated. (D) Time constant of spermine block/unblock obtained from single exponential fits to current relaxations (from experiments as in A). Block and unblock rates of both M169C and S212C channels are slowed ~100-fold after modification.

Most strikingly, at residues M169C and S212C, there was a dramatic slowing of the kinetics of spermine block and unblock (Fig. 7). With short voltage pulses, this slowing results in underestimation of the steady-

state block. However, when the voltage protocol is applied over a much longer time scale (note changes of time scale in top and bottom of Fig. 7, A and B), it is clear that the steady-state block, i.e., the potency, is not changed by MTSEA⁺ modification of either of these two residues, located at the bottom of the inner cavity and in the cytoplasmic vestibule, respectively. The implications of these findings are considered below.

DISCUSSION

Since the discovery of inward rectification, two questions, (1) where does the strong voltage dependence of rectification arise and (2) why does rectification depend so strongly on external [K⁺], have remained incompletely answered. The realization that polyamines cause inward rectification seemed to provide at least part of the answer (Nichols and Lopatin, 1997); the long linear molecules may enter deeply into the pore, moving through the field themselves and further displacing K⁺ ions through the field (Pearson and Nichols, 1998). Given high resolution structural views of K channel pores (Doyle et al., 1998; Jiang et al., 2002; Kuo et al., 2003), we can now consider the molecular basis of these phenomena.

The Role of the "Rectification Controller" in "Electrostatic Tuning"

In some Kir channels, the negative charge that is present at one particular location in M2 (D172 in Kir2.1) ensures very strong rectification, and neutralization of this residue weakens, but may not abolish, strong rectification (Nichols and Lopatin, 1997). In other channels, the introduction of a negative charge at the equivalent or neighboring residues can induce strong rectification in an otherwise only weakly rectifying channel (e.g., Kir1.1[N171D] and Kir6.2[N160D]; Lu and MacKinnon, 1994; Wible et al., 1994; Shyng et al., 1997; Lu et al., 2001). Since the initial studies on the role of this negative charge, no consensus has emerged as to whether the charge is actually involved in a binding site for polyamines, or allosterically affects binding to a site somewhere else. The fact that a negative charge is not required to generate reasonably strong rectification in Kir2.1 or Kir3.1 (Taglialatela et al., 1994; Lancaster et al., 2000) would seem to support the latter.

Guo and colleagues (Guo and Lu, 2003; Guo et al., 2003) have argued that although an acidic residue can be substituted at various sites in M2, the substitution must be made within a small region, and that D172 (residue 160 in Kir6.2) therefore exerts a localized electrostatic effect on the binding of cationic blockers. In addition, they demonstrated an energetic coupling between D172 and linear diamines, the degree of which was correlated with alkyl chain length, with maximal coupling at

DA9. They interpreted this finding to imply that one (tail) amine resides at a fixed location (where it interacts with E224 and E299 in the cytoplasmic vestibule), and that as the alkyl chain is extended, the other (head) amine first approaches and then extends beyond D172 in the inner cavity. Lack of energetic coupling to similar length monoamines was then explained by assuming that the single amine resides also at the E224/E299 location and it is the alkyl tail that protrudes into the inner cavity. It is not intuitively apparent why the monoamine would enter the water-filled inner cavity alkyl tail first. In addition, the effect of the D172 charge on the voltage dependence of block is still qualitatively similar for both the MA series and the diamine series, i.e., above a chain length of 5 or 6, valence is increased by 1–2 for both diamines and monoamines, when 172 is charged.

The hypothesis that the tail of linear diamines is fixed at the E224/E299 location and that the head resides into the inner cavity, projecting further upwards as chain length increases, predicts that moving the negative charge higher, or lower, in the inner cavity should shift the diamine chain length dependence of energetic coupling to longer or shorter lengths, respectively. We now show that a negative charge at multiple positions, from the cytoplasmic end of M2 up to the entrance of the selectivity filter, can generate strong polyamine-induced rectification, allowing us to directly test this hypothesis. We do not observe a peak at any single diamine length for residues in the center of the cavity, as reported in Kir2.1 (Guo et al., 2003), and an explanation for this discrepancy is not apparent at the present time. However, for residues at either end of the inner cavity (129, 164, and 168), there are very clear trends that are exactly opposite to those predicted by the above hypothesis, which instead argue for the alternative hypothesis (see below), that the head amine binds at the selectivity filter and the tail amine protrudes back into the inner cavity.

That placing a negative charge even as high as residue 129 (Fig. 1) is sufficient to generate strong rectification negates the idea that polyamine block at positive voltages involves the tail amine binding to negatively charged residues in the cytoplasmic vestibule (Guo and Lu, 2003; Guo et al., 2003), since the likely distance between the these residues and 129 is >40 Å (Kuo et al., 2003). The systematic introduction of positive charge in situ to cysteine side chains in a N160D background is also contrary to this idea. Introduction of positive charge at more superficial sites, i.e., below the inner cavity, reduces polyamine entry and exit rates (Fig. 7) but fails to decrease steady state rectification.

Where Does the Voltage Dependence of Polyamine Block Arise? The Riddle of Excess Charge Movement

Woodhull (1973) first proposed a model for voltage-dependent ion channel block that involved only the movement of the blocker itself into the electric field. The maximum valency of block in such a model is thus limited to that of moving the blocker entirely through the field. It is clear that for block by polyamines, and particularly diamines, there is a higher valence than can be accounted for by the blocker itself. Ruppersberg et al. (1994) suggested that excess charge movement associated with Mg^{2+} block of glutamate receptor channels may result from the associated obligate movement of permeant ions through the field, ahead of the blocking particle, and this mechanism has also been suggested to account for the excess charge movement associated with blockade of Kir channels (Pearson and Nichols, 1998). The demonstration that effects of external ion concentration on kinetics of blocker dissociation are highly specific to K^+, while the effects of internal cations are much less species dependent, suggests that interactions between permeant and blocking ions govern the properties of inward rectification (Oliver et al., 1998) and further suggest that blocker movement through the channel pore is coupled to movement of permeant ions within the selectivity filter (Hille and Schwarz, 1978; Oliver et al., 1998; Pearson and Nichols, 1998; Spassova and Lu, 1998).

Based on the realization that the Kir channel extends at least 30 Å below the "inner cavity" through a "cytoplasmic vestibule" (Lu et al., 1999b; Nishida and MacKinnon, 2002; Kuo et al., 2003) (Fig. 8), the above notion has been further developed with the hypothesis that polyamine molecules may block the channel within the cytoplasmic vestibule (Nishida and MacKinnon, 2002; Guo et al., 2003) or at the lower end of the inner cavity (Guo and Lu, 2003) and that the associated charge movement then results entirely from the "pushing" of a column of four or five K^+ ions (which implicitly extends from the selectivity filter all the way back into the "cytoplasmic pore") through the electric field. Guo et al. (2003) argue that true single filing of K^+ ions may not be necessary, since displacement may still occur as long as K^+ ions cannot exchange position with, or bypass, the blocker in the inner pore. Given the expected width of the inner cavity and cytoplasmic vestibule (>10 Å; Kuo et al., 2003), there seems no a priori reason to expect that this condition should exist, and an Ussing flux ratio exponent of 2.2 for Kir2.1 channels (Stampe et al., 1998), significantly lower than the value of 3.5 obtained for *Shaker* channels (Stampe and Begenisich, 1996), implies that only two or three ions permeate the pore in single file, most likely across the selectivity filter. Is it conceivable that several K^+ ions can be trapped in the inner cavity? This again seems problematical. The water-filled volume inside the inner cavity is in the order of 2 nm³ (10 Å $\times$ 10 Å $\times$ 20 Å). One K^+ ion in this region would have an effective concentration of ~1 M. If there were four negative charges on
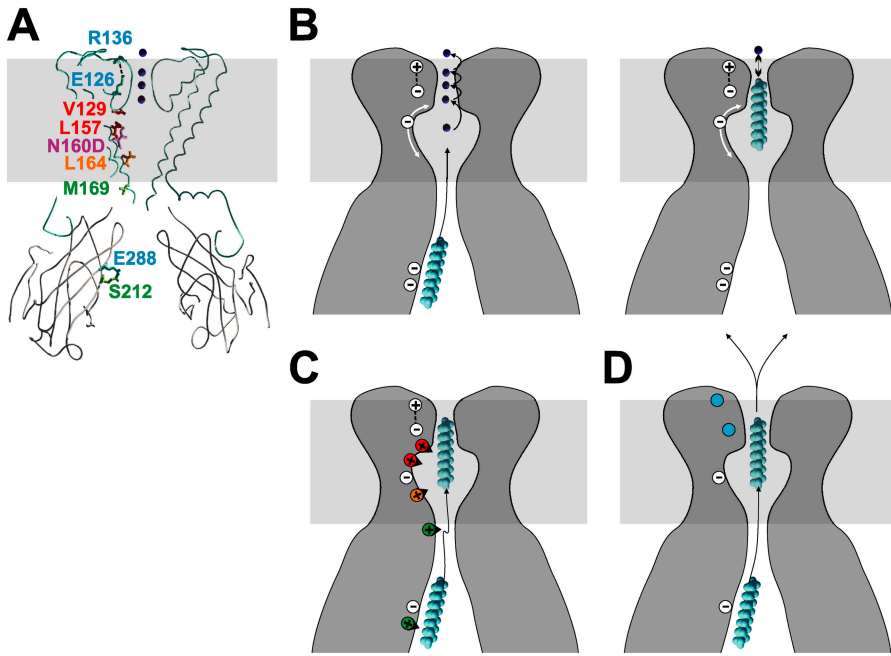
FIGURE 8. Mechanism of polyamine block. (A) Model of the Kir permeation pathway based on KirBac1.1 structure. On the Kir6.2[N160D] (purple) background, certain residues (red and orange) significantly decrease spermine potency when a positive charge is introduced by MTSEA$^+$ modification. Other residues (green) slow the spermine block rate but do not affect steady-state potency. Other critical residues are indicated in blue. (B) We propose that spermine (shown as space-filling model in extended conformation) interacts with cytoplasmic pore residues (left) en route to a blocking site at the top of the inner cavity, extending into the selectivity filter (right). In reaching the deep site, K$^+$ ions from Scav and the selectivity filter are displaced to the external solution, and only the outermost binding site(s) can be occupied by K$^+$, which can electrostatically interact with the blocking spermine. (C) MTSEA$^+$ modification of S212C or M169C (green) slows spermine entry to the deep blocking site, whereas modification of L164 (orange), L157C, or V129C (red) reduces the relative affinity at the deep site. (D) Destabilization of the selectivity filter structure, by disruption of the E126-R136 salt bridge (Yang et al., 1997; Dibb et al., 2003) (blue) removes the barrier to spermine permeation and abolishes rectification.

the wall of the inner cavity, this might be expected to allow more cations to concentrate in this volume, but in the absence of any M2 charges, e.g., in WT Kir6.2, or Kir2.1[D172N], there would be no such electrostatic concentrating effect. Nevertheless, in both of these channels, long diamines still cause relatively strong rectification, with valences approaching 2 (Fig. 6 A) or higher (Guo and Lu, 2003). Since there is no effect of the presence or absence of M2 negative charges on single channel conductance in these two channels (Shyng et al., 1997; Oishi et al., 1998), it seems unlikely that significant additional inner cavity K$^+$ accumulation occurs in the presence of a negative charge (Brelidze et al., 2003; Nimigean et al., 2003). By contrast, the incorporation of positive charges at L164C and L157C (or equivalent residues in Kir2.1) by modification with MTSEA$^+$ leads to progressive decrease in single channel conductance with each introduced charge (Lu et al., 1999a; Loussouarn et al., 2001; Phillips et al., 2003). These results suggest that the inner cavity already contains a saturating number of ions (i.e., 1) in the WT channel, and that fractional occupancy of the inner cavity is reduced when positive charges are introduced.

Selectivity Filter Binding of Polyamines: A Consistent Model of Polyamine-induced Rectification

Initial calculations of polyamine and permeant ion binding in 3D models of closed and open Kir channels, built by comparative modeling (Dibb et al., 2003; Rose

and Nichols, 2003), suggest that polyamines experience very low energetic barriers to entry to the inner cavity and only small barriers to entering the selectivity filter, but that significant barriers within the filter itself will preclude significant permeation. These calculations predict that longer diamines (and monoamines) are stable at progressively deeper sites in the filter (Rose and Nichols, 2003). In entering the filter, polyamines and diamines would themselves move significant amounts of charge into the electric field, as well as discharging K$^+$ ions from the inner cavity and selectivity filter to the outside, leaving only externally located K$^+$ binding sites free. Such a model thus provides an intuitive basis for both the external [K$^+$] dependence of block and the excess charge movement associated with block. In the preceding section, we have raised multiple questions regarding the notion that these organic blockers cause strong rectification by binding low down in the pore, outside the voltage field. What experimental consistency is there with the notion of polyamine block occurring in the selectivity filter? We will first consider the voltage and permeant ion dependence of block. Second, we will consider the results of additional mutagenic studies.

Calculations of the likely field strength in open and closed Kir channel pores (Jiang et al., 2002) indicate that 70–90% of the membrane field exists across the selectivity filter. Even in the closed KcsA channel, only ~30% of the field may drop across the inner cavity

(Jiang et al., 2002). Assuming that the electric field in Kir channels is relatively fixed and similar to that calculated for MthK (or KcsA), and that K^+ ion occupancy is also similar in the absence of polyamine, simple calculations can estimate the charge movement that is expected for polyamine or diamine movement to a binding site in the selectivity filter. If the polyamine head reaches S2, then the combined elementary charge movement from displacement of K^+ and entry of polyamine or diamine into the field should be $\sim$3-4, similar to the limiting voltage dependence estimated from fits of the Boltzmann equation to steady-state G–V relationships (Lopatin et al., 1995; Pearson and Nichols, 1998; Guo et al., 2003; Xie et al., 2003). Thus movement of K^+ ions from the selectivity filter and the inner cavity ion binding site (Scav), along with the charge carried into the field by the amine itself (Fig. 8 B), may be adequate to explain the charge movement associated with the block, without invoking the obligate movement of multiple ions from the inner cavity or cytoplasmic vestibule.

The proposed model provides an intuitive explanation for the strict dependence of rectification on external $[K^+]$ (Hagiwara and Takahashi, 1974; Lopatin and Nichols, 1996; Oliver et al., 1998). If the blocking amines occupy the selectivity filter and expel K^+ ions from all except the most external binding sites (Fig. 8), we may expect that the spermine residency will be sensitive to changes in external $[K^+]$. In a detailed theoretical consideration of a channel with a linear sequence of energy barriers and binding sites, in which ions move in single file through the channel, Hille and Schwarz (1978) demonstrated that many of the special flux properties of potassium channels could be explained if the barriers within the pore are less than those for exiting, and if there is repulsion between ions within the pore. They also showed that such theoretical models could qualitatively explain inward rectification in terms of block by an internal cation that entered one or more of the internal ion binding sites. However, there was a discrepancy between the valence of the blocker and the permeant ion dependence. In essence, a monovalent ion could block with steep dependence on the concentration of permeant ions on the trans side but could not then reproduce the steepness of block; conversely a polyvalent ion could reproduce the steep voltage dependence of block but not the dependence on trans permeant ion concentration. This modeling was performed with no knowledge of the nonpoint charge nature of the polyamine blockers nor of the channel pore structure, and it is far from clear how the details of ion–ion interaction will resolve in the complex structured environment of the K channel selectivity filter. In a subsequent examination of this specific issue, exploring the parameter space in more detail, but again without regard to the real structural constraints, we showed that steep voltage dependence and strict dependence on external $[K^+]$ are not necessarily mutually exclusive; by appropriate choice of energy profile, repulsion factor, and pore occupancy by permeant ions, both the valence and $[K^+]_{out}$ dependence of polyamine block can be modeled by interactions within a simple two-site, three-barrier model (Lopatin and Nichols, 1996).

The Role of Charged Residues Below the Inner Cavity

In addition to negative charges in M2, residues in the cytoplasmic vestibule (i.e., E224 and E299 in Kir2.1; Yang et al., 1995; Kubo and Murata, 2001) have also been shown to be important in inward rectification. High resolution Kir3.1 and KirBac1.1 cytoplasmic domain structures (Nishida and MacKinnon, 2002; Kuo et al., 2003) make it quite apparent that these residues are located in the permeation pathway, lining the cytoplasmic pore at a distance of $\sim$30 Å (or more depending on the separation of the pore domain from the cytoplasmic domain) below the D172 charges.

Whether the E224/E299 residues provide binding sites for blocking polyamine (Guo and Lu, 2003; Guo et al., 2003) or act to facilitate entry to the deeper sites (Xie et al., 2002, 2003) is unclear. In Kir6.2, channel block is generally well fit by a simple two-state scheme (Phillips and Nichols, 2003). The situation is more complicated in Kir2 channels, which invariably show a prominent additional weakly voltage-dependent component (Lopatin et al., 1995). Xie and colleagues (Xie et al., 2002, 2003) have suggested that this component results from the interaction of polyamines at sites within the cytoplasmic vestibule, facilitating the subsequent entry of spermine to the site of strong rectification. We would hypothesize that this peripheral interaction may provide a spermine-concentrating effect, i.e., these charges will reduce the apparent K_D for spermine at a deeper site by effectively concentrating the blocker outside the inner cavity (Fig. 8 B), as rings of charges do for K^+ permeation itself in MthK and BK channels (Brelidze et al., 2003; Nimigean et al., 2003). This would then explain why their neutralization can reduce the apparent K_D at the deep site for polyamines and diamines (Guo and Lu, 2003). However, the relatively restricted space within the cytoplasmic vestibule of Kir channels may underlie more specific interactions with polyamines, which may explain the length-dependent reduction of single channel conductance by diamines observed by Xie et al. (2003) in Kir2.1. The negatively charged residues in the cytoplasmic vestibule are E224 and E299 in Kir2.1 (only one equivalent charge, E288, is present in Kir6.2). Neutralization of one or both of these residues appears to reduce single channel conductance (Xie et al., 2002), suggesting that this region

may be involved in concentrating permeant ions, as well as controlling polyamine entry to and exit from deeper binding sites, since neutralization also greatly slows polyamine block (and unblock) kinetics (Kubo and Murata, 2001; Xie et al., 2002, 2003). Being outside the membrane electric field, such a barrier could explain the voltage independence of the forward blocking transition (Guo and Lu, 2003), as well as the greatly reduced entry and exit rates once a positive charge is introduced at these sites in Kir6.2 (Fig. 8 C).

The Role of Charged Residues Above the Inner Cavity

An additional group of residues that contribute to polyamine block of Kir channels provides further consistency with the notion of polyamine binding in the selectivity filter, rather than in the cytoplasmic pore as underlying strong rectification. Immediately before the GYG sequence (forming the K^+ selectivity filter) of the H5 region of Kir channels there is a glutamate residue that is predicted to make a salt bridge with an arginine immediately beyond the GYG sequence (Fig. 8 A). In Kir2.1 (a homotetramer), mutation of the glutamate (E148) results in a nonfunctional channel (Yang et al., 1997). However, channel activity can be rescued by mutation of the paired arginine (i.e., the double mutant, E138R/R148E), but the mutant channel is no longer selective for K^+, and loses inward rectification (Yang et al., 1997). We have recently demonstrated that essentially the same behavior is observed in Kir3.1/3.4 channels (Dibb et al., 2003); mutation of the equivalent charged residues in Kir3.4 causes a dramatic loss of ion selectivity and of inward rectification. These residues are unlikely to make contact with polyamines or permeant ions themselves, but instead may serve to control the flexibility of the selectivity filter (Dibb et al., 2003). The increased flexibility of the selectivity filter that results from breaking the salt bridge appears to cause loss of both K^+ ion selectivity and tight polyamine binding in the filter (Fig. 8 D).

Conclusions

Charges throughout the Kir6.2 channel pore, from the cytoplasmic vestibule right up to the selectivity filter, control inward rectification. Introduction of negative charges at residues throughout the inner cavity significantly strengthen block by spermine and linear diamines, providing stronger energetic contribution to block by shorter diamines as the charge moves closer to the selectivity filter. Introduction of positive charges at the bottom of, or below, the inner cavity slow the kinetics of polyamine block, but do not change steady-state rectification. By contrast, introduction of positive charges at the entrance to the selectivity filter cause dramatic loss of polyamine affinity. The results are consistent with a model in which strong rectification results from blocking of the permeation pathway by diamines and polyamines high in the inner cavity and selectivity filter. Blocking in the latter site provides a ready explanation for both the uniquely steep voltage dependence of polyamine block and the dependence on external $[K^+]$, the defining features of classical inward rectification.

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