Neuron, Vol. 14, 1047–1054, May, 1995, Copyright © 1995 by Cell Press

Control of Rectification and Permeation by Residues in Two Distinct Domains in an Inward Rectifier K^+ Channel

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Summary

Inwardly rectifying K^+ channels conduct more inward than outward current as a result of voltage-dependent block of the channel pore by intracellular Mg^{2+} and polyamines. We investigated the molecular mechanism and structural determinants of inward rectification and ion permeation in a strongly rectifying channel, IRK1. Block by Mg^{2+} and polyamines is found not to conform to one-to-one binding, suggesting that a channel pore can accommodate more than one blocking particle. A negatively charged amino acid in the hydrophilic C-terminal domain is found to be critical for both inward rectification and ion permeation. This residue and a negatively charged residue in the putative second transmembrane segment (M2) contribute independently to high affinity binding of Mg^{2+} and polyamines. Mutation of this residue also induces Mg^{2+}- and polyamine-independent inward rectification and dramatically alters single-channel behavior. We propose that the hydrophilic C-terminal domain comprises part of the channel pore and that involvement of both hydrophilic and hydrophobic domains in pore lining may provide a molecular basis for the multi-ion, long-pore nature of inwardly rectifying K^+ channels.

Introduction

Inwardly rectifying K^+ channels exist in various excitable and nonexcitable cells, including muscles (Katz, 1949; Sakmann and Trube, 1984), neurons (Constanti and Galvan, 1983), eggs (Hagiwara and Takahashi, 1974), and endothelial cells (Silver and DeCoursey, 1990), and are important for maintaining the resting membrane potential near the K^+ equilibrium potential (E_K) and for permitting long depolarizing responses (Hille, 1992). They conduct more efficiently when the membrane potential is negative to E_K than when positive to E_K, thus allowing more inward K^+ current than outward current. Many types of inward rectifiers have been found, some rectifying more strongly than others (Hille, 1992). Rectification in native channels is reported to be due in part to voltage-dependent block by cytoplasmic Mg^{2+} (Matsuda et al., 1987; Vandenberg, 1987; Matsuda, 1988; Ishihara et al., 1989) and in part to a voltage-dependent intrinsic gating (Ishihara et al., 1989; Silver and DeCoursey, 1990).

Several types of inwardly rectifying K^+ channels have recently been cloned and expressed in Xenopus laevis oocytes or cell lines; among them are the strongly rectifying IRK1 (Kubo et al., 1993a) and GIRK1 (Dascal et al., 1993; Kubo et al., 1993b) and the weakly rectifying ROMK1 (Ho et al., 1993) and rcK_{ATP}-1 (Ashford et al., 1994). These channels form a new channel-gene superfamily that has a unique architecture: in contrast to voltage-gated K^+ channels, inwardly rectifying K^+ channels possess only two putative transmembrane segments, M1 and M2, which correspond to transmembrane regions S5 and S6 of voltage-gated K^+ channels. The C-terminal half of the hydrophobic domain (S5, H5, S6, and S4–S5 loop) in voltage-gated K^+ channels constitutes the major part of the pore (for review, see Sather et al., 1994). By analogy, the corresponding hydrophobic domain (M1, H5, and M2) of inwardly rectifying K^+ channels is expected to include pore-lining structures (Ho et al., 1993; Kubo et al., 1993a; Nichols, 1993). In addition, the hydrophilic C-terminal domain is also implicated in specifying certain pore properties because it accounts for some differences between ROMK1 and IRK1 (Taglialatela et al., 1994). However, it is not clear which region of the C-terminal domain contributes to its effect or how it is involved.

Studies on cloned inwardly rectifying K^+ channels indicate that inward rectification is mainly a result of voltage-dependent block of the channel pore by intracellular Mg^{2+} (Lu and MacKinnon, 1994; Nichols et al., 1994; Stanfield et al., 1994; Taglialatela et al., 1994) and polyamines (Ficker et al., 1994; Lopatin et al., 1994; Fakler et al., 1995), resulting in tight coupling of gating to ion permeation. Block by internal polyamines is believed to be largely responsible for the so-called intrinsic gating found in native channels (Ficker et al., 1994; Lopatin et al., 1994). The strongly rectifying IRK1 is much more sensitive to Mg^{2+} and polyamines than the weakly rectifying ROMK1 (Ficker et al., 1994; Lopatin et al., 1994; Lu and MacKinnon, 1994; Taglialatela et al., 1994). Mutagenesis studies have shown that an amino acid in M2 (D172 in IRK1 and N171 in ROMK1) affects Mg^{2+} and polyamine binding (Ficker et al., 1994; Lopatin et al., 1994; Lu and MacKinnon, 1994; Stanfield et al., 1994). However, other structural elements must also play a key role, since mutation of this residue in IRK1 had little effect on inward rectification (Stanfield et al., 1994; Wible et al., 1994). A candidate is the hydrophilic C-terminal domain, since it appears to contribute to Mg^{2+} block (Taglialatela et al., 1994). We investigated the structural determinants of rectification and ion permeation in the strongly rectifying IRK1 channel by examining a series of point mutations in the hydrophilic C-terminal domain as well as in the hydrophobic M2 domain. We present evidence that a negatively charged residue in each domain is critical for inward rectification and ion permeation, and that both domains contribute to the inwardly rectifying K^+ channel pore.

Results

Alteration of Inward Rectification by Mutations in C-Terminal and M2 Domains

Two members of the inwardly rectifying K^+ channel family,

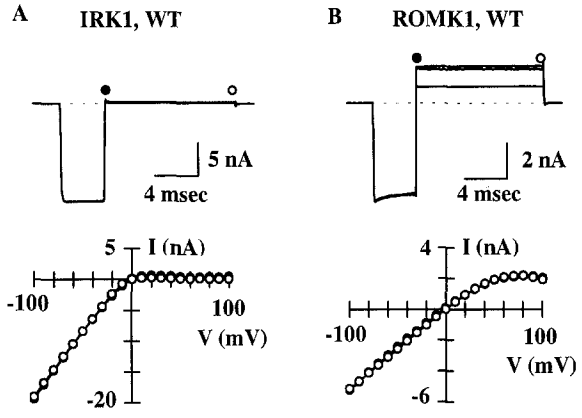

Figure 1. Distinct Inward Rectification of IRK1 and ROMK1

Representative macroscopic currents recorded in cell-attached patches and current–voltage (I–V) relations for wild-type IRK1 (A) and ROMK1 (B). Current was elicited from a 4 ms prepulse of −80 mV to 12 ms test potentials of −100 to +100 mV in 10 mV increments. Current traces at +100, +60, and +20 mV are superimposed and shown. Both instantaneous (closed circles) and steady-state (open circles) I–V relations are plotted.

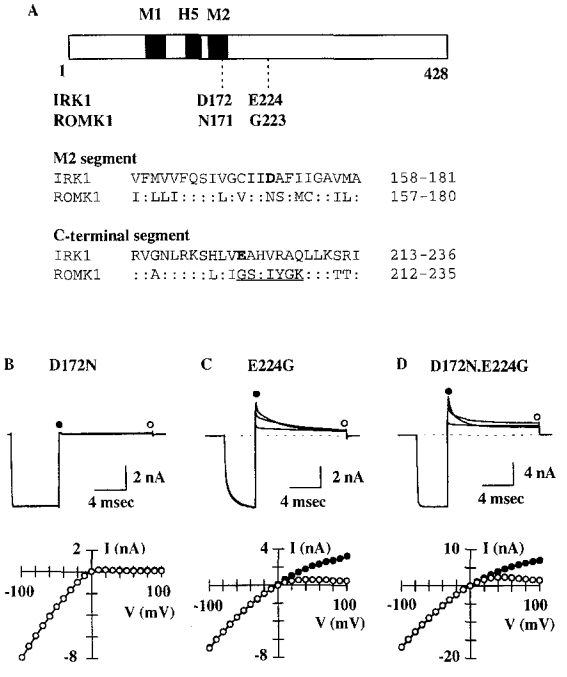

Figure 2. Mutations in C-Terminal and M2 Domains Attenuate Inward Rectification

(A) Schematic representation of proposed transmembrane topology of IRK1 (amino acid residues 1–428) and sequence alignment of the M2 region and a segment in the C-terminal domain of IRK1 and ROMK1, with residue numbers given on the right. The underlined sequence in ROMK1 indicates the proposed Walker type-A sequence. Amino acids D172 and E224 are in boldface. Colons indicate identical residues.

(B–D) Representative macroscopic currents recorded in cell-attached patches and I–V relations for D172N (B), E224G (C), and D172N–E224G (D). Current was elicited from a 4–6 ms prepulse of −80 mV (−100 mV for [D]) to 12 ms test potentials of −100 to +100 mV in 10 mV increments. Current traces at +100, +60, and +20 mV are superimposed and shown. Both instantaneous (closed circles) and steady-state (open circles) I–V relations are plotted.

IRK1 and ROMK1, differ considerably in inward rectification when expressed in Xenopus oocytes. In cell-attached patch recordings, wild-type IRK1 showed strong inward rectification, allowing little outward current when membrane potential was positive to E_K (Figure 1A). In contrast, wild-type ROMK1 displayed weak rectification, permitting significant outward current (Figure 1B).

Figure 2A illustrates a schematic diagram of inwardly rectifying K^+ channels that consist of two putative transmembrane domains and an H5 region, flanked by two hydrophilic domains that are proposed to be cytoplasmic. It has been shown recently that an aspartate in M2 in IRK1 (Figure 2A) affects Mg^{2+} and polyamine binding (Ficker et al., 1994; Stanfield et al., 1994). However, the D172N mutant channel still exhibited strong rectification similar to that in wild-type IRK1 (compare Figure 2B to Figure 1A), indicating involvement of other key regions in rectification. Sequence comparison between IRK1 and ROMK1 (Figure 2A) reveals a significant difference in the putative cytoplasmic C-terminal domain in a region that contains a putative Walker type-A motif for phosphate binding in ROMK1, starting from residue G223 (Ho et al., 1993). The corresponding residue in IRK1 is E224 (Figure 2A). We postulated that E224 might contribute to rectification and tested its functional importance by replacing it with its counterpart in ROMK1. Indeed, E224G mutant channels in cell-attached patch conducted a large instantaneous outward current and a substantial steady-state outward current, and both inward and outward currents showed prominent voltage-dependent relaxation (Figure 2C). Although the D172N mutation had little effect by itself, the double mutant D172N–E224G passed more steady-state outward current than E224G (Figure 2D). The mutations did not alter ion selectivity, as all mutants were still highly selective for K^+ over Na^+ (data not shown). The reverse mutation in ROMK1, G223E, produced little effect on the extent of inward rectification compared with wild-type ROMK1 (data not shown) and was therefore not characterized further.

Inward Rectification Produced by Mg^{2+} or Spermidine

The major mechanism for inward rectification is voltage-dependent block of channel pore by cytoplasmic Mg^{2+} and polyamines. In wild-type IRK1, inward rectification was almost completely removed following excision of the membrane patch from cell-attached to inside-out configuration and wash of the cytoplasmic face with a Mg^{2+}- and polyamine-free solution (Figures 3A and 3B). Rectification was restored upon exposing the cytoplasmic side to 1 mM Mg^{2+} or 100 μM spermidine (SPD), a polyamine found in Xenopus oocytes in an estimated concentration of 600–800 μM (Osborne et al., 1989; Ficker et al., 1994; Figures 3A and 3B). At the concentrations used, block by Mg^{2+} or SPD was largely instantaneous (Figure 3B). However, block by 100 μM SPD was more steeply voltage dependent than that by 1 mM Mg^{2+} (Figure 3A), a notion further demonstrated in Figure 3C, which shows the relationship of nor-

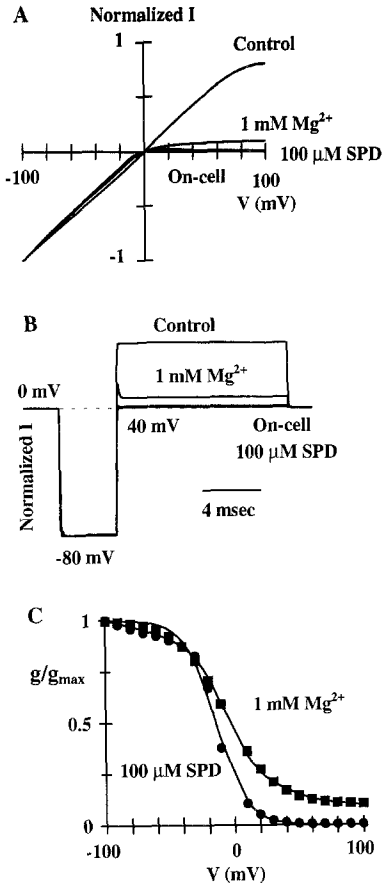

Figure 3. Inward Rectification Caused by Mg^{2+} and Spermidine in Wild-Type IRK1

(A and B) Normalized I–V relations (A) and current traces (B) recorded on the same patch in cell-attached configuration (on-cell) or in inside-out configuration in the control solution or in the presence of 1 mM Mg^{2+} or 100 μM spermidine (SPD). I–V records were generated by voltage ramps from −100 to +100 mV over a duration of 1.4 s, scaled to the same size at −100 mV, and averaged (five traces) for display. Current traces were elicited from a 4 ms prepulse of −80 mV to a 12 ms test potential of +40 mV and scaled to the same size at −80 mV. (C) Normalized chord conductance in the presence of 1 mM Mg^{2+} or 100 μM SPD plotted against membrane potential. Steady-state current at the end of a 12 ms test pulse was used for calculating the chord conductance; SEM is smaller than the symbols in all cases. Continuous curves are least-squares fit to equation 3. For Mg^{2+} block, V_h = −6.6 ± 0.6 mV and k = 18.6 ± 0.5 mV (n = 7); for SPD block, V_h = −14 ± 1 mV and k = 12 ± 0.9 mV (n = 5).

malized chord conductance calculated from steady-state current–voltage (I–V) relations as a function of membrane potential. Block by Mg^{2+} corresponds to an effective valence of 1.37 (± 0.03; n = 7), whereas that by SPD corresponds to 2.13 (± 0.16; n = 5).

Attenuation of Mg^{2+} Block by Mutations of E224 and D172

The attenuated inward rectification in E224G and D172N–E224G in cell-attached patches suggested a decreased sensitivity to Mg^{2+} and/or polyamine block. To test this, we examined these and additional mutations on channel block by Mg^{2+} or polyamines.

Steady-state block by Mg^{2+} in inside-out patches was achieved by voltage ramps from −100 to +100 mV over a duration of 0.35–2.8 s, depending on the time course of voltage-dependent relaxation of the channel (Figures 4A and 4B). The dose–response relationships of wild-type IRK1 and E224D show significant deviations from one-to-one binding (Figures 4C and 4D). Indeed, they could be best fitted by the sum of two Hill functions with a high affinity and a low affinity binding (Table 1; Figure 4C). Dose–response data of other mutants could be fitted to one Hill equation, with the Hill coefficient varying from 0.6 to 1 (Table 1; Figures 4C and 4D).

The Mg^{2+} sensitivity was reduced by several mutations of E224 (Table 1; Figure 4). With the exception of aspartate, replacement of E224 with other residues decreased Mg^{2+} affinity by 7- to >175-fold, with an order of potency of K > Q > S > G (the K_D for E224K was estimated as >3000 μM; data not shown). This dependence on the chemical nature as well as size of the side chain suggests involvement of more than a pure electrostatic interaction. In contrast to a previous report (Wible et al., 1994), the D172N mutation also reduced Mg^{2+} affinity by 5-fold, comparable to the effect of the E224G mutation (7-fold). The double mutation D172N–E224G decreased Mg^{2+} sensitivity even more, by ~33-fold (Table 1; Figure 4D). The fraction of electrical field (δ) sensed by Mg^{2+} (Woodhull, 1973) is 0.71 (± 0.03; n = 3) for the high affinity binding in wild-type IRK1 and 0.75 (± 0.02; n = 3) for D172N–E224G (Figure 4E), indicating that the mutation did not significantly alter the voltage dependence of block.

Attenuation of Polyamine Block by Mutations of E224 and D172

Figure 5 shows the sensitivity of wild-type and mutant channels to three different species of polyamines, putrescine (PUT), SPD, and spermine (SPM), all found in Xenopus oocytes (Osborne et al., 1989; Ficker et al., 1994; Lopatin et al., 1994). In this series of experiments, we focused on the single mutants D172N and E224G and the double mutant D172N–E224G to examine the individual and combined contributions of D172 and E224 to polyamine binding. The dose–response relationships of wild-type IRK1 and E224G for all three polyamines could be best fitted by the sum of two Hill functions with different affinities (Table 1). By contrast, the D172N mutant conformed to one-to-one binding (Table 1).

Mutation of E224 and D172 decreased the sensitivity to polyamines (Table 1; Figure 5). The affinity of different polyamines for wild-type IRK1 correlates with the number of positive charges they carry: the more the charge, the higher the affinity (Figure 6A). Likewise, the effect of mutations on polyamine binding also depends on the number of charges on the blocker: the more the charge, the more severe the mutant phenotype (see Figure 5). Whereas the affinity of PUT was reduced by 9-, 8-, and 93-fold (relative to the high affinity binding in wild-type IRK1) in D172N, E224G, and D172N–E224G mutants, the affinity of SPD was decreased by 415-, 113-, and 6 × 10^4-fold and that of SPM by 346-, 1111- and 1.2 × 10^6-fold, respectively.

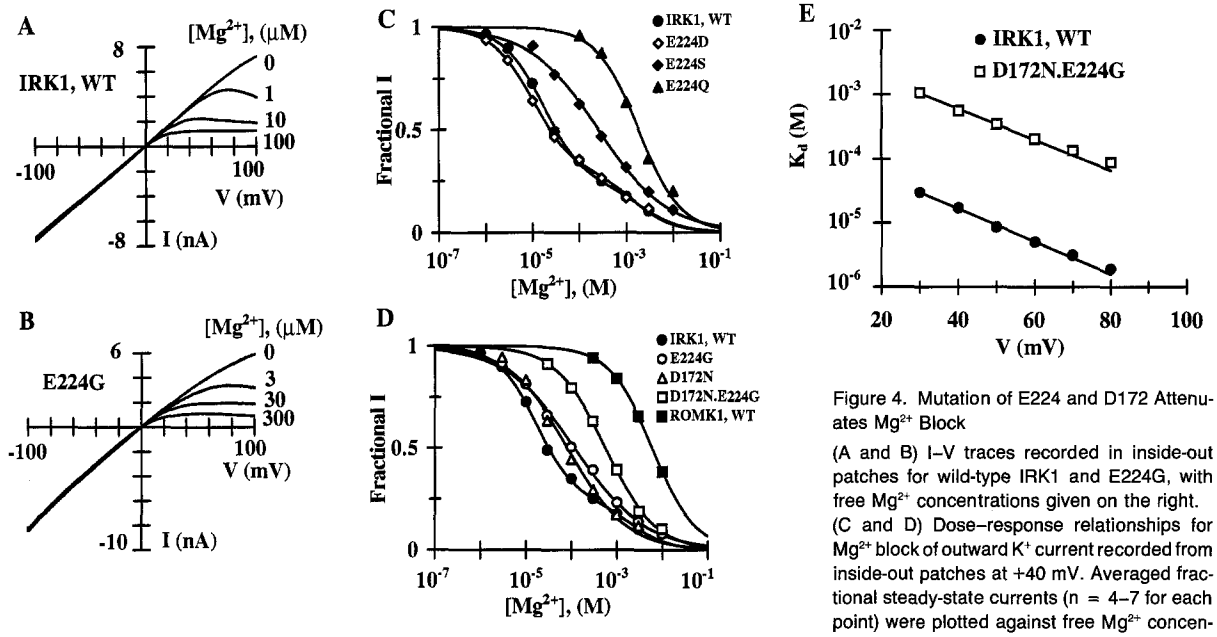

Figure 4. Mutation of E224 and D172 Attenuates Mg^{2+} Block

(A and B) I–V traces recorded in inside-out patches for wild-type IRK1 and E224G, with free Mg^{2+} concentrations given on the right. (C and D) Dose–response relationships for Mg^{2+} block of outward K^+ current recorded from inside-out patches at +40 mV. Averaged fractional steady-state currents (n = 4–7 for each point) were plotted against free Mg^{2+} concentration; SEM is smaller than the symbols in all cases. Smooth curves are least-squares fits to equation 1 or 2.

(E) Apparent K_D plotted as a function of membrane voltage for wild-type IRK1 and D172N–E224G. Lines represent least-squares fits to equation 4 with $K_D(0)$ of 174 μM for wild-type IRK1 and 5.5 mM for D172N–E224G.

Additivity of Binding Energy Changes

Interestingly, for each blocker, the mutational effect of the double mutant is close to the sum of the two individual mutants as predicted from additive changes in binding energy (Figure 6A). This is further demonstrated by the analysis (see Wells, 1990) in Figure 6B, which plots the change in binding energy for each blocker caused by the D172N–E224G double mutation as a function of the sum of binding energy changes induced by the D172N and E224G single mutations. A near perfect linear correlation (slope = 1.08; correlation coefficient = 0.995) between the two confirms a simple additivity and suggests that D172 and E224 contribute simultaneously and independently to binding of Mg^{2+} and polyamines.

Alteration of K^+ Permeation

Since gating in inwardly rectifying K^+ channels is tightly coupled to ion permeation (Hille, 1992), the critical role of E224 in binding of pore blockers suggests that it may reside inside the pore and that its side chain may be exposed to the ion conduction pathway. Indeed, mutation of E224 strongly affected K^+ permeation (Figure 7). Whereas the I–V relation of macroscopic current was almost linear for wild-type IRK1 when the inside-out patch was exposed to identical Mg^{2+}- and polyamine-free K^+ solution on both sides of the membrane (Figure 7A), several mutations of E224 caused blocker-independent inward rectification (n = 4–20), the extent of which varied with the chemical nature and size of the side chain. The simplest explanation is

Table 1. Affinities of Wild-Type and Mutant Channels for Mg^{2+} and Polyamines

	K_D (μM)			
Channel Type	Mg^{2+}	PUT	SPD	SPM
IRK1 (wild type)[a]	17.1 (0.76)	7.5 (0.77)	0.008 (0.7)	0.0009 (0.86)
	2170 (0.24)	807 (0.23)	2.87 (0.3)	0.61 (0.14)
E224D[a]	9.9 (0.7)	ND	ND	ND
	1437 (0.3)			
E224S[b]	258 [0.59]	ND	ND	ND
E224Q[b]	1848 [0.97]	ND	ND	ND
E224G[a]	117 [0.57][b]	61 (0.73)	0.9 (0.68)	1 (0.9)
		4672 (0.27)	253 (0.32)	9×10^5 (0.1)
D172N[b]	82 [0.68]	66 [0.89]	3.32 [0.99]	0.311 [1.07]
D172N–E224G[b]	559 [0.8]	700 [0.89]	504 [0.74]	1090 [0.39]
ROMK1 (wild type)[b]	5952 [0.92]	7992 [0.85]	3016 [0.74]	1611 [0.54]

[a] Dose–response data were fitted to equation 1. I_1 and I_2 are in parentheses.
[b] Dose–response data were fitted to equation 2. Hill coefficient is in brackets.
ND; not determined; PUT, putrescine; SPD, spermidine; SPM, spermine.

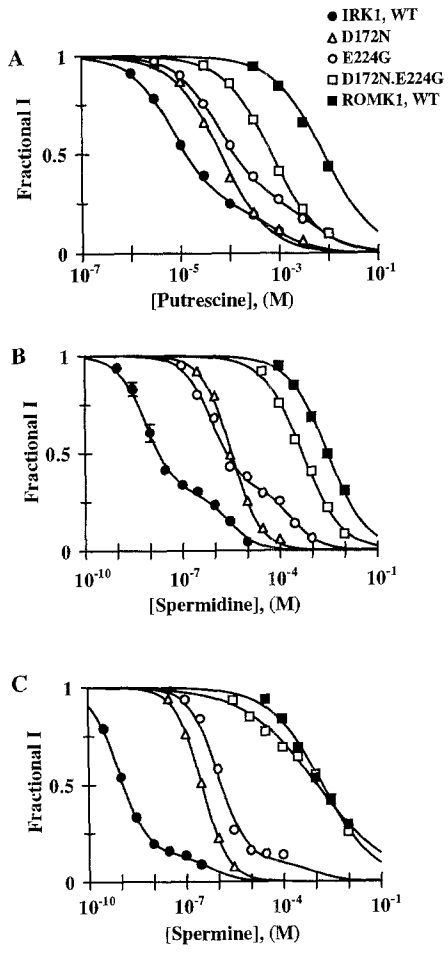

Figure 5. Mutation of E224 and D172 Attenuates Polyamine Block

Dose–response relationships of block of outward current at +40 mV in inside-out patches by putrescine (A), SPD (B) and spermine (C). Averaged fractional steady-state currents (n = 4–7 for each point) were plotted as a function of polyamine concentration; SEM is smaller than the symbols unless indicated. Smooth curves are least-squares fits to equation 1 or 2.

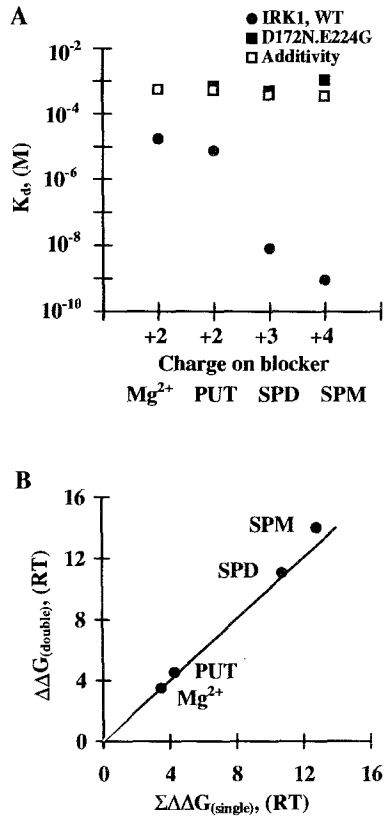

Figure 6. Independent Contribution from D172 and E224

(A) Fitted K_D values for different pore blockers plotted against the number of positive charges on the blocker. Open squares represent predicted K_D for D172N–E224G, assuming simple additivity of binding energy change from D172N and E224G single mutations, according to the equation

$$K_{(D172N-E224G)} = K_{(D172N)}K_{(E224G)} / K_{(WT)},$$

The high affinity K_D is used for wild-type IRK1 (WT).

(B) Plot of changes in blocker binding energy for the D172N–E224G double mutant versus the sum for the D172N and E224G single mutants.

$$\Delta\Delta G = RT\{\ln[K_{(mutant)} / K_{(WT)}]\},$$

using the high affinity K_D for wild-type IRK1. Solid line has a slope of 1.

that these mutations altered the energy profile of the ion permeation pathway through electrostatic and other mechanisms (such as steric hindrance). Consistent with this notion, mutation of E224 induced drastic changes in single-channel behavior, as illustrated in Figure 7B for E224G, which demonstrated very rapidly flickering single-channel openings with reduced conductance (n = 6). The rapid flickering persisted in inside-out patches exposed to pure KCl solutions on both sides, indicating altered interactions between permeating K^+ ions and the channel pore.

Discussion

Mechanism of Inward Rectification

The mechanism of inward rectification has fascinated ion channel biophysicists since the discovery of inwardly rectifying K^+ channels in muscles by Katz (1949). Armstrong (1969) noted a resemblance between the voltage-dependent block of voltage-gated K^+ channels by internal tetraethyl ammonium and the voltage-dependent gating of inward rectifiers and postulated that inward rectification could arise from block by an internal blocking particle. Hille and Schwarz (1978) simulated inward rectification using a three-site, single-file model for the pore with a monovalent intracellular blocking cation. In native channels, several cations, including Mg^{2+}, Ca^{2+}, and Na^+, have been found to produce voltage-dependent block (Matsuda et al., 1987; Vandenberg, 1987; Matsuda, 1988, 1993; Ishihara et al., 1989; Matsuda and Cruz, 1993), but only Mg^{2+} may be of physiological significance. However, some native channels exhibit steep rectification in the absence of intracellular Mg^{2+}, prompting the hypothesis that an open–close gating intrinsic to the channel also underlies inward

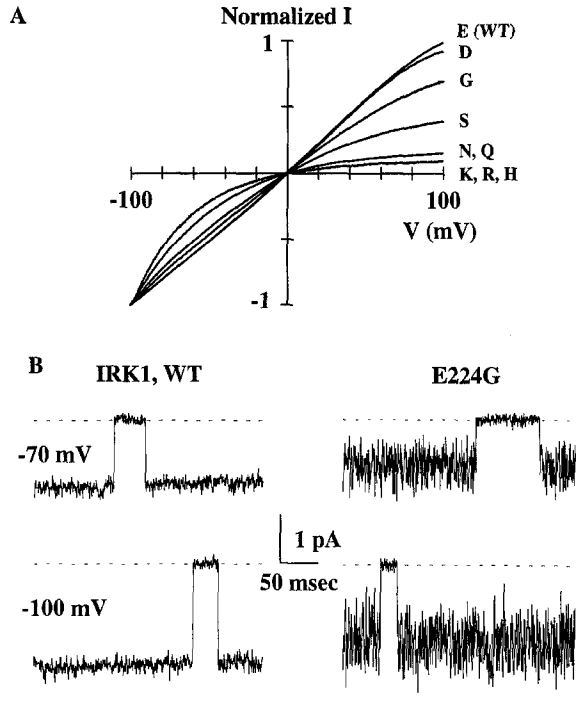

Figure 7. Mutation of E224 Affected K^+ Permeation

(A) I–V traces recorded from inside-out patches exposed to the Mg^{2+}- and polyamine-free bath solution on both sides of the membrane, with the amino acid at position 224 (E in wild-type IRK1) given on the right. I–V records were generated by voltage ramps from −100 to +100 mV over a duration of 1.4 s. Currents were scaled to the same size at −100 mV, and three to five traces were averaged for display.

(B) Representative single-channel records at −70 and −100 mV from cell-attached patch recordings for wild-type IRK1 and E224G. Dotted line indicates closed level.

rectification (Ishihara et al., 1989; Silver and DeCoursey, 1990).

Recent studies on cloned inwardly rectifying K^+ channels have shown that, besides being blocked by internal Mg^{2+} (Lu and MacKinnon, 1994; Nichols et al., 1994; Stanfield et al., 1994; Taglialatela et al., 1994), cloned channels can also be blocked by polyamines, a family of multiply positively charged molecules found in many cells (Ficker et al., 1994; Lopatin et al., 1994; Fakler et al., 1995). There is little intrinsic gating in IRK1 expressed in oocytes, since the steady-state I–V relation is almost linear over a voltage range of −100 to +80 mV, and the outward current at potentials positive to E_K shows little time-dependent gating in inside-out patches exposed to a Mg^{2+}- and polyamine-free solution (see Figure 3). This, however, does not necessarily imply that intrinsic gating does not exist in some native channels, since the molecular composition of native channels is unknown. In voltage-gated K^+, Na^+, and Ca^{2+} channels, gating can be specifically modified by an accessory protein such as a β subunit (for review, see Isom et al., 1994).

A surprising finding of our study is that Mg^{2+} and polyamine block of wild-type IRK1 (and some mutant) channels does not conform to one-to-one binding, particularly at high concentrations. Instead, the dose–response data could be best fitted by assuming two binding processes with different affinities. One interpretation is that there are two different populations of channels with distinct affinities for the blockers. This seems unlikely to occur with a cloned channel that is supposed to form homomultimers. Another possibility is that a single inwardly rectifying K^+ channel pore can accommodate more than one blocking particle, particularly at high concentrations, resulting in significant negative cooperativity generated by negative interactions (such as electrostatic repulsions and steric hindrance) between the blockers. Block by multiple particles provides an explanation for the steep voltage dependence of inward rectification in native as well as cloned channels. It also provides an explanation for the Mg^{2+}- and Ca^{2+}-induced subconductance states observed in some native channels (Matsuda, 1988; Matsuda and Cruz, 1993). Interestingly, the D172N mutant appears to show one-to-one binding to polyamines. This may indicate that the binding configuration is altered in the mutant so that the channel pore can be occluded by just one blocking molecule.

Molecular Determinants of Inward Rectification

Recent mutagenesis studies have shown that the identity of the amino acid at a position in the M2 region is important for inward rectification. This position is occupied by aspartate in IRK1 but by asparagine in ROMK1. Substitution of aspartate for asparagine converted the weakly rectifying ROMK1 into a strong rectifier (Lu and MacKinnon, 1994; Wible et al., 1994) and increased the affinity of Mg^{2+} and polyamine block (Ficker et al., 1994; Lopatin et al., 1994; Lu and MacKinnon, 1994). We demonstrate here that the reverse mutation, D172N, in IRK1 decreased the affinity of Mg^{2+} and polyamine block, in qualitative agreement with recent reports (Ficker et al., 1994; Stanfield et al., 1994). However, in comparison with the concentration of Mg^{2+} and polyamines in oocytes (Osborne et al., 1989; Ficker et al., 1994; Lopatin et al., 1994), the affinity of the mutant channel is still high enough to produce strong inward rectification similar to that in wild-type IRK1.

Our results also demonstrate that a residue, E224, in the hydrophilic C-terminal domain is crucial for inward rectification and ion permeation. Mutation of E224 caused the channel to conduct significant time-dependent outward current and attenuated the affinity of Mg^{2+} and polyamine block. This is achieved mainly by decreasing the on-rate of the blockers (unpublished data). Interestingly, mutation of E224 induced Mg^{2+}- and polyamine-independent rectification, which we think results from alteration of ion permeation rather than from an open–close gating of the channel. The flickery single-channel openings in E224G, which occur in the absence of any blocking molecules on either side of the channel pore, further suggest alteration of K^+ binding and permeation in the pore. Apparently, interactions between E224 with the blockers and K^+ involve an electrostatic mechanism, as suggested by the correlation between binding affinity and number of charges on a blocker. But other mechanisms seem also to contribute, since the effect of mutations on Mg^{2+} block as well as ion permeation depends on both the chemical nature and the

size of the substituting residue. In this regard, E224 differs from the residue (default or substitutions) at position 171 in ROMK1, whose effect can be accounted for by simple electrostatic principles (Lu and MacKinnon, 1994).

Although mutation of E224 produced profound effects in IRK1, mutation of its counterpart in ROMK1 to glutamate had no effect on inward rectification. This is not completely unexpected, since the analogous position is occupied by a glutamate residue in rcK$_{ATP}$-1, a cloned ATP-sensitive K^+ channel exhibiting weak rectification as ROMK1 (Ashford et al, 1994). This observation suggests differences in the pore structure of IRK1 and ROMK1 and the existence of multiple determinants for Mg^{2+} and polyamine block. Indeed, additional structural elements besides D172 and E224 must contribute to inward rectification, since mutation of both residues together to their counterparts in ROMK1 fell short of converting blocker sensitivity and gating phenotype completely (see Table 1 and Figure 2D). Participation of residues from multiple distinct domains in blocker binding in IRK1 is in contrast to binding of divalent cations in voltage-gated Ca^{2+} channels, in which 4 glutamates from the H5 region form a single high affinity binding site (Yang et al., 1993).

Structural Organization of Inwardly Rectifying K^+ Channel Pore

The profound effects produced by mutation of E224 and D172 on various pore properties related to ion conduction suggest that part of the hydrophilic C-terminal domain encompassing E224, as well as the hydrophobic M2 domain (Lu and MacKinnon, 1994; Stanfield et al., 1994), contributes to the channel pore in IRK1. Because the H5 region of inwardly rectifying K^+ channels is highly homologous to that of voltage-gated K^+ channels and may possess several K^+-binding sites (unpublished data), it is also expected to contribute. Thus, the pore of inwardly rectifying K^+ channels may be composed of multiple distinct domains. As expected for a hydrophilic domain, the entire C-terminus has been stably expressed in bacteria and purified as a water-soluble protein (Huang et al., submitted). The region harboring E224 is particularly hydrophilic (out of 23 residues from position 213 to 235, 9 are charged and 4 polar); therefore, it seems unlikely to fold back into the membrane. The simple additivity of binding energy change induced by mutation of E224 and D172 suggests that these 2 residues interact weakly with each other and thus are possibly physically far apart in space (Wells, 1990). Such distributed binding determinants may favor interaction with polyamines, particularly SPD and SPM, which are linear molecules with multiple dispersed positive charges. Contribution of regions from the hydrophilic C-terminal domain in addition to the H5 region and hydrophobic M2 domain to channel pore may offer a molecular correlate for the predicted long pore of inwardly rectifying K^+ channels (Hille and Schwarz, 1978; Hille, 1992).

Experimental Procedures

Molecular Biology

Site-directed mutations were generated by PCR cassette mutagenesis and confirmed by sequencing. To obtain high level expression, wild-type IRK1 and all mutants were subsequently subcloned into expression vector pGEMHE (a generous gift from Dr. Emily Liman), which contains 5′ and 3′ untranslated regions of a Xenopus β-globin gene separated by a polylinker containing multiple cloning sites (Liman et al., 1992). Current was recorded from Xenopus oocytes 2–20 days after injection of 1–10 ng of cRNA transcribed in vitro using the T7 RNA polymerase.

Electrophysiology

Patch-clamp recordings were performed using the Axopatch 200A amplifier (Axon Instruments). Oocytes were bathed in a Mg^{2+}- and polyamine-free control solution containing 110 mM KCl, 10 mM HEPES, 9 mM EGTA, 1 mM EDTA, and 200 mM $CaCl_2$ (pH adjusted to 7.3 with KOH; total K^+, ~140 mM). Recording glass pipettes pulled from pyrex glass tubes (Corning) were filled with either the control bath solution or a solution of 130 mM KCl, 10 mM HEPES, and 3 mM $MgCl_2$ (pH adjusted to 7.3 with KOH; total K^+, ~140 mM) and had resistances of 0.3–1.5 MΩ for macropatch or 6–11 MΩ for single-channel recording. Currents were generated by either voltage ramps or steps with varying durations (0.35–4 s) for different channels, depending on the time course of the voltage-dependent relaxation. At the end of all experiments, the membrane patch, excised into inside-out configuration, was exposed to a solution containing 10 mM free Mg^{2+} to induce complete current rundown. Subsequently obtained records were used for off-line leak subtraction. Current records were filtered at 100 Hz to 5 kHz and digitized at 250 Hz to 50 kHz for macropatch recordings, and filtered at 1 kHz and digitized at 2.5 kHz for single-channel recordings. Experiments were done at room temperature (22°C–24°C).

Free Mg^{2+} concentration was obtained by adding to the control bath solution appropriate amounts of $MgCl_2$, calculated using stability constants of 8.83 for EDTA and 5.28 for EGTA binding of Mg^{2+} (Martell and Smith, 1974). Polyamines were added to the control bath solution immediately before the experiment and used for <3 hr. In calculating percentage block, current was normalized to the same size at −100 mV to minimize the effect of rundown.

Data Analysis

Unless indicated, dose–response data for wild-type IRK1, E224D, and E224G were fitted to the sum of two Hill equations:

$$I / I_{Con} = I_1 / \{1 + ([X] / K_{D1})\} + I_2 / \{1 + ([X] / K_{D2})\} \quad (1),$$

where [X] is the free Mg^{2+} or polyamine concentration, I_1 and I_2 are the fractional currents of the two components ($I_1 + I_2 = 1$), and K_{D1} and K_{D2} are the apparent dissociation constants. Data for other channels were fitted to the Hill equation:

$$I / I_{Con} = 1 / \{1 + ([X] / K_D)^n\} \quad (2),$$

where n is the Hill coefficient, and [X] and K_D have the same meaning as above.

The voltage dependence of channel activation was determined from the relationship of chord conductance with voltage according to the Boltzmann equation:

$$g / g_{max} = (1 - g_{min}) / \{1 + \exp[(V - V_h) / k]\} + g_{min} \quad (3),$$

where V_h is the half-activation voltage and k is the slope factor.

The voltage dependence of Mg^{2+} block was examined using the Woodhull (1973) model according to the equation:

$$K_D(V) = K_D(0)\exp(-2\delta FV / RT) \quad (4),$$

where $K_D(0)$ is the apparent dissociation constant at 0 mV membrane potential, δ is the fractional electrical distance from the cytoplasmic side, and F, R, and T have their usual thermodynamic meanings (Hille, 1992).

Data are presented as mean ± SEM (number of observations).

Acknowledgments

We thank S. Hebert and A. Brown for ROMK1, E. Liman for pGEMHE vector, S. Fried for DNA sequencing, and C. L. Huang, E. Reuveny, A. Tinker, and B. Zhao for comments on an earlier version of the manuscript. This work was supported by a grant from the National Institutes of Health and a fellowship from the Muscular Dystrophy Association (J. Y.). Y. N. J. and L. Y. J. are Howard Hughes Investigators.

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Received January 30, 1995; revised March 8, 1995.

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