# Contributions of a Negatively Charged Residue in the Hydrophobic Domain of the IRK1 Inwardly Rectifying $K^+$ Channel to $K^+$-Selective Permeation

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ABSTRACT Inwardly rectifying $K^+$ channels are highly selective for $K^+$ ions and show strong interaction with ions in the pore. Both features are important for the physiological functions of these channels and pose intriguing mechanistic questions of ion permeation. The aspartate residue in the second putative transmembrane segment of the IRK1 inwardly rectifying $K^+$ channel, previously implicated in inward rectification gating due to cytoplasmic $Mg^{2+}$ and polyamine block, is found in this study to be crucial for the channel's ability to distinguish between $K^+$ and $Rb^+$ ions. Mutation of this residue also perturbs the interaction between the channel pore and the $Sr^{2+}$ blocking ion. Our studies suggest that this aspartate residue contributes to a selectivity filter near the cytoplasmic end of the pore.

## INTRODUCTION

Inwardly rectifying $K^+$ channels are found in excitable as well as in nonexcitable cell types. They serve a variety of cellular functions, including the control of neuronal excitability and heart rate, insulin release from the pancreas, and the flow of salt across the kidney epithelium (Hille, 1991; Wang et al., 1992). Underlying this wide range of cellular functions is the highly selective $K^+$ permeation of inwardly rectifying $K^+$ channels. Because these channels are active at membrane potentials near the potassium equilibrium potential ($E_K$) (Hodgkin and Horowicz, 1959; Hagiwara and Takahashi, 1974), they keep the resting potential of a cell near $E_K$ and may contribute to $K^+$ secretion (Hille, 1991; Wang et al., 1992). Regulation of inwardly rectifying $K^+$ channel activities via neurotransmitters, hormones, or the metabolic state of the cell alters the membrane potential, thereby modulating the activity of voltage-dependent calcium channels in pancreatic $\beta$ cells or the excitability of neurons and muscles (Ashcroft and Ashcroft, 1989; Hille, 1991).

The physiological requirement of inwardly rectifying $K^+$ channels is to allow $K^+$-selective permeation near $E_K$, but not to cause massive $K^+$ loss from the cell. This is fulfilled by inward rectification gating; these $K^+$ channels allow much greater $K^+$ influx below $E_K$ than $K^+$ efflux above $E_K$. Inward rectification of these channels is caused by a block of the channel pore by cytoplasmic cations such as $Mg^{2+}$ ions (Matsuda et al., 1987; Vandenberg, 1987) and polyamine ions (Lopatin et al., 1994; Ficker et al., 1994; Fakler et al., 1995), which exert only very mild effects on other types of $K^+$ channels, such as the voltage-gated $K^+$ channels.

The high $K^+$ selectivity of inwardly rectifying $K^+$ channels and their susceptibility to inward rectification gating by pore blocking ions is manifested by their ability to distinguish between permeant ions such as $K^+$ and $Rb^+$ (Hagiwara and Takahashi, 1974; Silver et al., 1994), and the easily detectable interaction of the channel pore with monovalent and divalent cations (Gay and Stanfield, 1977; Standen and Stanfield, 1978, 1980; Ohmori, 1978; Biermans et al., 1987; Harvey and Ten Eick, 1989). Indeed, these characteristics indicate that inwardly rectifying $K^+$ channels are among the most selective of $K^+$ channels (Hille, 1991), which in general possess long pores that allow multiple $K^+$ ions to go through in single file (Hille and Schwarz, 1978). The individual $K^+$ binding sites in the pore impart the channel with the ability to discriminate different ions. The presence of multiple $K^+$ ions in the pore probably results in sufficient electrostatic repulsion to facilitate their dissociation from the binding sites, thereby ensuring large $K^+$ flux.

In this study, we report that an acidic residue (D172) in the second hydrophobic segment (M2) of the IRK1 inwardly rectifying $K^+$ channel (Kubo et al., 1993a) is responsible for the low permeability ratio $P_{Rb}/P_K$ (about one-third), when the channel is exposed to $K^+$ ions on the extracellular side and $Rb^+$ ions on the cytoplasmic side of the membrane. In addition to the previously reported effect on channel block by $Mg^{2+}$ (Stanfield et al., 1994; Yang et al., 1995) and polyamines (Lopatin et al., 1994; Ficker et al., 1994; Yang et al., 1995), we show that mutations of D172 affect the rate of $Sr^{2+}$ block because of binding of $Sr^+$ to a site roughly halfway into the membrane electric field; removing the negative charge increases the off rate, whereas addition of a methyl group to the side chain reduces the affinity of the $Sr^{2+}$ binding site by increasing the off rate as well as decreasing the on rate. These findings indicate that

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this aspartate residue is intimately involved in interactions between permeant or blocking ions and the channel pore. A preliminary account of these results has been reported previously (Reuveny et al., 1995).

## MATERIALS AND METHODS

### Molecular biology

Single amino acid changes were produced either by site-directed mutagenesis or by using the polymerase chain reaction technique. In some cases, a high level of channel expression was achieved by subcloning the channel coding sequence into the pGEMHE vector, a high expression vector containing the 5′ and 3′ untranslated sequence of the *Xenopus laevis* β-globin gene (Liman et al., 1992). All mutations were verified by sequencing of the region containing the mutation.

### Electrophysiology

Oocytes were isolated as described previously (Kubo et al., 1993b) and injected with 47 nl solution containing in vitro transcribed 10–100 ng/μl cRNA. The macroscopic currents were recorded using the excised macropatch technique modified after the method of Collins et al. (1992). Thick-wall borosilicate glass pipettes (Corning) were pulled to 5–10 μm initial diameter. The pipettes were later broken to a 40–50 μm diameter. This was achieved by pushing the pipette tip into a melted glass and quickly cooling it, causing the pipette to break from the glass to form a tip with a uniform edge, followed by extensive fire polishing to the desired diameter (15–30 μm). This procedure gave electrodes with steep descent to maximize speed of recording. Pipettes were coated as described (Collins et al., 1992). For single-channel recordings pipettes were filled with 150 mM KCl, 2 mM $MgCl_2$, and 10 mM HEPES (pH 7.1). For macro inside-out patches pipettes were filled with 150 mM KCl or 150 mM RbCl, 10 mM HEPES, 10 mM EDTA, and 1 mM EGTA (pH 6.9–7.0). The latter solution was also used as an internal solution in the macropatch recordings. In experiments where the internal rubidium concentrations were lower than 150 mM, sucrose was used as a substitute. All recordings were done using the EPC-7 amplifier (List) and pClamp acquisition software. For macropatches data were filtered at 3–10 kHz (eight-pole Bessel) and digitized at 10–50 kHz.

Cell-attached single-channel recording of IRK1 wild-type and mutant channels were performed at room temperature. Single-channel recordings and analysis were performed as described previously (Kubo et al., 1993b). Currents were recorded continuously on a video cassette tape using a VR-10b analog-to-digital converter (Instrutech). For analysis, segments of data were transferred to a PC computer filtered at 1–2 kHz and analyzed using pClamp software. Means of open and closed time distributions were compiled and fitted to a single exponential time course. The mean open and closed times truly reflect the blocking and unblocking rates of $Sr^{2+}$, because the kinetics for channel opening and closing in the absence of $Sr^{2+}$ are three to four orders of magnitude slower (Kubo et al., 1993a). Because $Sr^{2+}$ binding to the channel is a first-order 1:1 binding reaction (Shioya et al., 1993), we calculated the blocking (closed) and unblocking (open) rate constants from the intercept of the fitted exponential curve at 0 mV. Assuming that external $Sr^{2+}$ has to overcome an energy barrier to reach the binding site (Shioya et al., 1993), the slope of the blocking rate gives the electrical distance from the extracellular side of the membrane to the peak of the energy barrier, $\delta_a$, according to the equation slope = $K \exp(z\delta_a V_m F/RT)$ (Woodhull, 1973), in which $K$ is inversely proportional to the energy barrier the $Sr^{2+}$ ion has to face entering the pore, $z$ is the valence of $Sr^{2+}$, $V_m$ is the membrane potential, and $F$, $R$, and $T$ have their usual meanings (Hille, 1991). Similarly, the unblocking rate, $\delta_b$, gives the electrical distance between the energy barrier and the $Sr^{2+}$ binding site. The total electrical distance, $\delta$, from the external side of the membrane to the $Sr^{2+}$ binding site is $\delta = \delta_a + \delta_b$.

The permeability ratios were measured under a biionic condition (potassium or rubidium on the extracellular or intracellular sides) and calculated using a modified form of the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin and Katz, 1949), $P_{Rb}/P_K = \exp(zFE_{rev}/RT)$, in which $E_{rev}$ is the reversal potential, $z$ is the valence of the ion, and $F$, $R$, and $T$ have their usual meanings (Hille, 1991). The driving force (DF) for channels exposed to various concentrations of $Rb^+$ was calculated using $DF = RT/F \log([Rb]_{in}/[Rb]_{out}) + V_m$, where $V_m$ is the electrical potential across the membrane.

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

## RESULTS

### Selectivity of IRK1 channels for permeant ions in the internal solution is altered by the D172N mutation

To determine the ratio of permeability to internal $Rb^+$ versus $K^+$ ions, we exposed inside-out membrane patches from IRK1-expressing *Xenopus* oocytes to 150 mM RbCl on the cytoplasmic side and 150 mM KCl on the extracellular side of the membrane, and measured the reversal potential (see Materials and Methods). The membrane had to be depolarized to +29 ± 6 mV to achieve zero net current, i.e., a balance of $K^+$ influx with $Rb^+$ efflux (Fig. 1 *A*). Compared to the reversal potential of 0 mV for channels exposed to symmetric KCl solutions on both sides, the positive reversal potential of 29 mV of IRK1 channels exposed to external $K^+$ and internal $Rb^+$ indicates that these channels have greater permeability to internal $K^+$ ions than internal $Rb^+$ ions with a permeability ratio (i)$P_{Rb}/P_K$ of 0.35 ± 0.06 ($n$ = 6) (Fig. 1 *E*).

Substitution of the aspartate residue in the M2 segment of IRK1 with glutamate (D172E) did not alter the permeability ratio (0.36 ± 0.04, $n$ = 4) (Fig. 1 *E*). By contrast, the asparagine-for-aspartate mutation (D172N) increased it to 0.68 ± 0.06 ($n$ = 8) (Fig. 1, *B* and *E*), indicating that the negative charge of this residue is crucial for the selectivity between $Rb^+$ and $K^+$ ions. In the absence of the negative charge, however, a dependence of selectivity on the size of the side chain became evident: (i)$P_{Rb}/P_K$ was increased to a much greater extent in the D172N mutant than in the D172Q mutant (0.46 ± 0.02, $n$ = 5), when glutamine was used to substitute for aspartate (Fig. 1 *E*). Thus, both the size and the charge of the side chain at position 172 affect the ability of the channel to distinguish between these two types of permeant ions.

The ROMK1 channel of the inwardly rectifying $K^+$ channel family shows weak inward rectification and has an asparagine (N171) at the position corresponding to D172 of IRK1 (Ho et al., 1993). Similar to the D172N mutant of IRK1, the ROMK1 channel has low selectivity ((i)$P_{Rb}/P_K$ = 0.62 ± 0.02, $n$ = 8) (Fig. 1, *C* and *E*). Although replacing D172 of IRK1 with asparagine converted the permeability ratio of IRK1 to that of ROMK1, replacing the corresponding asparagine of ROMK1 with aspartate (N171D) did not significantly enhance the selectivity of the mutant ROMK1 channel ((i)$P_{Rb}/P_K$ = 0.58 ± 0.03, $n$ = 2) (Fig. 1 *E*), indicating that other residues in IRK1 and ROMK1 also contribute to their difference in ion selectivity.

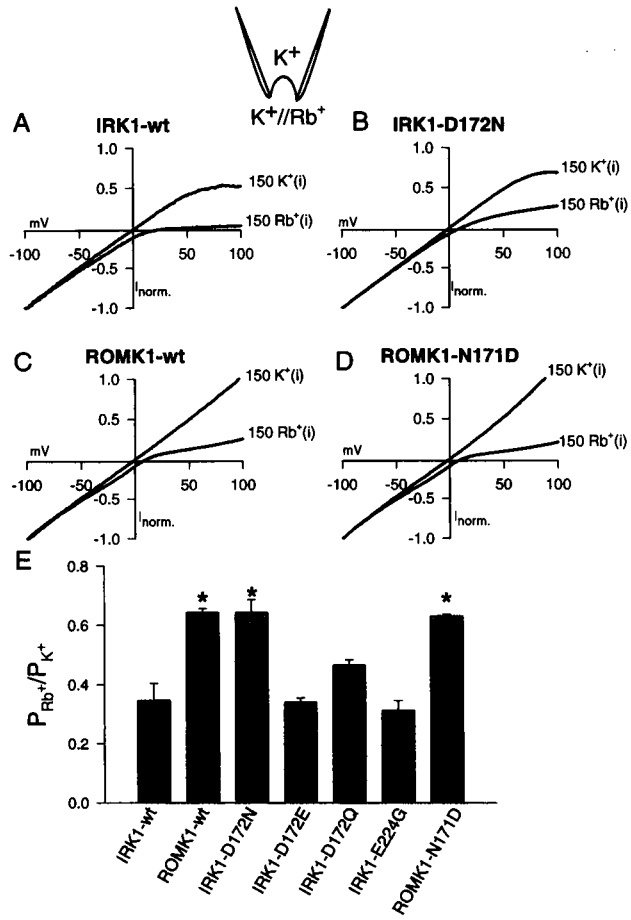


FIGURE 1 Effects of mutations in IRK1 and ROMK1 on selectivity for internal $Rb^+$ versus $K^+$ measured under external $K^+$. Comparison of normalized current-voltage relations recorded from inside-out excised macropatches for IRK1-wt (*A*), IRK1-D172N mutant (*B*), ROMK1-wt (*C*), and ROMK1-N171D mutant under either 150 mM symmetrical $K^+$ or the biionic condition of $K^+$ on the extracellular side and $Rb^+$ on the intracellular side of the membrane (as noted). Membrane potential was changed continuously at a rate of 5 mV/ms from −100 to +100 mV. (*E*) Mean (±SEM) permeability ratio ((i)$P_{Rb}{}^+/P_K{}^+$) calculated from the reversal potential for IRK1-wt, ROMK1-wt, and different mutant channels (see experimental procedure). Asterisks indicate significant difference from the permeability ratio of wild-type IRK1 ($p < 0.05$).

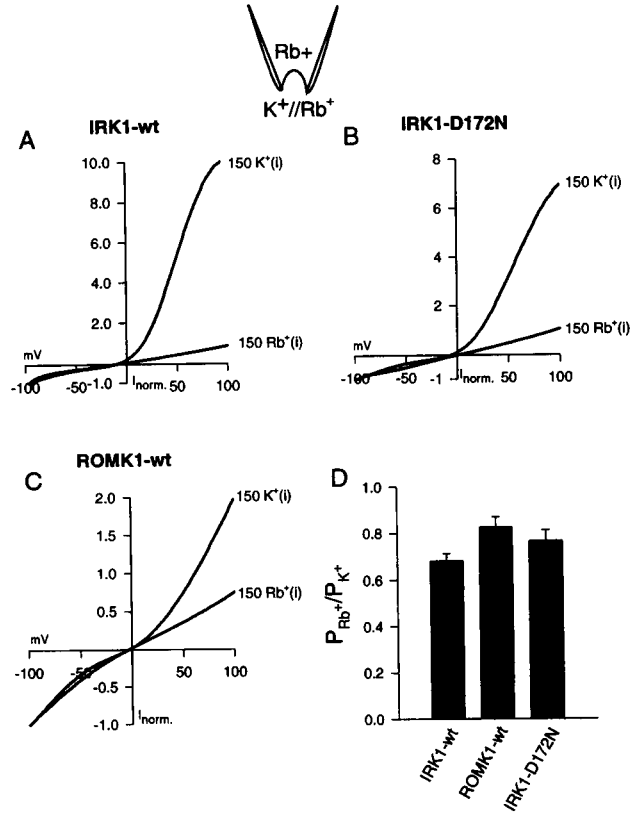


FIGURE 2 Effects of mutations in IRK1 and ROMK1 on selectivity for internal $Rb^+$ versus $K^+$ measured under external $Rb^+$ versus internal $K^+$. Comparison of normalized current-voltage relations (at −100 mV) recorded from inside-out excised macropatches for IRK1-wt (*A*), IRK1-D172N mutant (*B*), and ROMK1-wt (*C*) under either 150 mM symmetrical $K^+$ or the biionic condition of $Rb^+$ on the extracellular side and $K^+$ on the intracellular side of the membrane (as noted). Membrane potential was changed continuously at a rate of 5 mV/ms from −100 to +100 mV. (*D*) Mean permeability ratio ((o)$P_{Rb}{}^+/P_K{}^+$) calculated from the reversal potential (see experimental procedure).

## Differential effects of permeant ions from the cytoplasmic side on kinetics of $Rb^+$ influx

Different results were obtained when IRK1 channels in excised membrane patches were exposed to divalent-free 150 mM RbCl solution on the extracellular side of the membrane. The permeability ratio in the reverse biionic condition, external $Rb^+$ versus internal $K^+$ ((o)$P_{Rb}/P_K$ = 0.68 ± 0.03, $n$ = 11), is different from that in the biionic condition of external $K^+$ versus internal $Rb^+$ ((i)$P_{Rb}/P_K$ = 0.35 ± 0.06, $n$ = 6) (Fig. 1 and Fig. 2, *A* and *D*), which is similar to previous findings for voltage-gated $K^+$ channels (Wagoner and Oxford, 1987; Heginbotham and MacKinnon, 1993; Pérez-Cornejo and Begenisich, 1994). This observation raises the possibility that the structure of the inwardly rectifying potassium channel pore is asymmetric and may be accounted for by the presence of multiple permeant ion binding sites, as in the case of inwardly rectifying $K^+$ channels of different cell types (Hille, 1991) and of the ROMK1 channel expressed in *Xenopus* oocytes (Lu and MacKinnon, 1994a). These observations also raise the possibility that the D172N mutation affects the portion of the channel pore near the cytoplasmic surface. Indeed, this mutation greatly reduced the ability of the channel to discriminate between $Rb^+$ and $K^+$ ions on the cytoplasmic side of the membrane but had little effect on the ability of the channel to discriminate between these two permeant ions on the extracellular side of the membrane; (o)$P_{Rb}/P_K$ = 0.76 ± 0.05 ($n$ = 6) for D172N (Fig. 2 *B*), as compared to 0.68 ± 0.03 ($n$ = 11) for the wild-type IRK1 channel (Fig. 2 *A*) and 0.82 ± 0.05 ($n$ = 5) for ROMK1 (Fig. 2, *C* and *D*). This possibility is also consistent with the recent finding that when the N171 residue of ROMK1 is replaced with histidine, the mutant ROMK1 channel becomes sensitive to pH in the internal solution (Lu and MacKinnon, 1994b).

Not only does the IRK1 channel show lower selectivity for $Rb^+$ and $K^+$ ions on the extracellular side, as compared to its high selectivity for these permeant ions on the cytoplasmic side of the membrane; the type of permeant ion present in the internal solution determines the kinetics of $Rb^+$ influx as well. The inward $Rb^+$ currents show "inactivation"; the rate and the extent of this inactivation depend on the type of permeant ion present in the internal solution (Fig. 3). The inactivation of $Rb^+$ inward current through the IRK1 channel is much more pronounced when the internal solution contains $Rb^+$ (Fig. 3 *B*) rather than $K^+$ ions (Fig. 3 A). Furthermore, with $Rb^+$ in the internal solution, the rate of $Rb^+$ inward current inactivation is enhanced at more hyperpolarized potentials, showing a steep voltage dependence at potentials below −90 mV (Figs. 3 *B* and 4 *A*), as is the extent of inactivation (Fig. 3, *B* and *E*). Because no divalent cations are present in the solution on either side of the membrane, the inactivation of $Rb^+$ inward current cannot be due to voltage-dependent block by divalent cations (Mitra and Morad, 1991). The rate of inactivation of the inward $Rb^+$ current at hyperpolarized potentials was a function of the driving force for $Rb^+$ ions rather than the membrane potential per se; decreasing the internal $Rb^+$ ion concentration shifted the voltage dependence of inactivation along the voltage axis so that its dependence on the driving force (DF) remained unchanged (Fig. 4 *B*). The extent of inactivation of the $Rb^+$ inward currents ($1 - I_{ss}/I_{peak}$), on the other hand, is significantly reduced by decreasing the concentration of $Rb^+$ ions in the internal solution to 34 mM (Fig. 4 *C*). The D172N mutation increased the rate of inactivation but decreased the extent of the inactivation (Fig. 3, *D*, *E*, *F*).

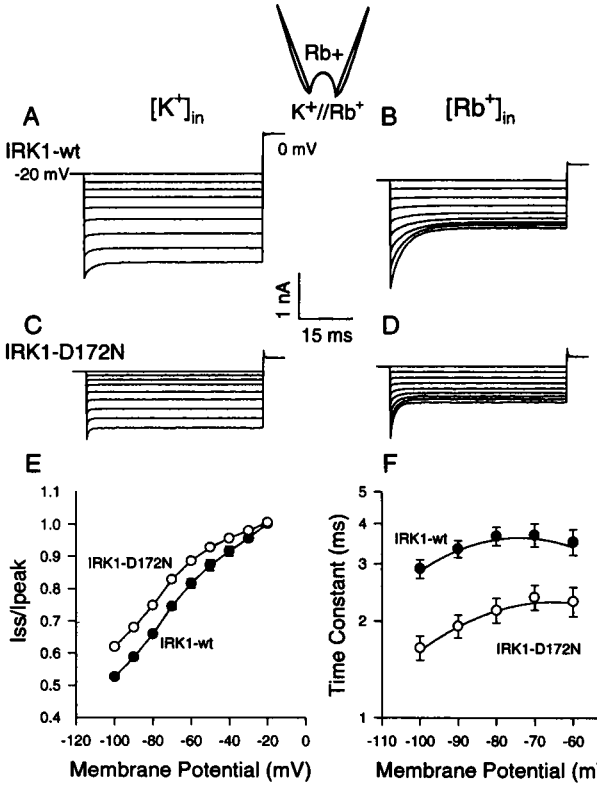


FIGURE 3 The effect of internal $Rb^+$ ions on inward $Rb^+$ currents. Inward $Rb^+$ currents through IRK1-wt and IRK1-D172N mutant recorded with either $K^+$ (*A* and *C*) or $Rb^+$ (*B* and *D*), in the internal solution, respectively. Inward currents were elicited from a holding potential of −20 mV by step hyperpolarizations up to −100 mV in 10-mV increments. (*E*) Mean ratio of the steady-state ($I_{ss}$) to peak ($I_{peak}$) current amplitudes for IRK1 (●) and IRK1-D172N (○) mutant channel at different membrane potentials. (*F*) Mean of time constants obtained from fitting current traces to monoexponential time course at symmetrical $Rb^+$ for IRK1-wt (●) and IRK1-D172N (○) mutant channel at different membrane potentials.

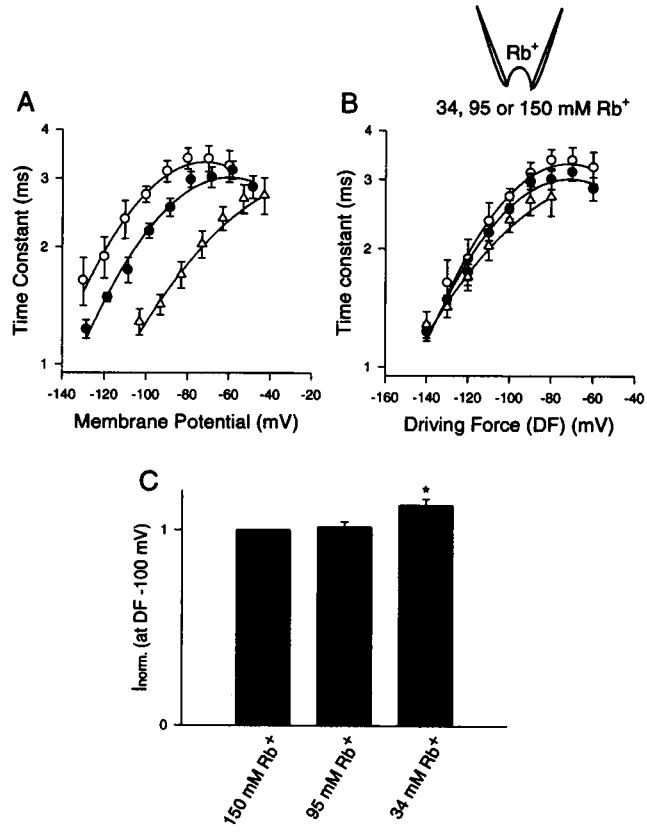


FIGURE 4 The time course of IRK1 inward current inactivation varies with driving force for $Rb^+$ ions. (*A*) Voltage dependence of mean inactivation rates of inward $Rb^+$ currents at 150 mM (○), 95 mM (●), and 34 mM (△) internal $Rb^+$. (*B*) Data similar to that in *A* but plotted as a function of the calculated driving force (DF) for $Rb^+$ ions (see experimental procedure). (*C*) Normalized mean ratios of steady state ($I_{ss}$) to peak ($I_{peak}$) current amplitudes (at DF-100 mV) with different concentration of internal $Rb^+$ ions. The ratios were normalized to that for 150 mM internal $Rb^+$.

## Voltage-dependent channel block by external $Sr^{2+}$

In addition to the effect of the D172N mutation on the selectivity between internal $K^+$ versus $Rb^+$ ions and the block by internal cations (Stanfield et al., 1994; Ficker et al., 1994), we asked whether this mutation also affects the block of channel pore by external ions. We chose to examine $Sr^{2+}$ block of the open IRK1 channel because individual events

of $Sr^{2+}$ block and unblock can be readily resolved and distinguished from gating of the IRK1 channel, which normally stays open for more than 500 ms. Indeed, this analysis revealed that the binding and dissociation of $Sr^{2+}$ from its binding site are affected differently by altering the size and/or charge of D172 (Fig. 5). The presence of 10 mM $Sr^{2+}$ in the extracellular solution caused the IRK1 channel to flicker between the conducting state and the blocked state (Fig. 5 *A*). The $Sr^{2+}$ block was voltage dependent (Fig. 5, *E* and *F*), as reported previously (Standen and Stanfield, 1978; Shioya et al., 1993). The blocking rate increased (Fig. 5 *E*), whereas the unblocking rate decreased with increasing hyperpolarization (Fig. 5 *F*). This influence of membrane electric field over the interaction between the positively charged $Sr^{2+}$ ion and its binding site may be explained by assuming that the $Sr^{2+}$ binding site is within the pore, so that it detects a fraction ($\delta$) of the electrical field across the membrane. This electrical distance, $\delta$, was not significantly different between the wild-type IRK1 channel (0.56 ± 0.06, $n = 3$) and the mutants D172N (0.63 ± 0.02, $n = 3$), D172E (0.58 ± 0.03, $n = 4$) and D172Q (0.70 ± 0.05, $n = 3$).

The blocking rate for $Sr^{2+}$ appears to depend on the size of the side chain for the residue at position 172 (Fig. 5 *E*).

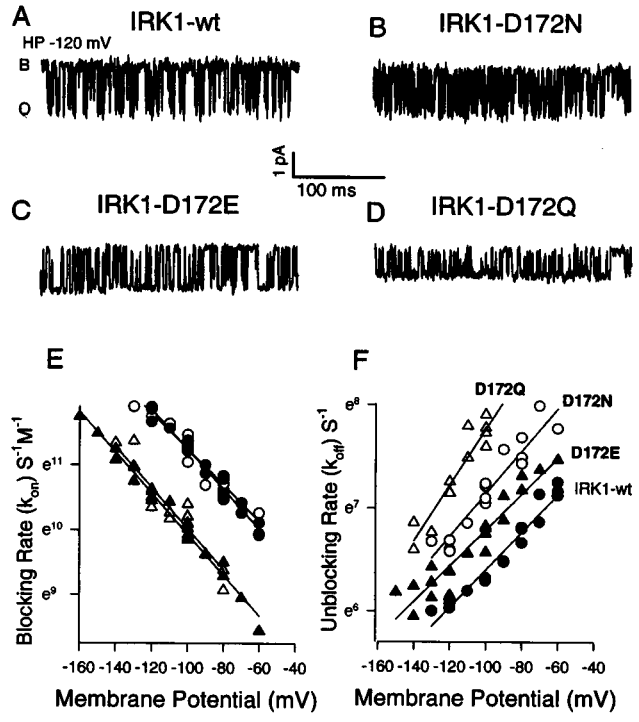


FIGURE 5 Single channel recordings of IRK1 and mutant channels in the presence of extracellular $Sr^{2+}$ ions. Cell-attached single-channel current traces in the presence of 10 mM external $Sr^{2+}$ recorded from IRK1-wt (*A*), IRK1-D172N (*B*), IRK1-D172E (*C*), and IRK1-D172Q (*D*) mutant channels at a holding potential of −120 mV. The open channel state is plotted as a downward deflection for all traces. (*E*) Voltage dependence of $Sr^{2+}$ blocking rates for IRK1-wt (●), IRK1-D172N (○), IRK1-D172E (▲), and IRK1-D172Q (△) mutant channels. (*F*) Voltage dependence of $Sr^{2+}$ unblocking rates; symbols as in *E*. For *E* and *F* individual measurements from each channel are plotted and fitted to a straight line by linear regression (*solid lines*).

The blocking rate in the absence of an electric potential across the membrane ($k_{on(0)}$ at 0 mV) is faster for the wild-type IRK1 channel (3.61 ± 0.32 × $10^3$ $s^{-1}M^{-1}$, $n = 3$) and the D172N mutant (4.79 ± 0.57 × $10^3$ $s^{-1}M^{-1}$, $n = 3$) than for the D172E mutant (1.25 ± 0.1 × $10^3$ $s^{-1}M^{-1}$, $n = 4$) and the D172Q mutant (1.11 ± 0.32 × $10^3$ $s^{-1}M^{-1}$, $n = 3$). By contrast, the unblocking rate ($k_{off(0)}$ at 0 mV) increased significantly ($p < 0.05$) upon removal of the negative charge from the residue at position 172, from 3.87 ± 0.46 × $10^3$ $s^{-1}$ ($n = 3$) for the wild-type IRK1 channel to 1.07 ± 0.21 × $10^4$ $s^{-1}$ ($n = 3$) for D172N, and from 4.25 ± 1.7 × $10^3$ $s^{-1}$ ($n = 4$) for D172E to 2.95 ± 0.41 × $10^4$ $s^{-1}$ ($n = 3$) for D172Q (Fig. 5 F).

## DISCUSSION

Potassium-selective permeation in the IRK1 inwardly rectifying $K^+$ channel appears to involve the aspartate residue (D172) in the second hydrophobic segment; removing the negative charge via the D172N mutation greatly reduced the ability of the channel to distinguish between internal $K^+$ and $Rb^+$ ions, as tested in the stringent biionic condition. In addition to this effect on $Rb^+$ selectivity, and the previously reported effects on inward rectification gating presumably due to channel block by $Mg^{2+}$ (Stanfield et al., 1994; Lu and MacKinnon, 1994b; Yang et al., 1995) and polyamines (Lopatin et al., 1994; Ficker et al., 1994; Yang et al., 1995) from the cytoplasm, we find that both the size and the charge of this aspartate residue influence the affinity of a $Sr^{2+}$ binding site in the pore that is roughly in the center of the membrane electric field. These results strongly suggest that D172 is in the permeation pathway and contributes to a selectivity filter that discriminates between the two different permeant ions, $K^+$ and $Rb^+$. The plausible location of this selectivity filter in the channel pore and the multiion nature of the inwardly rectifying $K^+$ channel will be discussed here.

### A selectivity filter that distinguishes between cytoplasmic $Rb^+$ and $K^+$ ions

The ability of IRK1 channel to discriminate between $Rb^+$ and $K^+$ ions on the cytoplasmic side of the membrane is manifested in two different ways. First, this channel exhibits much higher selectivity for these permeant ions on the cytoplasmic side ((i)$P_{Rb}/P_K$ = 0.35) than on the extracellular side ((o)$P_{Rb}/P_K$ = 0.68). Second, inward current carried by $Rb^+$ ions exhibits prominent inactivation when $Rb^+$ ions but not when $K^+$ ions are present in the intracellular solution. The first observation indicates that a selectivity filter that can distinguish between $K^+$ and $Rb^+$ is present near the cytoplasmic end of the pore. The second phenomenon may be accounted for either by interactions between channel pore and the permeant ions at the cytoplasmic mouth of the pore, or by a separate mechanism for $Rb^+$ to cause channel inactivation, as discussed below.

### Possible mechanisms for the dependence of inactivation of $Rb^+$ inward current on internal $Rb^+$ concentration

Inwardly rectifying $K^+$ channels exhibit several features indicative of a long pore harboring multiple $K^+$ ions (Hille and Schwarz, 1978). It is expected for such a long pore that when the permeant ion concentration at the cytoplasmic side is sufficiently high, ions at the cytoplasmic opening of the pore may impede inward ion flux by occupying binding sites in the pore (back flow), thereby reducing conductance (Hille and Schwarz, 1978; Lu and MacKinnon, 1994a). In light of such properties of a multiion long pore, one possible explanation for the apparent inactivation of the inward $Rb^+$ current is the block of permeation by $Rb^+$ ions that have exited the pore and accumulated in or near the vestibule of the channel. When the rate of ions exiting the pore is faster than the rate of ion clearance from the vestibule, due to either local interaction of $Rb^+$ with the channel protein or geometric constraints, local $Rb^+$ ion concentration will increase. This will result in more pronounced pore block (i.e., less $Rb^+$ inward current) until a steady state is reached, when the reduced $Rb^+$ influx supplies as many $Rb^+$ ions to the cytoplasmic vestibule as the $Rb^+$ ions moving from this vestibule to the bulk solution. Consistent with this hypothesis, the rate of inactivation of $Rb^+$ inward current varies with the driving force for $Rb^+$ ions (Fig. 4 *B*).

An alternative explanation for the observed inactivation of $Rb^+$ inward current would be a $Rb^+$-mediated gating machinery analogous to what has been proposed for the calcium-mediated inactivation of calcium channels (Eckert and Chad, 1984). In this scenario, $Rb^+$ ions that exit the pore and accumulate in or near the vestibule are able to interact with the channel and induce conformational changes that promote inactivation. As in calcium channels, this inactivation is selective; $Rb^+$ is more effective than $K^+$ in causing inactivation.

Single-channel analysis could allow one to determine whether the inactivation of $Rb^+$ inward current is due to such a gating machinery or a partial channel block due to back flow of $Rb^+$ ions accumulating at the cytoplasmic mouth of the pore. Unfortunately, the single-channel $Rb^+$ currents turned out to be too small to be resolved from the noise. It thus remains possible that the inactivation of $Rb^+$ inward current is due to a partial block of the channel pore by $Rb^+$, $Rb^+$-mediated inactivation, or both.

The dependence of ion permeation on the type of ion species present on the other side of the conduction pathway is also found for the cloned *Shaker* $K^+$ channel. $Cs^+$ outward currents through this channel are much smaller when external solution contains $Na^+$ rather than $K^+$ ions (Heginbotham and MacKinnon, 1993). It remains to be determined whether similar mechanisms are involved in these phenomena.

### Structural elements implicated for the multiion $K^+$ channel pore

Doubling of the ratio of permeability to internal $K^+$ versus $Rb^+$ is achieved by substitution of aspartate 172 of IRK1 with asparagine (D172N), indicating that this negatively charged residue in the second hydrophobic segment (M2) is involved with the $K^+$-selective permeation. Although the D172N mutation converted the high $K^+$ selectivity of IRK1 to the low $K^+$ selectivity of ROMK1, replacing asparagine 171 of ROMK1 with aspartate (N171D) did not significantly alter the selectivity for $K^+$ relative to $Rb^+$, suggesting that other structural elements also contribute to the difference between IRK1 and ROMK1 in ion selectivity. The M2 segment that harbors D172 of IRK1 corresponds to the S6 segment of voltage-gated $K^+$ channels (Kubo et al., 1993a). The S6 region in the *Shaker* $K^+$ channel has been shown to contribute to $K^+$ conductance and channel block by internal $Ba^{2+}$, tetraethylammonium, and 4-aminopyridine (Choi et al., 1993; Kirsch et al., 1993; Aiyar et al., 1994; Lopez et al., 1993; Shieh and Kirsch, 1994; Taglialatela et al., 1994). In addition, two other regions of voltage-gated $K^+$ channels, the H5 (or the P region) segment and the S4-S5 loop, contribute to $K^+$ permeation, because mutations in these regions affect the $K^+$ and $Rb^+$ conductance, $K^+$ selectivity, and block by external cations and peptide toxins (MacKinnon and Miller, 1989; MacKinnon and Yellen, 1990; Hartmann et al., 1991; Kavanaugh et al., 1991; Yellen et al., 1991; Yool and Schwarz, 1991; Heginbotham et al., 1992; Kavanaugh et al., 1992; Kirsch et al., 1992; De Biasi et al., 1993; Slesinger et al., 1993; Taglialatela et al., 1993, 1994; Heginbotham et al., 1994; Hidalgo and MacKinnon, 1995; Lü and Miller, 1995; Pascual et al., 1995). It remains to be determined whether the corresponding regions of inwardly rectifying $K^+$ channels are also involved in $K^+$ selectivity.

Besides residues within the hydrophobic domain, an acidic residue in the C-terminal hydrophilic domain of IRK1, glutamate 224 (E224), has been found to contribute to $K^+$ permeation and to channel block by internal $Mg^{2+}$ ions and polyamines (Yang et al., 1995). Replacing glutamate 224 of IRK1 with glycine (E224G), the corresponding residue in ROMK1, however, did not alter (i)$P_{Rb}/P_K$ ($0.32 \pm 0.03$, $n = 3$), unlike the D172N mutation of IRK1. This observation is consistent with the notion that D172 and E224 in IRK1 are physically distant from each other (Yang et al., 1995) and probably contribute independently to the multiion pore.

In conclusion, consistent with the multiion long-pore model of the inwardly rectifying $K^+$ channel, multiple structural elements have been implicated in $K^+$ permeation of the IRK1 channel. In particular, the contribution of D172 of the M2 region is significant because both the size and the charge of this residue play a critical role in determining the selectivity to $K^+$ versus $Rb^+$ permeation as well as the interaction of the channel with the blocking $Sr^{2+}$ ion in the pore.

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