Stable cation coordination at a single outer pore residue defines permeation properties in Kir channels

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Abstract In epithelial Kir7.1 channels a non-conserved methionine in the outer pore region adjacent to the G-Y-G selectivity filter (position +2) was found to determine unique properties for permeant and blocking ions characteristic of a K^+ channel in a single-occupancy state. The monovalent cation permeability sequence of Kir7.1 channels expressed in *Xenopus* oocytes was $Tl^+ > K^+ > Rb^+ > NH_4^+ > Cs^+ > Na^+ > Li^+$, but the macroscopic conductance for Rb^+ was ~8-fold larger than for the smaller K^+ ions, and decreased ~40-fold with the conserved arginine at the +2 position (Kir7.1M125R). Moreover, in Kir7.1 Rb^+ restored the typical permeation properties of other multi-ion channels indicating that a stable coordination of permeant ions at the +2 position defines the initial step in the conduction pathway of Kir channels.

Key words: Inwardly rectifying K channel; Kir7.1; Kir2.1; Ion selectivity; Anomalous mole fraction; Multi-ion pore

1. Introduction

Ion channels allow the passage of ions with remarkable selectivity and at open channel flux rates as large as 10^8 ions per second. The crystal structure of the *Streptomyces lividans* KcsA channel resolved at 3.2 Å now provides some insight into how most prokaryotic and eukaryotic K^+ channels may perform this amazing task [1,2]. Structural data together with steric and energetic considerations indicate that the highly conserved G-Y-G signature sequence in the P region amidst an 18 Å long filter region plays a major role in K^+ selectivity. It is thought that hydrated K^+ ions initially exchange their water molecules by the electronegative carbonyl oxygens of the selectivity filter's backbone whereby the dehydration energy is maximally compensated only for the right size K^+, but not Na^+ ions. The hydrophobic filter tunnel, which itself is adjusted to a canal of 3 Å width by a sheet of aromatic amino acids in the surrounding pore helices, then accommodates two K^+ ions at a distance of 7.5 Å (a third partially hydrated K^+ ion resides in a wider aqueous cavity near the inner mouth of the channel). One common view for the high throughput rates of the multi-ion pore is that, driven by the high electrochemical K^+ gradient across the membrane, electrostatic repulsion between the two K^+ ions lowers the binding affinity of the first, more distal K^+ ion. This results in its displacement out of the selectivity filter into bulk water with the second one taking its place [3–5].

In inwardly rectifying K^+ (Kir) channels residues in the outer mouth of the pore may also be involved in ion selectivity and permeation. A positively charged arginine two residues downstream of the selectivity filter (+2 position) is conserved in all Kir subunits, except in the recently described Kir7.1 [6–9]. In Kir2.1 this arginine is thought to form an exposed salt bridge together with a glutamate in the M1–H5 linker and thus stabilizes the selectivity filter and pore region [10]. Kubo [11] first reported slowing of current activation and a negative shift of the *I/V* relationship in Kir2.1R148Y mutants reasoning that this site was involved in the interaction with K^+, gating and block of Kir2.1. In the following it was demonstrated that this site, which is just outside the membrane electrical field, was accessible to external H^+, and acts as an external barrier for divalent cations such as Mg^{2+} [12,13]. Using Kir7.1 channels which naturally harbor a methionine residue at the +2 position we provide evidence in this report that this site acts as a K^+/Rb^+ switch, is crucially involved in the stable coordination and permeation of monovalent ions, and defines the initial step in the conduction pathway of the pore.

2. Materials and methods

The human Kir7.1 cDNA [6], the rat Kir2.1 cDNA [14], and mutant constructs thereof, which were engineered by 'Quickchange' site-directed mutagenesis (Stratagene, La Jolla, CA, USA), were subcloned into the polyadenylating transcription vector pSGEM (a gift of M. Hollmann). Defolliculated *Xenopus laevis* oocytes were injected with ~6 ng each of Kir channel cRNA and were incubated at 20°C in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM HEPES, pH 7.4) supplemented with 100 μg/ml gentamicin and 2.5 mM sodium pyruvate. Forty-eight hours after injection two electrode voltage-clamp measurements were performed with a TURBO TEC-10 C amplifier (npi, Tamm, Germany) and currents recorded with an EPC9 patch-clamp amplifier (Heka Electronics, Lambrecht, Germany). Stimulation and data acquisition were controlled by PULSE/PULSEFIT software (Heka). Oocytes were placed in a small volume perfusion chamber with a constant flow of ND96 or high K^+ solution (96 mM KCl, 2 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM HEPES, pH 7.4). In testing ion selectivity K^+ in the external solution was replaced by Tl^+, Rb^+, NH_4^+, Cs^+, Na^+, and Li^+. Data are presented as mean ± S.D. (number of cells).

3. Results and discussion

Kir7.1 channels give rise to currents that markedly differ from all other Kir channels in their extremely small unitary conductance of ~50 fS, the shallow dependence of conductivity on external K^+ ($[K^+]_e$), the non-saturating dependence

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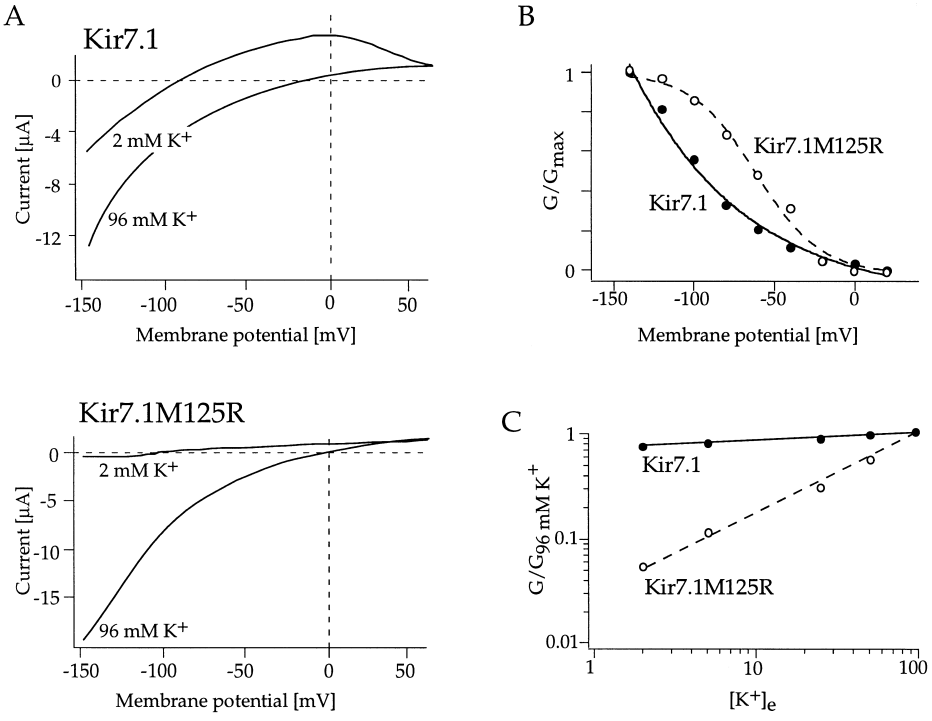

Fig. 1. Whole-cell voltage clamp responses of *Xenopus* oocytes expressing Kir7.1 and mutant Kir7.1M125R channels. A: Macroscopic currents in response to 500 ms voltage ramps from −150 to +60 mV in 2 mM and 96 mM external K^+ ($[K^+]_e$) as indicated. B: Normalized G/G_{max} slope conductances of Kir7.1 (●) and Kir7.1M12R (○) are plotted versus the membrane voltage and fit by a single exponential and Boltzmann function, respectively. C: Double-logarithmic plot of the chord conductances as a function of $[K^+]_e$. Conductances were measured at G_{max} and normalized with respect to G_{96K+}. Data were fit to $G = m[K^+]_e)^n$, where m and n are variables.

of the slope conductances at more hyperpolarized voltages and low sensitivity to the open channel blocker Ba^{2+} [6,7]. A candidate site in Kir7.1 to account for some aspects of this striking functional difference is a non-conserved methionine at position +2 (M125) with respect to the K^+ channel pore signature. Fig. 1 briefly demonstrates the current characteristics of Kir7.1 and mutant Kir7.1M125R (carrying a conserved arginine at the +2 position) as determined from voltage ramps in low (2 mM) and high (96 mM) $[K^+]_e$. The voltage dependence of the measured slope conductances for Kir7.1 was non-saturating as illustrated by the monoexponential fit of the G–V curve. In Kir7.1M125R channels, however, the G–V relationship followed a typical Boltzmann function with saturation (Fig. 1B) which indicates a deviation from the independence principle by ion–ion or ion–pore interactions. Moreover, in Kir7.1 the chord conductance was virtually independent of $[K^+]_e$, but in Kir7.1M125R this relationship followed a slope of ~0.6 in the double-logarithmic plot (Fig. 1C). Thus the steep dependence typical of other Kir channels (generally proportional to the square root of $[K^+]_e$) was restored in the mutant channel. These criteria were a first indication that the +2 position in the outer mouth of the Kir7.1 pore critically determined the features of single-file pores simultaneously occupied by multiple K^+ [15].

If the conserved arginines were involved in the stabilization of the selectivity filter [10] some of the aberrant features of Kir7.1 may be explained by a distortion of the pore. Thus we measured whether such a conformational change would be unmasked by altering the permeating ion species. With internal K^+ (~100 mM) and externally varied monovalent cations we determined under quasi-biionic conditions both the maximum chord conductances (G_{max}) and reversal potentials for Li^+, Na^+, K^+, Rb^+, Cs^+, NH_4^+, and Tl^+ (Fig. 2 and Table 1). The permeability ratios (PX^+/PK^+) for Kir7.1 channels were calculated from the reversal potentials E_{rev} (Fig. 2A) according to the Goldman–Hodgkin–Katz equation (see Table 1) and yielded a permeability sequence $Tl^+ > K^+ > Rb^+ > NH_4^+ > Cs^+ > Na^+ > Li^+$. In Kir7.1M125R channels this permeability sequence was maintained with a slight decrease in

Table 1
PX^+/PK^+ permeability ratios and GX^+/GK^+ conductance ratios in wild type and mutant Kir7.1 and Kir2.1 channels

Channel	Kir7.1	Kir7.1M125R	Kir2.1	Kir2.1R148M
PLi^+/PK^+	0.013	0.015	0.043	0.02
PNa^+/PK^+	0.026	0.032	0.033	0.02
PK^+/PK^+	1	1	1	1
PRb^+/PK^+	0.62	0.37	0.52	0.69
PCs^+/PK^+	0.13	0.048	0.21	0.07
PNH_4^+/PK^+	0.36	0.25	n.d.	n.d
GLi^+/GK^+	0.4	0.01	0.007	0.06
GNa^+/GK^+	0.52	0.03	0.02	0.08
GK^+/GK^+	1	1	1	1
GRb^+/GK^+	7.57	0.19	0.13	6.38
GCs^+/GK^+	0.3	0.01	0.007	0.1
GNH_4^+/GK^+	2.18	0.2	n.d.	n.d.

PX^+/PK^+ permeability ratios were calculated from the reversal potentials of whole-oocyte currents generated by voltage ramps between −150 and +60 mV according to the Goldman–Hodgkin–Katz equation ($PX^+/PK^+ = [K^+]_e/[X]_e \exp[(zE_{rev}F)/RT]$), with ($[X^+]_e$ being the concentration of the monovalent ion, E_{rev} the reversal potential, and z, F, R, T having their usual meanings).
GX^+/GK^+ are the ratios of chord conductances measured with the same protocol. Tl^+ values were only determined for Kir7.1 due to deterioration of oocytes under high Tl^+. PTl^+/PK^+ was 1.43, GTl^+/GK^+ was 3.66.
n.d., not determined.

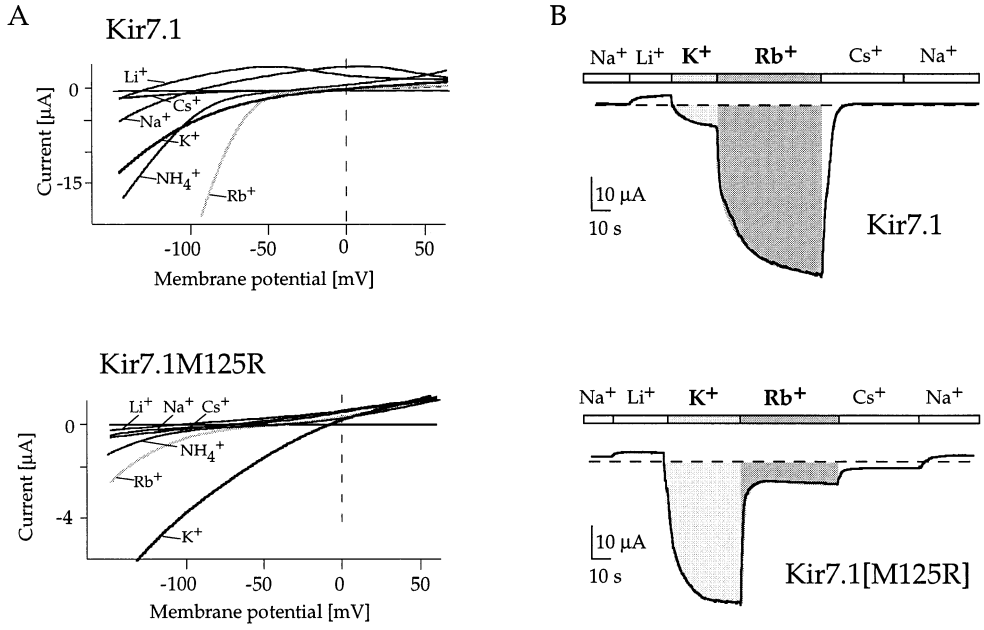

Fig. 2. A: Kir7.1 and Kir7.1M125R currents in response to 500 ms voltage ramps from −150 mV to +60 mV reveal change of reversal potentials E_{rev} for the monovalent cations Na^+, Li^+, K^+, Rb^+, NH_4^+, and Cs^+. B: Equimolar substitution of external monovalents in oocytes clamped to $V_h = -100$ mV shows peak currents for Rb^+ in Kir7.1 and K^+ in Kir7.1M125R channels.

PX^+/PK^+ for Rb^+, Cs^+, and NH_4^+ (Table 1), and was also quite similar in other Kir channels, e.g. steeply rectifying Kir2.1 and mutant Kir2.1R148M channels in which the conserved arginine was exchanged for a methionine at the +2 position. The GRb^+/GK^+ ratios, however, which were not reflected in the reversal potential and pointed to additional ion binding sites in the pore, revealed a substantial degree of discrimination for monovalents. Most remarkably, the Rb^+ conductivity of Kir2.1 and other Kir channels with an arginine at +2 was ~6–8-fold lower than for K^+, but in Kir7.1 channels exceeded that for K^+ by a factor of 7.5 (6.36 for Kir2.1R148M; Fig. 2B). The Rb^+ conductivity decreased ~40-fold with respect to K^+ when the arginine was introduced into Kir7.1M125R, and increased ~50-fold when

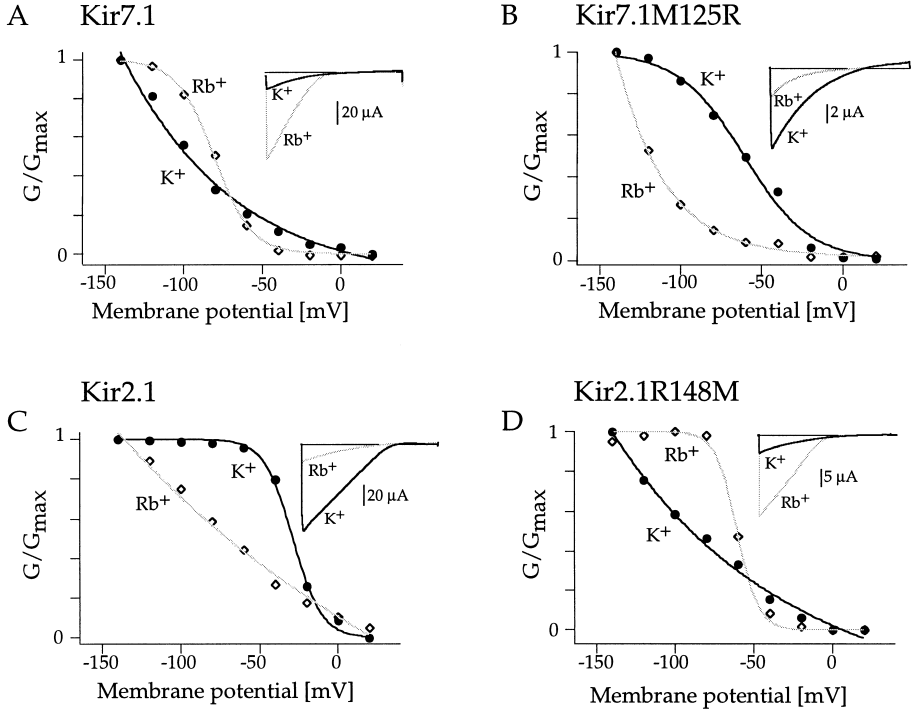

Fig. 3. Normalized G/G_{max} slope conductances of Kir7.1 (A), Kir7.1M12R (B), Kir2.1 (C), and Kir2.1R148M (D) are plotted versus the membrane voltage with either K^+ (●) or Rb^+ (◇) as permeant cation. Data are fit by a single exponential or Boltzmann function. Insets depict original current recordings to voltage ramps between −150 mV and +60 mV.

the arginine was replaced in Kir2.1R148M – which was a perfect illustration of the crucial role of the +2 position in cation coordination. In different Kir channel proteins the absence of the positive charge at +2 thus appeared to represent an additional energy barrier for K^+ ions (Stokes radius 1.33 Å), but not for the larger Rb^+ ions (Stokes radius 1.48 Å).

In addition to the effect on the absolute conductance values, it was noticeable that with Rb^+ as the permeant ion, the voltage dependence of the conductance in both Kir7.1 and Kir2.1R148M saturated in a similar way as in Kir7.1M125R and Kir2.1 when K^+ was used as permeant ion (Fig. 3). Vice versa, with the +2 arginine present, Rb^+ invoked non-saturating conductance behavior (Fig. 3B,C). Also, whereas in Kir7.1M125R (slope 0.45 versus 0.71) and Kir2.1 channels (0.47 versus 0.64) Rb^+ gave rise to a slightly more shallow dependence of conductance on $[X^+]_e$ than K^+, the Rb^+ conductance in Kir7.1 was approximately proportional to $[Rb^+]_e^{1.1}$ (data not shown) which exceeded the value for K^+ by an order of magnitude and was interpreted as an additional indicator of the effective ion coupling in multi-ion pores.

The best line of evidence for the multiple-occupancy theory of ion channels was provided by demonstrating the anomalous mole fraction effect. This phenomenon is thought to originate from competition in the pore lumen between permeant ions when they are mixed together, i.e. their different efficiencies in relieving neighboring ions from their binding sites. We noted that in mutant Kir7.1M125R channels the conductance ratio $GK^+ + Rb^+/GK^+$ plotted against the ratio of ionic concentrations $[K^+]/[K^+]+[Rb^+]$ was biphasic and went through a minimum at a mole fraction of ~0.38 measured between −100 and −120 mV (Fig. 4), as shown for single-file multi-ion pores. In strong contrast, no anomalous mole fraction effect occurred in Kir7.1 and the conductance ratio declined monotonically with the concentration ratio. This is most commonly explained by a model in which the two ion species do not bind simultaneously in the channel.

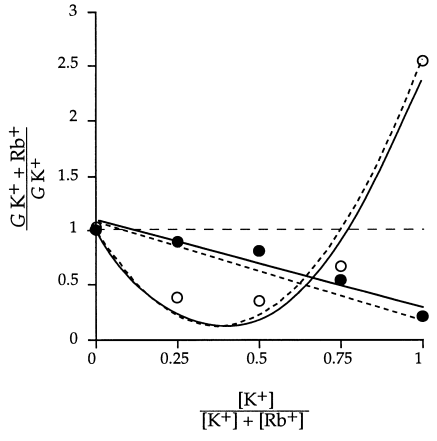

Fig. 4. Anomalous mole fraction behavior of Kir7.1M125R mutant channels. Macroscopic Kir7.1 and Kir7.1M125R currents were recorded from *Xenopus* oocytes superfused with solutions containing different proportions of K^+ and Rb^+ (total cation concentration 98 mM). Conductance ratios $GK^+ + Rb^+/GK^+$ are plotted against the concentration ratios $[K^+]/[K^+]+[Rb^+]$ (mole fraction) for Kir7.1 (●) and Kir7.1M125R (○) and fitted by linear regression and a second order polynomial function, respectively ($V_h = 100$ mV, solid lines; $V_h = -120$ mV, dashed lines).

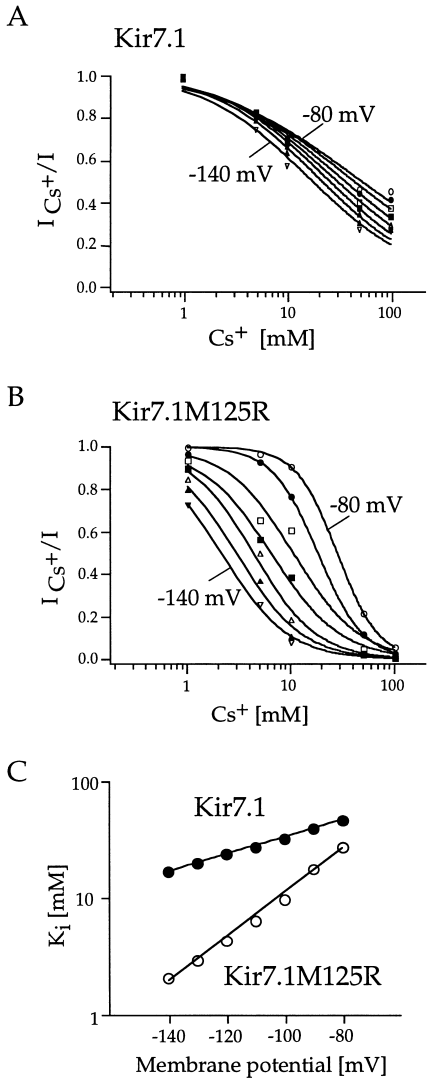

Fig. 5. Analysis of the voltage dependence of Cs^+ block of wild type and mutant Kir7.1 channels. A,B: The ratio of macroscopic current amplitudes of Kir7.1 and Kir7.1M125R in the presence and absence of blocker is plotted against the Cs^+ concentration for potentials between −140 mV and −80 mV. The least squares fits are derived from $I_{Cs+} = A/1+([Cs^+]_e/K_i)$ where A is a variable and K_i is the Cs^+ concentration that causes half-maximal block. C: The logarithms of K_i values (in mM) for Kir7.1 (●) and Kir7.1M125R (○) are plotted as a function of the membrane potential and fitted by linear regression.

From these measurements Kir7.1 channels appeared to function in a single-occupancy state in which the pore contains only one permeant ion at a time. This mechanistic view found support by a closer inspection of another characteristic Kir channel feature, the strong voltage-dependent block of the open channel pore by extracellular Ba^{2+} and Cs^+. In Kir7.1 the K_i values for block by Ba^{2+} and Cs^+ at −100 mV were 670 μM and 26.9 mM, respectively, and thus one to two orders of magnitude higher than for other Kir channel members [6,7,14,16]. In addition to the low sensitivity, we found the Ba^{2+} and Cs^+ block of Kir7.1 to be only weakly voltage-dependent. By plotting the degree of Cs^+ block against the Cs^+ concentration and determining K_i values for different potentials a 10-fold change in K_i was shown to correspond to a change in membrane potential of 123 mV (Fig. 5). Ac-

cording to Woodhull [17] the dissociation constant K_i for a blocking ion that binds to a site within the membrane electrical field is given as $K_i = K_i(0)\exp(-\delta z V_m F/RT)$, where $K_i(0)$ is the zero-voltage dissociation constant, δ is the location of the binding site as a fraction of the membrane voltage, z is the ionic valence and *R, T, F* have their usual meanings of thermodynamics [17]. Using this equation for simple voltage-dependent block, we determined a theoretical fractional electrical distance δ of 0.09 for the Cs^+ blocking site. Thus it appeared as if Cs^+ would hardly sense the membrane electrical field and bind to a superficial site of the channel. In contrast, with the arginine present in Kir7.1M125R channels, a 10-fold change in K_i occurred every 52 mV (36 mV for Kir2.1 [14]), which corresponded to a sensed membrane field fraction of 0.82. A plausible interpretation of these results is that in Kir7.1 with the arginines absent, the open-channel blocker Cs^+ preferentially binds to a low-affinity site just outside the electrical field different from other Kir channels in which Cs^+ binds halfway in the pore [18,19].

As revealed by several conductance characteristics of permeant and blocking ions we conclude that the electrostatic interactions of K^+ ions as they move inside the Kir7.1 pore are not in accordance with the theory of multi-ion occupancy of conventional Kir channels. The lack of the positively charged arginine at position +2 in Kir7.1 outside the electrical field creates a higher energy barrier for stabilizing the pore occupancy by K^+, but favors an optimal conformation of the permeation pathway for bonding with the larger Rb^+ ions. Instead of reflecting fluctuations of the pore radius, our data support the view that the positive charge at +2 elevates an energy minimum for permeating K^+ ions and that effective repulsion between permeating K^+ ions bound to adjacent pore sites may be the primary cause of high K^+ throughput rates [13]. Based on the double-occupancy theory, i.e. the selectivity filter being simultaneously occupied by two K^+ ions [1], we propose that in Kir7.1 permeating K^+ ions may bind at an additional site. In a plausible sequel of events one K^+ ion (and possibly also the channel blockers Ba^{2+} and Cs^+) would initially be accommodated at a newly defined energy minimum in the outer mouth near M125 [13]. K^+ ions pausing at this remote site would then only weakly interact with K^+ ions bound more distally in the selectivity filter whose transfer rates into the inner water-filled vestibule would consequently be low. The rate-limiting interaction of K^+ ions with the outer pore binding site renders the channel to appear in a pseudo single-occupancy state, with low unitary conductance levels and virtual independence of $[K^+]_e$. In Kir7.1M125R mutants, the +2 position no longer retains a K^+ ion in the outer vestibule, but promotes K^+ binding to two conventional high-energy binding sites in the selectivity filter proper. In this true double-occupancy state, the second K^+ ion experiences destabilization and high exit rates which account for the >20-fold increase in single channel conductance [7]. We can also explain that with Rb^+ conventional Kir channel features are restored in Kir7.1 if the larger Rb^+ ions do not find an energy minimum at the external site to balance the loss of hydration water. Thus they are not accommodated at this site and directly move on to bind to the first of two high-affinity pore binding sites. Vice versa, in Kir channels with the positive charge at +2 (Kir2.1 or Kir7.1M125R), Rb^+ ions may fall into an energy minimum and pause at the external site. This conception finds support from an intriguing observation made on Kir7.1G129E mutants in which we replaced a second non-conserved residue in the outer pore at position +6 by a conserved negative charge (data not shown). With K^+ as the permeant ion Kir7.1G129E failed to conduct detectable currents (see also [7]) and we argue that the additional negative charge in the outer vestibule prevents K^+ ions from finally moving into the pore. However, when Rb^+ ions that by-pass this site were used as charge carriers, prominent currents with classic properties were restored in Kir7.1G129E mutants.

As a consequence from these considerations mutations in other regions of the channel protein, that perturb the energy levels of K^+ binding sites and thus control access and exit rates, are likely to influence ion permeation. In Kv channels residues in the P loop proper [20–22], as well as transmembrane helices [23] which determine the pore tunnel diameter, have also been recognized as a mild K^+/Rb^+ switch. So is the aspartate at position 172 of Kir2.1 which faces the cytoplasmic side of the pore and controls Mg^{2+} binding and rectification [24]. In the outer mouth of the Kv1 pore the +3 position, involved in the binding of external K^+, TEA^+, and CTX [25,26], also affects permeation, e.g. when a positive charge is introduced the single channel conductance declines as expected for a reduction of the net negative surface charge on the outside of the membrane [27]. A decrease of the unitary conductance is also conceivable when steric hindrance for permeating ions is created by increasing the side chain volume of residues facing the pore, as documented for Kir6.1/6.2 channels. The two isoforms differ at the +4 position and allow an ion passageway of 10 Å and 5 Å, respectively, in diameter [28]. Thus, like the +2 position these residues in the outer mouth also fix the appropriate conformation of the permeation pathway to control the quantity of permeating K^+ ions. However, from cysteine scanning analysis [29,30], and when the Kir7.1 primary sequence is transposed onto the 3D structure of KcsA, the +2 position appears positioned with its side chain facing away from the vestibule where it participates in the stabilization of the outer pore mouth [10]. Except for native Kir7.1 and mutant Kir2.1R148M/Y channels this site hardly accepts other amino acids and channel activity is lost in several mutants [11,12,28]. Although structural data are not yet available it is conceivable that the lack of arginine in Kir7.1 distorts the outer pore structure to accommodate binding of a K^+ ion, but retains a condition of normal permeation for Rb^+. The +2 position is identified as the first example that cation fit in the outer pore +2 position crucially defines permeation properties in Kir channels.

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