

K⁺-dependent gating of K_{ir}1.1 channels is linked to pH gating through a conformational change in the pore

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1. We have used giant patch-clamp recording to investigate the interaction between pH gating and K⁺-dependent gating in rat K_{ir}1.1 (ROMK) channels heterologously expressed in *Xenopus* oocytes.
2. Gating by intracellular protons (pH gating) and extracellular K⁺ ions (K⁺-dependent gating) is a hallmark of K_{ir}1.1 channels that mediate K⁺ secretion and control NaCl reabsorption in the kidney. pH gating is driven by protonation of an intracellular lysine residue (K80 in K_{ir}1.1). K⁺-dependent gating occurs upon withdrawal of K⁺ ions from the extracellular side of the channel. Both gating mechanisms are thought to interact allosterically.
3. K⁺-dependent gating was shown to be strictly coupled to pH gating; it only occurred when channels were in the pH-inactivated closed state, but not in the open state. Moreover, K⁺-dependent gating was absent in the non-pH-gated mutant K_{ir}1.1(K80M).
4. Channels inactivated by K⁺-dependent gating were reactivated upon addition of permeant ions to the extracellular side of the membrane, while impermeant ions failed to induce channel reactivation. Moreover, mutagenesis identified two residues in the P-helix (L136 and V140 in K_{ir}1.1) that are crucial for K⁺-dependent gating. Replacement of these residues with the ones present in the non-K⁺-gated K_{ir}2.1 abolished K⁺-dependent gating of K_{ir}1.1 channels without affecting pH gating.
5. The results indicate that pH gating and K⁺-dependent gating are coupled to each other via structural rearrangements in the inner pore involving the P-helix.

Weak inward rectifier K⁺ channels (K_{ir}) that allow for bidirectional K⁺ transport across secretory epithelia are essential for a number of physiological processes. Thus, in kidney, K_{ir} channels are responsible for K⁺ secretion in the cortical collecting duct (CCD) and the distal convoluted tubule (DCT) and by recycling K⁺ into the lumen for reabsorption of NaCl via the Na⁺–K⁺–2Cl[–] cotransporter in the thick ascending loop of Henle (Wang *et al.* 1992; Hebert, 1998).

ROMK1 was the first K_{ir} channel to be cloned (Ho *et al.* 1993); it shares the prototypic membrane topology of this family of K⁺ channels with intracellular N- and C-termini and two transmembrane domains (M1 and M2) flanking a well-conserved pore-forming region (P-region). The ROMK protein exists in three splice variations, designated ROMK1–3 (K_{ir}1.1a–c; K_{ir}1.1b shortened by 19 amino acids and K_{ir}1.1c extended by seven amino acid residues with respect to K_{ir}1.1a) (Shuck *et al.* 1994; Yano *et al.* 1994; Boim *et al.* 1995). Although differentially expressed in renal tubular cells (Boim *et al.* 1995), all splice variants encode weak inward-rectifier K⁺ channels

with essentially identical functional properties. A number of K_{ir}1.1 mutations leading to impairment or loss of channel activity have been identified in patients suffering from hyperprostaglandin E syndrome (the antenatal variant of Bartter syndrome; Seyberth *et al.* 1985) underlining the physiological importance of proper ROMK function (Simon *et al.* 1996; Derst *et al.* 1997).

As with their native counterparts, cloned K_{ir}1.1 channels are gated by intracellular pH (pH_i); they close in response to acidification and open upon alkalization (Bleich *et al.* 1990; Wang *et al.* 1990; Tsai *et al.* 1995). The corresponding concentration–response curve displays a steep dependence of channel activity on pH_i with a midpoint around neutral pH. As the sensor for intracellular protons, a lysine residue located N-terminal to M1 (K80 in K_{ir}1.1a) and common to all pH-gated inward-rectifier channels (K_{ir}1.1 and K_{ir}4) has been identified (Fakler *et al.* 1996; Schulte *et al.* 1999). This residue displays a strong shift in its apparent pK_a, which results from close electrostatic interaction with two highly conserved arginines in the N- and C-terminus (R41 and R311 in K_{ir}1.1a); all three

residues are arranged in a triad that seems well conserved among K_{ir} channels (Schulte *et al.* 1999).

In addition to gating by pH_i , activity of $K_{ir1.1}$ channels critically depends on the presence of extracellular K^+ (K_o^+) in the millimolar range (Doi *et al.* 1996). Removal of K_o^+ leads to a decrease in channel activity, and reapplication of K_o^+ restores channel activity in a concentration-dependent manner. The time course of this K^+ -dependent gating (or K^+ gating) is markedly affected by pH_i . While K^+ -dependent inactivation is slow around neutral pH_i , it is speeded up considerably by intracellular acidification (Doi *et al.* 1996). Moreover, chimeras formed between $K_{ir1.1}$ and non- K^+ -gated $K_{ir2.1}$ suggest that K^+ -dependent gating is linked to the P-loop in the $K_{ir1.1}$ sequence (Doi *et al.* 1996). Thus, gating by extracellular K^+ and intracellular pH are thought to be coupled to each other via an allosteric mechanism.

Here, we studied the interdependence of gating by pH_i and K_o^+ in cloned $K_{ir1.1a}$ channels and investigated the structural determinants underlying the allosteric coupling of these gating mechanisms.

METHODS

Mutagenesis and cRNA synthesis

Site-directed mutagenesis with rat $K_{ir1.1a}$ was performed as described by Fakler *et al.* (1995) and verified by sequencing. For heterologous expression in *Xenopus* oocytes, all constructs were subcloned into the pBF expression vector (B. Fakler, unpublished work). Capped K_{ir} wild-type and mutant cRNAs were synthesized *in vitro* using the mMESSAGE mMACHINE kit (Ambion, Austin, TX) and stored as stock aliquots at -70°C .

Heterologous expression in *Xenopus* oocytes

Oocytes were surgically removed under anaesthesia from adult females (which were humanely killed after the final oocyte collection) and manually dissected. Dumont stage VI oocytes were injected with the appropriate cRNA solution (approximately 50 nl each), treated with collagenase type II (Sigma, 0.5 mg ml^{-1} for 15 min) and incubated at 19°C for 2–5 days prior to use. Animal care and experiments followed approved institutional guidelines at the University of Tübingen.

Electrophysiology

Two-electrode voltage-clamp measurements were done at room temperature as described by Doi *et al.* (1996). Briefly, electrodes were pulled from thick-wall borosilicate glass, and filled with 3 M KCl; they had resistances of 0.1–0.8 M Ω . Currents were recorded with a TurboTec 01C amplifier (npi electronic GmbH, Tamm, Germany) and sampled at 1 kHz with an ITC16 AD board (HEKA Elektronik, Lambrecht/Pfalz, Germany). The bath chamber was continuously perfused with solutions composed of (mM): 10 Hepes, 1.8 CaCl_2 and either 90 KCl + 30 NaCl (90 K_o^+) or 120 NaCl (0 K_o^+), pH 7.3.

Giant-patch recordings in inside-out configuration were performed as previously described (Fakler *et al.* 1995). Patch pipettes pulled from thick-wall borosilicate glass had resistances of 0.3–0.5 M Ω when filled with pipette solution (120 K_o^+ : 120 mM KCl, 10 mM Hepes, 0.18 mM CaCl_2 , pH 7.3; 120 Na_o^+ , 120 Rb_o^+ , 120 Cs_o^+ and 120 NMDG $_o^+$ were prepared by equimolar replacement of K^+ with the respective ion). Currents recorded in response to voltage ramps or steps were sampled at 1 kHz with an EPC9 amplifier (HEKA

Elektronik) with analog filter set to 3 kHz. For single-channel experiments, small patch pipettes were pulled from quartz glass capillaries and filled with standard 120 K_o^+ or 120 Rb_o^+ solution (resistance 3–7 M Ω). Currents were sampled at 10 kHz and filtered at 1 kHz.

Giant outside-out patches were obtained as follows: after establishing cell-attached configuration (seal resistances of $> 3\text{ G}\Omega$) with a giant patch pipette (pulled as described above, filled with K_i^+ (see below) + 10 μM PIP $_2$ to prevent channel rundown), the patch was ruptured and the pipette was slowly withdrawn from the oocyte allowing the membrane to form a bell-shaped extension which eventually cut off as a giant outside-out patch. Intra- and extracellular solutions were composed as follows (mM): $K_i^+/\text{Rb}_i^+/\text{NMDG}_i^+$: 10 Hepes, 10 EGTA, 0.1 DTT and 120 KCl/120 RbCl /120 NMDG, adjusted to the given pH; 0 K_o^+ /100 K_o^+ : 10 Hepes, 1.44 MgCl_2 , 10 EGTA and 100 NaCl/100 KCl, pH 7.2. Solutions were applied to excised patches via a multibarrel pipette as described by Fakler *et al.* (1995).

Data evaluation

Relative permeability of $K_{ir1.1}$ wild-type and mutant channels for K^+ , Rb^+ and Cs^+ was determined by linear regression according to a linearized form of the Goldman-Hodgkin-Katz equation: $P_{\text{ion}1}/P_{\text{ion}2} = [\text{ion} 1]/[\text{ion} 2]\exp\{V_{\text{rev}}(zF/RT)\}$. Reversal potentials (V_{rev}) were determined in excised inside-out patches at zero current with voltage ramps from -100 to 100 mV ; the patch pipette contained 30 mM Rb^+ ; concentrations of K^+ and Cs^+ were varied on the intracellular side (10, 30 and 100 mM for Cs^+ , 3, 10, 30 and 100 mM for K^+).

Relative conductance for Cs^+ ($g_{\text{Cs}}:g_{\text{K}}$) was determined from the asymptotic slope conductance of macroscopic currents (100 mM K^+ /100 mM Cs^+) in excised patches. The single channel conductance was calculated from linear fits to single channel currents ($\geq 50\text{ ms}$ periods of channel open state) and given as means \pm S.D. of n patches.

Parameters characterizing pH gating of $K_{ir1.1}$ channels were determined as described by Schulte *et al.* (1999). The apparent affinity of $K_{ir1.1}$ channels for K_o^+ was determined in inside-out patches by measuring relative current recovery from pH-induced inactivation with varying K_o^+ (1, 3, 10, 30 and 100 mM) in the pipette; data points were plotted as means \pm S.D. (of 5 experiments) and fitted with a logistic function.

All computational work was done on a Macintosh G3 using commercial software (IGOR, WaveMetrics, Lake Oswego, OR, USA) for fitting.

RESULTS

K^+ -dependent gating occurs in the pH-inactivated state

Figure 1 illustrates K^+ -dependent gating as observed in *Xenopus* oocytes expressing $K_{ir1.1}$ channels. When oocytes were incubated in K^+ -free solution for $> 2\text{ h}$, no currents were recorded in response to voltage ramps (from -120 to 50 mV) in K^+ -free solution (0 K_o^+). However, upon application of 90 mM K^+ to the extracellular side (90 K_o^+), $K_{ir1.1}$ -mediated currents appeared that increased over a period of minutes (Fig. 1A, upper recording; Doi *et al.* 1996). This slow K^+ -dependent increase and decrease in current was also observed in giant outside-out patches excised from such oocytes (Fig. 1B, upper recording). While at 0 K_o^+ $K_{ir1.1}$ currents (measured in response to voltage ramps from -100 to 50 mV) were small, they increased with time to more

than 10-fold (13.2 ± 3.3 , $n = 3$) after switching K_o^+ to 100 mM (the instantaneous increase in current amplitude following switch to the 100 K_o^+ solution is due to the respective shift in the K^+ reversal potential, E_K). Conversely, withdrawal of K_o^+ from the patch decreased the current amplitude to the level observed at the beginning of the experiment (Fig. 1*B*, upper recording). In such experiments, the amplitude of single $K_{ir}1.1$ channels remained unchanged indicating that the increase in current was due to a change in channel activity (data not shown; Sackin *et al.* 2001).

Different from wild-type, K^+ -dependent gating was not observed in $K_{ir}1.1(K80M)$ channels where pH gating is abolished (Fakler *et al.* 1996; Schulte *et al.* 1999). As shown in Fig. 1*A* and *B* (lower recordings), mutant channels exhibited robust currents in 0 K_o^+ (outward directed over

the whole voltage range due to the negative E_K). The switch to high K_o^+ did not change channel activity, but rather led to an instantaneous increase in current amplitude resulting from the shift in E_K (Fig. 1*B*, lower recording).

The lack of K^+ gating in $K_{ir}1.1(K80M)$ channels suggested that K^+ -dependent gating is linked to pH gating. This was further investigated in a series of patch experiments, where 0 K_o^+ was used as an extracellular solution and pH and K^+ concentration of the intracellular solution were varied; currents were recorded at a potential of -80 mV that was stepped to 40 mV for 40 ms every second. In an initial experiment, the channel pore was depleted of K^+ ions at a constant pH_i of 8.0 by switching the intracellular solution from K_i^+ to K^+ -free NMDG $_i^+$. As shown in Fig. 2*A*, $K_{ir}1.1$ -mediated outward currents were constant at pH_i 8.0 and K^+ depletion did not induce K^+ -dependent inactivation as

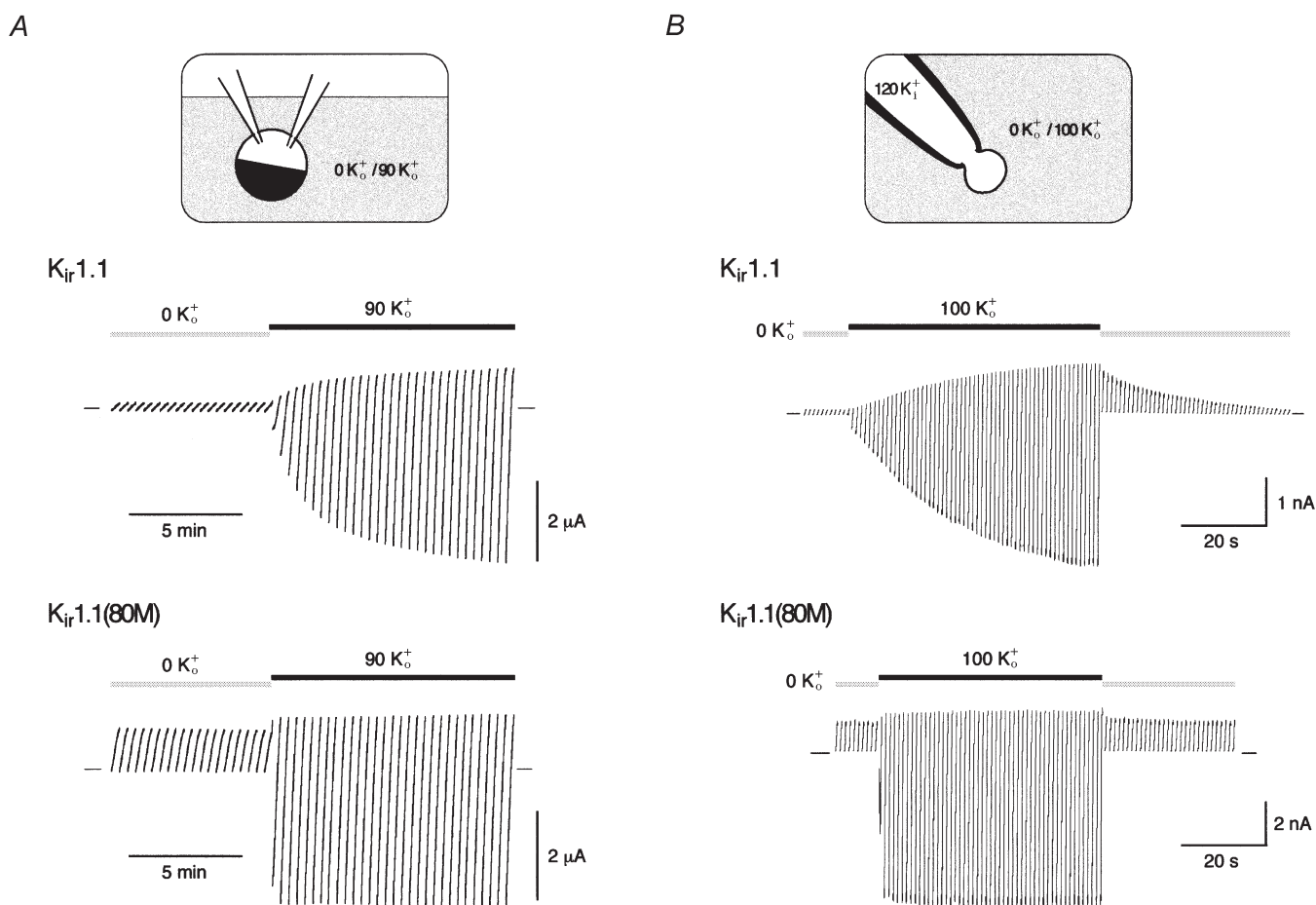


Figure 1. K^+ -dependent gating of $K_{ir}1.1$ channels

A, currents mediated by $K_{ir}1.1$ (upper trace) or $K_{ir}1.1(K80M)$ channels (lower trace) recorded in oocytes preincubated in K^+ -free solution for more than 2 h. Change in bath solution from 0 K_o^+ to 90 K_o^+ as indicated by horizontal bars; currents were recorded in response to voltage ramps from -120 to 50 mV (in 20 s), and zero current is indicated by small bars. *B*, currents recorded in giant outside-out patches from oocytes expressing $K_{ir}1.1$ (upper trace) or $K_{ir}1.1(K80M)$ channels (lower trace) and treated as in *A*. Currents are response to voltage ramps from -100 to 50 mV (in 400 ms) at pH_i 7.1; application of extracellular solutions as indicated. Note the difference between slow increase in $K_{ir}1.1$ currents due to recovery from K^+ -dependent inactivation and rapid increase in current amplitude due to the shift in E_K resulting from the change in $[K^+]_o$.

envisaged by the instantaneous recovery of currents upon switching the intracellular solution back to K_i^+ . In a second set of experiments, channels were pH inactivated by intracellular acidification (application of K_i^+ at pH_i 6.0). Different from K^+ -depletion alone, $K_{ir}1.1$ channels failed to recover in the absence of K_o^+ (Fig. 2*B*) whereas in the presence of high K_o^+ , pH-induced inactivation was fully reversible (Schulte *et al.* 1998; Figs. 2*D* and 4*A*).

Furthermore, in $K_{ir}1.1(K80M)$ channels intracellular acidification together with K^+ depletion failed to induce K^+ -dependent inactivation (Fig. 2*C*).

These results suggest that K^+ -dependent inactivation only occurs when channels are in the pH-inactivated closed state, while channels in the open state are not susceptible to this process.

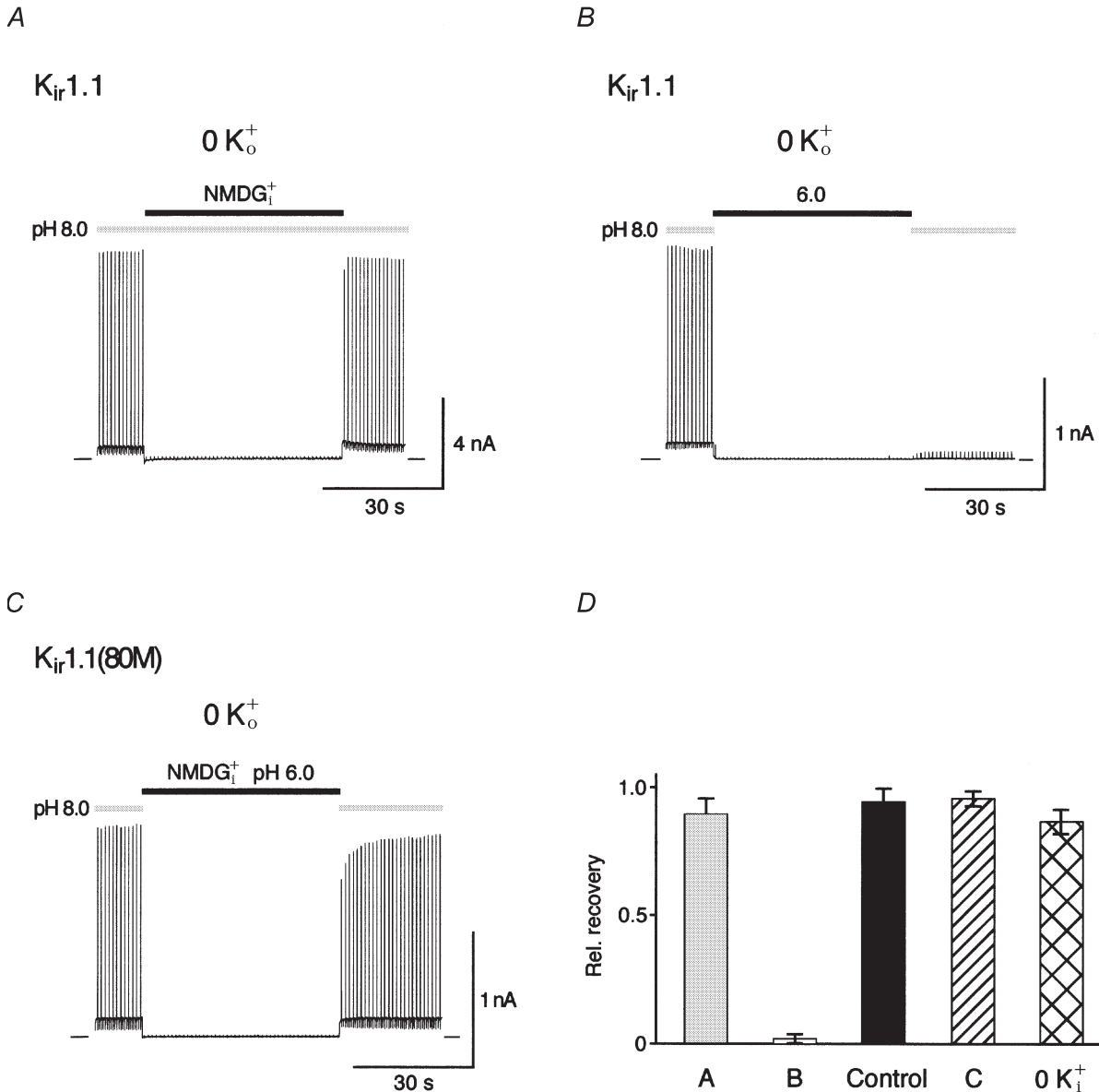


Figure 2. K^+ -dependent gating is linked to pH gating

A, $K_{ir}1.1$ currents recorded in the absence of extracellular K^+ ($0 K_o^+$) from a giant inside-out patch with intracellular K^+ switched from 120 mM (K_i^+) to 0 mM (NMDG_i⁺) and back at a constant pH_i of 8.0. Current was recorded at a membrane potential of -80 mV stepped to 40 mV for 40 ms every second. Note the complete recovery of currents after switching K^+ back to 120 mM. B, recovery experiment as in A but with pH_i switched from 8.0 to 6.0 and back at constant intracellular K^+ of 120 mM. Note the lack of current recovery after pH-induced inactivation in the absence of K_o^+ . C, currents mediated by $K_{ir}1.1(K80M)$ channels recorded in an experiment as in A but with simultaneously changing intracellular K^+ and pH_i (NMDG_i⁺ at pH_i of 6.0). D, relative recovery of currents (measured at 40 mV) from experiments as in A–C given as means \pm S.D. of ≥ 6 experiments; control refers to experiments as in B, but with 120 mM K_o^+ ; $0 K_i^+$ refers to experiments as in B but with 120 mM K_o^+ and 0 mM K_i^+ .

K^+ -dependent gating exhibits selectivity for permeant ions

K^+ gating was further characterized by determining the time course of K^+ -dependent inactivation. This was done by recording currents in the absence of K_o^+ at pH_i 8.0 before and after acidification of the channels for an increasing period of time; inactivation was quantified by normalizing the recovered current with respect to that observed prior to acidification. For acidification a pH_i of 5.0 was used, where all channels were pH inactivated within less than 0.5 s (data not shown). As shown in Fig. 3, the time course of K^+ -dependent inactivation could be approximated by a monoexponential yielding a time constant of 8.2 s.

Next, the sensitivity of K^+ -dependent gating to changes in ion species was tested. This was done in recovery experiments where K^+ was replaced by various alkali ions on the extracellular side of the membrane. As shown in Fig. 4, recovery of $K_{ir}1.1$ channels from pH-induced inactivation was almost complete in 120 mM K_o^+ , Rb_o^+ and Cs_o^+ , whereas no recovery was observed in 120 mM Na_o^+ . This pattern roughly paralleled the permeability sequence of $K_{ir}1.1$. While K^+ , Rb^+ and Cs^+ were permeant (relative permeabilities of $P_K : P_{Rb} : P_{Cs} = 1 : 0.6 : 0.039$, for details see Methods), Na^+ did not penetrate through the channel. The extent of recovery was independent of the absolute permeance, since Cs^+ induced full recovery although it was more than 160-fold less permeant than K^+ (data not shown).

These findings suggest that K^+ -dependent gating may be governed by occupancy of an ion binding site located in the channel's selectivity filter.

K^+ -dependent gating is determined by the P-helix

Among K_{ir} channels, K^+ gating was only observed with $K_{ir}1.1$ and $K_{ir}4$ channels, but not with other subtypes including $K_{ir}2.1$ (Doi *et al.* 1996; Pearson *et al.* 1999). Comparison of primary sequence uncovered three positions

within the highly conserved stretch around the selectivity filter that were different between $K_{ir}1.1$ and $K_{ir}2.1$ (Fig. 5). Residues V140 and L136 in the P-helix of $K_{ir}1.1$ were tested for their ability to affect K^+ -dependent gating after replacing them with the respective residues of the $K_{ir}2.1$ sequence. As shown in Fig. 6, both mutants, $K_{ir}1.1(L136I)$ and $K_{ir}1.1(V140T)$, expressed K^+ -selective channels ($P_K : P_{Rb} : P_{Cs} = 1 : 0.63 : 0.053$ and $1 : 0.67 : 0.013$ for $K_{ir}1.1(L136I)$ and $K_{ir}1.1(V140T)$, respectively) that were gated by intracellular pH indistinguishable from wild-type channels (Fig. 6A and B). In contrast to wild-type, however, these channels exhibited complete recovery from pH-induced inactivation independent of the presence of extracellular K^+ (Fig. 6). Thus, in either case the mutation in the P-helix abolished K^+ -dependent gating.

Together with the results shown above these findings suggest that the P-helix is involved in the conformational changes underlying K^+ -dependent gating and its coupling to pH gating.

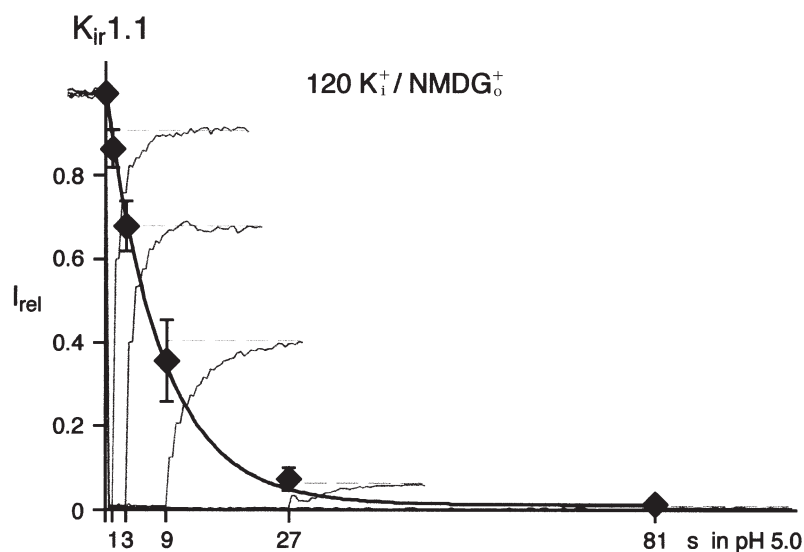
DISCUSSION

Regulation of channel activity by extracellular K^+ has been described for native (Giebisch, 1998) and cloned (Doi *et al.* 1996) ROMK ($K_{ir}1.1$) channels as well as for $K_{ir}4.1$ (Pearson *et al.* 1999). The mechanism underlying this gating process, however, has remained poorly understood. The results presented here demonstrate that K^+ -dependent gating is strictly coupled to pH gating, only occurring when channels are pH inactivated. Mechanistically, K^+ gating is thought to result from dissociation of K^+ ions from the channel and conformational changes in the inner pore involving the P-helix.

K^+ -dependent gating of $K_{ir}1.1$ was observed in both whole oocytes and excised outside-out patches (Fig. 1). The kinetics of K^+ -dependent inactivation/reactivation as determined in excised patches, however, were significantly faster than those measured in whole oocytes with the two-electrode voltage-clamp technique. These

Figure 3. Time course of K^+ -dependent inactivation

Time course of K^+ -dependent inactivation monitored by the current recovery as obtained from experiments as in Fig. 2B. K^+ at pH 5.0 was applied for periods of 1, 3, 9, 27 and 81 s. Overlaid are current traces from such experiments normalized to the initial outward current amplitude at pH 8.0 (only currents at 40 mV are shown); data points are means \pm S.D. of 5 experiments. Line is monoexponential fit to the data that yielded a time constant of 8.2 s.



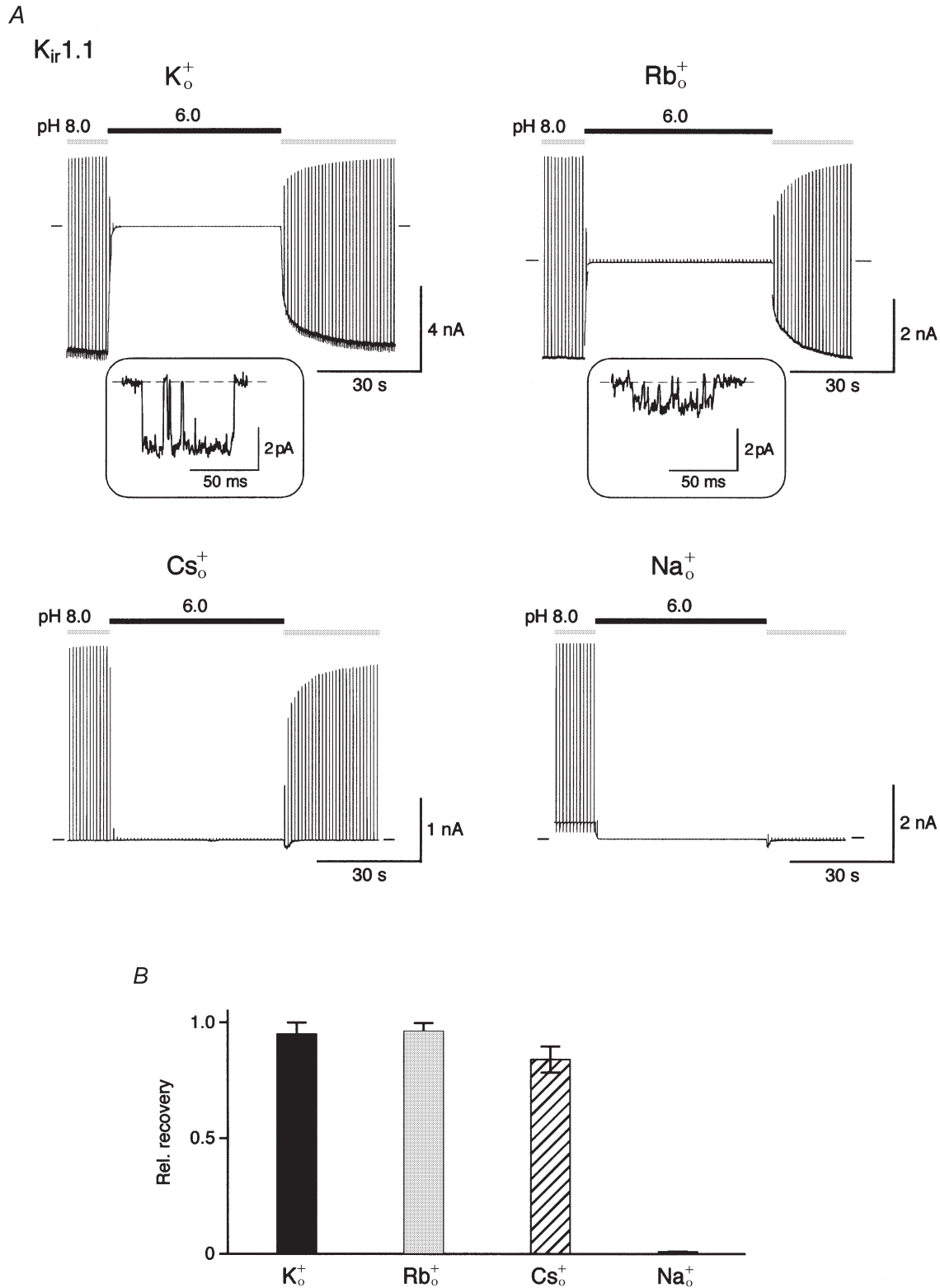


Figure 4. K⁺-dependent gating is selective for permeant ions on the extracellular side

A, recovery experiments as in Fig. 2*B* with 120 mM K⁺ on the intracellular side (K_i⁺) and 120 mM K⁺ (K_o⁺), Rb⁺ (Rb_o⁺), Cs⁺ (Cs_o⁺) or Na⁺ (Na_o⁺) in the recording pipette. Insets: K_{ir}1.1 single channel currents recorded from inside-out patches at -100 mV in symmetrical 120 mM K⁺ (left) and 120 mM Rb⁺ (right); single channel conductances were 32 ± 0.5 pS (*n* = 5) and 11 ± 0.7 pS (*n* = 6), respectively; closed level indicated by the dashed line. *B*, relative recovery of currents from experiments as in *A* (acidification periods of 50 s); bars represent means ± S.D. of 5–12 experiments.

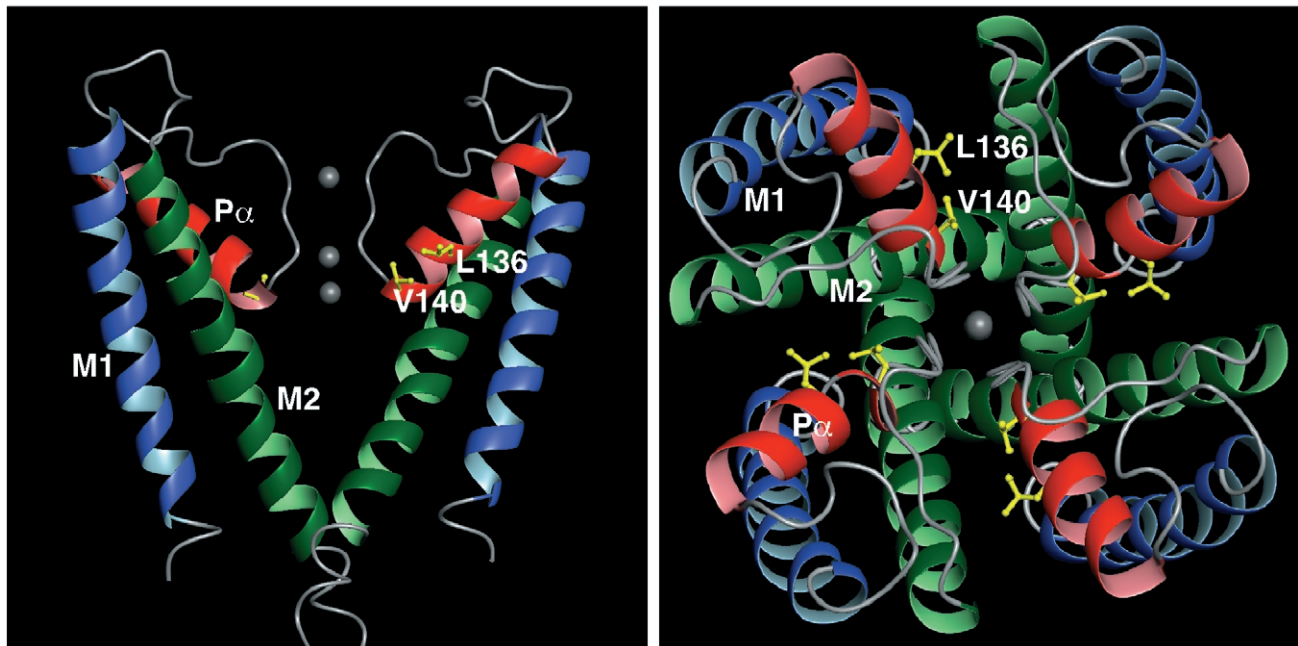
differences are most likely to be due to the large differences in solution exchange observed between both experimental configurations.

Basically, K^+ gating describes a gating reaction that occludes the channel pore as a result of dissociation of K^+ ions from the channel (Doi *et al.* 1996). Different from most other gating processes, however, K^+ -dependent gating did not act on the open channel, but rather affected $K_{ir}1.1$ channels when they were in the pH-inactivated closed state. Thus, K^+ gating was neither observed at pH 8.0, even when the pore was completely depleted from K^+ (Fig. 2A),

nor present in $K_{ir}1.1(K80M)$ mutant channels in which pH gating was abolished by disruption of the pH sensor (Figs 1 and 2C). Consequently, the conformational change(s) associated with pH gating may be regarded a prerequisite for K^+ -dependent gating.

A further characteristic feature of K^+ gating is its ion selectivity. Thus, while the permeant monovalents Rb^+ , NH_4^+ or Cs^+ were equally as potent as K^+ in preventing K^+ -dependent inactivation, the impermeant Na^+ and $NMDG^+$ failed to keep the pore open (Fig. 4; Doi *et al.* 1996). As permeation is governed by binding site(s) in the channel's

A



B

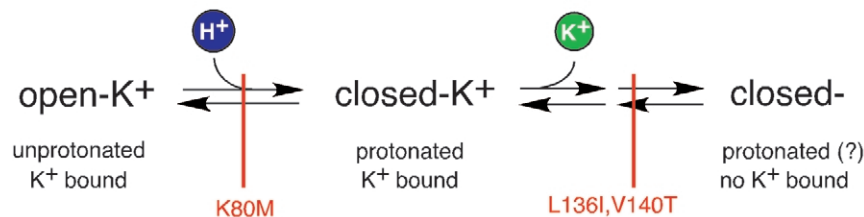


Figure 7. Structural model of the KcsA pore and sequential model of $K_{ir}1.1$ gating by pH_i and K^+

A, structural model of the KcsA pore as determined by X-ray crystallography (Doyle *et al.* 1998) viewed from the side (left panel, only two subunits shown for clarity) and the top (right panel); transmembrane segments in green (M1) and blue (M2) and the P-helices ($P\alpha$) in red; K^+ binding sites are indicated by grey spheres. Side chains of residues 70 and 74 homologous to residues 136 and 140 in $K_{ir}1.1$ were replaced by I and V and highlighted. Note that the side chains are oriented towards the neighbouring subunits rather than to the ion conducting pathway. B, sequential model of $K_{ir}1.1$ gating by pH_i and K^+ as deduced from the experiments presented here. 'open- K^+ ' represents active channels; 'closed- K^+ ' is pH-inactivated closed state with K^+ bound to the pore binding site(s); 'closed-' is K^+ -inactivated closed state; possible intermediate states are omitted for simplicity. These channel conformations are linked through gating transitions (pH gating and K^+ -dependent gating, symbolized by arrows) which can be blocked independently by point mutations (indicated in red).

selectivity filter (Doyle *et al.* 1998; Stampe *et al.* 1998), it seemed likely that K^+ gating may be accompanied by conformational changes in the channel pore similar to what was reported as a 'pore collapse' for C-type inactivation of voltage-gated K^+ channels (reviewed by Yellen, 1998). In line with this interpretation, a number of residues (F148C, Q152E and E151D) located in the extracellular portion of the $K_{ir}1.1$ pore were recently suggested to affect K^+ -dependent gating (Sackin *et al.* 2001). Additionally, we identified two residues in the P-helix, I136 and V140, that markedly affected K^+ -dependent gating. Replacement of either site with the respective residue from non- K^+ -gated $K_{ir}2.1$ eliminated K^+ -dependent gating without any effect on pH gating (Fig. 6). $K_{ir}1.1(V140T)$, which enhances block by extracellular Ba^{2+} (Zhou *et al.* 1996), showed a higher single channel conductance (data not shown; Zhou *et al.* 1996), increased relative permeability for K^+ (see above; Choe *et al.* 2000) and altered single channel kinetics (data not shown; H. Sackin, personal communication), whereas in $K_{ir}1.1(L136I)$ these parameters were almost identical to $K_{ir}1.1$ wild-type. Thus, the presence or absence of K^+ -dependent gating could not be linked to any of these single channel parameters.

Interestingly, the P-helix was recently shown to move during pH gating in KcsA, a two segment-type K^+ channel from *Streptomyces lividans* (Perozo *et al.* 1998; Heginbotham *et al.* 1999). When viewed in the structural context of the KcsA crystal (Doyle *et al.* 1998), side chains of L136 and V140 (positions 70 and 74 in KcsA) appeared to interact with neighbouring subunits rather than with the ion binding sites (Fig. 7A). This is consistent with the idea that P-helix mutations act by preventing the slow gating reaction observed upon K^+ depletion of the pore (Fig. 3).

Figure 7B summarizes the results in a kinetic model of $K_{ir}1.1$ gating by pH_i and K^+_o . Thus, channels undergo at least two consecutive conformational changes. In a first step, reflecting pH gating and triggered by protonation of K80 ($pK_a = 6.8$; Schulte *et al.* 1999), channels enter a closed state with K^+ ions present at the respective binding sites (closed- K^+). Upon dissociation of these ions, K^+ -dependent gating associated with structural rearrangements involving the P-helix drives channels into a second closed state (closed-). Equilibrium of this second transition is defined by the apparent affinity of closed channels for K^+_o ($K_D = 1.6$ mM; see Methods). As result of this sequential mechanism, the apparent pK_a of ROMK should be shifted to higher values at physiological K^+_o , which has indeed been reported (Sackin *et al.* 2001). This model also explains the pH_i dependence of K^+ gating kinetics (Doi *et al.* 1996). K^+ -dependent inactivation becomes very slow at alkaline pH_i as the fraction of channels susceptible to this reaction (closed- K^+) is reduced, whereas recovery is governed entirely by the slow transition from (closed-) to (closed- K^+), independent of pH_i .

Thus, the interaction of gating by K^+_o and pH_i may play an important role in the regulation of ROMK activity under physiological conditions.

- BLEICH, M., SCHLATTER, E. & GREGER, R. (1990). The luminal K^+ channel of the thick ascending limb of Henle's loop. *Pflügers Archiv* **415**, 449–460.
- BOIM, M. A., HO, K., SHUCK, M. E., BIENKOWSKI, M. J., BLOCK, J. H., SLIGHTOM, J. L., YANG, Y., BRENNER, B. M. & HEBERT, S. C. (1995). ROMK inwardly rectifying ATP-sensitive K^+ channel: II. Cloning and distribution of alternative forms. *American Journal of Physiology* **268**, F1132–1140.
- CHOE, H., SACKIN, H. & PALMER, L. G. (2000). Permeation properties of inward-rectifier potassium channels and their molecular determinants. *Journal of General Physiology* **115**, 391–404.
- DERST, C., KONRAD, M. & KÖCKERLING, A. (1997). Mutations in the ROMK gene in antenatal Bartter syndrome are associated with impaired K^+ channel function. *Biochemical and Biophysical Research Communications* **203**, 641–645.
- DOI, T., FAKLER, B., SCHULTZ, J. H., SCHULTE, U., BRÄNDLE, U., WEIDEMANN, S., ZENNER, H. P., LANG, F. & RUPPERSBERG, J. P. (1996). Extracellular K^+ and intracellular pH allosterically regulate renal $K_{ir}1.1$ channels. *Journal of Biological Chemistry* **271**, 17261–17266.
- DOYLE, D. A., CABRAL, J. M., PFUETZNER, R. A., KUO, A., GULBIS, J. M., COHEN, S. L., CHAIT, B. T. & MACKINNON, R. (1998). The structure of the potassium channel: molecular basis of K^+ conductivity and selectivity. *Science* **280**, 69–77.
- FAKLER, B., BRÄNDLE, U., GLOWATZKI, E., WEIDEMANN, S., ZENNER, H. P. & RUPPERSBERG, J. P. (1995). Strong voltage-dependent inward-rectification of inward rectifier K^+ channels is caused by intracellular spermine. *Cell* **80**, 149–154.
- FAKLER, B., SCHULTZ, J. H., YANG, J., SCHULTE, U., BRÄNDLE, U., ZENNER, H. P., JAN, L. Y. & RUPPERSBERG, J. P. (1996). Identification of a titratable lysine residue that determines sensitivity of kidney potassium channels (ROMK) to intracellular pH. *EMBO Journal* **16**, 4093–4099.
- GIEBISCH, G. (1998). Renal potassium transport: mechanism and regulation. *American Journal of Physiology* **274**, F817–833.
- HEBERT, S. C. (1998). Roles of Na-K-2Cl and NaCl cotransporters and ROMK potassium channels in urinary concentration mechanism. *American Journal of Physiology* **275**, F325–327.
- HEGINBOTHAM, L., LEMASURIER, M., KOLMAKOVA-PARTENSKY, L. & MILLER, C. (1999). Single *Streptomyces lividans* K^+ channels: functional asymmetries and sidedness of proton activation. *Journal of General Physiology* **114**, 551–560.
- HO, K., NICHOLS, C. G., LEDERER, W. J., LYTTON, J., VASSILEV, P. M., KANAZIRSKA, M. V. & HEBERT, S. C. (1993). Cloning and expression of an inwardly rectifying ATP-regulated potassium channel. *Nature* **362**, 31–38.
- PEARSON, W. L., DOURADO, M., SCHREIBER, M., SALKOFF, L. & NICHOLS, C. G. (1999). Expression of a functional Kir4 family inward rectifier K^+ channel from a gene cloned from mouse liver. *Journal of Physiology* **514**, 639–653.
- PEROZO, E., CORTES, D. M. & CUELLO, L. G. (1998). Three-dimensional architecture and gating mechanism of a K^+ channel studied by EPR spectroscopy. *Nature Structural Biology* **5**, 459–469.

- SACKIN, H., SYN, S., PALMER, L. G., CHOE, H. & WALTERS, D. E. (2001). Regulation of ROMK by extracellular cations. *Biophysical Journal* **80**, 683–697.
- SCHULTE, U., HAHN, H., KONRAD, M., JECK, N., DERST, C., WILD, K., WEIDEMANN, S., RUPPERSBERG, J. P., FAKLER, B. & LUDWIG, J. (1999). pH gating of ROMK (Kir1.1) channels: Control by an Arg-Lys-Arg triad disrupted in antenatal Bartter syndrome. *Proceedings of the National Academy of Sciences of the USA* **96**, 15298–15303.
- SCHULTE, U., HAHN, H., WIESINGER, H., RUPPERSBERG, J. P. & FAKLER, B. (1998). pH-dependent gating of ROMK (Kir1.1) channels involves conformational changes in both N and C termini. *Journal of Biological Chemistry* **273**, 34575–34579.
- SEYBERTH, H. W., RASCHER, W., SCHWEER, H., KÜHL, P. G., MEHLS, O. & SCHÄRER, K. (1985). Congenital hypokalemia with hypercalciuria in preterm infants: a hyperprostaglandinuric tubular syndrome different from Bartter syndrome. *Journal of Pediatrics* **107**, 694–701.
- SHUCK, M. E., BOCK, J. H., BENJAMIN, C. W., TSAI, T. D., LEE, K. S., SLIGHTOM, J. L. & BIENKOWSKI, M. J. (1994). Cloning and characterization of multiple forms of the human kidney ROMK potassium channel. *Journal of Biological Chemistry* **269**, 24261–24270.
- SIMON, D. B., KARET, F. E., RODRIGUEZ-SORIANO, J., HAMDAN, J. H., DIPIETRO, A., TRACHTMAN, H., SANJAD, S. A. & LIFTON, R. P. (1996). Genetic heterogeneity of Bartter's syndrome revealed by mutations in the K⁺ channel, ROMK. *Nature Genetics* **14**, 152–156.
- STAMPE, P., ARREOLA, J., PEREZ-CORNEJO, P. & BEGENISICH, T. (1998). Nonindependent K⁺ movement through the pore in IRK1 potassium channels. *Journal of General Physiology* **112**, 475–484.
- TSAI, T. D., SHUCK, M. E., THOMPSON, D. P., BIENKOWSKI, M. J. & LEE, K. S. (1995). Intracellular H⁺ inhibits a cloned rat kidney outer medulla K⁺ channel expressed in *Xenopus* oocytes. *American Journal of Physiology* **37**, C1173–1178.
- WANG, W., SACKIN, H. & GIEBISCH, G. (1992). Renal potassium channels and their regulation. *Annual Review of Physiology* **54**, 81–96.
- WANG, W., SCHWAB, A. & GIEBISCH, G. (1990). Regulation of small-conductance K⁺ channel in apical membrane of rat cortical collecting tubule. *American Journal of Physiology* **259**, F494–502.
- YANO, H., PHILIPSON, L. H., KUGLER, J. L., TOKUYAMA, Y., DAVIS, E. M., LE BEAU, M., NELSON, D. J., BELL, G. I. & TAKEDA, J. (1994). Alternative splicing of human inwardly rectifying K⁺ channel ROMK1 mRNA. *Molecular Pharmacology* **45**, 854–860.
- YELLEN, G. (1998). The moving parts of voltage-gated ion channels. *Quarterly Reviews of Biophysics* **31**, 239–295.
- ZHOU, H., CHEPILKO, S., SCHÜTT, W., CHOE, H., PALMER, L. G. & SACKIN, H. (1996). Mutations in the pore region of ROMK enhance Ba²⁺ block. *American Journal of Physiology* **271**, C1949–1956.

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