pH-dependent Gating of ROMK (Kir1.1) Channels Involves Conformational Changes in Both N and C Termi

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ROMK channels (Kir1.1) are members of the superfamily of inward rectifier potassium channels (Kir) and represent the channels underlying K+ secretion in the kidney. As their native counterparts, Kir1.1 channels are gated by intracellular pH, with acidification leading to channel closure. Although a lysine residue (Lys80) close to the first hydrophobic segment M1 has been identified as the pH sensor, little is known about how opening and closing of the channel is accomplished. Here we investigate the gating process of Kir1.1 channels exploiting their state-dependent modification by water-soluble oxidants and sulfhydryl reagents. Mutagenesis of all intracellular cysteines either alone or in combination revealed two residues targeted by these reagents, one in the N terminus (Cys308) and one in the C terminus (Cys308) of the channel protein. Both sites reacted with the thiol reagents only in the closed state and not in the open state. These results indicate that pH-dependent gating of Kir1.1 channels involves movement of protein domains in both N and C termini of the Kir1.1 protein.

Potassium homeostasis is controlled by secretion of K+ ions across the apical membrane of cortical collecting duct cells in the kidney. As the channels responsible for K+ secretion, low conductance (35 pS) inwardly rectifying K+ channels with particular high sensitivity to changes in intracellular pH (pH_i) were identified (1, 2). Intracellular acidification in the physiological range reversibly reduced open probability of these channels and is thought to account for the subsequent decrease in K+ secretion (1). Thus, the sensitivity of the apical K+ channel to pH_i is assumed to play a key role in K+ homeostasis (2, 3).

Efforts to identify channel molecules responsible for renal K+ secretion resulted in cloning of ROMK1 (4) and its splice variants ROMK2 and ROMK3 (5, 6). Splicing results in variable length of the respective N termini with ROMK2 shortened by 19 amino acids and ROMK3 exhibiting an extension of 7 amino acids with respect to ROMK1. ROMK channels are members of a superfamily of structurally and functionally related K+ channel proteins (Kir channels, for review see Refs. 7–9). As the channels with paracellular transport, low conductance Kir channels are assembled in renal tubular cells (5, 18). As their native counterparts, Kir1.1 channels are gated by pH_i with acidification leading to channel closure (19–21). The steady-state current-pH_i relation shows a pH_i value for half-maximal activation (pH_{1/2}) of 6.9 and a Hill coefficient of around 3, indicating cooperativity of the gating process (19). As the determinant responsible for sensing pH_i we have identified a lysine residue (Lys80) close to M1 whose protonation triggers pH-dependent gating (19). Thus, the sensor of pH-dependent gating is known, whereas the processes involved in opening and closing of Kir1.1 channels are largely unknown. Here we investigate the pH-dependent opening and closing of Kir1.1 channels using state-dependent chemical modification of cysteine residues (22, 23) intrinsic to the Kir1.1 sequence.

MATERIALS AND METHODS

Mutagenesis and cRNA Synthesis—All mutants were prepared with standard techniques (24) and subcloned into pBluescript; and the mutation was verified by sequencing. Capped cRNAs specific for Kir1.1 wild type or mutant channels were synthesized in vitro using SP6 polymerase (Promega, Heidelberg, Germany) and stored in stock solutions at –70 °C.

Preparation and Injection of Oocytes—Xenopus oocytes were surgically removed from adult females and manually dissected. About 50 ml of a solution containing cRNA was injected into Dumont stage VI oocytes. Oocytes were treated with collagenase type II (Sigma, 0.5 mg/ml) and incubated at 19 °C for 2–4 days prior to use.

Cysteine Modification—DTT, DTNB and ATP (sodium salt) were purchased from Sigma, MTSES was obtained from Toronto Research Chemicals (North York, ON, Canada), and PIP2 was obtained from Boehringer (Mannheim, Germany). For DTT and DTNB, stock solutions (100 mM) were made and stored at −20 °C; the final dilutions were used for about 8 h. MTSES was freshly prepared prior to each experiment and used within 20 min. PIP2 (100 μM) was dissolved by 30 min of sonification in the cytoplasmic solution (25, 26).

Electrophysiology and Data Evaluation—Electrophysiological recordings were performed 3–7 days after injection using the giant patch-clamp technique as described previously (17). Briefly, pipettes were made from thick walled borosilicate glass and were filled with 0.3–0.6 M K+ (tip diameter of 20–30 μm) and were filled with 120 mM KCl, 10 mM HEPES and 1.8 mM CaCl2. Currents were sampled at 10 kHz and were corrected for capacitance transients with an EPC9 amplifier (HEKA electronics, Lambrecht, Germany), with analog filter set to 3 kHz (−3 db). Cytoplasmic solution was applied to excised patches via a multibarrel pipette and had the following composition: 100 mM KCl, 10 mM HEPES, 10 mM K2EGTA, (total K+ of 120 mM); pH was adjusted to the

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1 B. Fakler, unpublished observation.
2 The abbreviations used are: DTT, dithiothreitol; DTNB, 5,5′-dithio-bis(2-nitro-benzoic acid); MTSES, (2-sulfonatoethyl)-methanethiosulfonate; PIP2, phosphatidylinositol-4,5-bisphosphate; Cu-Phen, Cu(II)-1,10-phenanthroline.
values indicated by titration with HCl or KOH, respectively. All experiments were performed at room temperature (approximately 23 °C).

Fractional recovery of oxidized channels by DTT (relative recovery) was calculated as follows: relative recovery = \( \frac{I_{\text{pH8.0(after)}}}{I_{\text{pH8.0(before)}}} \), where \( I_{\text{pH8.0(after)}} \) indicates the steady-state current amplitude at pH 8.0 in the presence of DTT, \( I_{\text{pH8.0(before)}} \) indicates current amplitude at pH 8.0 after acidification, and \( I_{\text{pH8.0(after)}} \) indicates current amplitude at pH 8.0 before acidification. The values are presented as mean ± S.D. of \( n \) experiments.

RESULTS

**K_{r1.1} Channels Are Redox-sensitive**—The pH-dependent gating of K_{r1.1} channels as recorded in giant inside-out patches from Xenopus oocytes expressing K_{r1.1} channels. Intracellular acidification (pH 6.0) and alkalinization (pH 8.0) is indicated by gray and black bars, respectively. Inset, pH-induced inactivation at expanded time scale. B, Mg-ATP (left panel, 1 mM) and PIP\(_2\) (right panel, 100 \( \mu \)M) failed to recover channel activity. Application of Mg-ATP and PIP\(_2\) as well as acidification and alkalinization are indicated by bars. Voltage protocol as in A. Calibration of time and current are indicated, zero current level as marked by small horizontal bars.

Because the incomplete recovery was reminiscent of “channel run-down,” a phenomenon well known for K_{r} channels, Mg-ATP (28, 29) and the anionic phospholipid PIP\(_2\) (25, 26, 30), both reported to counteract run-down, were tested. As shown in Fig. 1B, neither reagent was able to restore channel activity. Addition of DTT (100 \( \mu \)M) or reduced glutathione (5 mM) to the pH 8.0 solution led to complete recovery even after prolonged acidification (\( n = 12 \), Fig. 1C). This suggests that oxidation occurs during acidification, which subsequently prevents channels from recovery from pH inactivation.

**Recovery from pH Inactivation Is Different with Oxidizing and Modifying Reagents**—Redox sensitivity of K_{r1.1} channels was further investigated with reagents that differentially interact with sulfhydryl-groups. Cu(II)-1,10-phenantroline (Cu-Phen), which induces formation of disulfide bonds, largely reduced the fraction of channels that spontaneously recovered upon realkalinization (Fig. 2A). Addition of DTT to the pH 8.0 solution still resulted in complete recovery from pH inactivation.
tion (Fig. 2A, n = 2). In contrast, when reagents that chemically modify cysteine residues such as MTSES or DTNB were applied at acidic pH, DTT failed to recover channels from pH inactivation (Fig. 2, B and C, n > 10). The increase in current observed upon realalkalinization in the experiments in Fig. 2 (B and C) most likely represented unmodified channels because no recovery was seen when application of DTNB was extended to periods longer than ~2 min (not shown). These results indicate that Cu-Phen-induced oxidation is reversible, whereas modification by DTNB or MTSES irreversibly locks channels in a closed state.

**Modification of Channels by DTNB Is State-dependent**—To more closely characterize modification by DTNB and its relation to pH-dependent gating, experiments were performed where DTNB was applied at pH 8.0 either before or after pH-induced inactivation, *i.e.* when channels were either in the open or pH-inactivated state. As shown in Fig. 3 (A and B), DTNB did not affect open channels at pH 8.0, nor did preapplication of DTNB change subsequent pH gating (n = 3).

In contrast, when DTNB was applied during DTT-induced recovery from pH inactivation, the recovery process was promptly stopped (Fig. 3C, n = 5). As before (Fig. 3A), DTNB did not induce inactivation in this experiment. Thus, under identical conditions, channels were only susceptible to chemical modification when they were pH-inactivated prior to DTTN application.

This coupling of chemical modification to pH inactivation was confirmed by experiments with a mutation of K\textsubscript{ir}1.1, K\textsubscript{ir}1.1(K80M), in which the pH sensor has been removed and which therefore did not exhibit pH-dependent gating (19). As shown in Fig. 3 (D–F), no effect of DTNB was observed, neither at basic nor at acidic pH (Fig. 3, D and F, n = 4). The reversible decrease in current amplitude seen at pH 6.0 reflects a weak pH dependence also known for pH-sensitive K\textsubscript{ir}2.1 (IRK1) channels and at least in part due to block of channels by hydrogen ions (19). Taken together, these results indicate that K\textsubscript{ir}1.1 channels are targeted by DTNB in a state-dependent manner, *i.e.* channels are modified in the pH-inactivated state but not in the open state.

**State-dependent Modification Occurs at Residues in the N and C Termini**—This state dependence together with the fact that DTNB specifically modifies cysteine residues was exploited to see which domains of the K\textsubscript{ir}1.1 protein move during pH-dependent gating.

For this purpose all cysteines in the K\textsubscript{ir}1.1 sequence were replaced by alanine or serine (Fig. 4A). Mutations in the N and C termini outside the “core region” (hydrophobic domains M1 and M2 and the P region) resulted in functional channels gated by intracellular pH, whereas no currents were observed upon expression of the two Cys → Ala/Ser exchanges in the P region (C121A/S and C153A/S).

The functional mutations were tested for recovery from pH inactivation in the presence of DTT after they were inactivated by a 50-s application of the pH 6.0 solution either in the absence (control) or presence of DTNB. As shown in Fig. 4 (B and C), all mutants recovered very similar to WT under control conditions, and DTNB modification was not abolished by either of the single C to A/S exchanges. However, the fractional recovery from inactivation after DTNB modification observed in C49A and C308A was significantly larger than for WT or any of the other mutants (Fig. 4B) and led to the assumption that DTNB modification may occur at one cysteine. This was indeed verified, because in the double mutant K\textsubscript{ir}1.1(C49A,C308A) recovery from pH inactivation in the presence of DTT was complete, independent of whether DTNB was added to the inactivation solution (Fig. 5).

These results show that the cysteine residues modified by sulphydryl reagents in a state-dependent manner are Cys\textsuperscript{19} in the N terminus and Cys\textsuperscript{208} in the C terminus of the K\textsubscript{ir}1.1 protein. Moreover, the results indicate that pH-dependent gating in these channels is accompanied by structural rearrangements in both intracellular N and C termini.

**DISCUSSION**

The results presented here show that pH-dependent opening and closing of K\textsubscript{ir}1.1 channels is accompanied by conformational changes of the channel protein involving movement of domains in the intracellular N and C termini. This movement was visualized by state-dependent modification of Cys\textsuperscript{19} and Cys\textsuperscript{208}, which were susceptible to reaction with DTNB in the pH-inactivated state, whereas no modification was observed for open channels.

Besides modification by DTNB or MTSES, channels were also oxidized in a state-dependent manner. Cu-Phen applied at pH 6.0 prevented channels from recovery from inactivation, an effect that could be fully reversed by application of the reducing agent DTT (Fig. 2). But although oxidation and reduction were induced by agents specific for formation and reduction of disul-
fide bonds, no significant alteration of the redox sensitivity was observed in either of the Cys → Ala/Ser mutations. Moreover, oxidation was also observed in the double-mutant Kir1.1(C49A,C308A), which completely abolished state-dependent modification by DTNB (Fig. 5) as well as in the triple mutants Kir1.1(C308A,C355A,C358A) and Kir1.1(C49A,C175A,C308A). When all cysteines outside the core region were replaced by alanine, no channel activity was detected upon functional expression. Thus, we could not identify the cysteine(s) involved in oxidation nor exclude a role for a protein putatively associated with ROMK.

The reducing agents DTT and reduced glutathione were required for complete recovery from pH inactivation in excised inside-out patches. In living cells, however, the redox sensitivity most likely has no physiological implication, because reduced glutathione should be available in millimolar concentrations. Moreover, experiments with nitric oxide (NO, SIN-1, and S-nitrosocysteine as NO donors), which replaced DTNB in activation of cyclic nucleotide-gated channels (31, 32), did not reveal any effect on pH-dependent gating of Kir1.1 channels. The structural rearrangements that occur during pH gating in both N and C termini of Kir1.1 represent the first direct evidence for conformational changes in the Kir channel family. Work on GIRK channels (Kir3.0 subfamily) showed that protein domains in both N and C termini are involved in interaction with G-proteins (33–35). These domains mediate binding of Gβγ or Gaβγ to the Kir subunit. It is not yet known which parts of the molecule move during G-protein-induced channel gating.

Gating induced by changes in extracellular pH (pK_a around 2.5) has recently been related to conformational changes in the two-segment type potassium channel from Streptomyces lividans (KscA, (36)). Using EPR spectroscopy, Perozo and co-workers uncovered structural rearrangements at the C-terminal end of the second transmembrane helix (37, 38) that were hypothesized to change the width of the inner vestibule and to thus control ion permeation. Whether such a mechanism also underlies gating in Kir1.1 channels with rearrangements in N and C termini as secondary processes or whether these cytoplasmic domains form the actual gate of the channel remains to be elucidated. Furthermore it remains to be elucidated whether the conformational changes resulting from pH-dependent gating may affect processes reported to affect channel activity such as phosphorylation by protein kinase A (39, 40) or interaction with anionic phospholipids (26).

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