Localization of the pH Gate in Kir1.1 Channels

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ABSTRACT We used cysteine-modifying reagents to localize the pH-sensitive gate in the renal inward-rectifier K^+ channel Kir1.1a (ROMK1). Cytoplasmic-side methanethiosulfonate (MTS) reagents blocked K^+ permeation in native Kir1.1 channels, expressed in *Xenopus* oocytes. Replacement of three cysteines in the N-terminus, C-terminus, and transmembrane domains eliminated this sensitivity to MTS reagents, as measured with inside-out macropatches. Reintroduction of one cysteine at 175-Kir1.1a in the second transmembrane domain allowed blockade of the open channel by the MTS reagents MTSEA, MTSET, and MTSES and by Ag^+. However, closure of the channel by low pH protected it from modification. Cysteine was also introduced into position G223, which is thought to line the cytoplasmic pore of the channel. MTSET blocked G223C in both the open and closed state. In contrast, MTSEA reduced G223C single-channel conductance from 40 to 23 pS but did not produce complete block. We conclude that cytoplasmic acidification induces a conformational change in the channel protein that prevents access of cysteine-modifying reagents, and presumably also K^+ ions, to the transmembrane pore from the cytoplasm. This is consistent with localization of the Kir1.1 pH gate at the helix bundle crossing near the cytoplasmic end of the transmembrane pore.

INTRODUCTION

Inward-rectifier K^+ channels are regulated by cytoplasmic factors including ATP and ADP concentrations (Kir6), G-protein subunits (Kir3), and intracellular pH (Kir1 and 4) (1–3). Although the voltage-dependent gating of K_v channels is widely thought to take place at the cytoplasmic end of the transmembrane pore (4–6), the site of gating in Kir channels has not been unequivocally established.

Structural studies of bacterial Kir channels indicate that in the closed state, the transmembrane helices may converge to constrict the pore at the cytoplasmic end, similar to the picture envisioned for Kv channels (7). In the putative open state, these helices move apart to allow passage of K^+ and other permeant ions (8). However, the structure of a channel in protein crystals does not necessarily reflect the native state in the membrane. Nonetheless, several physiological studies of functional channels support the idea of gating at the so-called helix bundle crossing. Phillips et al. (9) showed that closure of Kir6.2 channels by ATP could trap intracellular blockers in the pore, suggesting that the gate lies at a point between the cytoplasm and the transmembrane pore. Sackin et al. (10) studied mutations at the putative bundle-crossing site and showed that reduction in the size of the hydrophobic amino-acid side chain prevented Kir1 channels from closing in response to low cytoplasmic pH.

On the other hand, Xiao et al. (11) found that the PIP2-induced changes at the helix bundle crossing were not sufficient to explain channel closure in Kir2. They suggested that the selectivity filter at the extracellular end of the pore could be an important gating site. A similar conclusion was reached regarding the gating of cyclic-nucleotide-dependent cation channels (12). Furthermore, Proks et al. (13) reported that ATP-dependent closure of Kir6.2 channels did not abolish access of cytoplasmic-side Ba^{2+} to the selectivity filter.

With respect to Kir1.1, several studies have demonstrated that closure of channels by low cytoplasmic pH is inhibited by high extracellular K^+ concentrations (14–16). This is consistent with the idea that low pH induces a rearrangement of the selectivity filter, closing its conduction path, and that the presence of K^+ in the filter protects against this collapse.

In the current study, we have used cysteine-modifying reagents to assess the location of the pH-dependent gate of Kir1.1 channels expressed in *Xenopus* oocytes. The results support a location of the gate at the helix bundle crossing near the cytoplasmic end of the transmembrane pore.

METHODS

Generation of a MTS-insensitive background and introduction of cysteine

Site-directed mutants were made using the Pfu enzyme (Stratagene, La Jolla, CA) according to manufacturer's instructions. Primers were synthesized by Operon Technologies (Alameda, CA). Sequences were confirmed using an ABI 377XL automated DNA sequencer at The Cornell University Bio Resource Center (Ithaca, NY).

Channel expression

ROMK1 plasmids were linearized with *Not*I restriction enzyme, and cRNAs were transcribed with T7 RNA polymerase using mMESSAGE mMACHINE kit (Ambion, Austin, TX). cRNA pellets were dissolved in nuclease-free water and stored at −70°C before use. Oocytes harvested from *Xenopus*

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laevis were incubated in OR2 solution with 2 mg/ml collagenase type II (Worthington, Lakewood, NJ), and 2 mg/ml hyaluronidase type II (Sigma Chemical, St. Louis, MO) and incubated with gentle shaking for 60 min at room temperature. Before the injection, oocytes were incubated in OR2 solution for 1 h at 19°C. Defolliculated oocytes were selected and injected with RNA. Following the injection, they were stored at 19°C in Leibovitz's L15 medium (Invitrogen, Carlsbad, CA) for 1–2 days before measurements were made.

Electrophysiology

Before patch-clamping, the oocyte vitelline membrane was mechanically removed in a hypertonic solution containing 200 mM sucrose. Macroscopic patch-clamp pipettes were prepared from Fisherbrand hematocrit capillary glass (Fisher Scientific, Pittsburgh, PA) using a three-stage puller, coated with Sylgard (Dow Corning, Midland, MI), and fire-polished with a microforge. Pipettes were filled with solution containing the same components as the standard bath solution; pipette resistances ranged from 0.5 to 1 MΩ.

Macroscopic inside-out patches were studied in a chamber that allowed the bathing solutions to be changed within seconds. After seals formed in STD solution (mM: 110 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, pH 7.4), inside-out patches were obtained by lifting the electrodes into FVPP solution (mM: 110 KCl, 4 NaF, 3 Na_3VO_4, 10 $Na_4P_2O_7$, 5 HEPES, 5 EDTA, pH 7.4) to prevent channels from running down. Intracellular pH was changed by switching into FVPP solutions with different pH values. In experiments with Ag^+ the Na_3VO_4 and $Na_4P_2O_7$ were omitted to prevent precipitation; 1.4 μM $AgNO_3$ was added to solutions containing 90 KNO_3, 20 KF, 65 mM EDTA, and 10 mM HEPES (pH 7.4 or 6.0 for open and closed state modifications), yielding a free Ag^+ concentration of 1.5 nM (12). The $AgNO_3$ stock solution was made every week and was kept in the dark at −20°C. We used an open syringe system to allow the MTS or Ag^+ reagents to be added only after a successful patch was formed. This resulted in a dead time for solution exchange of several seconds.

MTS reagents were purchased from Toronto Research Chemicals (Downview, ON) and were stored at −20°C as powder. Each day a stock solution was prepared, stored on ice, and diluted to final concentration immediately before use. For dithiothreitol (DTT) (Sigma Chemical), 100 mM stock solutions were made and stored at −20°C; the final dilution was used for ~4 h.

Currents were recorded using a model EPC7 patch-clamp amplifier (Medical Systems, Greenvale, NY). Records were sampled at 4 kHz using an ITC-16 interface (Instrutech, Mineola, NY) and Pulse and Pulsefit software (HEKA Elektronik, Southboro, MA). For analysis, data were filtered at 1 KHz.

Whole-cell conductances were measured in intact oocytes using a two-electrode voltage clamp (OC-725, Warner Instrument, Hamden, CT). Intracellular pipettes had a resistance of 0.5–1 MΩ when filled with 3 M KCl. pH titration curves in intact oocytes were obtained by changing the extracellular pH with permeant acetate-buffered solutions to control intracellular pH (17,18).

RESULTS

Fig. 1 illustrates the phenomenon of gating of Kir1.1 channels by intracellular pH using inside-out macropatches. Channels were expressed in *Xenopus* oocytes, and patches containing 10–300 channels were excised into FVPP solution. Under these conditions channel activity was stable for 5–10 min and was rapidly inhibited by lowering the pH on the cytoplasmic side of the patch. As previously reported (19), reversal of the effect of acidification was slow unless a reducing agent was present in the bath (Fig. 1 *A*). We therefore included 100 μM DTT in subsequent experiments to facilitate recovery from low pH (Fig. 1 *B*).

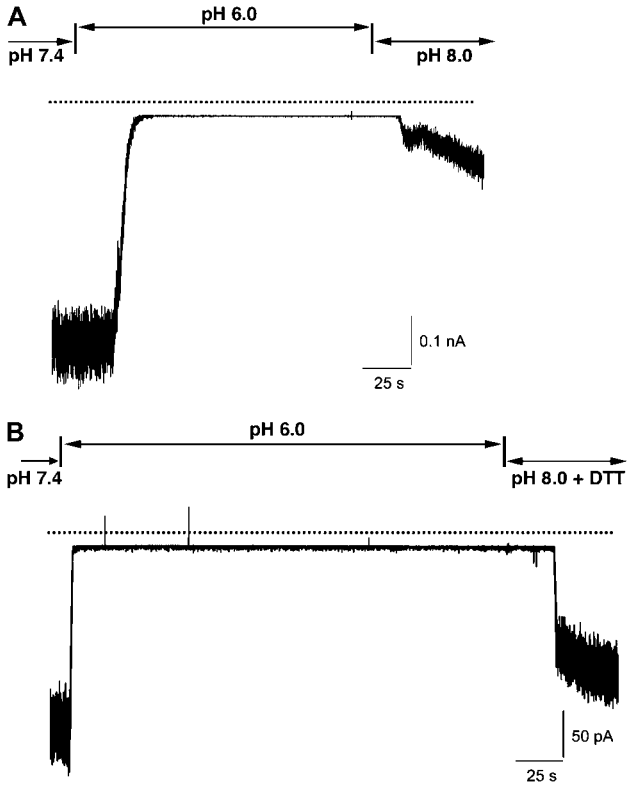

FIGURE 1 Activity of Kir1.1 channels in excised inside-out macropatches. Patches were formed on oocytes expressing wild-type Kir1.1 and excised in the inside-out configuration. The bathing medium was exchanged for FVPP solution with pH 7.4, and the membrane voltage was clamped to −80 mV. The dashed line represents the zero-current level. There were 50–100 active channels in the patch. Lowering the pH to 6.0 abruptly abolished the channel activity and reduced the current to the baseline level indicating the leak current. (*A*) Channel activity returned slowly toward baseline when the pH was increased to 8.0. (*B*) Recovery was greatly accelerated when DTT (100 μM) was included in the bath solution. Traces are representative of five or more experiments. Mean fractional recoveries measured 50 s after switching to pH 8.0 were 0.19 ± 0.01 without DTT and 0.85 ± 0.04 with DTT.

To assess the effects of modifying reagents on individual cysteine residues, we needed to first engineer a Kir1.1 channel that was insensitive to the MTS reagents. As shown in Fig. 2 *A*, application of 1 mM MTSET to the cytoplasmic side of the wild-type channel produced a prompt and complete inhibition of current. The reagent also irreversibly blocked wild-type channels while they were in the closed state (Fig. 2 *B*). In the experiment of Fig. 2 *B*, cytoplasmic-side pH was lowered to 6.0, MTSET was applied for 60 s and washed off, followed by an increase in bath pH to 8.0 in the presence of DTT. No channel activity was recovered, indicating that the channels had irreversibly reacted with MTSET.

Based on previous results (19), we focused on the removal of three cysteines that are expected to be accessible from the cytoplasm and that were shown to affect modification by the cysteine reagent DTNB. These were C49 in the cytoplasmic N-terminus, C175 in the second transmembrane domain, and C308 in the cytoplasmic C-terminus. We found that any two

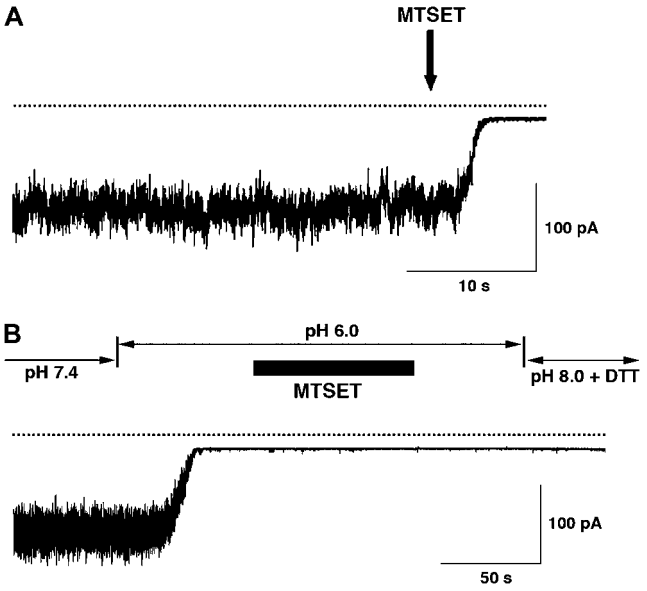

FIGURE 2 Effects of MTSET on wild-type Kir1.1. (*A*) MTSET (1 mM) added (*arrow*) to the bath of an inside-out patch containing WT channels produced an abrupt inhibition of channel activity. (*B*) Addition of MTSET to a patch in which the channels were first closed by low pH. Subsequent removal of the reagent and increase of the pH to 8.0 in the presence of DTT did not recover channel activity, indicating that MTSET had irreversibly inhibited the channels when they were in the closed state. Traces are representative of three or more experiments. The fractional recovery of current was 0.010 ± 0.005.

of these residues could be mutated to alanine but that C-to-A substitution of all three led to an almost complete loss of activity. However, after screening a number of combinations, we found that the triple mutant C49V/C175S/C308V (termed $ROMK_{YZ}$) retained activity close to that of WT. Furthermore, the channels were insensitive to 1 mM MTSET but were still closed by low cytoplasmic pH (Fig. 3). This mutant served as a background for the subsequent series of experiments.

Reintroduction of a cysteine at 175 (to form $ROMK_{YZ}$ S175C) placed an MTS-reactive cysteine just above the putative gate formed by L179 at the helix-bundle crossing (see Fig. 3 *A*). This mutant expressed robust pH-sensitive K^+ currents, as measured in the two-electrode voltage clamp, where intracellular pH was controlled with permeant acetate buffers (17), as indicated in Fig. 4. The apparent pK_a of the WT channel after correction for differences in external and internal pH using the empirical relationship $pH_i = 0.595\ pH_o + 2.4$ (17) was 6.61. Those for the $ROMK_{YZ}$ S175C mutant (6.81) and for the other major construct studied here, $Kir1.1_{YZ}$ G223C (6.80) were slightly higher. In all cases the relationship between channel activity and pH was steep, with Hill coefficients >1. This indicates that the mutant and WT channels are gated by the same pH-dependent processes.

The effect of MTS reagents on $ROMK_{YZ}$ S175C is shown in Fig. 5. Representative experiments in Fig. 5 *B* indicate that, at 1 mM and high pH, the positively charged MTS reagents MTSET and MTSEA completely blocked $ROMK_{YZ}$ S175C despite a difference in the size of their amine groups. The negatively charged MTSES reagent reduced channel activity but did not produce a complete block.

To assess the rates of modification, we reduced the concentration of the reagents so that the time course of current inhibition could be resolved. The responses to 5 μM MTSEA and 50 μM MTSET in the open state are shown in Fig. 6. In both cases there was a lag period caused by dead space in the solution-exchange system, followed by a decline in inward current that could be well described by a single exponential function. A 10-fold larger concentration of MTSET was required to achieve rates of modification comparable to that of MTSEA. The first-order reaction rate constants calculated from the time constants and concentrations used were $2.0 \pm 0.3 \times 10^4\ s^{-1}M^{-1}$ for MTSEA and $1.1 \pm 0.2 \times 10^3\ s^{-1}M^{-1}$ for MTSET (Table 1).

Initially, we examined the state-dependent block of the $ROMK_{YZ}$ S175C channel using a high concentration (1 mM) of MTS reagents (Fig. 7). As shown in Fig. 7 *A*, low-pH-induced closure of the channels protected the C175 site against modification by MTSET, so that realkalinization after removal of MTSET restored 60% of the original current. This was comparable to the recovery of current after acidification seen in time controls not subjected to MTS reagents (see Fig. 9). This implies that low pH produces a change in the structure of the channel between the intracellular solution and the transmembrane cavity, effectively abolishing MTSET access to the cavity. Even if MTSET inhibited 20% of channel activity at low pH, a reasonable upper limit from the data of Fig. 7 *A*, this would imply a maximum modification rate of only $2\ s^{-1}M^{-1}$ under these conditions. This is 500-fold slower than the open-state modification rate.

In contrast, the same 1 mM exposure to MTSEA appeared to block the channels in both the open and closed states because only a small fraction of the original current could be recovered after realkalinization following exposure and washout of MTSEA (Fig. 7 *B*). However, these reagent concentrations were far in excess of what was required to block the channels in the open state, so we reassessed state-dependent block using lower concentrations of MTSEA. As shown in Fig. 8, exposure to MTSEA at a concentration of 5 μM for 50 s was sufficient to almost completely inhibit channels in the open state. However, exposure of channels in the closed state (pH 6) to the same concentration of the reagent for up to 200 s had no detectable effect. The results of Fig. 8 show that channel closure protects the C175 residue from modification by cytoplasmic-side MTSEA as long as the MTSEA concentration is in the range of 5 μM.

We repeated these experiments using Ag^+ as a cysteine-modifying reagent. This has the advantage of being similar in size to K^+ and thus a better reporter of the state of the K^+ permeation path. Initial results indicated that 1–10 nM Ag^+, similar to the MTS reagents, inhibited $ROMK_{YZ}$ S175C but not $ROMK_{YZ}$ channels and that the inhibition was very slowly reversible in the presence of DTT (not shown). The

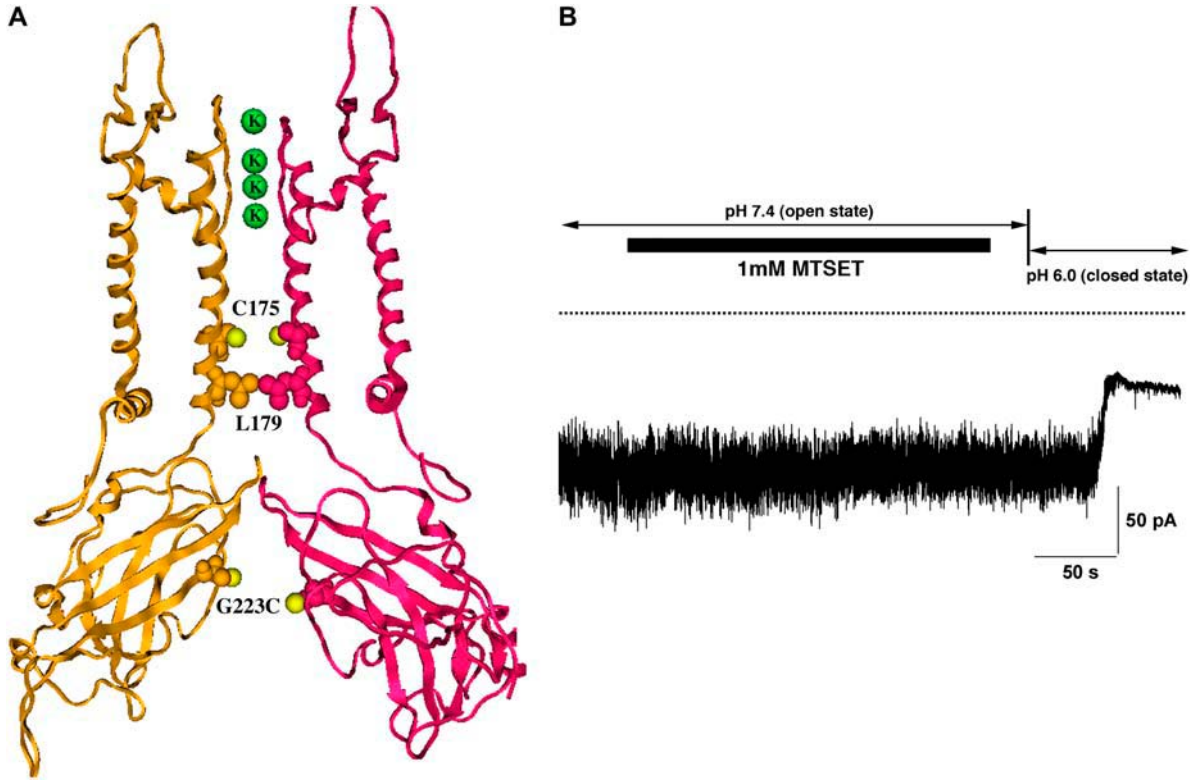

FIGURE 3 Effects of MTSET on a triple-cysteine mutant. (*A*) Homology model of Kir1.1a showing the transmembrane pore cysteine at C175, the putative pH gate at L179, and the cytoplasmic pore cysteine at 223C formed by the mutation: Kir1.1a-G223C. Two of the four subunits are drawn in the figure. (*B*) An MTSET-insensitive channel ($ROMK_{YZ}$) was constructed by making three mutations on the Kir1.1a wild-type: C49V/C175S/C308V. A current trace from an inside-out patch containing $ROMK_{YZ}$ shows insensitivity to MTSET. The channels retained their inhibition by low pH. Trace is representative of four experiments. The current measured 60 s after addition of MTSET was 0.94 ± 0.04 of the initial value.

apparent rate constant for Ag^+ inhibition was much greater than that for the MTS reagents. With 1.5 nM Ag^+ the reduction in current was approximately exponential with a time constant of around 4 s (Fig. 9 *A*). The mean rate constant was $1.3 \pm 0.4 \times 10^8\ M^{-1}s^{-1}$, suggesting a modification rate that was diffusion limited. When Ag^+ was applied for 50 s after closure of the channels by low pH, the current recovered rapidly in response to high pH plus DTT (Fig. 9 *B*).

These results are summarized in Fig. 10. After exposure of the channels to low cytoplasmic-side pH (6.0) in the absence of MTS reagents, an average of ~60% of the initial current could be recovered by increasing the pH to 8.0 in the presence of 100 μM DTT. Exposure of the patch to MTSET (1 mM), MTSEA (5 μM), or Ag^+ (1.5 nM) during the period in which the channels were completely closed (as judged from the lack of visible openings in the current record) did not significantly alter the fractional recovery. Hence, channel closure via low pH diminished access of MTS reagents to the C175 residue in the transmembrane cavity. However, at high concentrations (1 mM), MTSEA could modify this residue, even when the channel was in the closed state.

One possible explanation for the discrepancy between the two compounds is that MTSEA is somewhat hydrophobic, presumably because some of the amine groups will be un-ionized at neutral pH (20). The uncharged MTSEA molecules could then partition into the membrane and might reach the C175 side chain through a hydrophobic pathway that would not be available to MTSET. The finding that the channels could be modified during the exposure to the reagent at pH 6.0, where the number of un-ionized molecules should be minimal, argues against this scheme. Nevertheless, we tested the idea using the premise that if the compounds were taking a route through the membrane, they should also be able to reach C175 from outside the cell. As shown in Fig. 11, neither MTSET nor MTSEA significantly affected currents through $ROMK_{YZ}$ S175C channels when added to the extracellular solution. Although we cannot completely rule out the possibility that the two reagents take different paths to modify the same side chain, we regard it as unlikely.

Finally, we evaluated the reactivity of a cysteine introduced into the cytoplasmic pore. This is a region of the protein formed by parts of the N- and C-termini that extend into the cytoplasm, thereby lengthening the permeaton path of the channel (7). The G223 position in Kir1.1a is homologous to the E224 residue of Kir2.1 (IRK1) that enhances inward rectification, presumably by interacting with cationic blockers in the cytoplasmic pore (21,22). Expression levels of this channel were lower than for the other constructs, but

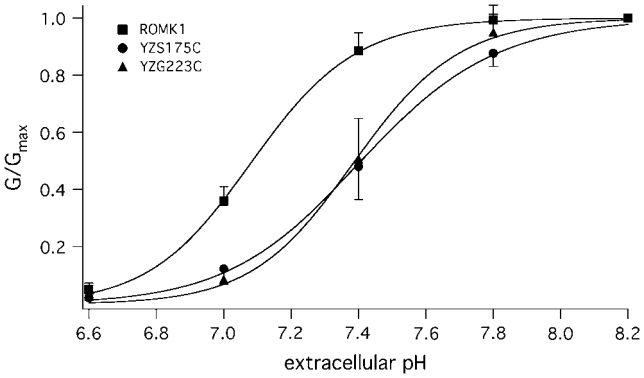

FIGURE 4 pH sensitivity of WT channels and the mutants $ROMK_{YZ}$ S175C and $ROMK_{YZ}$ G223C. Whole-cell currents in oocytes expressing the three channel types were measured by two-electrode voltage clamp. Currents were measured at a voltage of −100 mV and were corrected for currents measured in the presence of 5 mM Ba^{2+}. Internal pH was varied by changing the external pH in the presence of 55 mM acetate. Abscissa indicates the extracellular pH used to control internal pH. Data were normalized to the value measured at pH 8.2 and are presented as means ± SE for four or more oocytes. Solid lines represent best fits with the Hill equation: WT, $pK_a = 7.08$, $n = 1.7$. $Kir1.1_{YZ}$ S175C $pK_a = 7.41$, $n = 1.6$. $Kir1.1_{YZ}$ G223C $pK_a = 7.39$, $n = 1.7$

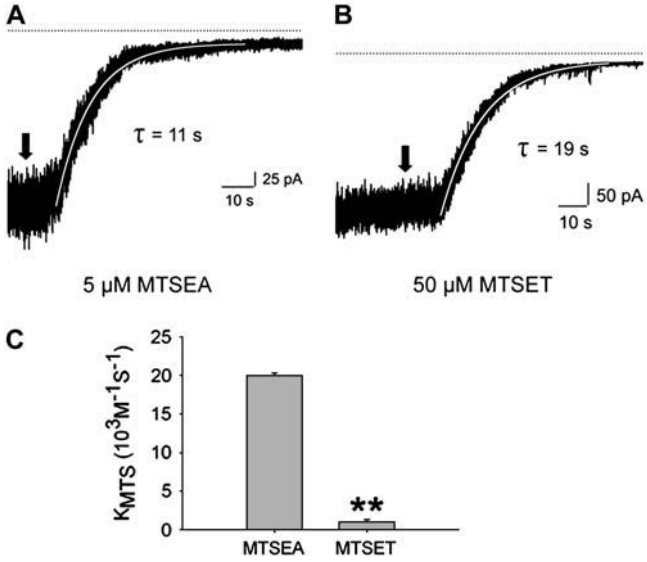

FIGURE 6 Modification rates of position 175 in the open state. Inside-out macropatches from oocytes expressing on $ROMK_{YZ}$ S175C were exposed to 5 μM MTSEA (*A*) or 50 μM MTSET (*B*). Arrows denote the times at which the solutions were switched. After a lag of several seconds that is accounted for by the dead space of the exchange system, the currents decayed following an exponential time course (*white lines*) with time constants of 11 s and 19 s, respectively. (*C*) First-order rate constants for modification. Data represent means ± SE for three independent measurements . ** indicates that the difference in the values is statistically significant ($p < 0.05$).

macroscopic currents could be monitored in favorable batches of oocytes.

Placing a cysteine in the cytoplasmic pore ($ROMK_{YZ}$G223C) allowed complete block of the open state by cytoplasmic-side application of 50 μM MTSET. The modification rate of this cysteine was similar to the modification rate for a cysteine placed at 175 (Table 1). However, in contrast to C175, $ROMK_{YZ}$G223C was also modified by MTSET in both the open and the closed state (Fig. 12 *A*). $ROMK_{YZ}$G223C was also partially inhibited in the open state by MTSEA, where MTSEA reduced the single-channel chord conductance at −100 mV from 40 ± 1 to 23 ± 1 pS ($n = 3$) (Fig. 12 *B*). The fractional decrease in single-channel conductance (0.42) was somewhat less than that of the total current (0.60). We did not investigate the actions of MTSEA on the closed state of this channel.

DISCUSSION

The major conclusion of these studies is that low intracellular pH alters the accessibility of cysteine-modifying reagents to the transmembrane pore from the cytoplasmic side. The C175 residue lies on the inner helix, within the transmembrane pore, just above the so-called bundle crossing, analogous to the convergence of inner transmembrane helices depicted in the crystal structure of KirBac1 (7). The rates of modification of the channel at this site by MTSET were at least 500-fold lower in the closed state than in the open state. This suggests that K^+ entry into the pore from the cytoplasm would also be

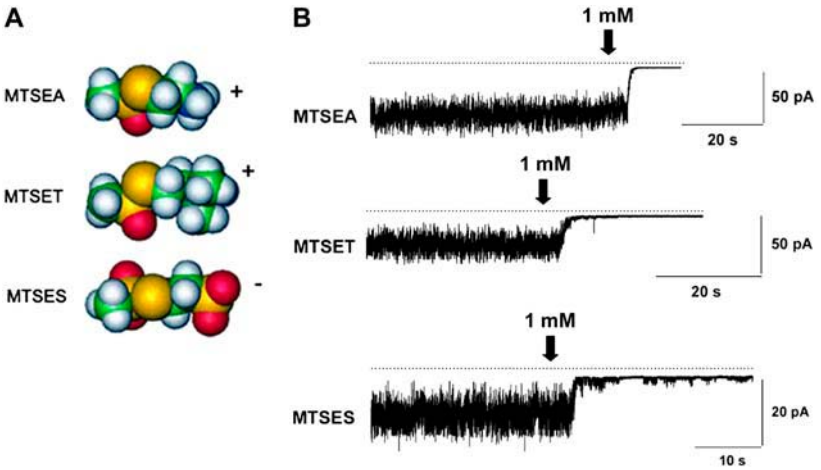

FIGURE 5 Effects of MTS reagents on $ROMK_{YZ}$ S175C channels in the open state (high intracellular pH). (*A*) Structures of the three MTS reagents MTSET, MTSEA, and MTSES (from Kaplan et al. (27)). (*B*) All three reagents when added to the bath at 1 mM inhibited K^+ currents. The negatively charged MTSES did not completely abolish channel activity and was not studied further. Traces are representative of four or more experiments. No activity above baseline could be detected with MTSEA or MTSET. The average fractional current remaining after addition of MTSES was 0.12 ± 0.01.

TABLE 1 Rates of modification by cysteine-modifying regents ($s^{-1}M^{-1}$)

	MTSEA	MTSET	Ag^+
C175	$2.0 \pm 0.3 \times 10^4$	$1.1 \pm 0.2 \times 10^3$	$1.3 \pm 0.4 \times 10^8$
C223	-	$1.3 \pm 0.3 \times 10^3$	

Data represent means ± SE for three to four experiments.

impeded by closure of a pH gate at the bundle crossing. This was confirmed by the use of Ag^+ as a modifying reagent.

The three different modifying reagents used here have different advantages and disadvantages as reporters of accessibility of specific cysteines. MTSET is strongly hydrophilic, being almost completely dissociated in aqueous solution (20). It is the largest of the reagents, however, and could conceivably be excluded from a permeation path that was still wide enough for K^+ to permeate. MTSEA is smaller but is less hydrophilic and can permeate cell membranes to some extent (20). Ag^+ is similar in size to K^+. It has also been

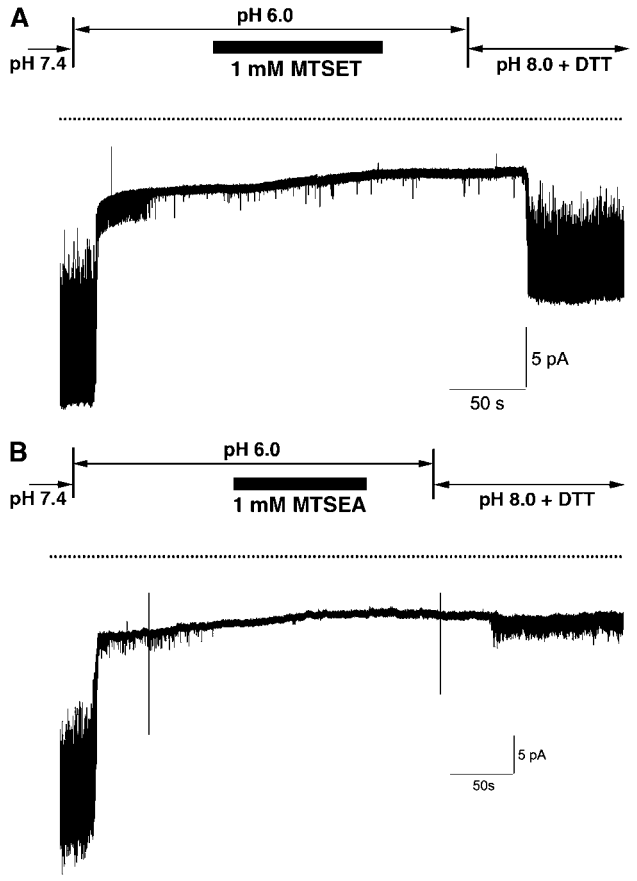

FIGURE 7 Modification of position 175 in the closed state. $ROMK_{YZ}$ S175C channels in inside-out patches were inhibited by lowering pH to 6.0. MTS reagents were added for 2 min. After the reagents were washed out, the pH was raised to 8.0 in the presence of 100 μM DTT. MTSET (1 mM, *upper panel*) did not inhibit channel activity when the channels were closed, but 1 mM MTSEA (*lower panel*) did. Traces are representative of three or more experiments (see Fig. 10).

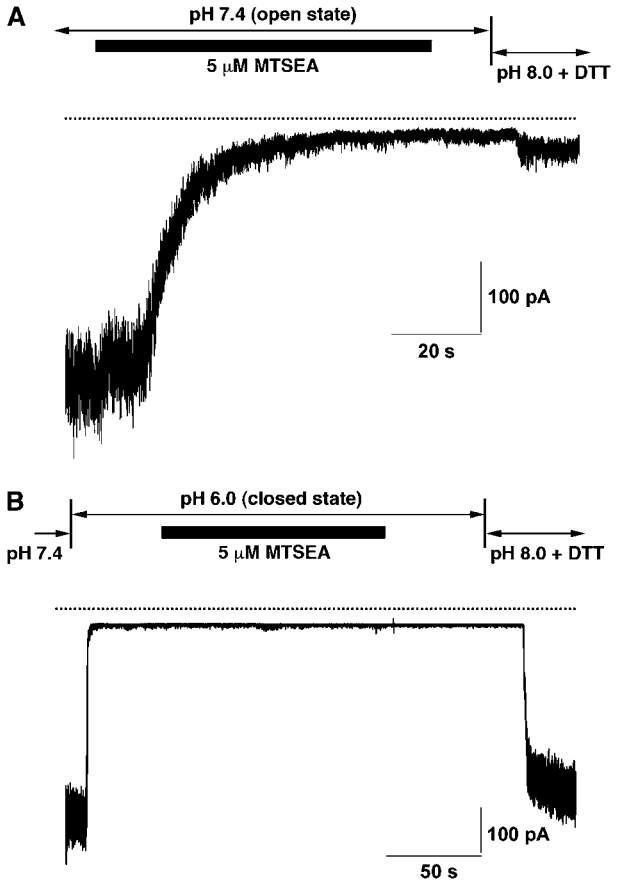

FIGURE 8 Effects of low concentrations of MTSEA on currents through $ROMK_{YZ}$ S175C. (*A*) Application of 5 μM MTSEA to an inside-out patch reduced current with an exponential time course. The time constant was 8 s. (*B*) Application of 5 μM MTSEA for 50–120 s to patches in which the channels were closed by low pH did not significantly inhibit channel activity. Traces are representative of three or more experiments (see Fig. 10).

shown to react with cysteines in a membrane environment, possibly by associating with trace Cl^- ions to form a neutral pair (11). The fact that the reaction of all three of these reagents with C175 was reduced in the closed state strongly suggests that pH controls a gate that lies between the transmembrane central cavity and the cytoplasm. However, these results do not exclude the possibility that other parts of the channel, such as the selectivity filter, also participate in gating.

At very high concentrations and long exposure times, MTSEA, but not MTSET, was able to modify the cytoplasmic pore cysteine (C175-Kir1.1a) even when the channel was in the closed state. We do not understand the basis of this modification. MTSEA has been considered to be a less reliable probe of the aqueous permeation pathway in ion channels because of its ability to partition into the hydrophobic environment of the membrane (20). It is therefore possible that MTSEA enters the pore in part through a route other than that taken by K^+ ions. The lack of effect of MTSEA from outside the cell argues against this mechanism but does not completely exclude it. Another possibility is that the channels

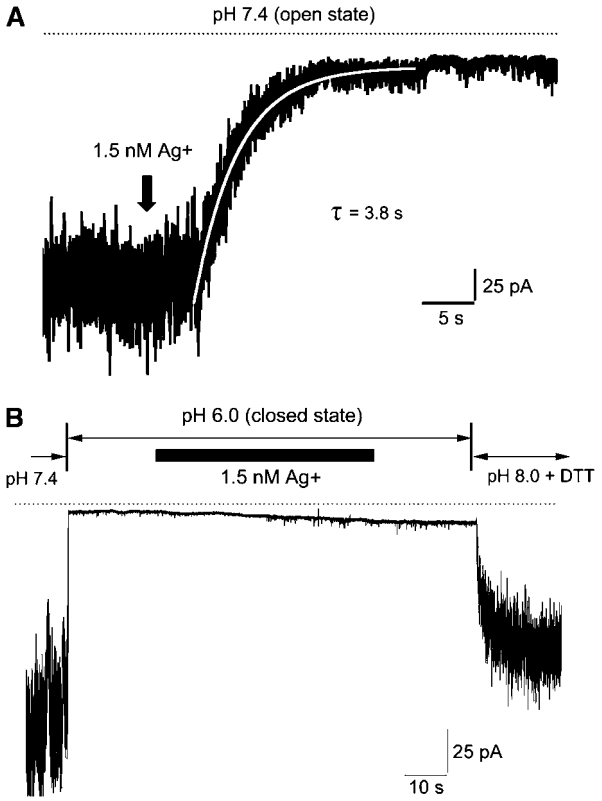

FIGURE 9 Effects of Ag^+ on currents through $ROMK_{YZ}$ S175C. (*A*) Application of 1.5 nM Ag^+ to an inside-out patch reduced current with an exponential time course. The time constant was 3.8 s. (*B*) Application of 1.5 nM Ag^+ for 50 s to patches in which the channels were closed by low pH did not significantly inhibit channel activity. Traces are representative of three or more experiments (see Fig. 10).

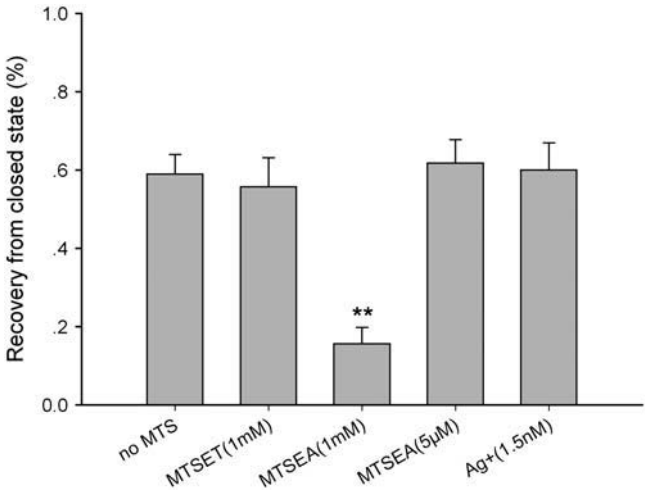

FIGURE 10 Recovery of $ROMK_{YZ}$ S175C current after low pH (expressed as percentage of initial value). Inside-out macropatches were exposed to pH 6.0 solution on the cytoplasmic side for 4–6 min with or without addition of cysteine-modifying reagents. The recovery was measured as the ratio of the maximal current obtained after raising pH in the presence of 100 μM DTT divided by the initial current before acidification. The mean percentage recovery of current is expressed as mean ± SE for four or more experiments under each condition. ** indicates that the difference in value relative to no MTS is statistically significant ($p < 0.05$).

opened very briefly under the conditions employed. A closed channel modification rate of only 30 $s^{-1}M^{-1}$ could account for these data. Because the open channel modification rate was 2×10^4 $s^{-1}M^{-1}$, if pH-closed channels actually had a P_o of 0.0015, this would be sufficient to permit MTSEA to modify C175 in the ''closed'' state. Modification of these very low P_o channels by MTSET would be much slower because of its lower (open-state) modification rate. Although we could not detect any channel openings during exposure to MTSEA, we cannot exclude very brief (<1 ms) open events that might be occurring during the ''closed state''. Finally, it is possible that the pore does not close completely at the cytoplasmic end, permitting very slow entry of both MTS reagents and K^+. Although the channel conductance appears to be close to zero in the pH-closed state, again it would be difficult to detect a conductance that is 100- to 1000-fold smaller than that of the open state (40 pS).

Among other inward rectifier K^+ channels, Kir6.2 also appears to be gated, in this case by intracellular ATP, through changes in access to the pore across the helix bundle crossing (9). This conclusion was also based on the trapping of spermine, an intracellular blocking ion, within the pore when the channels were closed by ATP. In contrast, studies of MTS reagent accessibility to the transmembrane pore of Kir2.1 indicated that gating of those channels by changes in PIP_2 levels did not alter the rates of modification by MTSET and MTSEA (11). This finding suggested that the selectivity

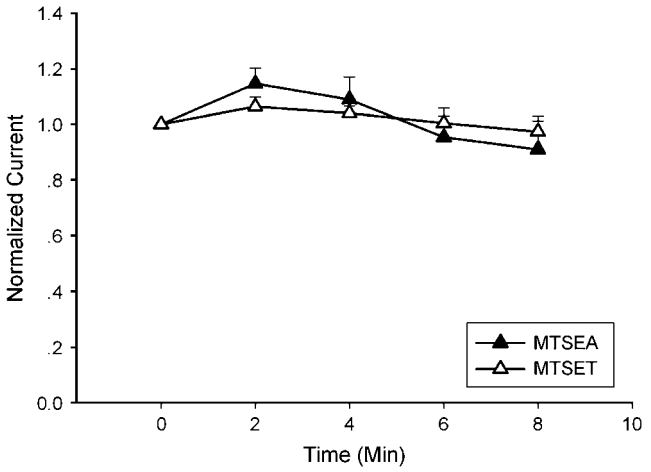

FIGURE 11 Effects of extracellular MTS reagents on channel activity. Whole-cell currents in oocytes expressing $ROMK_{YZ}$ S175C were measured by two-electrode voltage clamp. Currents were measured at $V_m = -100$ mV and normalized to the value at $t = 0$. MTSET or MTSEA (1 mM) was added to the bath, and currents were recorded for 8 min. Neither reagent caused a measurable change in current over this time period. Therefore, neither reagent could modify the C175 residue from the outside solution. Data represent means ± SE for three to four experiments.

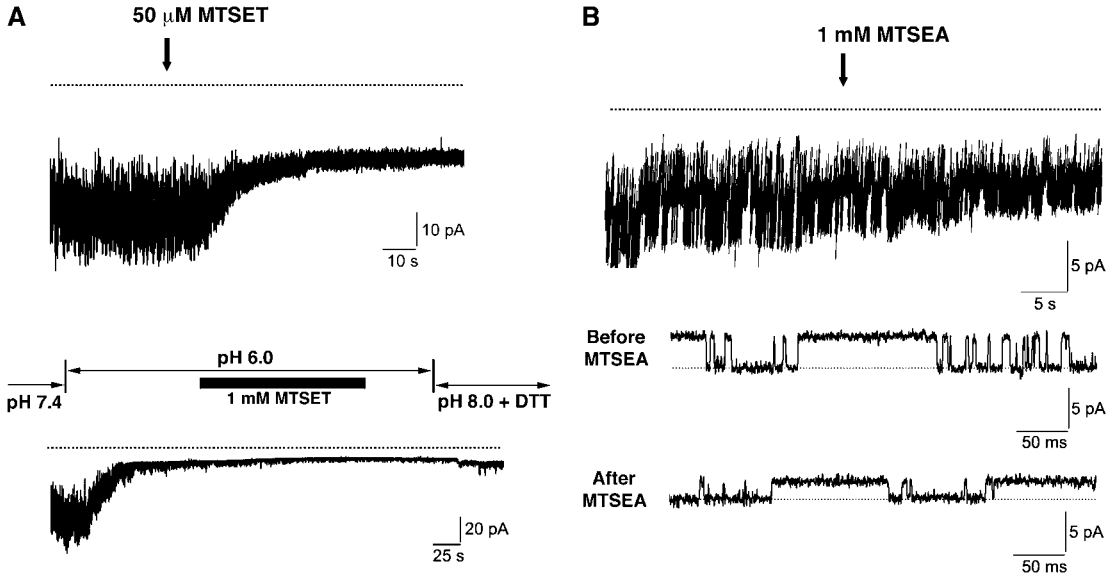

FIGURE 12 Effect of MTS reagents on $ROMK_{YZ}$ G223C channels. (*A*) MTSET (50 μM) modified the 223C position, with a time constant of 11 s, when the channels were in the open state. At a concentration of 1 mM, MTSET also modified the 223C residue in the closed state. The fractional recovery after addition of pH 8.0 plus DTT was 0.33 $\pm$ 0.05. (*B*) MTSEA (1 mM) reduced but did not abolish currents. In a patch with only a single active channel, MTSEA reduced the single-channel conductance of $Kir1.1_{YZ}$ G223C. Traces are representative of three or more experiments. The current after MTSEA was 0.40 $\pm$ 0.05 of the initial value.

filter might play a more important role in the gating process. It is possible that Kir1 and Kir2 channels are gated differently. Alternatively, gating by changing the PIP2 content may involve mechanisms distinct from those involved in pH-dependent gating.

In contrast to results with the transmembrane pore, access to the cytoplasmic pore did not seem to be as strongly affected by channel closure. MTSET, which could not modify a cysteine above the bundle crossing ($ROMK_{YZ}$S175C) in the closed state, was able to modify a cytoplasmic pore cysteine ($ROMK_{YZ}$G223C) in both open and closed states. Although MTSET was able to modify the cysteine at C223 and completely block permeation through the cytoplasmic pore, MTSEA was only able to reduce the single-channel conductance of this mutant. Because both reagents carry the same +1 charge, the difference probably arises from the size of the molecules. The smaller amine groups on MTSEA presumably permit more space for K^+ and water to pass through the cytoplasmic pore than the more bulky trimethylammonium group of MTSET. The reduced conductance in the case of the MTSEA-modified channel may result from the extra positive charges. A similar change in charge at another position of the cytoplasmic pore also reduced the single-channel conductance to a similar extent (23).

The ability of MTS reagents to block the cytoplasmic pore by modifying cysteines at this site is not the same for all Kir channels. Both MTSEA and MTSET completely blocked Kir2.1 conductance when modifying E224C, homologous to Kir1.1a-G223C (24). In contrast, neither of these reagents affected the conductance of the homologous Kir6.2-S212C, although larger MTS compounds did have an effect (25). Results with Kir1.1 were intermediate between these two behaviors, with MTSET producing complete block but MTSEA only a partial block. The simplest interpretation of these data is that the cytoplasmic pore is widest at this point in Kir6.2, intermediate in Kir1.1, and narrowest in Kir2.1. This might relate to the single-channel conductance of the channels, which has the same sequence Kir6.2 > Kir1.1 > Kir2.1. This would be consistent with the idea that conductances in these channels reflect the properties of the cytoplasmic pore as well as those of the selectivity filter (23,26). It is also intriguing that the channels with the two largest cytoplasmic pores are weak rectifiers compared with Kir2.1. It is possible that a narrow pore strengthens interactions between this part of the channel and intracellular blockers such as polyamines.

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