Regulation of ROMK by Extracellular Cations

H. Sackin,* S. Syn,* L. G. Palmer,† H. Choe,† and D. E. Walters‡

Departments of *Physiology and Biophysics and ‡Biochemistry and Molecular Biology, The Chicago Medical School, North Chicago, Illinois 60064, and †Department of Physiology and Biophysics, Weill Medical College of Cornell University, New York, New York 10021 USA

ABSTRACT The effect of external potassium (K) and cesium (Cs) on the inwardly rectifying K channel ROMK2 (K_{ir}1.1b) was studied in *Xenopus* oocytes. Elevating external K from 1 to 10 mM increased whole-cell outward conductance by a factor of 3.4 ± 0.4 in 15 min and by a factor of 5.7 ± 0.9 in 30 min (n = 22). Replacing external Na by Cs blocked inward conductance but increased whole-cell conductance by a factor of 4.5 ± 0.5 over a period of 40 min (n = 15). In addition to this slow increase in conductance, there was also a small, rapid increase in conductance that occurred as soon as ROMK was exposed to external cesium or 10 mM K. This rapid increase could be explained by the observed increase in ROMK single-channel conductance from 6.4 ± 0.8 pS to 11.1 ± 0.8 pS (10 mM K, n = 8) or 11.7 ± 1.2 pS (Cs, n = 8). There was no effect of either 10 mM K or cesium on the high open probability (P_o = 0.97 ± 0.01; n = 12) of ROMK outward currents. In patch-clamp recordings, the number of active channels increased when the K concentration at the outside surface was raised from 1 to 50 mM K. In cell-attached patches, exposure to 50 mM external K produced one or more additional channels in 9/16 patches. No change in channel number was observed in patches continuously exposed to 50 mM external K. Hence, the slow increase in whole-cell conductance is interpreted as activation of pre-existing ROMK channels that had been inactivated by low external K. This type of time-dependent channel activation was not seen with IRK1 (K_{ir}2.1) or in ROMK2 mutants in which any one of 6 residues, F129, Q133, E132, V121, L117, or K61, were replaced by their respective IRK1 homologs. These results are consistent with a model in which ROMK can exist in either an activated mode or an inactivated mode. Within the activated mode, individual channels undergo rapid transitions between open and closed states. High (10 mM) external K or Cs stabilizes the activated mode, and low external K stabilizes the inactivated mode. Mutation of a pH-sensing site (ROMK2-K61) prevents transitions from activated to inactivated modes. This is consistent with a direct effect of external K or Cs on the gating of ROMK by internal pH.

INTRODUCTION

K secretion into the lumen of the renal cortical collecting tubule (CCT) and thick ascending limb (TAL) is largely mediated by the ROMK family of weak inward rectifier K channels (Palmer et al., 1997). At the intracellular level, ROMK is down-regulated by internal acidity (Tsai et al., 1995; McNicholas et al., 1998; Doi et al., 1996), protein kinase C (Wang and Giebisch, 1991), and a Mg-dependent dephosphorylation process (McNicholas et al., 1994). Maintenance of ROMK channel activity requires phosphorylation of at least two of three protein kinase A (PKA) consensus sites, one on the N-terminal and two on the C-terminal domains (Xu et al., 1996; MacGregor et al., 1998).

ROMK is also sensitive to extracellular regulation, most notably regulation by external (luminal) K. Increasing external [K] from 1 to 10 mM increases whole-cell outward conductance over a period of 20–30 min (Doi et al., 1996). Because normal variations in dietary K produce luminal K concentrations in this range (Malnic et al., 1966), a K-dependent apical ROMK conductance may be physiologically relevant for K regulation by the kidney.

Voltage-gated K channels also exhibit a sensitivity to external K, and at least one of the residues responsible is in the pore region at position 11 (see Fig. 1). For example, mutations at location T449 (pore position 11, Fig. 1) in Shaker Kv affect the sensitivity of C-type inactivation to extracellular K, as well as the sensitivity to TEA (Schlief et al., 1996; Pardo et al., 1992; Baukrowitz and Yellen, 1995). Mutations at A428 (pore position 11) in yeast YKC1 (pore position 11) decreased K sensitivity, slowed activation kinetics, and speeded up deactivation (Vergani et al., 1998). In hippocampal RCK4 (Kv1.4) channels, the lysine at pore position 11 (K533) appears to be the crucial residue that confers sensitivity to external K (Pardo et al., 1992).

The external K sensitivity of inward rectifiers such as ROMK may also arise from specific features of the channel protein. Early studies indicated an important role for the ROMK core, defined as the two transmembrane segments and the intervening pore (Doi et al., 1996). Recently, the report that external K sensitivity could be conferred on Kir 7.1 by replacing a Met at pore position 10 (M125) with a positively charged Arg suggests the importance of this position (Döring et al., 1998). The aim of the present study was to investigate how these residues, and others, control the interaction between ROMK and external cations such as K and Cs.

Received for publication 1 March 2000 and in final form 14 November 2000.

Address reprint requests to Dr. Henry Sackin, Department of Physiology and Biophysics, The Chicago Medical School, 3333 Green Bay Road, North Chicago, IL 60064. Tel.: 847-578-8329; Fax: 847-578-3265; E-mail: sackinh@mis.finchcms.edu.

0006-3495/01/02/683/15 $2.00

MATERIALS AND METHODS

Construction of chimeras and point mutants of ROMK

Chimeras were constructed using the splicing by overlap extension method (Horton et al., 1989) as described in Choe et al. (1999), using parts of either ROMK2 (GenBank accession L29403) or IRK1 (GenBank accession

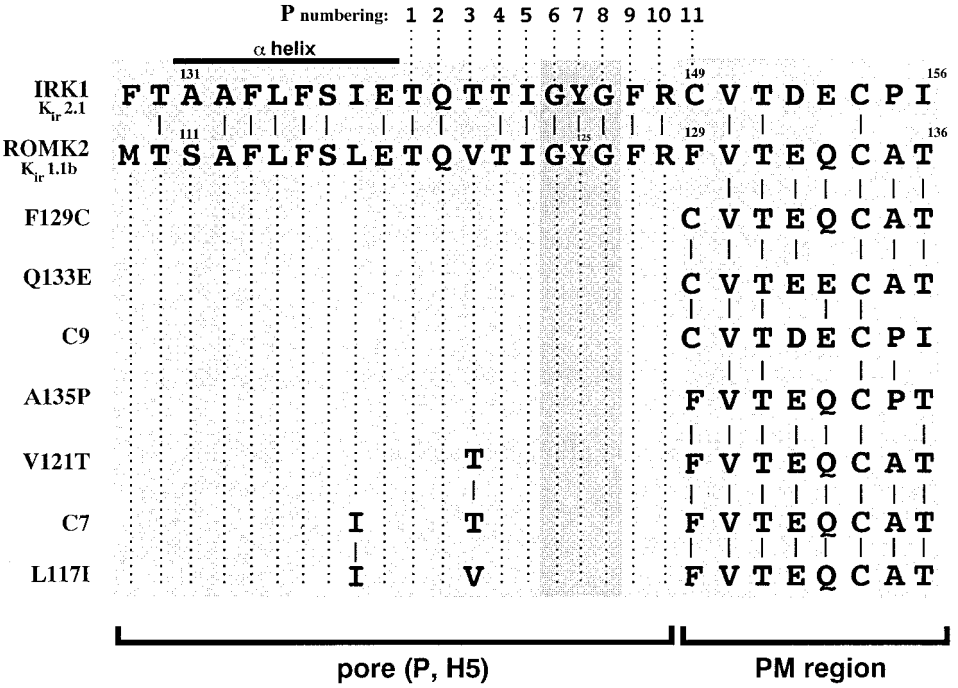

FIGURE 1 Sequence alignment in the pore (P) and PM regions of the inward rectifiers ROMK2, IRK1, C7, and C9 and the ROMK2 point mutants F129C, Q133E, A135P, V121T, and L117I. Solid vertical bars connect identical residues. Dotted lines indicate the same residue at that position. The location numbers refer to positions in ROMK2 (ROMK1 position = ROMK2 + 19). The P numbers refer to positions relative to the conserved Thr (P1) at the end of the pore helix.

X73052). Point mutations in both ROMK2 and the C9 chimera were engineered with a polymerase chain reaction QuikChange site-directed mutagenesis kit, where primers were synthesized by Integrated DNA Technologies (Coralville, IA). Nucleotide sequences between two restriction enzyme sites were checked using an ABI Prism 377XL automated DNA sequencer at The University of Chicago Cancer Research Center (Chicago, IL).

Expression of channels

Plasmids were linearized with *Not*I restriction enzyme and transcribed in vitro with T7 RNA polymerase in the presence of the GpppG cap using mMESSAGE mMACHINE kit (Ambion, Austin, TX). Synthetic cRNA was dissolved in water and stored at −70°C before use. Stage V-VI oocytes were obtained by partial ovariectomy of female *Xenopus laevis* (NASCO, Ft. Atkinson, WI) anesthetized with tricaine methanesulfonate (1.5 g/L, adjusted to pH 7.0). Oocytes were defolliculated by incubation in OR2 solution (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, adjusted to pH 7.5 with NaOH) containing 2 mg/ml collagenase type II and 2 mg/ml hyaluronidase type II (Sigma Chemical Co., St. Louis, MO) for 90 min and (if necessary) another 90 min in a fresh enzyme solution at 23°C. Oocytes were injected with 0.5–1 ng of cRNA and incubated at 19°C in 2X diluted Leibovitz medium (Life Technologies, Grand Island, NY) for 1–4 days before measurements were made.

Electrophysiology

All experiments described were conducted at room temperature (21 ± 2°C) on ROMK2 (or mutants/chimeras of ROMK2) expressed in *Xenopus* oocytes.

Whole-cell experiments

Oocytes were initially bathed in a solution containing (in mM): 1 KCl, 109 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, and 5 HEPES at pH 7.4. External K was elevated to 10 mM by equimolar replacement of 9 mM Na by K. Activation by external Cs was accomplished by equimolar replacement of 109 mM Na by Cs, at a constant K concentration of 1 mM. The osmolarity of all experimental solutions ranged between 230 and 270 mosmol.

Currents were recorded with the two-electrode voltage clamp (TEVC), and conductances refer to total whole-cell conductance (G) = gNP_o, where g = single-channel conductance, N = total number of channels expressed, and P_o = channel open probability.

Whole-cell conductance was measured by recording the current during application of six or seven command voltages of 50-ms duration centered around the resting potential of the oocyte. Between each cycle of command voltages, the oocyte was left unclamped in the open-circuit (zero-current) condition. Except for the protocol used to generate the data of Fig. 3 (in which the effects of outward current were specifically examined), net membrane current was zero during the interval between conductance measurements.

Because the whole-cell I-V relations were approximately linear at small outward currents (see Fig. 2), outward conductances were tabulated at an oocyte potential of V_m = 25 mV + E_{rest}, where E_{rest} is the resting (zero-current) potential of the oocyte. These conductances were then normalized to the initial conductance in 1 mM external K and plotted as a function of time following elevation of external K from 1 to 10 mM or during replacement of Na by Cs at constant external K. At the termination of the experiment, leak conductance was determined by a 10-min exposure to either 1 or 5 mM Ba solutions, containing 1 mM K.

Delays attributable to diffusion and unstirred layers around the oocyte were estimated from the time course of the change in resting potential following a change in bath solution. Elevation of bath K from 1 to 10 mM (or replacement of Na by Cs) depolarized the resting potential of the oocyte by approximately 30 mV in less than 1 min. Return of the bath solution to 1 mM K (or exchange of Cs by Na) repolarized the membrane with approximately the same time course. The similarity and reproducibility of these resting potential changes implies that alterations in bath K must have reached at least 90% of the oocyte membrane within 1 min after the solution change.

Single-channel experiments

Patch-clamp pipettes were pulled from PG165T-7.5 borosilicate glass (Warner Instruments Corp., Hamden, CT) using a two-stage process (L/

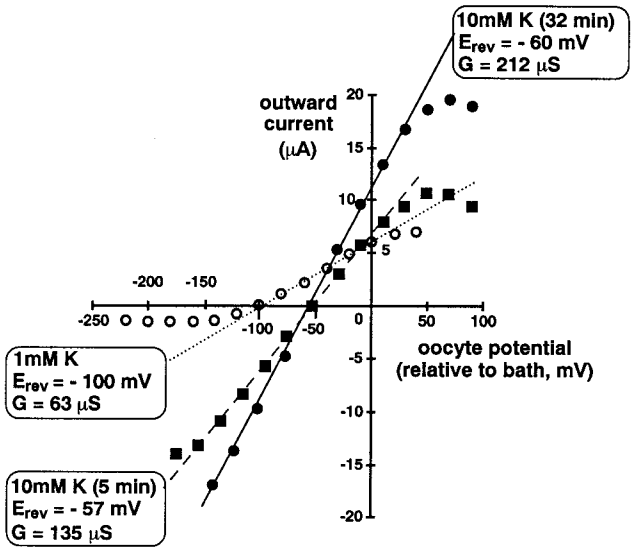

FIGURE 2 Raising external K increases ROMK whole-cell outward conductance. Representative whole-cell current voltage relations were measured at three times in the protocol: 1) 1mM K bath (○, · · ·); 2) 5 min after increasing bath K from 1 to 10 mM (■, - - -); and 3) 32 min after the increase in bath K to 10 mM (●, - - -). E_{rev} is the reversal potential for each condition, and G denotes the outward conductance for each of the three conditions.

M-3P-A puller) and coated with Sylgard (Dow Corning, Midland, MI). In all oocytes used for patch clamping, the vitelline membrane was first removed with forceps following a brief exposure to hypertonic (450 mosmol/L) solution. Patch-clamp currents were recorded in the cell-attached mode using pipettes filled with one of the three external bath solutions (all containing 2 mM $CaCl_2$, 1 mM $MgCl_2$, buffered with 5 mM HEPES to pH 7.4): 1) 1 mM KCl, 109 mM NaCl; 2) 10 mM KCl, 100 mM NaCl; or 3) 1 mM KCl, 109 mM CsCl.

To facilitate resolution of outward K currents, oocytes were bathed in 100 mM KCl, 10 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and buffered with 5 mM HEPES to pH 7.4. This depolarized the oocyte membrane potential to an average of -4 ± 1 mV ($n = 37$), as measured in the TEVC.

Pipette resistances ranged from 5 to 10 MΩ. Currents were recorded with a Dagan 8900 patch-clamp amplifier and stored, unfiltered, on videotape. For off-line analysis, current records were replayed from videotape, filtered at 300 Hz, and sampled at 5 kHz, using an Atari-based data acquisition system (Instrutech, Port Washington, NY). Current-voltage relations were constructed from clear channel currents at different holding potentials, where the voltage across the patch (inside minus outside) was defined as the cell membrane potential minus the pipette holding potential (relative to the bath). Single-channel conductances were obtained from the *I-V* relations for each experiment using a least-squares fit of the data in the linear region of outward currents. In patches having only a single channel, the open probability was calculated from channel dwell times using the TAC analysis program (Instrutech).

All statistical comparisons were conducted using Statview 5.05 software.

In experiments designed to look directly at the appearance of single channels following exposure to elevated external K, oocytes were preincubated in either 1 or 50 mM K for at least 40 min. In both cases the patch pipette contained 50 mM KCl. The time (in minutes) until channel appearance was measured from the moment of seal formation. For the 1 mM K condition, seal formation represents the first exposure of the patch to elevated K.

Variable expression rates with different oocyte populations made it technically difficult to interpret initially quiescent patches. Hence, patches that were completely without channel activity were excluded from the sample. Patches initially containing more than four active channels were also excluded because it was difficult to reliably determine changes in channel number with more than four channels in the patch.

Active channels were counted using inward currents produced by positive pipette potentials. Use of inward (rather than outward) currents eliminated much of the ambiguity in counting active channels because the P_o of ROMK is significantly less than one for inward currents (Chepilko et al., 1995), a condition necessary to observe the state where all channels in a multi-channel patch are closed.

RESULTS

Following the initial observation by Doi et al. (1996), ROMK2 currents expressed in *Xenopus* oocytes, preincubated in 1 mM external K for >30 min, exhibited a low whole-cell outward conductance that increased upon exposure to either 10 mM external K or 109 mM external cesium. The basic observation is illustrated by the whole-cell *I-V* relations of Fig. 2, obtained with the two-electrode voltage clamp.

The open circles of Fig. 2 depict the *I-V* relation in 1 mM external K. The average resting potential under these conditions was -101 ± 2 ($n = 19$) mV, consistent with an internal K of at least 70 mM. With 1 mM K in the bath, outward conductance was linear for cell potentials between −100 and 0 mV. Inward conductance was small and indistinguishable from the conductance of uninjected oocytes.

Subsequent elevation of bath K from 1 to 10 mM increased both inward and outward whole-cell conductance and linearized the whole-cell *I-V* relation between −150 and +50 mV. A typical *I-V* relation after 5 min of elevated K is illustrated by the solid squares of Fig. 2. Macroscopic linearity of ROMK *I-V* relations is a consequence of single-channel rectification being balanced by a slight voltage dependence of P_o (Chepilko et al., 1995). Over the next 30 min both outward and inward ROMK whole-cell conductance progressively increased (solid circles, Fig. 2) until a maximum conductance was achieved after ~40 min. A complete time course for the response to bath K elevations is shown in Fig. 3. Currents and conductances were measured by stepping (for 50 ms) the command voltage in 20-mV increments above and below the resting potential as described in Materials and Methods. Whole-cell outward conductance was measured at a membrane potential approximately +25 mV more positive than the resting potential, which averaged -68 ± 2 ($n = 19$) mV in 10 mM K. Values were then normalized to the outward conductance in 1 mM K, measured at the same potential. The ROMK whole-cell conductance increased steadily over a period of 20 min and reached a plateau 30–40 min after the increase in K (solid line, Fig. 3).

Return of the bath K from 10 to 1 mM reversed the increase in outward conductance. However, the time course of the conductance reversal depended on current flow through the channel (Fig. 3). In these experiments, channels

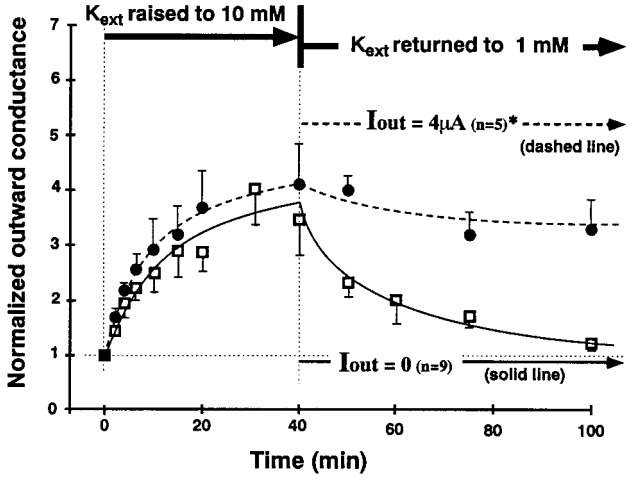

FIGURE 3 Effect of outward current on stabilization of ROMK open state during return to low external K. Channels, initially activated by 10 mM external K, were returned to a 1 mM bath K under conditions of either 1) no outward current (□, ——) or 2) maintained outward current produced by a 40-mV outward driving force (●, - - -). Outward current for this latter case averaged 4 μA and was produced by a net driving force: $V - E_K = -60 - (-100) = +40$ mV. Whole-cell outward conductances were all measured at a clamped membrane potential of −40 mV, using 50-ms pulses. Momentary driving force = +20 mV for 10 K condition and +60 mV for the 1 K condition.

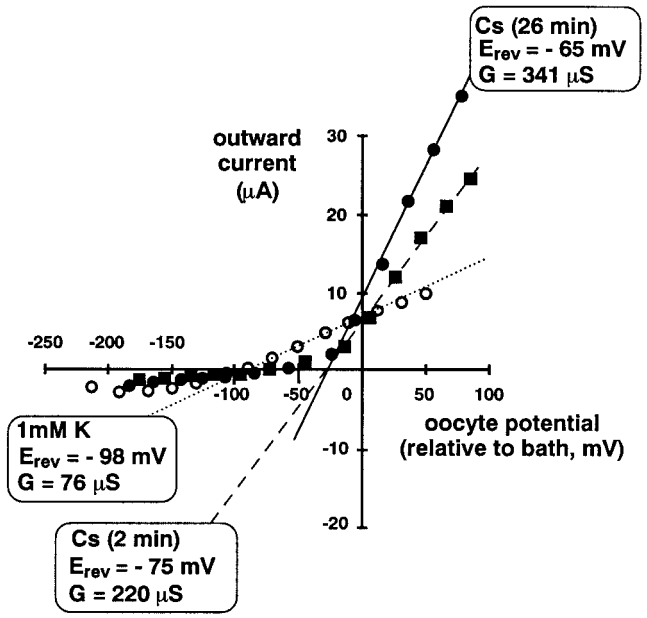

FIGURE 4 External Cs increases ROMK whole-cell outward conductance. Representative whole-cell current voltage relations were measured at three times in the protocol: 1) 1mM K bath (○, ···); 2) 2 min after replacing 109 mM Na by Cs at constant 1 mM K (■, - - -);, and 3) 26 min after replacing Cs by Na (●, - - -). E_{rev} is the reversal potential for each condition, and G denotes the outward conductance for each of the three conditions.

were first activated by 10 mM external K for 40 min, at which time the bath was returned to 1 mM K under one of two conditions: 1) no outward current, except for brief 50-ms pulses to measure conductance every 10 or 20 min (Fig. 3, open squares, solid line), or 2) sustained outward current (solid circles, dashed line). In the latter condition, outward current was maintained by clamping the membrane potential more positive than E_K. In this case (solid circles), outward conductance declined much more slowly and still had not returned to initial levels after more than an hour. This suggests that outward current stabilizes the open state of the channel.

Part of the increase in conductance following exposure to 10 mM external K could be explained by small changes in single-channel conductance (see below). However, this cannot account for the large, slow increase in conductance that occurs over a 40-min exposure to 10 mM K.

The effect of external Cs on outward conductance was also examined (Fig. 4). Equimolar replacement of 109 mM external Na by Cs progressively increased ROMK outward conductance over a 30-min period, analogous to what was observed with 10 mM external K (Fig. 2). The dashed line (solid squares, Fig. 4) represents the outward slope conductance after 2 min, and the solid line (closed circles) the outward conductance after 26 min. External Cs produces a clear increase in outward conductance that increases over a period of ~30–40 min. In contrast to the 10 K condition (Fig. 2) the *I-V* curves with Cs display a marked curvature, intersecting the voltage axis at an average of −67 ± 4 mV (n = 15). This is a consequence of block of inward K current by external Cs (solid squares and circles, Fig. 4). Outward currents at $V < -10$ are also reduced by external Cs, but those at positive voltages are clearly enhanced.

The Cs-induced increase in ROMK outward conductance did not appear to be related to replacement of Na by Cs. When Cs addition was accompanied by sucrose removal rather than Na replacement, there was only a slight difference in the time course of the increase in slow conductance (Fig. 5). Although Na is known to block some K channels, relief of Na block can account for no more than a small percentage of the increase in ROMK conductance. However, the magnitude and time course of the effect of Cs on outward ROMK conductance did depend on the external concentration of Cs (Fig. 6). With only 10 mM Cs in the bath, the half-time of the response was significantly increased, and the maximal conductance increase was attenuated. This suggests that the concentration of Cs at the outer mouth of the channel is a limiting factor for stimulation of outward K conductance.

To assess the effects of raising K on single-channel properties, patch-clamp experiments were carried out with either 1 mM K (Fig. 7) or 10 mM K (Fig. 8) in the patch pipette. With 1 mM external K, outward ROMK currents have a high open probability (P_o = 0.97 ± 0.01; n = 12) and a low single-channel conductance (g = 6.4 ± 0.8 pS; n = 16). There was no change in the P_o for outward currents

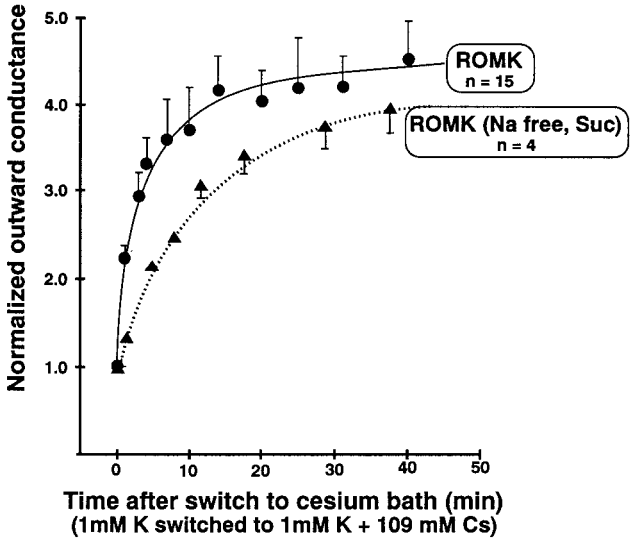

FIGURE 5 Effect of sucrose versus sodium removal on Cs stimulation of outward conductance. During addition of 109 mM Cs at time 0, osmotic equivalents of either Na (●) or sucrose (▲) were removed. The initial conductances in 1 mM K were 102 ± 33 μS for the Na replacement experiments or 35 ± 2 μS for the sucrose replacement experiments. Conductance was measured at net outward current, with the cell potential clamped at 25 mV more positive than the resting potential. Data were normalized to the conductance in 1 mM K, measured at the same potential.

when the pipette (external) solution was changed to either 10 mM K or 109 mM Cs (Figs. 8 and 9). However, single-channel conductances in 10 mM K and Cs external solutions

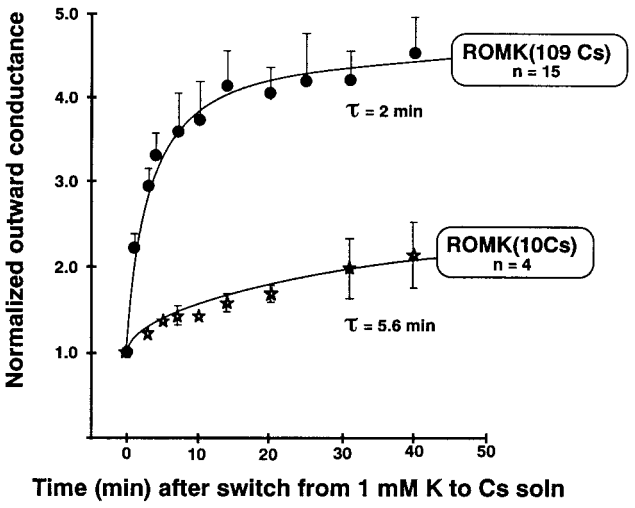

FIGURE 6 Effect of external Cs concentration on stimulation of outward conductance. Time course of whole-cell conductance following introduction of either 109 mM Cs (●) or 10 mM Cs (★). The initial conductances in 1 mM K were 102 ± 33 μS for the 109 mM Cs experiments and 260 ± 50 μS for the 10 mM Cs experiments. Conductance was measured at net outward current, with the cell potential clamped at 25 mV more positive than the resting potential. Data were normalized to the conductance in 1 mM K, measured at the same potential. τ denotes time to half-maximal response.

were slightly, but significantly, higher than in 1 mM K solutions (Table 1). This effect on single-channel conductance was significantly smaller than the increase in whole-cell conductance that occurred over a 40-min period after external K or Cs was raised. This suggests that the latter phenomenon involves increases in the number of conducting K channels.

Cell-attached patches were also used to determine whether high external K increased the number of individual channels. Oocytes were preincubated in 1 mM K solutions, similar to the two-electrode experiments. In nine experiments, formation of a gigaohm seal with a patch pipette containing 50 mM K solution resulted in the appearance of additional channels with characteristics similar to those present from the beginning of the recording. The average time for the appearance of the first additional channel was 4.5 ± 1 min.

A representative result is shown in Fig. 10. At the start of the recording, the patch contained a single ROMK channel that is readily identified by its characteristic conductance and kinetics. At approximately 4 min after seal formation, there was an abrupt transition to a second level. These two channels remained active for the duration of the recording (17 min in this case). In three of nine patches, channel activation was preceded by a brief burst of activity to the higher level (see Fig. 10).

In seven experiments, the number of active channels remained unchanged following exposure to 50 mM K in the pipette (Table 2). In addition, seven control experiments were conducted in which seals were formed on oocytes incubated for at least 30 min in 50 mM K instead of 1 mM K. In these experiments there was no increase in the number of active channels in the patch, suggesting that channel activation requires a period of low K exposure (Table 2).

The effect of external K on channel activation was structure dependent. The strong inward rectifier IRK1 (Kir2.1) is homologous to ROMK but shows an entirely different response to external K. IRK1 whole-cell currents exhibited strong rectification in either 1 or 10 mM external K (Fig. 11). Furthermore, IRK1 outward conductance (near its reversal potential) was less affected than ROMK by external K concentration, and the effect was complete within 5 min (Fig. 11). The dramatic difference in the response of these two homologous channels to external K suggests that external cation sensitivity depends on one or more of the amino acids that are different in ROMK and IRK. We therefore decided to investigate which of the differences, particularly those in the extracellular portion of the proteins, might be responsible. Alignment of ROMK2 and IRK1 (Fig. 1) from the pore (P) to the second transmembrane segment (encompassing the P and PM regions) suggests a number of possible sites for mutagenesis.

First, the entire PM region of ROMK2 was replaced by its IRK1 homolog (chimera C9). This abolished the slow increase in whole-cell conductance seen with wild-type

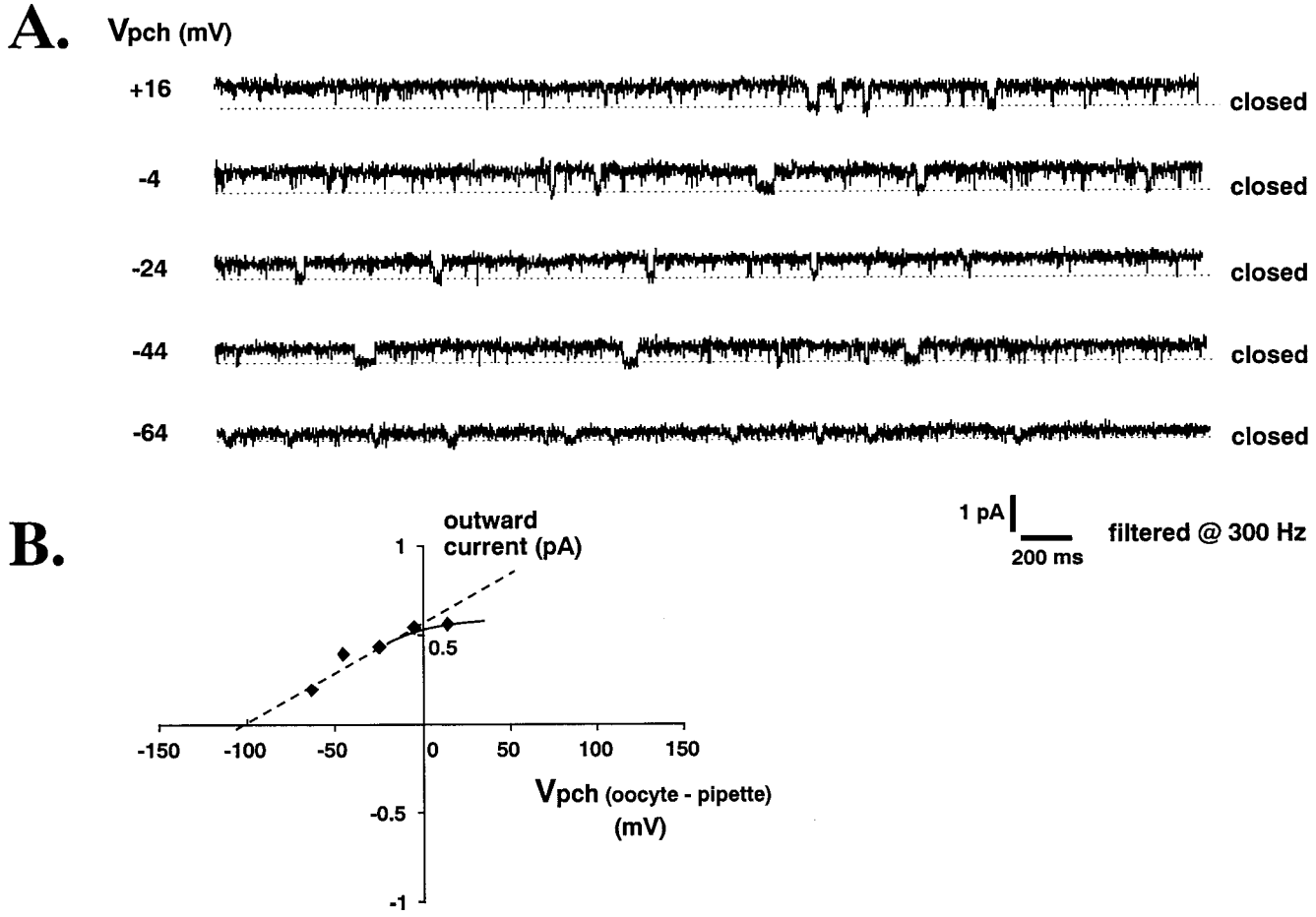

FIGURE 7 Outward ROMK2 single-channel currents with 1 mM external (pipette) K. (*A*) Current records at different holding potentials from a cell-attached patch on a *Xenopus* oocyte. Bath contained 100 mM KCl, which depolarized the oocyte membrane potential to an average of −4 ± 1 mV. V_{pch} is the potential difference across the patch of membrane (cytoplasmic minus external). (*B*) Current-voltage relation for the currents in *A*. Single-channel conductance (5.6 pS) was determined by a linear fit to the nonrectifying portion of the current-voltage relation. The extrapolated reversal potential was −103 mV. This is in good agreement with the average resting potential of the macroscopic (TEVC) measurements of −102 ± 2 mV.

ROMK (Fig. 12). However, the C9 chimera still displayed a small, rapid conductance increase that could be explained by a rapid effect of 10 mM K on single-channel conductance (Fig. 8; Table 1).

Addition of external Cs (Fig. 13) produced effects similar to addition of 10 mM K (Fig. 12). In contrast to the wild-type ROMK, external Cs produced only an abrupt increase in the outward conductance of C9, which then remained constant after the first 4 min (open squares, Fig. 13).

We then asked which of the residues within the PM region were most critical. In the sequence alignment of Fig. 1, the C9 chimera differs from ROMK2 by only five residues in the PM region. Replacement of the F129 residue of ROMK2 (148 in ROMK1) with Cys, the equivalent amino acid in IRK1, effectively eliminated the slow increase of outward conductance seen with wild-type ROMK, leaving only the initial, rapid jump in conductance (stars, dashed line, Fig. 13). The time course of F129C outward conductance was similar to that of C9, except that the initial increase in conductance was slightly larger.

Not all residues in the PM region were essential for the slow increase in outward whole-cell conductance. Replacing the two residues at the (C-terminal) end of the PM region (A135 or T136) by their IRK1 homologs did not affect the slow increase in outward conductance. The results of one of these mutations, ROMK2-A135P, is shown in Fig. 14. On the other hand, replacing the Gln residue at (ROMK2) position 133 by glutamic acid completely abolished the slow increase in conductance (solid squares, Fig. 14). The point mutation E132D produced an effect similar to Q133E. Hence residues at the start (N-terminal end) of the PM region seem more essential for sensing external K.

In addition to residues in the PM region (Fig. 1), two residues that differ in the ROMK-IRK pore region (L117 and V121) also affected the slow increase in outward conductance. As indicated in Fig. 15, replacing either L117 or

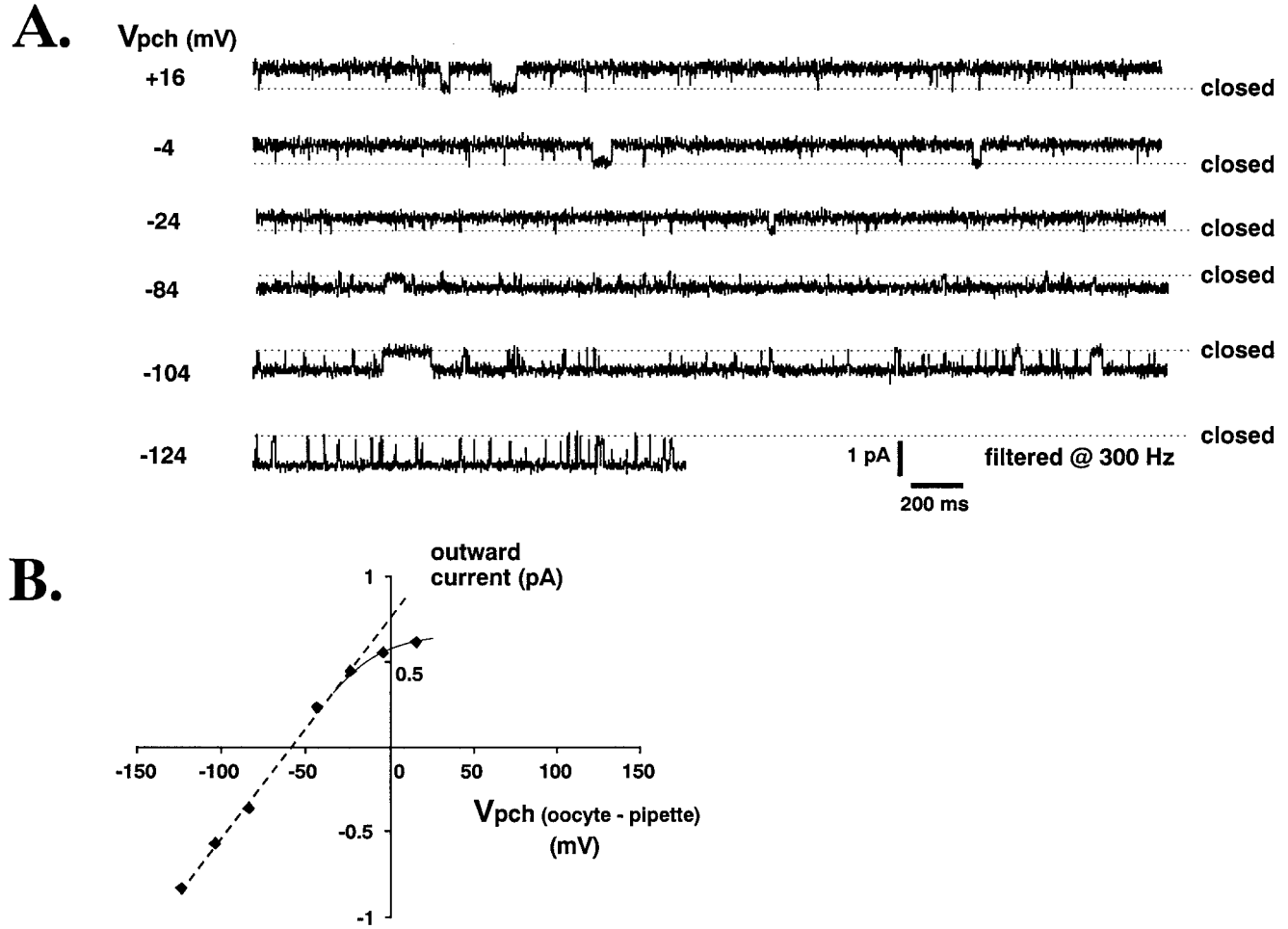

FIGURE 8 ROMK2 single-channel currents with 10 mM external (pipette) K. (*A*) Current records at different holding potentials from a cell-attached patch on a *Xenopus* oocyte. Bath contained 100 mM KCl, which depolarized the oocyte membrane potential to an average of -4 ± 1 mV. V_{pch} is the potential difference across the patch of membrane (cytoplasmic minus external). (*B*) Current-voltage relation for the currents in *A*. For this experiment, the single-channel conductance was 12.9 pS, as determined by a linear fit to the nonrectifying portion of the current-voltage relation. Reversal potential $= -59$ mV, which can be compared with the average resting potential of the whole oocyte in 10 mM K (TEVC measurement) of -68 ± 2 mV.

V121 in ROMK2 with its IRK1 homolog eliminated the slow increase in conductance produced by external Cs, although the rapid response was unaffected. Replacing both residues with their corresponding IRK1 homologs (chimera C7) produced a response that was not significantly different from either mutation alone (Fig. 15). Hence, the effect of the two point mutations is not additive. Either mutation abolishes the slow, cation-induced increase in conductance, but neither eliminates the rapid stimulation, which presumably arises from an immediate change in single-channel conductance.

It is not surprising that the point mutation V121T alters the sensitivity of ROMK to external K because this mutation was previously found to enhance the sensitivity of ROMK to external Ba and Cs (Zhou et al., 1996), increase single-channel conductance, and alter ion selectivity (Choe et al. 2000). The additional finding that the L117I mutation also eliminated the slow increase in outward conductance was unexpected as the L117I mutant did not have an increased affinity for external Ba (Zhou et al., 1996) or an altered single-channel conductance (Choe et al., 2000). The importance of the V121 and L117 residues for sensing external K was also reported by U. Schulte and colleagues (American Physiological Society conference, Snowmass, CO, 1999, and personal communication).

The K61 residue of ROMK2 (K80 in ROMK1) is known to function as a pH sensor (Doi et al., 1996; Choe et al., 1997). Replacing K61 by its IRK1 homologue (Met) abolished the slow increase in outward conductance (Fig. 16). This reinforces the link between internal pH and external K, as originally suggested by Doi et al. (1996).

The relationship between the K-induced slow increase in outward conductance and the pH sensitivity of the channel was further explored by comparing the pH sensitivity of ROMK and the C9 chimera (Fig. 17). In previous studies, acetate-buffered bath solutions were used to control oocyte

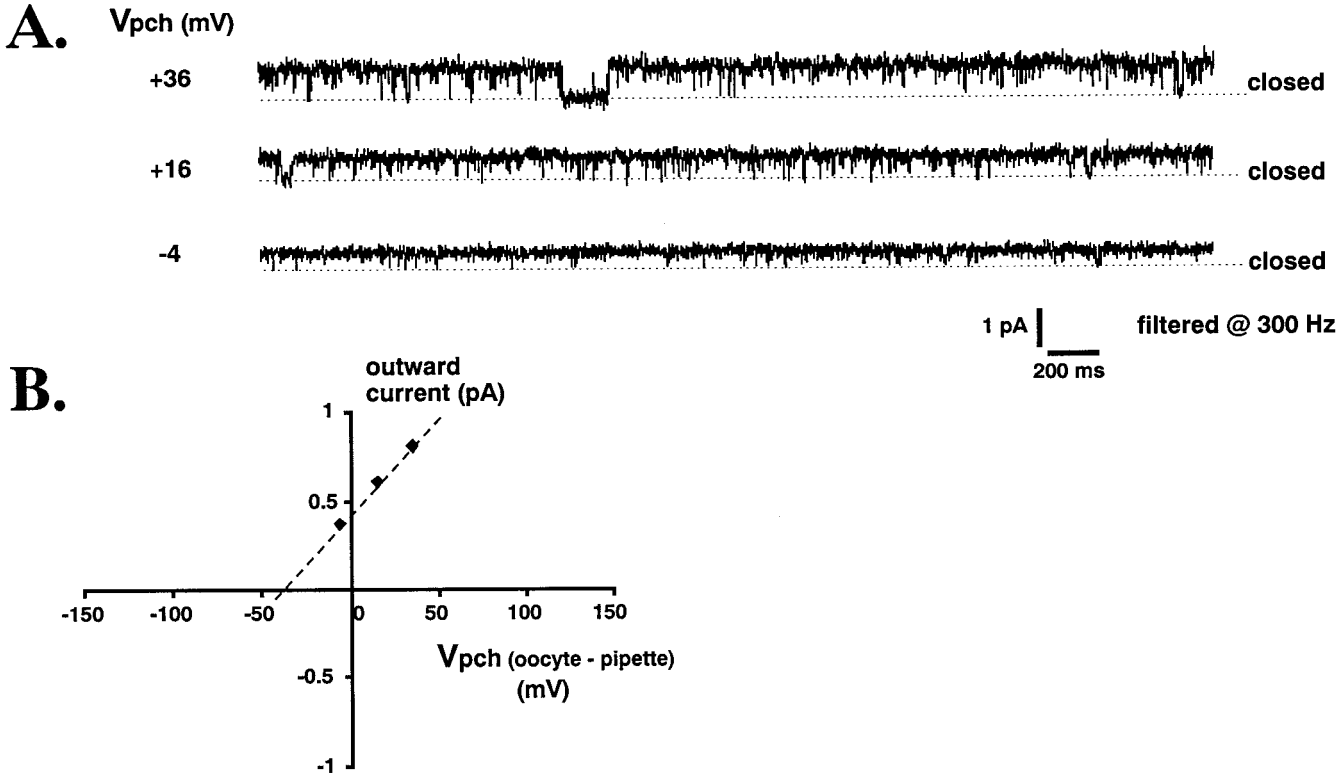

FIGURE 9 Outward ROMK2 single-channel currents with 109 mM external (pipette) Cs. (*A*) Current records at different holding potentials from a cell-attached patch on a *Xenopus* oocyte. Bath contained 100 mM KCl, which depolarized the oocyte membrane potential to an average of -4 ± 1 mV. V_{pch} is the potential difference across the patch of membrane (cytoplasmic minus external). (*B*) Current-voltage relation for the currents in *A*. For this experiment, the single-channel conductance was 11.1 pS, as determined by a linear fit to the current-voltage relation. The linear extrapolated reversal potential was -40 mV. This is significantly less than the average resting potential of the macroscopic (TEVC) measurements of -66 ± 4 mV.

pH via permeation of uncharged acetic acid, although this often required as long as 20 min to achieve a stable intracellular pH (Choe et al., 1997). With acetate solutions, an external pH of 7.4 corresponded to a normal internal pH of 7.32 (Leipziger et al., 2000). In contrast, use of an impermeant buffer had no effect on internal pH and confirmed that external pH does not affect ROMK conductance (Tsai et al., 1995; Choe et al., 1997)

Results of these pH experiments are summarized in Fig. 17. When the external bath contains at least 10 mM K, both

TABLE 1 Comparison of single-channel and whole-cell data at zero membrane potential

External solution	Single-channel, steady state			Whole-cell			
				2 min after solution change		Steady state	
	P_o	g(pS)	i(pA)	G (μS)	I (μA)	G (μS)	I (μA)
1 mM K	0.97 ± 0.01	6.4 ± 0.8	0.6 ± 0.05			94 ± 13	9.5 ± 1
10 mM K	0.98 ± 0.01	11.1 ± 0.8*	0.7 ± 0.06	115 ± 16†	8.8 ± 1†	373 ± 43†‡	20.3 ± 3†‡
Cs	0.97 ± 0.01	11.7 ± 1.2*	0.4 ± 0.03*	170 ± 21†	5.5 ± 1†	380 ± 74†‡	9.8 ± 2‡

All measurements were obtained from either patches or whole oocytes expressing ROMK2.

P_o denotes ROMK2 single-channel open probability; g denotes single-channel outward conductance; i denotes single-channel current at zero patch potential; G denotes whole-cell (macroscopic) outward conductance at zero membrane potential; and I denotes whole-oocyte current at zero membrane potential.

For single-channel data, $n = 8$ for each group.

*Significantly different from corresponding value in 1 mM K external solution (unpaired data; $p < 0.005$).

For whole-cell data, the 10 mM K and Cs conditions both had 20 measurements each. In all cases currents were first measured in 1 mM K, permitting paired comparisons. The data for 1 mM K includes all 40 measurements.

†Significantly different ($p < 0.005$) from paired 1 mM K value.

‡Significantly different ($p < 0.005$) from paired value under the same condition 2 min after the solution change.

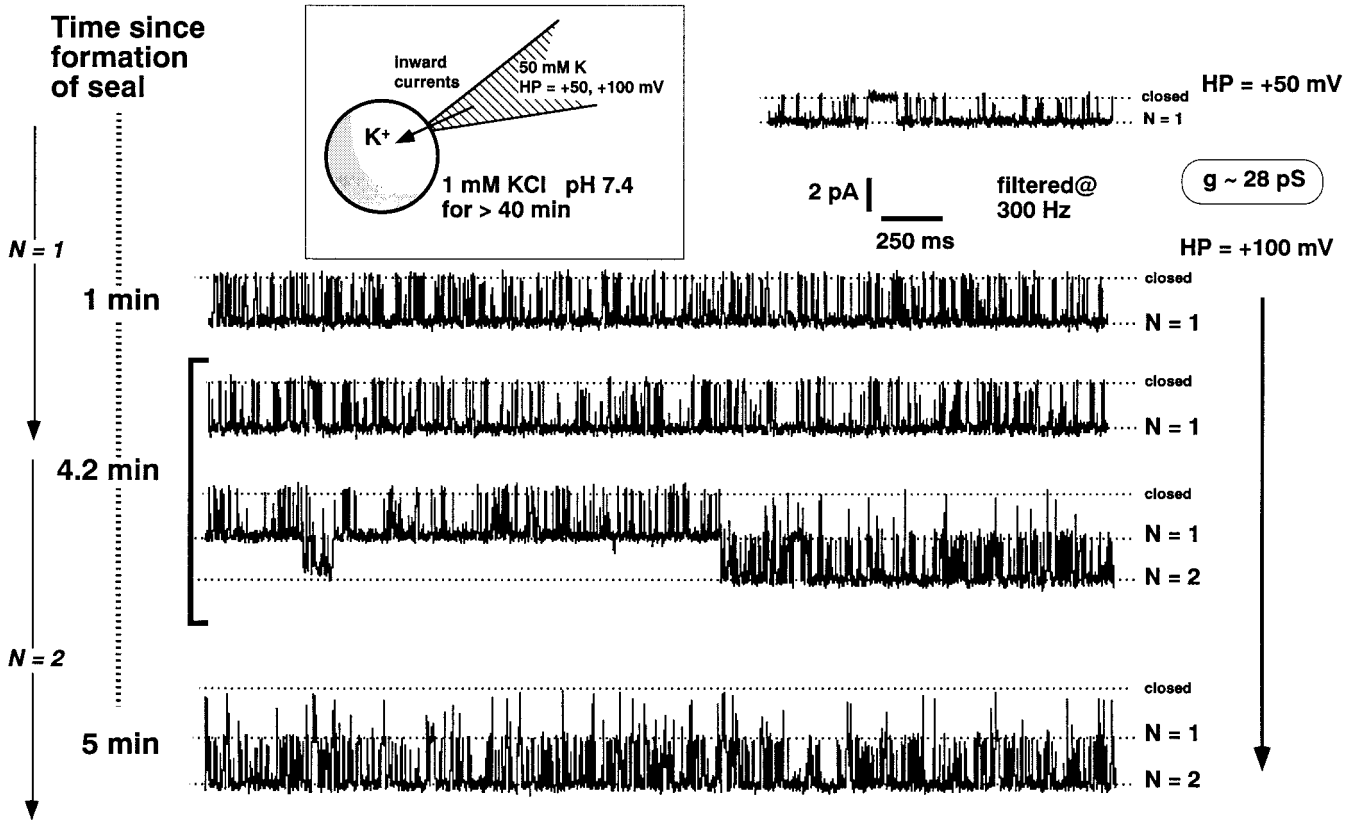

FIGURE 10 One of nine patches in which exposure to elevated external K increased the number of active channels. In this cell-attached recording, a ROMK2 oocyte was pre-incubated in 1 mM K for 40 min before patch-clamping with a pipette containing 50 mM KCl. Time was measured from the moment of seal formation, which in these experiments represents the first exposure of the patch to elevated external K.

ROMK and the C9 chimera exhibit a steep sensitivity to internal pH over the range of 6.7–7.1. However, reduction of external [K] to 1 mM shifts the apparent pKa of ROMK to more alkaline values ($n = 4$). The exact pKa of ROMK2 in 1 mM K could not be determined because salts of permeant weak acids were able to acidify, but not alkalinize, the oocyte (Choe et al., 1997).

Reduction of external [K] produced a much smaller alkaline shift in apparent pKa for C9 than for ROMK (Fig. 17). At normal oocyte pH (7.4 on the abscissa of Fig. 17), C9 channels are activated at both 1 and 10 mM K. Hence, raising external K at pH 7.4 would produce only a small increase in conductance due to channel activation. The remainder of the conductance increase reported in Fig. 12 for C9 can be attributed to an increase in single-channel conductance (Figs. 7 and 8).

In contrast, ROMK channels are mostly inactivated in 1 mM K (open squares, dotted line of Fig. 17), so that raising external K at normal oocyte pH would significantly increase whole-cell conductance by shifting the ROMK operating point from the dotted line to the dashed line (Fig. 17).

TABLE 2 Appearance of single channels in patches exposed to high external K

Bath solution	Initial N	Fraction of patches showing an increase in N	Δt (min) for N to $N + 1$
1 mM K	2.3 ± 0.3	9/16	4.5 ± 1.1
50 mM K	1.9 ± 0.4	0/7	

Oocytes expressing ROMK2 were exposed to bath solutions of either 1 or 50 mM K for at least 45 min before patching. In all cases the pipette solution contained 50 mM K, and patch currents were recorded for between 30 and 40 min. N denotes the number of active channels in the patch; Δt denotes the time after seal formation when one additional channel appeared in the patch (transition N to $N + 1$). Only patches that initially contained between one and four channels were included in the table.

DISCUSSION

Elevation of external K or replacement of Na by Cs produced a rapid and then a slow increase in the outward K conductance of the weak inward rectifier, ROMK (Figs. 12 and 13). A priori, any increase in whole-cell outward conductance (G) could be caused by increases in single-channel conductance (g), number of channels (N), or the open probability of existing channels (P_o).

The rapid phase of the whole-cell conductance increase is consistent with the small increment in single-channel con-

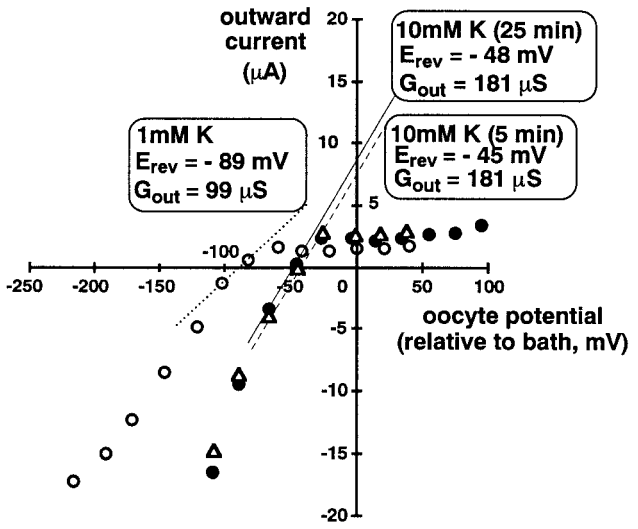

FIGURE 11 Effect of external K on IRK1 whole-cell outward conductance. Representative whole-cell current voltage relations measured in 1mM external K (○, - - -) and at 5 min (△, – – –) and 25 min (●, ——) after a change in bath solution to 10 mM K. Outward conductance (G_{out}), measured near the reversal potential (E_{rev}), showed no significant time dependence in 10 mM external K.

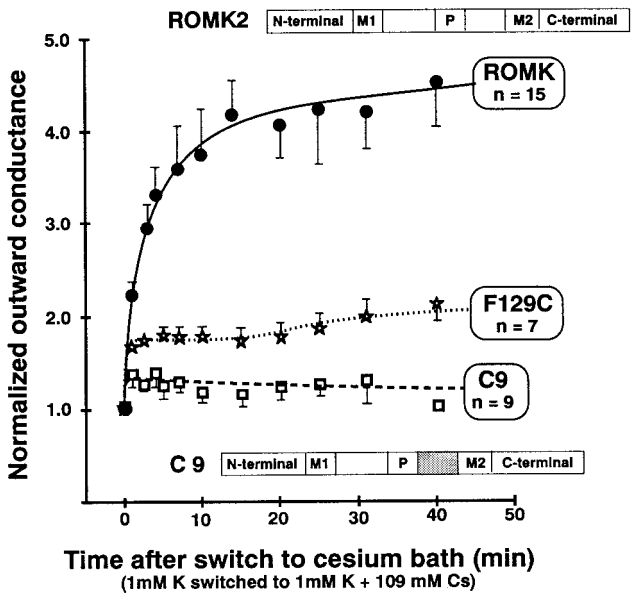

FIGURE 13 Importance of the F129 (11P) residue for external Cs-induced stimulation of outward conductance. Shown is the time course of whole-cell conductance following replacement of external Na by Cs. Wild type and mutants are defined in Fig. 1. The initial conductances in 1 mM K were as follows: ROMK2, 102 ± 33 μS; F129C, 113 ± 22 μS; C9, 144 ± 23 μS. Conductance was measured at net outward current, with the cell potential clamped at 25 mV more positive than the resting potential. Data were normalized to the conductance in 1 mM K, measured at the same potential.

ductance that was observed in both ROMK and all of the mutants tested. Table 1 summarizes the single-channel and whole-cell data for ROMK2. The mean single-channel ROMK2 conductance in 1 mM K (6.4 ± 0.8 pS; $n = 8$) is close to the value of outward single-channel conductance (8.7 ± 2.4 pS) for the native SK channel in rat CCT (Frindt and Palmer, 1989). This is consistent with the presumption that the CCT secretory K channel (SK) and the cloned ROMK2 are one and the same (Palmer et al., 1997).

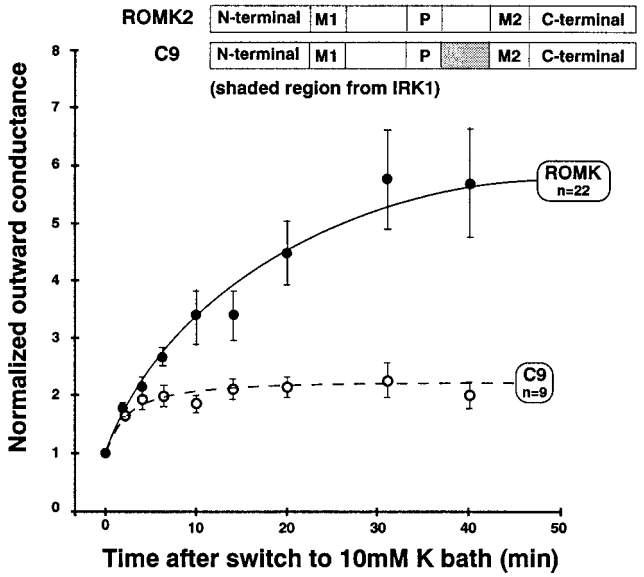

FIGURE 12 External K-induced stimulation of outward conductance. In the C9 chimera the PM region of ROMK2 was replaced by the homologous region of IRK1 (*shaded rectangle*). The initial conductances in 1 mM K were as follows: ROMK2, 80 ± 12 μS; C9, 202 ± 42 μS. Conductance in 10 mM K was measured at net outward current, with the cell potential clamped at 25 mV more positive than the resting potential. Data were normalized to the conductance in 1 mM K, measured at the same potential.

Elevating external K from 1 to 10 mM or adding 109 mM Cs increased outward single-channel conductance from 6.4 to 11.1 pS (10 mM K) or 11.7 pS (Cs). This 1.7–1.8-fold increase in single-channel conductance is close to the factor that whole-cell conductance increased after 2 min (Fig. 12 and Table 1). Note that in Table 1, the average 1 and 10 mM K whole-cell conductances are not paired data because half of the 1 mM K values correspond to the 10 mM K experiments and half correspond to the Cs experiments.

A slight increase in single-channel conductance would also explain the rapid, time-independent increase in whole-cell conductance seen with the C9 chimera (Fig. 12) and the ROMK2-F129C mutant (Fig. 13). Increases in single-channel conductance during elevation of external K are not terribly surprising as they may be the direct result of increasing the concentration of permeant ions (Lopatin and Nichols, 1996; Sakmann and Trube, 1984; Hagiwara and Takahashi, 1974; Kubo et al., 1993). In experiments with Kir 2.1, in which rectification was eliminated by removal of cytoplasmic Mg and polyamines, outward conductance was

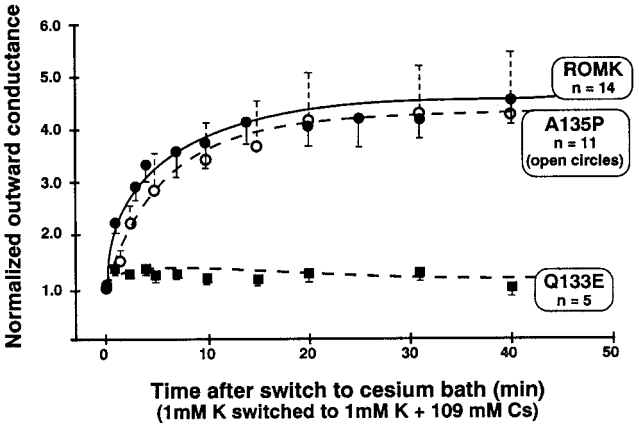

FIGURE 14 Effect of other mutations in the PM region. Shown is the time course of whole-cell conductance following elevation of external K. A135P (○) resembles wild-type ROMK2 (●) except that an Ala has been replaced by a Pro. Q133E resembles wild-type ROMK2 except that a Gln at position 133 has been replaced by a negatively charged Glu. See Fig. 1 for respective sequences. The initial conductances in 1 mM K were as follows: ROMK2, 102 ± 33 μS; A135P, 75 ± 15 μS; Q133E, 72 ± 10 μS. Conductance was measured at net outward current, with the cell potential clamped at 25 mV more positive than the resting potential. Data were normalized to the conductance in 1 mM K, measured at the same potential

found to depend on external [K] to the power of 0.2 (Lopatin and Nichols, 1996).

It is somewhat harder to explain how external Cs (which blocks inward K current) would increase single-channel ROMK conductance. One clue to the action of Cs is that at

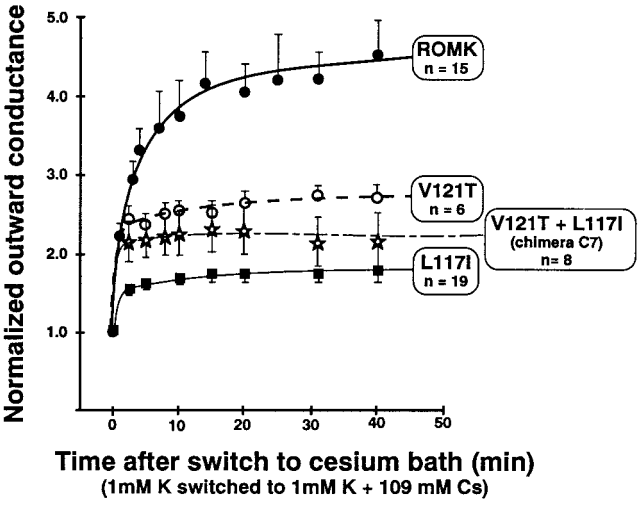

FIGURE 15 Importance of the pore region for external Cs-induced stimulation of outward conductance. V121T and L117I are two point mutations constituting the difference between the ROMK and IRK pore (P) regions. The initial conductances in 1 mM K were as follows: ROMK2, 102 ± 33 μS; V121T, 164 ± 35 μS; L117I, 106 ± 21 μS; C7, 140 ± 15 μS. Conductance was measured at net outward current, with the cell potential clamped at 25 mV more positive than the resting potential. Data were normalized to the conductance in 1 mM K, measured at the same potential (see Fig. 1 for mutant sequences).

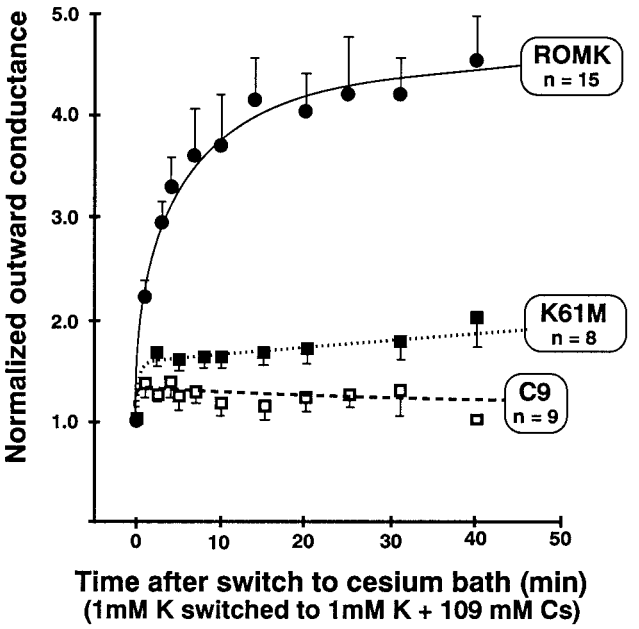

FIGURE 16 A mutation at the cytoplasmic pH gate (K61M) blocks slow activation. Shown is the time course of conductance as a function of time after replacement of external Na with Cs. The ROMK2 mutation K61M is known to abolish the pH sensitivity of ROMK. Results for the C9 chimera and wild-type ROMK are shown for comparison. Initial conductances in 1 mM K solutions were as follows: ROMK2, 102 ± 33 μS; C9, 144 ± 23 μS; K61M, 207 ± 57 μS. Conductance was measured at net outward current, with the cell potential clamped at 25 mV more positive than the resting potential. Data were normalized to the conductance in 1 mM K, measured at the same potential.

zero membrane potential, external Cs decreases both the single-channel and the initial whole-cell currents (Table 1), presumably by blocking the channel with kinetics that are too rapid to resolve (see record at −4 mV, Fig. 9). Thus the increased conductance could simply reflect a relief of Cs block as the membrane voltage is depolarized.

As indicated in Table 1, neither external 10 mM K nor Cs had an effect on the high P_o that characterizes ROMK outward currents. High P_o values were also seen with outward currents through renal SK channels at a variety of external K concentrations (Palmer et al., 1997; Frindt and Palmer, 1989). As a result, the most likely explanation for the stimulation of whole-cell conductance with external 10 mM K or Cs is an increase in the number of conducting channels (N). In RCK4 channels of hippocampal neurons, regulation by external K also appears to involve changes in the number of active channels, rather than single-channel conductance or mean open time (Pardo et al., 1992).

This hypothesis was further evaluated by examining whether 50 mM external K increased the number of active channels in cell-attached patches whose external surface was previously incubated in 1 mM K. As indicated in Fig. 10 and Table 2, raising external K increased the number of active channels in 9 of 16 patches, for patches that initially

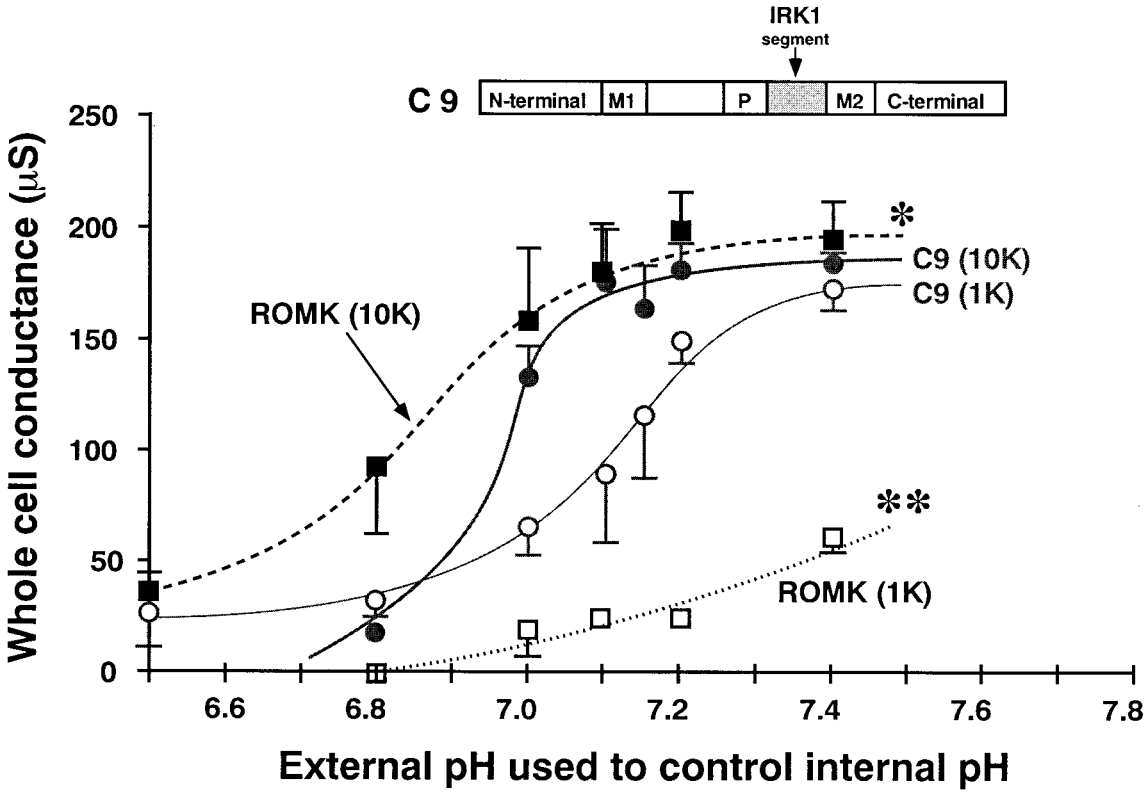

FIGURE 17 pH dependence of ROMK and C9 outward conductance at different external [K]. Oocytes expressing equal amounts of either ROMK2 or the chimera C9 were voltage clamped and a permeant acetate buffer was used to control intracellular pH. The abscissa is the pH of the external 55 mM sodium acetate (plus 50 mM NaCl plus 1 or 10 mM [K]) used to control intracellular pH. Oocyte total outward conductance was periodically measured at holding potentials approximately 25 mV more positive than the resting potential (-104 ± 3 mV in 1 mM K or -58 ± 3 mV in 10 mM K) during reductions in extracellular pH. □, ROMK2 in 1mM K; ■, ROMK2 in 10 mM K; ○, C9 in 1 mM K; ●, C9 in 10 mM K. Normal intracellular oocyte pH corresponds to an external pH of 7.4 in acetate solutions. At the asterisk on the ROMK (10 mM K) curve, K61 is believed to be deprotonated and the channel is activated (pKa $\approx$ 6.9). At the double asterisk on the ROMK (1 mM K) curve, K61 is believed to be protonated and the channel is inactivated.

contained one to three channels. None of these patches showed a decrease in the number of channels during a 40-min observation.

The percentage increase in macroscopic current following exposure to elevated external K (solid line, Fig. 12) appears to be higher than what would be predicted from the observed increase in single-channel number (Table 2). This can be partially explained by the exclusion of inactive patches from the data of Table 2 (see Materials and Methods). Appearance of de novo channel activity in previously quiescent patches would constitute a large (i.e., infinite) percentage increase in activity that might underlie the fivefold increase in macroscopic conductance (Fig. 12).

Prior exposure of the oocytes to 50 mM K for at least 30 min prevented any increase in the number of active channels. These results strongly suggest that the slow increase in whole-cell conductance seen with elevated external K is caused by a K activation of dormant (inactivated) channels.

We cannot rule out the possibility that increased channel activity is caused by insertion of cytoplasmic channels into the membrane. However, there are several arguments against this alternative hypothesis. First, single-channel experiments like the one in Fig. 10 never indicated simultaneous activation of two or more channels. If the mechanism of activation were vesicle fusion, there is no a priori reason to expect that vesicles fusing with the membrane should always contain only one channel. Second, transient bursts often preceded the appearance of new channel activity (Fig. 10). These are difficult to reconcile with a straightforward insertion of cytoplasmic channels. Third, the entire activation process is dependent on specific residues in both the pore and the PM (Fig. 1) region. A physical insertion process would require external K to interact (at a distance) with structural elements of cytoplasmically sequestered channels. Finally, the slowing of channel inactivation by maintained outward current (Fig. 3) favors an activation rather than an insertion hypothesis. The presumption is that outward current produces a high concentration of K near the putative sensor in the outer mouth of the pore, preventing inactivation. On the other hand, it is difficult to understand why channel current would affect retrieval of channels from the membrane.

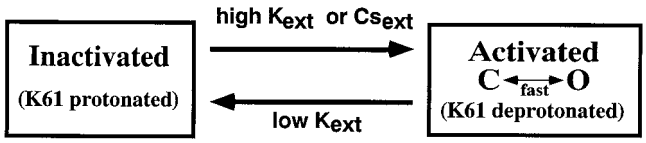

FIGURE 18 Simple model of ROMK2 activation by external K or Cs. Low external K and/or protonation of the pH gate (K61) drive wild-type channels into an inactivated state. Elevation of external K or Cs increases the likelihood of transitions into the activated state, in which the channel flickers rapidly between open and closed states. The ROMK2-K61M mutant cannot be protonated and is locked into the activated state. In the mutants F129C, E132D, Q133E, L117I, and V121T the effect of external cations is reduced.

Hence, our working hypothesis is that low ($\leq$1 mM) K causes a significant population of ROMK channels to enter an inactive or dormant state ($P_o \approx 0$). Subsequent elevation of external K (or addition of Cs) shifts these channels out of their inactive state, raising their P_o close to 0.9. The slow increase in outward whole-cell conductance results from more and more channels being pulled out of their dormant state. Such a direct regulation of conductance by external cations implies the existence of a K sensor somewhere in the structure of the channel protein.

One possibility is that extracellular cations such as K, Rb, or Cs prevent collapse of the channel which occurs during K removal. This would be analogous to the explanation proposed by Yellen and colleagues for the effect of external K on C-type inactivation of Kv channels, in which the selectivity filter of the pore is presumed to undergo a partial collapse at the extracellular end, which can be prevented or reversed by external cations that bind in this region of the pore (Liu et al., 1996; Ortega-Sáenz et al., 2000).

On the other hand, the observation that the slow (but not the fast) response to external cations required a Lys at position 61 (position 80 in ROMK1) suggests an important link between the slow increase in outward K conductance and pH gating of the channel (Doi et al., 1996), with deprotonation of the K61 residue of ROMK2 (K80 in ROMK1) being required for the (slow) increase in conductance depicted in Figs. 12–16. In this scenario, K removal shuts down the channel by altering its pH sensitivity.

External K (or Cs) and internal pH seem to operate as separate (but interacting) stimuli for channel activation. We do not believe that the effect of 1 mM external K is simply to acidify the oocyte. First, elevating external [K] activated wild-type ROMK whole-cell conductance even when intracellular pH was clamped by acetate-buffered solutions (Fig. 17). Second, experiments conducted with macropatches that allowed examination of external [K] at controlled cytoplasmic-side pH also indicated K activation of ROMK at constant pH (U. Schulte, personal communication).

An interaction between external K and internal pH is further supported by the data of Figs. 16 and 17. Complete activation of outward conductance seems to require an intact pH sensor, because the K61M mutant of ROMK2 lacks both a pH gate and a slow increase in conductance (Fig. 16). Conversely, the pH-sensing mechanism of the channel does not require the K-activation site, which (at normal pH) is absent in the C9 chimera. Yet C9 clearly responds to changes in internal pH. Hence, an internal pH sensor (at K61) is a necessary but not a sufficient condition for the slow activation of outward conductance by external K.

A simple model for the slow activation and inactivation of ROMK is illustrated in Fig. 18. Macroscopic conductance is directly proportional to the number of channels in the activated mode. Individual channels can switch between inactivated and activated modes over a period of 20–40min. Within the activated mode, channels undergo transitions between open and closed states on a time scale that is much faster than transitions between inactive and active modes. Protonation of the cytoplasmic K61 site drives channels into the inactivated mode, unless this site has been altered (e.g., the mutation K61M). Addition of extracellular K or Cs promotes the activated state.

A hypothetical structure of ROMK is depicted in Fig. 19, based on homologies with the known structure of KcsA (Doyle et al., 1998). Only two of the four subunits are shown, and the pore is assumed to contain three K ions, with a water molecule (labeled O) between the inner and outer K-binding sites. The outer binding site is depicted by the cyan sphere (labeled Cs^+, K^+ in Fig. 19), whereas the inner binding site (dark blue K^+) and the vestibule K ion (purple) are blurred to represent uncertainty in their precise location. M1 and M2 refer to the transmembrane helices, and the approximate positions of the critical residues are indicated.

We hypothesize that slow activation of ROMK outward conductance occurs when K or Cs occupies the outer binding site (cyan sphere, Fig. 19) and stabilizes the open configuration of the channel. The V121 and L117 residues lie close to the selectivity filter and could affect cation binding within. The V121T mutation was previously shown to increase single-channel conductance and Ba block, but the L117I mutation did not affect these parameters. The F129, E132, and Q133 residues are probably too far from the pore to alter cation binding directly. These amino acids could be involved in a conformational change in the outer part of the channel or the transmission of such a change to the cytoplasmic region, changing the pKa of K161. It was previously shown that this pKa seems to be affected by the charge of other amino acids on the cytoplasmic N-terminus of the channel protein (Choe et al., 1997).

The pKa of K61 (ROMK1-K80) has also been shown to be affected by arginine residues on the N- and C-termini (ROMK1-Arg 41 and Arg 311) (Schulte et al., 1999). It is possible that elevating external K or Cs causes a shift in ROMK that alters the interaction between these Arg and Lys residues, allowing the Lys to be deprotonated at normal pH (Fig. 17), a condition that stabilizes the activated state.

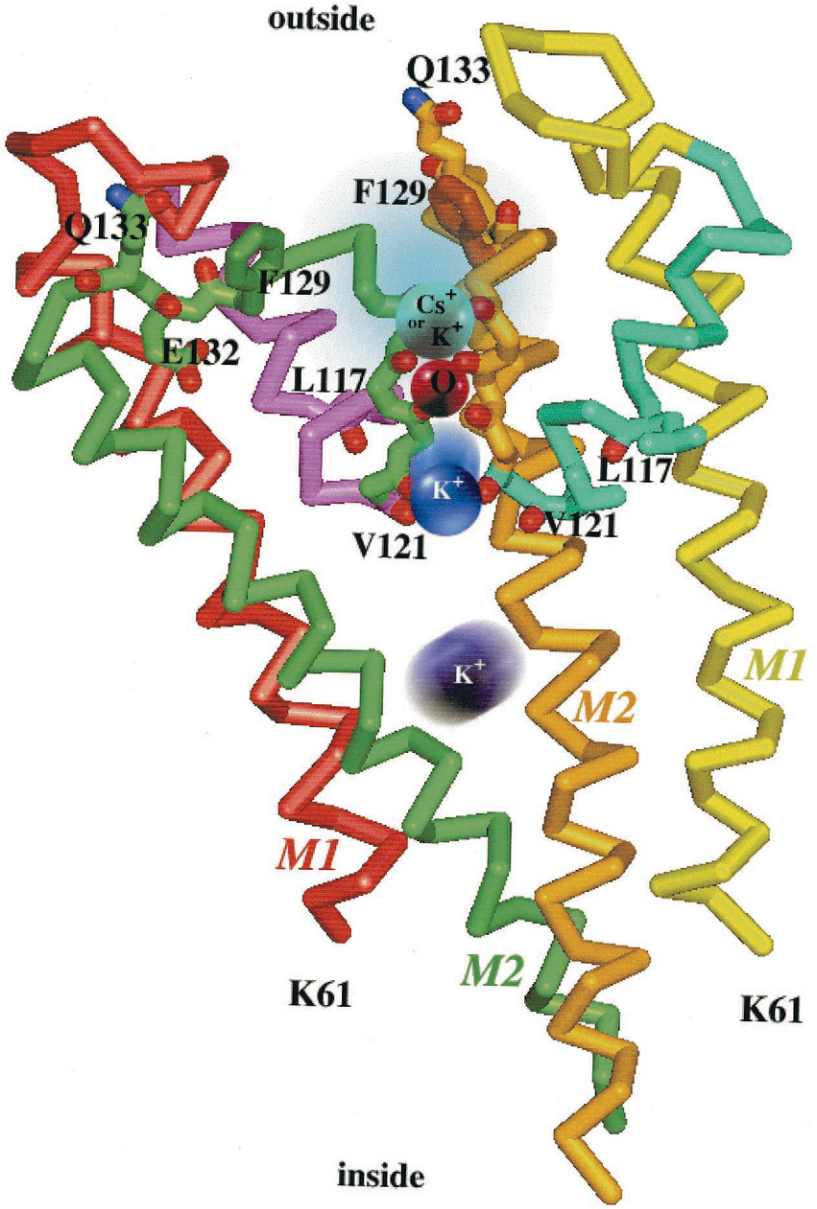

FIGURE 19 Molecular scheme for regulation of ROMK by external K or Cs. Model of ROMK2 is based on homology with the known crystal structure of KcsA (Doyle et al., 1998). Two of the four subunits, with transmembrane segments M1 and M2, are shown. By analogy with the KcsA data, the pore is assumed to contain at least three K^+ ions, where the K^+ at the inner binding site (dark blue) and the K^+ within the vestibule (purple) are blurred to represent uncertainty in their position. The cyan sphere represents a possible position for either Cs or K, and the smaller red sphere is a water interspersed between two cations. For visual clarity, each transmembrane segment and pore helix is shown in a different color. Critical ROMK2 residues F129, E132, Q133, L117, and V121, are indicated with their respective nitrogens (blue) and oxygens (red), as well as the carbonyl oxygens of the selectivity site. Only one of several possible orientations is shown for each of these side chains. The pH sensor at K61 is three residues cytoplasmic to the beginning of the M1 transmembrane segment.

In summary, we have identified specific ROMK residues that might constitute a functional K sensor that would permit the channel to regulate its activity in response to changes in external K. For example, low K in the lumen of the nephron would trigger ROMK inactivation, thereby reducing K secretion in the cortical collecting tubule and conserving cell K (and plasma K) during periods of K depletion. On the other hand, elevated luminal K (reflecting K loading) would shift more ROMK channels into the active state, enhancing renal K secretion into the urine.

We thank Dr. Uwe Schulte for many helpful discussion.

This work was supported by National Institutes of Health grants DK46950 (H. Sackin) and DK27847 (L.G. Palmer).

REFERENCES

Baukrowitz, T., and G. Yellen. 1995. Modulation of K^+ current by frequency and external $[K^+]$: a tale of two inactivation mechanisms. *Neuron.* 15:951–960.

Chepilko, S., H. Zhou, H. Sackin, and L. G. Palmer. 1995. Permeation and gating properties of a cloned renal K^+ channel. *Am. J. Physiol.* 268: C389–C401.

Choe, H., L. G. Palmer, and H. Sackin. 1999. Structural determinants of gating in inward-rectifier K^+ channels. *Biophys. J.* 76:1988–2003.

Choe, H., H. Sackin, and L. G. Palmer. 2000. Permeation properties of inward-rectifier potassium channels and their molecular determinants. *J. Gen. Physiol.* 115:391–404.

Choe, H., H. Zhou, L. G. Palmer, and H. Sackin. 1997. A conserved cytoplasmic region of ROMK modulates pH sensitivity, conductance, and gating. *Am. J. Physiol.* 273:F516–F529.

Doi, T., B. Fakler, J. H. Schultz, U. Schulte, U. Brandle, S. Weidemann, H. P. Zenner, F. Lang, and J. P. Ruppersberg. 1996. Extracellular K^+ and intracellular pH allosterically regulate renal Kir1.1 channels. *J. Biol. Chem.* 271:17261–17266.

Döring, F., C. Derst, E. Wischmeyer, C. Karschin, R. Schneggenburger, J. Daut, and A. Karschin. 1998. The epithelial inward rectifier channel Kir7.1 displays unusual K^+ permeation properties. *J. Neurosci.* 18: 8625–8636.

Doyle, D. A., J. M. Cabral, R. A. Pfuetzner, A. Kuo, J. M. Gulbis, S. L. Cohen, B. T. Chait, and R. MacKinnon. 1998. The structure of the potassium channel: molecular basis of K^+ conduction and selectivity. *Science.* 280:69–77.

Frindt, G., and L. G. Palmer. 1989. Low-conductance K channels in apical membrane of rat cortical collecting tubule. *Am. J. Physiol.* 256: F143–F151.

Hagiwara, S., and K. Takahashi. 1974. The anomalous rectification and cation selectivity of the membrane of a starfish egg cell. *J. Membr. Biol.* 18:61–80.

Horton, R. M., H. D. Hunt, S. N. Ho, J. K. Pullen, and L. R. Pease. 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene.* 77:61–68.

Kubo, Y., T. J. Baldwin, Y. N. Jan, and L. Y. Jan. 1993. Primary structure and functional expression of a mouse inward rectifier potassium channel. *Nature.* 362:127–133.

Leipziger, J., G. MacGregor, G. Cooper, J. Xu, S. Hebert, and G. Giebisch. 2000. PKA site mutations of ROMK2 channels shift the pH dependence to more alkaline values. *Am. J. Physiol.* 279:F919–F926.

Liu, Y., M. E. Jurman, and G. Yellen. 1996. Dynamic rearrangement of the outer mouth of a K^+ channel during gating. *Neuron.* 16:859–867.

Lopatin, A. N., and C. G. Nichols. 1996. [K+] dependence of open-channel conductance in cloned inward rectifier potassium channels (IRK1, Kir2.1). *Biophys. J.* 71:682–694.

MacGregor, G. G., J. Z. Xu, C. M. McNicholas, G. Giebisch, and S. C. Herbert. 1998. Partially active channels produced by PKA site mutation of the cloned renal K^+ channel, ROMK2 (kir1.2). *Am. J. Physiol. Renal Physiol.* 275:F415–F422.

Malnic, G., R. M. Klose, and G. Giebisch. 1966. Micropuncture study of distal tubular potassium and sodium transport in rat nephron. *Am. J. Physiol.* 211:529–547.

McNicholas, C. M., G. G. MacGregor, L. D. Islas, Y. Yang, S. C. Herbert, and G. Giebisch. 1998. pH-dependent modulation of the cloned renal K^+ channel, ROMK. *Am. H. Physiol. Renal Physiol.* 275:F972–F981.

McNicholas, C. M., W. Wang, K. Ho, S. C. Herbert, and G. Giebisch. 1994. Regulation of ROMK1 K^+ channel activity involves phosphorylation process. *Proc. Natl. Acad. Sci. U.S.A.* 91:8077–8081.

Ortega-Sáenz, P., R. Pardal, A. Castellano, and J. López-Barneo. 2000. Collapse of conductance is prevented by a glutamate residue conserved in voltage-dependent K channels. *J. Gen. Physiol.* 116:181–190.

Palmer, L. G., H. Choe, and G. Frindt. 1997. Is the secretory K channel in the rat CCT ROMK? *Am. J. Physiol.* 273:F404–F410.

Pardo, L. A., S. H. Heinemann, H. Terlau, U. Ludewig, C. Lorra, O. Pongs, and W. Stuhmer. 1992. Extracellular K^+ specifically modulates a rat brain K^+ channel. *Proc. Natl. Acad. Sci. U.S.A.* 89:2466–2470.

Sakmann, B., and G. Trube. 1984. Conductance properties of single inwardly rectifying potassium channels in ventricular cells from guinea-pig heart. *J. Physiol. (Lond.).* 347:641–657.

Schlief, T., R. Schönherr, and S. H. Heinemann. 1996. Modification of C-type inactivating Shaker potassium channels by chloramine-T. *Pflügers Arch.* 431:483–493.

Schulte, U., H. Hahn, M. Konrad, N. Jeck, C. Derst, K. Wild, S. Weidemann, J. Ruppersberg, B. Fakler, and J. Ludwig. 1999. pH gating of ROMK (Kir1.1) channels: control by an Arg-Lys-Arg triad disrupted in antenatal Bartter syndrome. *Proc. Natl. Acad. Sci. U.S.A.* 96: 15298–15303.

Tsai, T. D., M. E. Shuck, D. P. Thompson, M. J. Bienkowski, and K. S. Lee. 1995. Intracellular H^+ inhibits a cloned rat kidney outer medulla K^+ channel expressed in *Xenopus* oocytes. *Am. J. Physiol.* 268: C1173–C1178.

Vergani, P., D. Hamilton, S. Jarvis, and M. R. Blatt. 1998. Mutations in the pore regions of the yeast K^+ channel YKC1 affect gating by extracellular K^+. *EMBO J.* 17:7190–7198.

Wang, W. H., and G. Giebisch. 1991. Dual modulation of renal ATP-sensitive K^+ channel by protein kinases A and C. *Proc. Natl. Acad. Sci. U.S.A.* 88:9722–9725.

Xu, Z. C., Y. Yang, and S. C. Hebert. 1996. Phosphorylation of the ATP-sensitive, inwardly rectifying K^+ channel, ROMK, by cyclic AMP-dependent protein kinase. *J. Biol. Chem.* 271:9313–9319.

Zhou, H., S. Chepilko, W. Schutt, H. Choe, L. G. Palmer, and H. Sackin. 1996. Mutations in the pore region of ROMK enhance Ba^{2+} block. *Am. J. Physiol.* 271:C1949–C1956.