External K Activation of Kir1.1 Depends on the pH Gate

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ABSTRACT The inward rectifier Kir1.1 (ROMK) family is gated by both internal pH and external K, where the putative pH gate is formed by the convergence of leucine side chains, near the inner helical bundle crossing at L160-Kir1.1. However, it is unclear whether K activation is mediated at the pH gate or by another gate in the permeation path. In this study, we used the whole-cell conductance increase during rapid K elevation as a measure of K activation, assuming that activation is inherently slower than changes in channel conduction. Results indicate that structural disruption of the Kir1.1 bundle-crossing pH gate prevents both inactivation by low external K and reactivation by high external K.

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Previous studies have documented an interaction between internal pH and external K in the Kir1.1 inward rectifier family (1–6). However, the nature of this interaction is not well understood. Removal of external K inactivates Kir1.1 channels that have been closed by internal acidification. Reopening of these channels requires return of external K before internal alkalization (5,6). These results are compatible with either a single pH gate that responds to both internal pH and external K (6) or an internal pH gate in series with a K sensitive gate at the selectivity filter (5,7).

In this study, we explored this interaction between internal pH and external K by determining whether external K gating depends on a functional internal pH gate. We mutated the putative internal pH gate of Kir1.1b (ROMK2) by replacing the hydrophobic leucines at the bundle-crossing of the inner transmembrane helices by smaller glycine side chains (L160G-Kir1.1b). We also added a second mutation (A158L-L160G-Kir1.1b), which appeared to both increase expression efficiency and render the channel even less sensitive to internal pH.

Fig. 1 summarizes how these two mutations (*orange* and *green lines*, Fig. 1) diminish the normal pH sensitivity of Kir1.1 (ROMK, *blue triangles*, Fig. 1). The A158L mutation alone is not responsible for this lack of pH sensitivity and in fact causes the channel to shut down at more alkaline pH, probably because a *Leu* side chain at 158 restricts bending of the inner helix away from the permeation path during channel opening (8).

The goal of this study was to determine whether external K gating requires a functional pH gate at the bundle crossing. In this regard, we compared external K gating in wild-type ROMK and the A158L-L160G mutant by dissecting the time course of whole-cell conductance changes during rapid elevations of external [K], after different exposures to low [K].

A 30-min incubation of wt-ROMK2 in 1 mM external [K], at an internal pH of 7.04, both decreased single-channel conductance and inactivated the channels (*dashed arrow*, Fig. 2). Subsequent rapid elevation of external K produced a rapid 7 ± 2-fold increase of inward whole-cell conductance within 10 s, followed by a slower increase that occurred over the next 20 min (*red circles*, Fig. 2). Similar results were obtained for outward conductance (*cyan triangles*, Fig. 2), indicating that the slow time-course of K-activation was independent of current direction. External solution exchange, indicated by oocyte membrane potential, is essentially complete within 10 s (*inset*, Fig. 2).

Interpretation of these time-course data is based on the premise that channels that have been inactivated by prolonged

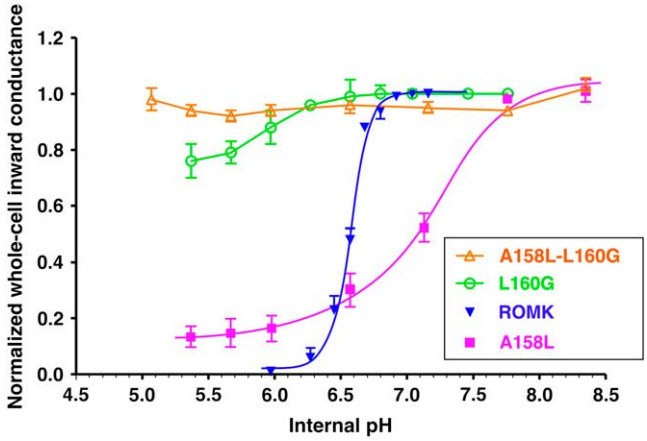

FIGURE 1 Steady-state pH gating of ROMK2 (pK_a = 6.6 ± 0.01, *blue triangles*), A158L (pK_a = 7.3 ± 0.07, *magenta squares*), and two mutants that do not pH-gate: L160G (*green circles*) and A158L-L160G (*orange triangles*). Each oocyte conductance was normalized to its maximum value at alkaline pH. Inward conductances were determined at negative 190 mV in whole oocytes using the two-electrode voltage-clamp. Internal pH was controlled with 100 mM K permeant acetate buffers (8).

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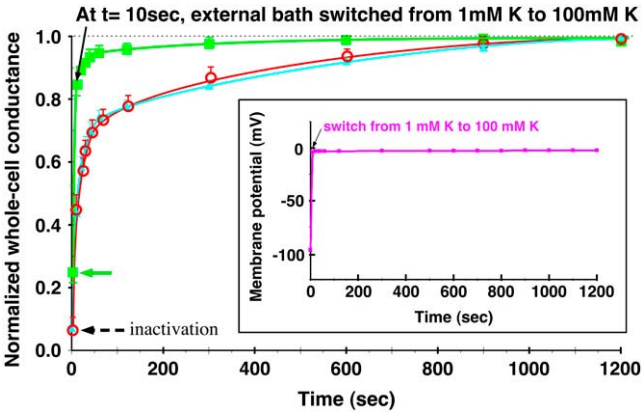

FIGURE 2 Normalized, whole-cell ROMK conductance during elevation of external K from 1 mM to 100 mM. Slow time courses (indicative of K activation) occur for both inward (*red circles*) and outward (*cyan triangles*) ROMK currents, after inactivation by a 30-min exposure to 1 mM K. ROMK channels that were exposed to 1 mM K for only 1 min (*green squares*) failed to inactivate (*horizontal green arrow*), and subsequent elevation of external K produced only a fast increase in inward conductance. Conductances were measured at negative 190 mV (inward) or positive 100 mV (outward) currents, using the two-electrode voltage-clamp in whole oocytes. Constant internal pH of 7.04 was maintained by permeant acetate buffered solutions at pH_o = 7.8. (*Inset*) Speed of solution change as determined from membrane depolarization.

exposure to low external K should exhibit a biphasic increase in whole-cell conductance, upon rapid reintroduction of external [K]. The fast component should reflect both the concentration dependence of single-channel conductance and the beginning of K activation, whereas the slower component should primarily reflect channel activation (gating)—presumed to be inherently slower than changes in single-channel conductance (permeation).

In contrast to results with a 30-min exposure to low K, a 1-min incubation in 1 mM K caused less of a decrease in whole-cell conductance (*green arrow*, Fig. 2). Subsequent reintroduction of 100 mM external [K] produced a 4 ± 1-fold rapid increase in whole-cell conductance within 10 s (*green squares*, Fig. 2), but without much of a slow change in whole-cell conductance, which would be indicative of gating. These results are consistent with 1 min of low K being sufficient to decrease single-channel conductance, but insufficient to inactivate the channels.

We applied the same protocol of 30-min incubation in 1 mM [K] followed by rapid K elevation to both wild-type ROMK2 and the pH-gate deficient mutant: A158L-L160G-ROMK2 (Fig. 3). Thirty minutes in 1 mM external [K] decreased A158L-L160G-ROMK2 whole-cell conductance (*green arrow*, Fig. 3), but did not inactivate this double mutant to the same extent as either wt-ROMK or the single mutant A158L (*dashed arrow*, Fig. 3).

Reintroduction of 10 mM K to A158L-L160G oocytes produced a 3 ± 1-fold rapid increase in whole-cell conductance within 10 s (*green squares*, Fig. 3), but no slow change in whole-cell conductance indicative of gating. This was consistent with the 10-mM K solution raising single-channel conductance, without producing an increase in the number of active channels. In contrast, wild-type ROMK (*orange circles*, Fig. 3) exhibited both an initial 6 ± 1-fold rapid increase in conductance, indicating an increase in single-channel conductance and a slow time course that primarily reflects external K activation. Qualitatively similar results were obtained for transitions between 1 mM and 100 mM K (not shown).

As a control, we examined the time course of A158L whole-cell inward conductance during the same 1–10 mM external K elevation (subsequent to a 30-min incubation in 1 mM K). As indicated by the blue line (*open circles*, Fig. 3), the single mutant A158L-Kir1.1b showed both fast and slow components of increasing inward whole-cell conductance, similar to wild-type ROMK. Hence, the absence of a slow component in the A158L-L160G conductance increase (*green squares*, Fig. 3) results from the L160G mutation rather than the A158L mutation.

The data of Figs. 2 and 3 were fit to a two-exponential association model,

$$G = G_{\max 1}(1 - \exp(-k_1 t)) + G_{\max 2}(1 - \exp(-k_2 t)),$$

where the fast time constant is assumed to represent the concentration dependence of single-channel conductance, and the slow time constant is assumed to reflect gating by external [K]. The average fast time constant of 6 ± 2 s was independent of channel type and was essentially determined by the bath exchange system. The set of simultaneously fitted parameters is given in Table 1.

The slower ROMK time constants, reflecting K activation, were similar for both the 1 → 100 mM transition (330 ± 55 s, Fig. 2) and the 1 → 10 mM transition (474 ± 74 s, *orange line*, Fig. 3). In addition, there was no significant difference between the slow phases of wt-ROMK and the single A158L mutant (873 ± 400 s, *blue line*, Fig. 3).

Since changes in external K can, in principle, affect both single-channel conductance and gating, the rapid phase of the ROMK time course in Figs. 2 and 3 probably contains an early phase of K activation. However, this is hard to separate from the rapid concentration-dependent increase in single-channel conductance. This is consistent with the K-activated oocytes of Figs. 2 and 3 having a seven- and sixfold increase in whole-cell conductance during the first 10 s, compared to the four- and threefold whole-cell conductance increase in oocytes whose channels did not inactivate in low K (*green arrows*, Figs. 2 and 3).

Kir1.1 (ROMK) is strongly sensitive to internal (but not external) pH, where low internal pH causes channel closure (1,4–6,9). The physical locus of the pH gate of Kir1.1 (ROMK) is thought to be at the bundle crossing of the inner transmembrane helices. Disruption of this gate by the

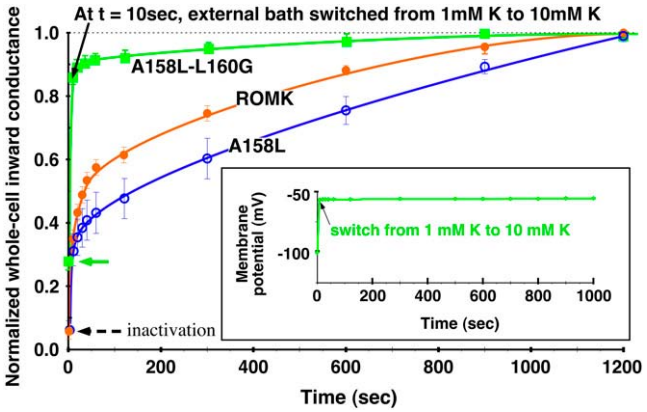

FIGURE 3 Comparison of ROMK and A158L-L160G normalized whole-cell inward conductance during elevation of external K from 1 mM to 10 mM, after a 30-min incubation in 1 mM K. Internal pH of 7.04 was maintained by permeant acetate buffered external solutions at $pH_o = 7.8$. Since A158L is alkaline-shifted (Fig. 1), the external K transition for this mutant was conducted at $pH_i = 8.35$. Slow-phase time courses (indicative of K activation) occur for both ROMK (*orange circles*) and the single A158L mutant (*blue circles*), but not for the pH-insensitive double mutant A158L-L160G (*green squares*), which exhibited only the rapid phase response to external K elevation. Whole-cell conductances were measured as in Fig. 2. (*Inset*) Time course of solution change.

L160G-Kir1.1b mutation renders the channel functionally insensitive to internal pH, especially when combined with the stabilizing mutation A158L (Fig. 1). Results of this study indicate that mutation of the inner pH gate also prevents inactivation and reactivation by external K.

This effect on K gating clearly arose from L160G and not A158L. Hence, the ''leaky'' pH gate produced by the L160G mutation not only prevented pH gating but also prevented inactivation of the channel by low external [K]. These results confirm a physical or functional linkage between internal pH gating and external K gating in the Kir1.1 family, similar to linkages seen between activation and inactivation in both KcsA (10) and *Shaker* (11) channels.

The simplest interpretation of our results is that a single (L160-Kir1.1b) gate at the bundle-crossing mediates both internal pH gating and external K gating. In this case, the L160G mutation would simultaneously prevent both pH gate closure and low K closure (inactivation). However, we cannot rule out the possibility that there is a selectivity filter gate in series with the bundle-crossing pH gate. If this were the case, the L160G pH gate mutation might block K sensitivity either by altering K occupancy or by allosterically preventing the selectivity filter gate from closing under low K conditions.

TABLE 1 Fitted parameters for rapid K elevation

	Min in 1 mM K	Δ[K] (mM)	Fast G_{max}	Fast τ (s)	Slow G_{max}	Slow τ (s)
ROMK	1	1→100	0.9 ± 0.1	3 ± 1	0.1 ± 0.08	59 ± 34
ROMK	30	1→100	0.7 ± 0.02	10 ± 1	0.3 ± 0.02	330 ± 55
ROMK	30	1→10	0.5 ± 0.02	9 ± 1	0.6 ± 0.03	474 ± 74
A158L-L160G	30	1→10	0.9 ± 0.1	3 ± 2	0.1 ± 0.1	363 ± 320

Time constants: $\tau = 1/k$ (see equation in text).

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