Partially active channels produced by PKA site mutation of the cloned renal K⁺ channel, ROMK2 (kir1.2)

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MacGregor, Gordon G., Jason Z. Xu, Carmel M. McNicholas, Gerhard Giebisch, and Steven C. Hebert. Partially active channels produced by PKA site mutation of the cloned renal K⁺ channel, ROMK2 (kir1.2). Am. J. Physiol. 275 (Renal Physiol. 44): F415–F422, 1998.—The activity of the cloned renal K⁺ channel (ROMK2) is dependent on a balance between phosphorylation and dephosphorylation. There are only three protein kinase A (PKA) sites on ROMK2, with the phosphorylated residues being serine-25 (S25), serine-200 (S200), and serine-294 (S294) (Z.-C. Xu, Y. Yang, and S. C. Hebert J. Biol. Chem. 271: 9313–9319, 1996). We previously mutated these sites from serine to alanine to study the contribution of each site to overall channel function. Here we have studied each of these single PKA site mutants using the single-channel configuration of the patch-clamp technique. Both COOH-terminal mutations at sites S200A and S294A showed a decreased open channel probability (Pₒ), whereas the NH₂-terminal mutation at site S25A showed no change in Pₒ compared with wild-type ROMK2. The decrease in Pₒ for the S200A and S294A mutants was caused by the additional presence of a long closed state. In contrast, the occurrence of the S25A channel was ~66% less, suggesting fewer active channels at the membrane. The S200A and S294A channels had different kinetics compared with wild-type ROMK2 channels, showing an increased occurrence of sublevels. Similar kinetics were observed when wild-type ROMK2 was excised and exposed to dephosphorylating conditions, indicating that these effects are specifically a property of the partially phosphorylated channel and not due to an unrelated effect of the mutation.

phosphorylation; sublevel; conductance state; phosphatase; Bartter’s syndrome

EXPRESSION CLONING from the kidney has isolated a candidate channel for the native secretory K⁺ channel found in the cortical collecting duct (CCD) (13). We and others are systematically determining and comparing the properties of the cloned ROMK channels to that of the previously published native CCD channel (34). Properties such as pH sensitivity (5, 8, 7, 26), channel modulation by ATP (28), inhibition by arachidonic acid (25), activation by protein kinase A (PKA) (27), and channel conductance, selectivity, and block (4, 21, 30, 37, 38) are similar between native and cloned ROMK channels.

Excised membrane patches expressing ROMK channels show a decrease in channel activity over time, a process called rundown. Dephosphorylation plays an important role in channel rundown (27), but it may not be the only factor involved, as loss of associated proteins or specific cellular milieu may alter channel properties upon patch excision (1). It has recently been shown that anionic phospholipids, especially phosphatidylinositol 4,5-bisphosphate, can reverse excision-induced rundown, implying that rundown may be a multifaceted process (9). A large proportion of the rundown of ROMK channels is Mg2⁺ dependent and prevented by the chelation of free Mg2⁺ in the solution bathing the internal side of the membrane (27). Channel activity can be restored by rapidly applying Mg2⁺-ATP and PKA, suggesting that the initial phase of rundown is attributable to a Mg2⁺-dependent dephosphorylation process (27, 28). The native low-conductance secretory K⁺ channel studied in the principal cell of the rat CCD likewise displays Mg2⁺-dependent rundown (17). Okadaic acid prevents a large component of rundown in excised native and ROMK channels, which indicates that rundown of the native renal K⁺ channel from rat CCD, and the cloned ROMK channel expressed in Xenopus oocytes results mainly from dephosphorylation of the channel by membrane-bound phosphatases (17, 27).

Recently, we have identified three serine residues on ROMK that are phosphorylated by PKA, serine-25 on the NH₂-terminal and serine-200 and serine-294 on the COOH-terminal (35). Any two of these three sites must be phosphorylated for channel function, but removal of a single site decreases the observed K⁺ current by 30–40%. The addition of a phosphate group to specific amino acids on ion channels is a common mechanism of channel regulation (19, 20). The simplest mechanism of regulation would be that phosphorylation is required to move the channel from an inactive state to an active one, but modes of action such as alterations in number of active channels at the membrane, the open probability of the single channel, and the single-channel conductance are common (15). Modification of either of these parameters will affect the observed whole cell current (19). Here we study the contribution of any single serine residue to ROMK2 channel function by rendering it inactive and by examining the biophysical properties of mutant ROMK channels where one of the three serine residues has been mutated from serine to alanine.

METHODS

Preparation of oocytes for patch-clamp experiments. Stage V-VI oocytes were isolated from Xenopus laevis frogs by partial ovariectomy under tricaine anesthesia. Oocytes were

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then defolliculated by treatment with 2 mg/ml collagenase (Boehringer Mannheim) in zero Ca\(^{2+}\) hypotonic solution (82.5 mM NaCl, 2.0 mM KCl, 1.8 mM MgCl\(_2\), and 5.0 mM HEPES, pH 7.4) combined with gentle agitation for 1 h. Following this incubation, oocytes were washed in ND-96 media (96 mM NaCl, 2.0 mM KCl, 1.8 mM CaCl\(_2\), 1.0 mM MgCl\(_2\), and 5.0 mM HEPES, pH 7.4). Thereafter, oocytes were maintained in supplemented ND-96 media (96 mM NaCl, 2.0 mM KCl, 1.8 mM CaCl\(_2\), 1.0 mM MgCl\(_2\), and 5.0 mM HEPES, pH 7.4; with 50 mg/ml gentamicin and 2.5 mmol/l sodium pyruvate). Twenty-four hours after defolliculation, oocytes were injected with 50 nl (12.5 ng) of ROMK or mutant cRNA using a pneumatic injector (Inject-a-ject, Genèvre, Switzerland). Experiments were performed on days 3–6 postinjection.

Oocytes were placed in a hypertonic solution (220 mM N-methylglucamine, 220 mM aspartic acid, 2.0 mM MgCl\(_2\), 10 mM EGTA, and 10 mM HEPES, pH 7.4) and allowed to shrink for 2–5 min. The vitelline membrane (VM) appeared as a transparent sphere around the oocyte, which was easily removed using fine watchmaker’s forceps (Dumont no. 5). Nylon mesh placed beneath the oocyte immobilizes and thereby facilitates VM removal. Immediately following VM removal, the oocyte was carefully transferred into a chamber (volume, 0.5 ml) mounted on an inverting microscope (Olympus IMT-2) and positioned so that the animal pole was freely accessible to patch pipettes.

Site-directed mutagenesis of ROMK2 PKA sites. Site-directed mutagenesis of ROMK2 PKA sites was performed in ROMK2/pSPORT according to the method of Kunkel (18) as described by Xu et al. (35). For each of the single mutations that modified the three potential PKA phosphorylation sites, three primers were used to change the indicated serine to alanine, as follows: S25, TCTTCCTTCTTTGGCCACCAGCCTTG; S200A, CTGCCAATCAGTAAGGCCTTCCTAAAGG; S294A, CCTCTGGGACATATGCCGTGCGGACC. Three silent restriction sites (S25A, Msc I; S200A, Stu I; S294A, Nde I) generated by these primers were used to confirm subcloning. cRNA was prepared as described previously (13). Briefly, the plasmid was linearized with Not I, and transcripts were generated by T7 RNA polymerase in the presence of capped GTP and nucleotide mixtures. We assume that the effect of the serine-to-alanine mutation on channel properties is produced by the lack of phosphorylation at serine-25, -200, and -294. Supporting this is the observation that dephosphorylated wild-type ROMK2 and the PKA site mutants exhibit similar kinetics, i.e., 44% subconductance that modified the three potential PKA phosphorylation sites, described by Xu et al. (35). For each of the single mutations unless otherwise stated in text.

RESULTS

Characterization of wild-type ROMK2 conductance properties. Figure 1 shows the single-channel properties of wild-type ROMK2 when expressed in Xenopus oocytes and studied using the cell-attached configuration of the patch-clamp technique. The current trace shows rapid spontaneous transitions between the open and closed states of the channel. The distribution of dwell times in each state could be described by a

![Diagram](https://example.com/diagram.png)

Fig. 1. Single-channel properties of wild-type ROMK2. The mean open and closed times are determined for ROMK2 cell-attached patches at ~80 mV (the applied pipette holding potential with respect to the inside of the membrane patch). There is only one open and closed state, with the mean open time (\(\tau_{\text{open}}\)) of 25 ms and the mean closed time (\(\tau_{\text{closed}}\)) of 1.4 ms. The open probability of the channel is relatively voltage independent.
Markov process. The open and closed time distributions were described well with a single exponential. The time constant for the open and closed histograms was 25.4 and 1.4 ms, respectively, at −80 mV (Fig. 1). These values are in agreement with the previously published data of another group (25) but differ with the published data from the Sackin and Palmer groups (5, 38) that report two closed states.

Wild-type ROMK2 rundown. The phosphorylation state of the wild-type ROMK2 channel is unstable in excised-inside out patches, and channels only exist in a phosphorylated state for a short time before further dephosphorylation occurs to render the channel inactive, a process called rundown. As rundown progresses, the level of channel phosphorylation is decreased below the minimum that is essential to support channel activity, and the channel open probability progressively declines to zero (Fig. 2A). Figure 2 shows the last few minutes of channel activity before complete channel closure from an excised single-channel patch. The patch was bathed in Mg$^{2+}$-free solution, a procedure that slows rundown (27, 28). This behavior is typical of channel rundown in Mg$^{2+}$-free conditions, but in the presence of Mg$^{2+}$, rundown is accelerated and occurs over a few tens of seconds (27, 28). This rundown may be caused by phosphatases that are not dependent on Mg$^{2+}$ such as protein phosphatases 1, 2A, or 2B. Before the final closure of the channel, both an increase in the frequency of subconductance states (Fig. 2B) and the presence of an additional long closed state are observed (Fig. 2C). The substrates (which conduct at 44% of the main conductance level) could represent intermediate conducting conformations in the partially phosphorylated channel and occur transiently in the wild-type channel immediately preceding rundown (asterisks on Fig. 2C). To examine the possibility that substrates are partially phosphorylated channels, we studied the effects of PKA site mutation on ROMK channel activity and kinetics.

Single-channel properties of PKA site mutant channels ROMK2 S25A, S200A, and S294A. Spontaneous channel activity was observed upon seal formation, and differences were immediately apparent between the wild-type ROMK2 and all the single-site mutant channels, suggesting that the serines in these PKA sites are phosphorylated in the resting state. The altered channel properties observed are due to the mutant channels that cannot support a full complement of phosphorylation. Current-voltage (I-V) relationships were derived for the main open level of the PKA site mutant channels (all channels showed subconductance states, but S200A and S294A displayed an increased presence of the 44% subconductance state). The mean inward conductance in the cell-attached configuration (measured between −100 mV and −40 mV) was 38.7 ± 1.6 pS (n = 3) for the S25A mutant, 37.1 ± 1.6 pS (n = 3) for the S294A mutant channel, and 34.3 ± 0.6 pS (n = 3) for S200A (measured by extrapolation to zero of the current observed at −80 mV). The I-V relationships and degree of inward rectification were similar for wild-type and the single PKA site mutant ROMK2 channels (Fig. 3B). In ROMK channels the decrease in outward current at positive holding potentials is mostly due to a voltage-dependent block by free intracellular Mg$^{2+}$ (29). The single-channel kinetics were determined at a single voltage of −80 mV for S25A, S200A, and S294A. The mean value of open and closed times for the three PKA site mutant channels are shown in Fig. 3B and were similar to each other and wild-type ROMK2 (Fig. 1). The presence of substrates would register as a closed event in 50% threshold analysis, but this did not affect the mean kinetic data, as substrates only amounted to a few of the thousands of events in the dwell time histograms (Fig. 3B).

Effect of COOH-terminal PKA site mutations (S200A and S294A) on channel properties. The open probabilities of the two COOH-terminal PKA site mutants were studied and compared with wild-type channels. The main difference of the mutant channels was a lower $P_o$ manifest as fluctuations in the $P_o$-time relationship due to the variable occurrence of a long closed state (Fig. 4A). In the cell-attached configuration, wild-type ROMK2 displayed a high single-channel open probabil-
ity with time ($P_0 = 0.92 \pm 0.01$, $n = 6$), but the two single PKA site mutant channels S200A and S294A had long closed intervals, in some cases lasting over 1 min. To assess the effect of the new observed gating mode on open channel probability, continuous recordings of 20 min from six different cell-attached patches containing between one and five channels were analyzed, and the mean $P_0$ was plotted against time. Figure 4A shows the open probability of S294A over 20 min. The mean activity was $0.52 \pm 0.01$ ($n = 6$). The S200A mutant gave a similar value, although it was measured only over 5–10 min ($0.57 \pm 0.11$, $n = 4$). It follows that S200A and S294A channels have a reduced single-channel open probability compared with wild-type ROMK2 channels as a result of the presence of additional long closed/inactive state(s).

These striking differences in the single-site mutant channels upon seal formation suggest that these serines are normally phosphorylated by PKA in the resting state in oocytes and that the absence of phosphorylation at these amino acids (S200 and S294) determines the altered channel activity. We studied S294A in excised patches, with no ATP or PKA added to the cytoplasmic bath solution, to distinguish whether the long closed state was an intrinsic property of the partially phosphorylated mutant channel or was due to a phosphorylation/dephosphorylation cycle. In such excised patches, S294A behaved similarly to the mutant channel in cell-attached patches, displaying repetitive long channel closures and sublevels; but rate of rundown of the channel was also accelerated.

Properties of the inactive state of the COOH-terminal mutant (S294A). Inactivation and activation are spontaneous in partially phosphorylated channels or single PKA site mutant channels. The properties of the partially active gating mode were studied further in the COOH-terminal PKA site mutant channel (S294A). The distributions of the long closed states from a single S294A channel clamped at $-80 \text{ mV}$ were described by a sum of two exponential components with time constants of 5.0 s and 29.8 s, and the mean burst of an active open state was represented by a single exponential with a time constant of 32.8 s (Fig. 4B). The occurrence of sublevels during spontaneous activation...
and inactivation of S294A (Fig. 5) is similar to the activity observed during rundown of wild-type ROMK2 (Fig. 2, B and C). The subconducting states appear to be partially active states with a lower conductance, which lead to and from the inactive state in the incompletely phosphorylated mutant channels as shown in Fig. 5, A and B. Thus the partially phosphorylated COOH-terminal ROMK2 mutant channels S200A and S294A are kinetically different from the fully phosphorylated ROMK2 channel due to the presence of long closed states and the increased presence of substates.

Effect of NH$_2$-terminal PKA site mutation (S25A) on channel properties. The single-channel activity of the NH$_2$-terminal mutant channel S25A was different from the COOH-terminal mutations. The S25A channels had no long closed states like S200A and S294A but resembled the wild-type ROMK2 channel. The open prob-

Fig. 5. Sublevels are intermediate conductance states during channel activation and inactivation in the S294A mutant channel. A: channel activity in a cell-attached S294A patch voltage clamped at −80 mV. In the expanded regions of the trace, partially conducting states can be observed before and after the channel activity. The predominant subconductance state is at 44% of the main level, but there may exist other small sublevels which form a staircase pathway for channel activation and inactivation (see asterisks). B: the condensed trace shows an isolated dwell in an inactive state of the same patch. The expanded regions below the trace show the sublevels forming steps down to and up from the inactive state. Dotted lines on the expanded trace represent the open state and the 44% conductance state. Solid line represents the closed state.

ability of S25A was constantly high over the 20-min investigation period used in this study. Figure 6A shows the calculated channel $P_o$ against time for three different patches averaged over 20 min (0.92 ± 0.01, $n = 3$) and was indistinguishable from ROMK2 channel activity ($P_o = 0.92 ± 0.01, n = 6$). The distribution of channel open and closed times was similar to ROMK2

Fig. 4. Mutant S294A channel has a reduced single-channel open probability. A: open probability of the wild-type ROMK2 and the single-site PKA mutant channel S294A. Measurements were made from the cell-attached configuration using normal 150 mM KCl bath and pipette solutions and was voltage clamped at −80 mV. Open probability was calculated every 10 s over 20-min experimental periods and plotted against time. Lines on A represent the mean of values from 6 such patches that contained between 1 and 5 channels. Values quoted as $N P_o/n$ or $P_o$ for the wild-type and S294A channels were calculated from the mean of 6 data sets. Mean $N P_o/n$ or $P_o$ value of the S294A mutant is 43% less than wild-type ROMK2 and can entirely explain the −40% reduction in whole cell current observed when studying this PKA site mutant using the two-electrode voltage clamp (35). In B, the top represents the distribution of durations in the closed inactive state (●, number of observations greater than the indicated duration). Data could not be described by a single exponential, and the broken line superimposed on the data represents the best fit to a sum of two exponential components with time constants of 5 s ($P = 35\%$ of the events) and 29.8 s ($P = 65\%$ of the events), where $P$ is proportion of each component in the function. In B, bottom represents the histogram of open burst durations (●, number of observations greater than the indicated duration), and the broken line represents the best fit to the data of a single exponential with a time constant of 32.8 s. The definition of a long closed state was an event longer than 500 ms starting from the open level; the definition for measurement of the long open burst was any duration that was longer than 500 ms starting from the closed state. The 500-ms cutoff from the open state was necessary to exclude long dwells in substates which would register as a closed event in 50% threshold analysis.
with a mean open time of 24.7 ms and mean closed time of 1.4 ms at −80 mV (Fig. 3B, top). The single S25A mutant channels displayed rare occurrences of the 44% substate similar to ROMK2 but contained no long inactive state. However, there was a noticeable lower channel observation rate. Figure 6B shows the number of active channels per patch calculated for ROMK2, one of the COOH-terminal mutants S294A, and the NH2-terminal mutant S25A. The average channel frequency was similar for ROMK2 and S294A, being 1.68 ± 0.27 (n = 6) and 1.59 ± 0.15 (n = 8), but was only 0.55 ± 0.15 (n = 5) for S25A (where each n represents a single day of experiments). In patches without channel activity, procedures that stimulate ROMK2 (or ROMK2 mutants) were applied. Addition of PKA and ATP or increasing the pH of the cytoplasmic solution to 7.8 failed to elicit channel activity in these patches.

**DISCUSSION**

Mechanism of ROMK2 modulation by phosphorylation. The PKA phosphorylation sites of ROMK2 on the NH2- and COOH-terminal regions modulate channel activity by different mechanisms. The phosphorylation sites on the COOH-terminal S200 and S294 controlled the open probability of the channel by regulating the stability of the open state. The PKA phosphorylation site on the NH2-terminal S25 determined the number of conducting channels observed at the plasma membrane. The phosphorylation state of the S25 site may be important in assembly of ROMK tetramers, or the phosphorylation site could be involved in the trafficking and retrieval of the channels to the membrane. The phosphorylation of a single serine (S256) on aquaporin-2 has been shown to increase dramatically the level of protein in the plasma membrane (10, 16). Although membrane trafficking is probably different between HEK cells and oocytes, we measured similar levels of ROMK protein for all three PKA site mutants in the plasma membrane of HEK cells (35). If S25A is controlling the stability of the open state, then it is an all or nothing effect.

The total observable membrane current produced by a population of channels (I) is dependent on

\[ I = nP_o \]

where n is the conducting channel number, \( P_o \) is the open probability of the channel and i is the single-channel amplitude (assuming driving force and selectivity do not change). In ROMK2, phosphorylation increases channel activity by utilizing all these parameters at different phosphorylation sites. Phosphorylation at site S25 will increase n, and phosphorylation at S200 and S294 will increase \( P_o \) (i.e., phosphorylation decreases the amount of substates and the frequency of observation of a population of stable substate channels; Ref. 24). The association of sublevels with partially phosphorylated channels and especially with transitions between the active and inactive states suggests that changes in the energy profile for ion permeation through the pore determine entry to and from the inactive state. The ion channel could be effectively dosed by an increase in the barrier/decrease in the well of the rate-limiting barrier to conduction.

This interpretation, together with the observations of others (3, 36) suggests that the process of ion permeation and the mechanisms of ion channel activation/inactivation are coupled and that the same process controls both. PKA phosphorylation is required to transfer ROMK from the inactive to active state allowing the channels to adopt lower free energy permeation profiles, but in this process there appear to be several partially conducting intermediates, which will be the subject of further investigation.

Relevance of partially phosphorylated channels to native CCD. The native low-conductance secretory K+ channel displays a high single-channel \( P_o \) suggesting high levels of endogenous phosphorylation. However, procedures that further stimulate channel phosphorylation increase channel activity by increasing the number
of active channels per patch (2), indicating that the NH₂-terminal PKA site (S25) is most likely unphosphorylated in the resting state. These observations are consistent with an interpretation that native channels are not maximally phosphorylated in the resting state. It is possible that incompletely phosphorylated ROMK channels may respond differently to regulatory stimuli (i.e., pH and ATP), offering yet another level of regulation of potassium secretion in the CCD. Of special interest is PKA site S200, which is in the vicinity of the ATP binding site on the COOH terminus. The phosphorylation state of S200 may influence the ability of ATP to bind and regulate channel activity. Several mutations in ROMK gene have been found in patients suffering from Bartter’s syndrome (6, 14, 31). Bartter’s syndrome is a severe potassium-wasting disease with diverse symptoms (11, 32). Two of the point mutations in the channel protein (A195 to V and S200 to R; Ref. 31) are at or near one of the PKA sites and may influence the phosphorylation of S200 and alter channel activity by the mechanisms shown here. The study of partially phosphorylated channels will provide insight into the pathophysiological mechanism of Bartter’s syndrome.

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REFERENCES


