Structural determinants and specificities for ROMK1-phosphoinositide interaction

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Zeng, Wei-Zhong, Horng-Huei Liou, U. Murali Krishna, J. R. Falck, and Chou-Long Huang. Structural determinants and specificities for ROMK1-phosphoinositide interaction. Am J Physiol Renal Physiol 282: F826–F834, 2002.—We have recently reported that direct interaction between phosphatidylinositol bisphosphate (PIP2) and the COOH-terminal cytoplasmic domain of ROMK1 is important for opening of the channel. We identified arginine-188 of ROMK1 as a critical residue for this interaction. Here, we further report that substitution of a neutral amino acid for lysine-181, arginine-217, or lysine-218 decreases single-channel open probability for the full-conductance state and increases the frequency of opening at a subconductance state. Compared with wild-type ROMK1 channels, these substitution mutants also display an increased sensitivity to the block by anti-PIP2 antibodies and to inhibition by intracellular protons. These results indicate that, like arginine-188, lysine-181, arginine-217, and lysine-218 are also involved in interactions with PIP2 and are critical for ROMK1 to open at full conductance. Using synthetic phosphoinositides containing phosphates at different positions in the head group, we also examined the specificities of phosphoinositides in the regulation of ROMK1 channels. We found that phosphoinositides containing phosphate at both positions 4 and 5 of the inositol head group have the highest efficacy in activating ROMK1 channels. These results suggest that phosphatidylinositol 4,5-bisphosphate is likely the important phosphoinositide in the regulation of ROMK1 channels in a physiological membrane milieu.

phosphatidylinositol bisphosphate; pleckstrin homology domain; inwardly rectifying potassium channels; giant patch-clamp recording; kidney

ROMK POTASSIUM (K+) CHANNELS, including ROMK1 and its isoforms ROMK2 and ROMK3 (2, 10, 13, 40), play important roles in the regulation of water and electrolyte transport. In the thick ascending limb (TAL) of Henle’s loop, the recycling of K+ ions across the low-conductance apical K+ channels is essential for NaCl reabsorption through the apical Na-K-2Cl cotransporter (38). In the cortical collecting ducts (CCD), secretion of K+ into urinary space is mediated by active transport of K+ into the cell through basolateral Na+-K+-ATPase, followed by passive movement of K+ into the tubular fluid through apical K+ channels (38). On the basis of the distribution of mRNA and proteins and biophysical characterization, it is known that ROMK1 and ROMK2 encode, at least in part, the low-conductance secretory K+ channels in CCD and TAL, respectively (10).

ROMK channels belong to a large family of structurally related inwardly rectifying K+ channels (26), including the strongly inwardly rectifying channels (IRK1) (18), the G protein-gated inwardly rectifying K+ channels (GIRK1) (19), and the ATP-sensitive K+ channels (KATP) in the pancreas and heart (17). The membrane topology of the inwardly rectifying K+ channels is predicted to have one NH2-terminal cytoplasmic domain, two hydrophobic segments that span the membrane as α-helices (M1 and M2), one pore-forming partial membrane-spanning region (H5), and one long cytoplasmic COOH-terminal tail (26). Functional inwardly rectifying K+ channels are believed to be formed by four polypeptide subunits. The two membrane-spanning segments (M1 and M2) from each polypeptide subunit are coassembled in a manner to form a tetrameric structure with fourfold symmetry around a central pore (H5 region) (26).

ROMK channels are regulated by multiple signaling pathways, including protein kinase A (PKA), protein kinase C (PKC), and intracellular pH (pHi), etc. (10, 14). These signaling pathways play important roles in the hormonal regulation of renal K+ channels. For example, the PKA pathway is important for regulation of the K+ channels in TAL and CCD by vasopressin (10, 38). Intracellular acidification reversibly reduces the open probability (Po) of ROMK (3, 4, 33) and the native CCD K+ channels (32, 37). The reduction in K+ secretion by acidification plays a key role in K+ homeostasis during metabolic acidosis (38).

Recently, we and others reported a novel mechanism for the regulation of ROMK and other inwardly rectifying K+ channels via direct interaction with the membrane phospholipid phosphatidylinositol bisphosphate.
(PIP_2) (7, 11, 15). Using cloned channels expressed in Xenopus laevis oocytes and in vitro biochemical binding assays, we found that PIP_2 directly binds to the COOH-terminal cytoplasmic domains of ROMK1 and other inwardly rectifying K⁺ channels (15). This direct lipid-channel interaction underscores the regulation of the channels by PIP_2 and the specific structural determinants of ROMK1 for interaction with PIP_2 (15). The importance of PIP_2 for inwardly rectifying K⁺ channels is further supported by many subsequent studies showing that PIP_2 influences the regulation of the channels by other signaling or gating molecules. For example, PIP_2 modulates the regulation of GIRK channels by G_α_i (15, 36) and intracellular Na⁺ ions (36), the regulation of K_ATP by intracellular ATP (1, 35), and the regulation of ROMK by PKA (23) and by pH (21).

Besides inwardly rectifying K⁺ channels, PIP_2 interacts and regulates the function of many other proteins, including signaling proteins that contain a pleckstrin homology (PH) domain (12, 27). PH domains are regions which are ~100 amino acids long that are present in many signaling proteins (27). Although the overall homology in an amino acid sequence is low, PH domains share conserved secondary and tertiary structures (8, 9, 16). The three-dimensional structure of several PH domains, analyzed by the use of nuclear magnetic resonance spectroscopy and/or X-ray crystallography, reveals an antiparallel β-sheet of seven strands and a COOH-terminal amphipathic α-helix (8, 9, 16). The PH domains have an electrostatically polarized surface surrounded by many positively charged residues (8, 9, 16). This positively charged surface forms the binding site for the anionic PIP_2. The electrostatic interaction occurs between the positively charged amino acid residues and the negatively charged head groups (phosphates) within the inositol 1,4,5-trisphosphate (IP_3) moiety of PIP_2.

The three-dimensional structure of the PIP_2-binding region in the COOH-terminal cytoplasmic region of inwardly rectifying K⁺ channels is not known. However, several studies using site-directed mutagenesis have examined structural determinants of the COOH-terminal region for PIP_2 binding. In studying IRK1, GIRK1, and K_ATP, others have identified several basic residues in the proximal COOH terminus critical for interaction with PIP_2 (7, 34, 39). We have previously identified arginine-188 (R188) of ROMK1 as important for binding PIP_2 (15). In the present study, we further examine the structural determinants of ROMK1 for interaction with PIP_2 and the specificity of ROMK1 for phosphoinositide isoforms.

**METHODS**

**Phosphoinositides.** Dipalmitoyl-PI(4, 5)P_2 and diC_8-phosphoinositides were synthesized as described previously (5, 6, 29, 30). PI(4,5)P_2 and PI(4)P purified from bovine brain were purchased from Roche-Boehringer Mannheim.

**Molecular biology.** Wild-type ROMK1 cDNA was in the pSPORT plasmid (13). Site-directed mutagenesis of ROMK1 was performed using a commercial mutagenesis kit (Quick-change from Stratagene, La Jolla, CA) and confirmed by nucleotide sequencing as previously described (15, 21, 23).

mCAP cRNAs of the wild-type and mutant ROMK1 channels were transcribed in vitro using T7 RNA polymerase.

**Giant patch-clamp recording.** Xenopus laevis oocytes were injected with ~5 ng of cRNA for the wild-type or mutant ROMK1, and giant patch-clamp recording was performed as previously described (15, 21, 23). The pipette (extracellular) solution contained (in mM) 100 KCl, 2 CaCl_2, and 5 HEPES (pH 7.4). The bath (cytoplasmic) solution contained (in mM) either 100 KCl, 5 HEPES (pH 7.4), 5 EGTA, and 1 MgCl_2 (Mg_2⁺ solution) or 100 KCl, 5 HEPES, 5 EDTA, 4 NaF, 3 Na_3VO_4 and 10 Na_2P_2O_7 (FVPP) solution as indicated for each experiment. Inward K⁺ currents (at ~30 mV holding potential, at 23–25°C) were recorded onto a chart recorder using an Axopatch 200B amplifier, pCLAMP software, and a Digidata 1200A digitizer (Axon Instruments, Foster City, CA). Chart recording strips were digitally scanned and analyzed in a computer for presentation. Each vial of anti-PIP_2 monoclonal antibody stock (Perseptive Biosystems, Framingham, MA) was reconstituted in 0.5 ml of distilled water and diluted 40-fold into experimental solutions to yield a final concentration of 40 nM. PIP_2 (Boehringer Mannheim) was diluted in water (1 mg/ml) and sonicated to form liposomes (15, 21, 23).

**Single-channel patch-clamp recording.** Patch-clamp pipettes (pulled from borosilicate glass, Warner Instrument, Hamden, CT) were filled with solutions containing (in mM) 100 KCl, 1 MgCl_2, 2 CaCl_2, and 5 HEPES (pH 7.4 with KOH). Pipette tip resistance ranged from 3 to 5 MΩ. The Mg_2⁺-free, Mg_2⁺-containing, and FVPP bath solutions were the same as used for giant patch-clamp recording. Single-channel currents were recorded with an Axopatch 200B patch-clamp amplifier (Axon Instruments), low-pass filtered at 1 kHz with the use of an eight-pole Bessel filter, sampled every 0.1 ms (10 kHz) with a Digidata-1200A interface digitizer, and stored directly onto a computer hard disk (20 GB) using pCLAMP7 software. Data were transferred to compact disks for long-term storage. For analysis, event list files were generated using the Petchan program and analyzed for P_0, amplitude, and dwell-time histograms using pCLAMP7 pSTAT (version 6.0.5, Axon Instruments). P_0 was analyzed on segments of continuous recordings (at least 5 min) from patches that contained only one active channel during the lifetime (~20 min) of the recording. P_0 for full- and subconductance opening in the same patch was determined using the criteria of threshold crossing of the current levels assigned for closed, sub- and full states (i.e., baseline for closed, current level 1 and current level 2 for sub- and full states, respectively). The current level for each state was assigned on the basis of visual inspection of a long segment of recordings for best fit and comparison with the current peaks in the amplitude histogram. For amplitude histogram analysis, only periods of recordings containing bursts of channel activity were used.

**RESULTS**

Effects of substitution of glutamine or alanine for lysine-181, arginine-217, and lysine-218 on PIP_2 interaction with ROMK1 channels. We have previously reported that regulation of ROMK1 and other inwardly rectifying K⁺ channels by PIP_2 involves direct binding of PIP_2 to a putative PIP_2-binding region in the COOH terminus of the channels (15). One of the residues involved in this interaction of ROMK1 with PIP_2 is the amino acid R188 (15). Substitution of glutamine for R188 (R188Q) reduces PIP_2-ROMK1 interaction, as
evidenced by a reduced binding of the COOH terminus of ROMK1 to [3H]PIP2 in vitro (15) and an increased sensitivity of the channels to inhibition by anti-PIP2 antibodies in the giant excised inside-out patches (15). To further examine the structural determinants of the putative PIP2-binding region, we systematically substituted a neutral amino acid (glutamine or alanine) for each of the positively charged amino acids within this region by site-directed mutagenesis. The interaction of each of the positively charged amino acids within this region with the putative PIP2-binding region, we systematically substituted a neutral amino acid (glutamine or alanine) for (WT) and leucine-181Q (K181Q), arginine-217A (R217A), and lysine-218A (K218A) showed an increased sensitivity to inhibition by anti-PIP2 antibodies. The $t_{1/2}$ of inhibition by anti-PIP2 antibodies for these mutants were 12 ± 1, 32 ± 2, and 28 ± 3 s, respectively (Fig. 1B, Table 1).

Effects of neutralization of the positive charge of amino acids K181, R217, and K218 on single-channel conductance and $P_o$. Reduction of PIP2-ROMK1 interaction by either Mg$^{2+}$-induced rundown or mutation of a PIP2-binding residue (as in R188Q) leads to a reduction in $P_o$ for the full-conductance state and the emergence of subconductance states (21). The effects of charge neutralization of putative PIP2-binding residues on single-channel $P_o$ and conductance were studied by cell-attached (on-cell) recording of oocytes expressing the channels. Recordings were analyzed from the start recorded in the cell-attached mode, followed by recordings in excised inside-out patches. After stabilization of currents in inside-out patches, anti-PIP2 antibodies were applied to the cytoplasmic face to study the effects on the channels. Figure 1B shows representative tracings of wild-type ROMK1 channels and those mutants with increased sensitivity to inhibition by anti-PIP2 antibodies. As shown in our previous report (15), the activities of wild-type ROMK1 channels and the R188Q mutant were inhibited by anti-PIP2 antibodies with a half-time ($t_{1/2}$) of 100 ± 7 and 14 ± 3 s, respectively. The mutants lysine-181Q (K181Q), arginine-217A (R217A), and lysine-218A (K218A) showed an increased sensitivity to inhibition by anti-PIP2 antibodies. The $t_{1/2}$ of inhibition by anti-PIP2 antibodies for these mutants were 12 ± 1, 32 ± 2, and 28 ± 3 s, respectively (Fig. 1B, Table 1). Figure 1C is a summary of the results of measurements of $t_{1/2}$ for wild-type ROMK1 and all of the substitution mutants. As shown, substitution of glutamine or alanine for other cationic amino acids [except lysine-181 (K181), R188, arginine-217 (R217), and lysine-218A (K218)] within this region did not affect channel sensitivity to anti-PIP2 antibodies (i.e., $t_{1/2}$ was not significantly different from that of wild-type). These results indicate that neutralization of the positive charge of amino acids K181, R217, K218, or R188 reduces the interaction of the channels with PIP2.

**Effects of neutralization of the positive charge of amino acids K181, R217, and K218 on single-channel conductance and $P_o$.** Reduction of PIP<sub>2</sub>-ROMK1 interaction by either Mg$^{2+}$-induced rundown or mutation of a PIP<sub>2</sub>-binding residue (as in R188Q) leads to a reduction in $P_o$ for the full-conductance state and the emergence of subconductance states (21). The effects of charge neutralization of putative PIP<sub>2</sub>-binding residues on single-channel $P_o$ and conductance were studied by cell-attached (on-cell) recording of oocytes expressing the channels. Recordings were analyzed from the start recorded in the cell-attached mode, followed by recordings in excised inside-out patches. After stabilization of currents in inside-out patches, anti-PIP2 antibodies were applied to the cytoplasmic face to study the effects on the channels. Figure 1B shows representative tracings of wild-type ROMK1 channels and those mutants with increased sensitivity to inhibition by anti-PIP2 antibodies. As shown in our previous report (15), the activities of wild-type ROMK1 channels and the R188Q mutant were inhibited by anti-PIP2 antibodies with a half-time ($t_{1/2}$) of 100 ± 7 and 14 ± 3 s, respectively. The mutants lysine-181Q (K181Q), arginine-217A (R217A), and lysine-218A (K218A) showed an increased sensitivity to inhibition by anti-PIP2 antibodies. The $t_{1/2}$ of inhibition by anti-PIP2 antibodies for these mutants were 12 ± 1, 32 ± 2, and 28 ± 3 s, respectively (Fig. 1B, Table 1).

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membrane patches containing only one channel during the lifetime of the recording.

Wild-type ROMK1 channels recorded on-cell from oocytes have a near-maximal single-channel $P_o$ of $0.91 \pm 0.08$, $n = 5$ (Fig. 2A; see also Table 1 and Ref. 21). The single-channel current-voltage relationship for channels recorded on-cell is weakly inwardly rectifying, with an inward slope conductance of $36 \pm 2.3$ pS (from 0 to $-100$ mV; $n = 5$; not shown). As reported previously by us and others, opening at subconductance states was rarely observed for wild-type channels during on-cell recording (21, 25). Figure 2B is a representative on-cell recording from oocytes expressing R188Q, showing a reduced $P_o$ for full-conductance opening ($0.14 \pm 0.01$; see also Table 1) and emergence of an opening at a subconductance state of $0.13 \pm 0.01$ (44% of the full, open state). The $P_o$ of the subconductance opening is $0.13 \pm 0.01$ (Table 1). Existence of the subconductance state is further revealed in the amplitude histogram analysis (Fig. 2B, bottom, where C, S, and O indicate closed, subconductance and full-conductance state, respectively).

Substitution of a neutral amino acid for K181, R217, and K218 also reduced full-conductance opening and increased frequency of subconductance opening for wild-type ROMK1 channels recorded on-cell from oocytes expressing R188Q, showing a reduced $P_o$ for full-conductance opening ($0.14 \pm 0.01$; see also Table 1) and emergence of an opening at a subconductance state of $1.1$ pS ($44\%$ of the full, open state). The $P_o$ of the subconductance opening is $0.13 \pm 0.01$ (Table 1). Existence of the subconductance state is further revealed in the amplitude histogram analysis (Fig. 2B, bottom, where C, S, and O indicate closed, subconductance and full-conductance state, respectively).

Substitution of a neutral amino acid for K181, R217, and K218 also reduced full-conductance opening and increased frequency of subconductance opening for

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Values are means ± SE. WT, wild-type; $P_o$ (F) and $P_o$ (S), full-conductance and subconductance channel opening, respectively; K, leucine; R, arginine; $t_{1/2}$, half-time for inhibition of anti-phosphatidylinositol bisphosphate antibody; $pK_a$, intracellular pH of 50% inhibition by intracellular acid; ND, not determined; NS, not significant. Bold indicates increased sensitivity to inhibition by anti-phosphatidylinositol bisphosphate antibodies. *$P < 0.05$ compared with WT (unpaired t-test).

Fig. 2. Effects of charge neutralization on single-channel open probability ($P_o$) and conductance. A–E: on-cell recording of a single channel at $-100$ mV for WT (A), R188Q (B), K181Q (C), R217A (D), and K218A (E) channels, respectively. Top, middle, and bottom: recording at a compressed time base, recording at an expanded time base, and amplitude histogram, respectively. In E, time and amplitude scale are shown on the right. For amplitude histogram analysis, multiple periods of recording containing bursts of channel activity were used. Bin width = 0.1 pA. Line indicates fit by the sum of 2 (WT) or 3 (mutants) Gaussian distributions. O, S, and C: full-conductance, subconductance and closed level, respectively.
ROMK1 (Fig. 2, C–E). The \( P_o \) values for full-conductance and subconductance opening were \( 0.1 \pm 0.01 \) and \( 0.07 \pm 0.005 \) for K181Q, 0.67 \( \pm \) 0.02 and 0.03 \( \pm \) 0.003 for R217A, and 0.77 \( \pm \) 0.03 and 0.04 \( \pm \) 0.002 for K218A, respectively (Fig. 2, Table 1). These results, taken together with the effects of anti-PIP\(_2\) antibodies, suggest that charge-charge interactions between PIP\(_2\) and K181, R217, and K218 as well as R188 are critical for ROMK1 to open at full conductance. Disruption of PIP\(_2\)-channel interaction favors ROMK1 to enter the subconductance and the closed states. Charge neutralization for other amino acids within the region of amino acids 180–227 of ROMK1 did not cause an increase in the frequency of the subconductance state (Table 1). With respect to the \( P_o \) for the full-conductance state, we observed a small but statistically significant reduction for mutants R184Q and R212A (0.68 \( \pm \) 0.02 and 0.79 \( \pm \) 0.02, respectively; see Table 1). The opening of ROMK1 is likely also controlled by other factors besides PIP\(_2\)-channel interaction. The reduction in \( P_o \) for the full-conductance state without a concomitant increase in \( P_o \) for that subconductance state raises the possibility that mutations of amino acids R184 and R212 reduce the opening of ROMK1 via these other factors rather than through affecting the channel’s interaction with PIP\(_2\). This idea is further supported by the lack of a shift in \( pHi \) sensitivity for these two mutants (see Fig. 3, Table 1).

Charge neutralization of K181, R217, or K218 increases the sensitivity of the channels to intracellular protons. Intracellular acidification leads to channel closure (3, 4). The regulation of ROMK1 by intracellular protons is influenced by PIP\(_2\)-channel interaction. For example, disruption of PIP\(_2\)-ROMK1 interaction by the R188Q mutation increases the sensitivity of the channels to inhibition by intracellular protons (21). We therefore further examined \( pHi \)-dependent regulation of those mutant ROMK1 channels with reduced PIP\(_2\) interaction, as revealed by the above experiments using anti-PIP\(_2\) antibodies and by single-channel analysis. Wild-type and mutant K181Q, R217A, and K218A channels were expressed in oocytes, and the activities of the channels were recorded in giant patch-clamp recordings. The activity of wild-type channels (measured as inward K\(^+\) currents at \(-30\) mV holding potential) was near maximal on-cell (where the resting \( pHi \) is \(-7.32\); see Ref. 20) and was not significantly increased when the cytoplasmic face of the excised inside-out membrane patches was alkalinized to \( pHi \) 9.4 (21). As shown in Fig. 3A, currents were relatively unchanged from \( pHi \) 9.4 to 7.4. Acidification in the cytoplasmic face below \( pHi \) 7.4, however, inhibited currents in a steep, \( pHi \)-dependent manner. The effective acidic dissociation constant (\( pK_a \)) for inhibition of wild-type ROMK1 by intracellular protons was 6.84 \( \pm \) 0.04 (mean \( \pm \) SE; \( n = 5 \); Fig. 3, A and E, Table 1). Unlike wild-type ROMK1, the activities of K181Q, R217A, and K218A mutants were already \(<50\%\) of the maximal level at \( pHi \sim 7.4 \), indicating that the channel’s sensitivity to inhibition by intracellular protons was i...
increased (Fig. 3, B–D). The effective pK_a values for these mutants were shifted toward alkaline pH: the values were 7.71 ± 0.03, 7.65 ± 0.03, and 7.59 ± 0.03 for K181Q, R217A, and K218A, respectively (Fig. 3E, Table 1). The direction of the alkaline shift in effective pK_a values for those mutants with normal PIP2 interaction (based on studies using anti-PI(4,5)P_2 antibodies and single-channel analysis) were not significantly different from that of wild-type ROMK1 (Table 1). These results, together with the results of single-channel studies and of the studies using anti-PI(4,5)P_2 antibodies, support the idea that amino acids K181, R188, R217, and K218 but not other amino acids within the region of amino acids 180–227 of ROMK1 are critical for interaction with PIP2.

Effects of phosphoinositides with different acyl chains on ROMK1 channels. It has been shown that phosphoinositides with different acyl chains or a different number and position of phosphates in the head groups have different efficacies in activation of some but not other members of inwardly rectifying K^+ channels (31). In the present study, we examined the effects of these different phosphoinositides on ROMK1. The effects of acyl chains on the efficacy of phosphoinositides to activate ROMK1 were examined using purified bovine brain PI(4,5)P_2 (containing mostly arachidonyl and stearoyl acyl chains) or synthetic PI(4,5)P_2 with palmitoyl-C_16 or 8-carbon (C_8) in positions SN-1 and SN-2 of glycerol.

Oocytes expressing ROMK1 were recorded by micro-patch patch-clamp recording. After the recording of channel activity in cell-attached (on-cell) mode, membrane patches were excised in Mg^2+-containing solutions to allow rundown of the channels. Different phosphoinositides were then applied to the cytoplasmic face of the inside-out membranes in the FVPP solution. The top panel of Fig. 4A is a recording from one of the representative experiments of application of the native PI(4,5)P_2. As shown, the activity of ROMK1 (shown as NP_o, the product of P_o and the no. of channels) in the excised patches decreased to near 0 over ~7 min. Application of purified bovine brain PI(4,5)P_2 (50 μM) fully activated channels over ~15 min. Thereafter, the activity of channels remained stable for at least 30 min despite extensive washing of the cytoplasmic face of membrane patches with FVPP solution ("washout" in Fig. 4A, top; only part of the recording of the washout period is shown). The average results of similar experiments using native PI(4,5)P_2 were summarized and are shown in the bottom panel of Fig. 4A. Compared with the native PI(4,5)P_2, C_8-PI(4,5)P_2 was less effective in

![Fig. 4. Effect of acyl chains of phosphoinositides on ROMK1 channel activity. A: effect of purified bovine brain PI(4,5)P_2. The activity (NP_o) of WT ROMK1 channels in oocytes was measured by micropatch patch-clamp recording. Current levels for closed state (C) and different numbers of open channels are indicated. NP_o (averaged every minute) was measured using a program as described previously (25). Membrane patches were excised into Mg^2+-containing solutions. After excision, the inside-out patches were placed in a perfusion chamber (with a volume capacity of ~50 μl), and the cytoplasmic face was perfused with FVPP solution containing sonicated PI(4,5)P_2 liposomes (50 μM). In each experiment, a total of ~500 μl of PI(4,5)P_2-containing FVPP solution was perfused over ~1 min. Thereafter, membrane patches were bathed in the same PI(4,5)P_2-containing FVPP solution until washout (see below). As shown, perfusion of PI(4,5)P_2 increased NP_o of the channels. Without PI(4,5)P_2, the FVPP solution by itself did not cause an increase in channel activity (not shown). After the activity of the channels stabilized in the presence of PI(4,5)P_2, membrane patches were washed with at least 5 ml of PI(4,5)P_2-free FVPP solution (washout). Bottom panels are means ± SE of the normalized P_o from 7 similar experiments. A different number of channels was recorded in each individual experiment. Normalized P_o for each individual experiment was calculated by dividing the measured NP_o by the number of channels observed in the on-cell configuration. B: effect of the synthetic C_8-PI(4,5)P_2. The experimental paradigm was the same as in A. *P < 0.05 by unpaired t-test, compared with the on-cell level.](http://ajprenal.physiology.org/)
activation of ROMK1. As shown in Fig. 4B, application of C₈-PI(4, 5)P₂ (50 μM) after channel rundown only activated channels to 53 ± 13% (n = 6) of the on-cell level. Compared with the native PI(4, 5)P₂ containing arachidonyl and stearyl acyl chains, C₈-PI(4, 5)P₂ is more soluble in aqueous solution (has a lower lipid-to-water partition ratio). The lower efficacy for C₈-PI(4, 5)P₂ suggests that incorporation of the lipid into the membrane is necessary for activation of the channels. Interestingly, the effects of C₈-PI(4, 5)P₂ on the channels were reversible by extensive washing of the inside-out membrane patches, presumably also due to an increased partition of the lipid in aqueous solution. The effects of C₁₆-PIP₂ (50 μM) on ROMK1 were not significantly different from those of the purified bovine brain PIP₂ (activated channels to 94 ± 13% of the on-cell level, n = 5; recordings not shown).

**Effects of phosphoinositides with different phosphate head groups on ROMK1 channels.** We first examined the effects of the number of phosphates by comparing PI(4)P and PI(4, 5)P₂. Both submaximal (5 μM) and maximal concentrations (50 μM) of purified bovine brain PI(4)P and PI(4, 5)P₂ were used. At 5 μM, PI(4)P and PI(4, 5)P₂ activated ROMK1 (after rundown in Mg²⁺ solutions) to 9.8 ± 5.4 and 89 ± 13% of the on-cell level, respectively (n = 4 for each). Maximal concentration of PI(4)P and PI(4, 5)P₂ activated ROMK1 to 20 ± 14 and 95 ± 12% of the on-cell level, respectively (n = 4 for each). We next examined the dependence of the position of the phosphate in the head group in the activation of ROMK1. To avoid the potential contribution from differences in the acyl chains among the different native phosphoinositide preparations, these questions were examined using synthetic C₈-phosphoinositides. The reversibility of the effects of diC₈-phosphoinositides on channels also helps to determine the specific effects caused by application of lipids. As shown in Fig. 5, A and C, C₈-PI(3, 4, 5)P₃ was as effective as C₈-PI(4, 5)P₂ in activating ROMK1 [activated channels to 54 ± 15 (50 μM, n = 5) and 27 ± 13% (5 μM, n = 4) of on-cell level vs. to 53 ± 13 (50 μM, n = 6) and 21 ± 9% (5 μM, n = 5), respectively, of on-cell level]. For comparison, C₈-PI(3, 4)P₂ was less effective [activated channels to 24 ± 12 (50 μM, n = 50) and 4.3 ± 2.1% (95 μM, n = 5) of the on-cell level; Fig. 5, B and C; P < 0.05, C₈-PI(3, 4)P₂ vs. C₈-PI(4, 5)P₂]. These results, together with the findings that PI(4)P is less effective than PI(4, 5)P₂, suggest that phosphates at positions 4 and 5 of the inositol moiety of phosphoinositides are important for interaction with ROMK1 channels.

**DISCUSSION**

PIP₂ participates in many crucial cellular functions. As an important signal-generating lipid precursor, PIP₂ hydrolyzes in response to activation of phospholipase C and gives rise to IP₃ and diacylglycerol (DAG). IP₃ and DAG, by increasing intracellular Ca²⁺ and activating PKC, respectively, regulate functions of many proteins. Recently, PIP₂ itself has emerged as a major signaling molecule (12). It is now known that PIP₂ interacts and regulates functions of a wide range of PH domain-containing signaling proteins, proteins involved in membrane transport, cytoskeletal proteins, and many ion channels and transporters (12, 27). Among the ion channels regulated by PIP₂ are members of the inwardly rectifying K⁺ channels (14).

The structure of the PIP₂-binding region of inwardly rectifying K⁺ channels is not known. We have shown previously that the COOH-terminal cytoplasmic domain of the inwardly rectifying K⁺ channels interacts with PIP₂ directly and the direct interaction is important for opening of the channels (15). One of the basic amino acids in the COOH terminus of ROMK1, R188, was found to be important for this binding with PIP₂ (15). In the present study, we further examined the structural elements of ROMK1 for interacting with...
The interactions between PIP2 and PIP2-binding proteins are predominantly electrostatic in nature (27). By systematically substituting neutral amino acids for positive charges in the proximal COOH terminus of the channel, we now report that (in addition to R188, K181, R217, and K218 of ROMK1) are also important for regulation of ROMK1 by PIP2. The marked reduction in PIP2 regulation of ROMK1 by a single mutation suggests that, like the interaction with the PH domains (9), the interaction of PIP2 with the multiple basic residues of ROMK1 are probably cooperative.

The structural elements of IRK1 and KATP (Kir6.2) for interaction with PIP2 have also been studied (7, 34, 39). An earlier study by Fan and Makielski (7) reported that mutation of R176 and R177 (equivalent to K187 and R188 of ROMK1) decreased the regulation of the channel by PIP2. A more recent study by Shyng et al. (34) identified five additional basic residues (R195, R206, K222, R230, and R314) of Kir6.2 important for regulation by PIP2. Focusing on amino acids 207–245 of IRK1, Zhang et al. (39) identified R218 and R228 as critical for regulation by PIP2. Some of the residues of IRK1 and Kir6.2 important for PIP2 regulation are conserved on ROMK1. These conserved residues include R176 and R206 of Kir6.2 (equivalent to R188 and R217 of ROMK1, respectively), and R218 of IRK1 (equivalent to R217 of ROMK1). However, other critical residues for regulation of these channels, such as R176 and R195 of Kir6.2 (equivalent to K187 and R206 of ROMK1), are not involved in PIP2 regulation of ROMK1. Although members of inwardly rectifying K+ channels share the same fundamental molecular structure, particularly in the transmembrane and the pore regions, the regulation of these channels by the intracellularly generated signaling molecules are quite different. For example, Kir6.2 channels are sensitive to inhibition by an increase in intracellular ATP (26). Intracellular acidification inhibits ROMK1 but not other inwardly rectifying K+ channels (3, 4, 26). Activation of PKA regulates ROMK1 (23) and Kir6.2 (22) but not IRK1. Thus it is perhaps no surprise that the structural elements of the PIP2-binding region residing in the intracellular COOH-terminal domain are only partially conserved among members of the inwardly rectifying K+ channels.

We also examined the effects of different phosphoinositide isoforms on the channels. The efficacy of PI(4)P to activate ROMK1 is ∼10–20% of that of PI(4, 5)P2. The efficacy of PI(4, 5)P2 and PI(3, 4, 5)P3 are about equal, and PI(3, 4)P2 is ∼45% as potent compared with the other two. In most cells, PI(4)P and PI(4, 5)P2 are about equal in abundance and constitute ∼1% of anionic phospholipids, respectively (12, 27). The abundance of the D3 isoforms of phosphoinositides PI(3, 4)P2 and PI(3, 4, 5)P3 is generally only a few percentage points of that of PI(4, 5)P2 in the basal state (27). Thus PI(4, 5)P2 is likely the most relevant phosphoinositide in the regulation of ROMK1 in the physiological membrane milieu. The specificity of ROMK1 for phosphoinositides is similar to that of IRK1 reported by Rohács et al. (31). This study by Rohács et al. also reported that, in contrast to IRK1, GIRK1/GIRK4 channels showed little specificity toward phosphates in position 3, 4, or 5 of the inositol head group. Compared with GIRK channels, ROMK1 and IRK1 have a much higher affinity for PIP2 (15). Whether the low specificity of GIRK for phosphates in the inositol ring of phosphoinositides is directly related to the lower affinity of the channel for PI(4,5)P2 requires further investigation.

How direct interaction with PIP2 regulates opening of the inwardly rectifying K+ channels remains elusive. One mechanism for PIP2 regulation of protein function is by influencing the localization of proteins via binding to PIP2-binding domains in the proteins (12, 14, 27). For example, the interaction of the β-adrenergic receptor kinase (via its PH domain) with PIP2 works synergistically with the G protein βγ-subunits (Gβγ) to facilitate translocation of the proteins to the plasma membrane (28). Many proteins bind PIP2 (27). Except for those proteins with PH domains, the majority of the PIP2-binding proteins have no obvious primary sequence and/or structural homology among the PIP2-binding regions (27). However, all of the putative PIP2-binding sites identified to date contain multiple cationic residues. PIP2 is a trivalent anion at the physiological pH. If the multiple cationic residues of PIP2-binding proteins are clustered near one exposed surface, the cooperative electrostatic interactions between these residues and PIP2 would likely provide a fairly stable interaction at the lipid-protein interface of a native membrane (27).

The three-dimensional structure of the COOH-terminal cytoplasmic domain of inwardly rectifying K+ channels is not yet available. Using cysteine-scanning mutagenesis, Lu et al. (24) recently reported that many residues in the region of amino acids 215–234 of IRK1 (equivalent to 214–233 of ROMK1) are accessible to methyl-thio-sulfhydryl reagents (24). Reaction with these reagents affects K+ ion permeation. These results suggest that at least part of this region of IRK1 (and conceivably the equivalent region of ROMK1) is accessible to permeant ions and forms part of the cytoplasmic entrance of the pore (24). PIP2-binding region of ROMK1 resides between this region of the cytoplasmic entrance of the pore and the second transmembrane domain. Thus it is conceivable that binding of the PIP2-binding region of ROMK1 to PIP2 in the inner leaflet of the plasma membrane regulates channel opening by stabilizing the structure of the cytoplasmic entrance of the pore. Our recent report that reduction of PIP2-channel interaction decreases single-channel P0 of the channels as well as favors ROMK1 channels to enter subconductance states (21) lends support to this hypothesis. Many hormones or growth factors alter PIP2 metabolism through activation of PLC, phosphoinositide kinases, and/or phosphatases (12, 14). The role of these hormones in regulating the activity of ROMK channels in the kidney via alteration of PIP2 channel interaction awaits future investigation.

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