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ORIGINAL PAPER

Protein kinase C mediated pH_i-regulation of ROMK1 channels via a phosphatidylinositol-4,5-bisphosphate-dependent mechanism

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Abstract The protein kinase C (PKC) pathway is important for the regulation of K^+ transport. The renal outer medullar K^+ (ROMK1) channels show an exquisite sensitivity to intracellular protons (pH_i) (effective pK_a approximately 6.8) and play a key role in K^+ homeostasis during metabolic acidosis. Our molecular dynamic simulation results suggest that PKC-mediated phosphorylation on Thr-193 may disrupt the PIP_2-channel interaction via a charge–charge interaction between Thr-193 and Arg-188. Therefore, we investigated the role of PKC and pH_i in regulation of ROMK1 channel activity using a giant patch clamp with *Xenopus* oocytes expressing wild-type and mutant ROMK1 channels. ROMK1 channels pre-incubated with the PKC activator phorbol-12-myristate-13-acetate exhibited increased sensitivity to pH_i (effective pK_a shifted to pH approximately 7.0). In the presence of GF109203X—a PKC selective inhibitor—the effective pK_a for inhibition of ROMK1 channels by pH_i decreased (effective pK_a shifted to pH approximately 6.5). The pH_i sensitivity of ROMK1 channels mediated by PKC appeared to be dependent of PIP_2 depletion. The giant patch clamp together with site direct mutagenesis revealed that Thr-193 is the phosphorylation site on PKC that regulates the pH_i sensitivity of ROMK1 channels. Mutation of PKC-induced phosphorylation sites (T193A) decreases the pH_i sensitivity and increases the interaction of channel-PIP_2.

Po-Tsang Huang and Chien-Hsing Lee contributed equally to this work.

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Taken together, these results provide new insights into the molecular mechanisms underlying the pH_i gating of ROMK1 channel regulation by PKC.

Keywords PKC · ROMK1 channel · PIP_2 · Intracellular proton · Effective pK_a · PMA

Abbreviations

PKC	Protein kinase C
ROMK1	Renal outer medullary potassium
pH_i	Intracellular pH
PIP_2	Phosphatidylinositol 4,5-bisphosphate
MD	Molecular dynamic

Introduction

The protein kinase C (PKC) pathway is important for the regulation of K^+ transport in cortical collecting ducts (CCDs) that is mediated by many PLC-activating hormones and growth factors, including prostaglandin E2, bradykinin, and epidermal growth factor [1, 2]. Renal outer medullary potassium (ROMK1) channels in the thick ascending limb mediate K^+ recycling across the apical membrane and are involved in K^+ secretion in CCDs [3]. Activation of PKC by phorbol 12-myristate 13-acetate (PMA) inhibits apical K^+ channels in CCDs [2]. Activation of PKC is likely the mechanism whereby PLC-activating hormones inhibit ROMK1 channels in the apical membrane of CCDs to prevent excess kaliuresis during natriuresis (occurs as a result of inhibition of Na^+ reabsorption at the proximal sites) [2, 4]. In addition, PKC-mediated phosphorylation is essential for regulating ROMK1 channel activity through the expression of channels in the cell surface [5] and the stimulation of protein tyrosine kinase (PTK) activity [6]. PKC-dependent pathways are involved in ROMK1 channel activity under conditions of dietary potassium restriction in the CCDs regulated by AngII [7]. Thus, PKC plays an important role in the regulation of ROMK1 channel activity under physiological and pathophysiological states.

ROMK1 channel activity is not only regulated by PKC-mediated phosphorylation, but is also highly sensitive to changes in cytosolic pH (pH_i) [8–12]. Intracellular acidification reversibly reduces the open probability (P_o) of native K^+ channels in CCDs as well as that of ROMK1 channels expressed in oocytes, with an effective pK_a value of ~6.8 [8–12]. This pH_i sensitivity provides an important mechanism for linking renal K^+ excretion with acid-base balance [13]. We have previously shown that the molecular mechanisms underlying PKA-mediated phosphorylation in regulating ROMK1 channels and pH_i gating defects are associated with hyperprostaglandin E syndrome/antenatal Bartter syndrome [9]. PKC has been reported to antagonize PKA activation of ROMK1 channels in CCDs [2, 14], but the molecular mechanisms for the regulation of pH_i in ROMK1 mediated by PKC remain poorly understood. Thus, in this study, we investigated the relationships between PKC-mediated phosphorylation and pH_i in the regulation of ROMK1 channel activity using molecule dynamics (MD) simulations and a giant patch clamp system.

Materials and methods

Homology modeling of ROMK1

Homology modeling was performed following previously described procedures [9]. Briefly, residues of the ROMK1 channel chosen according to the results of GCG paired sequence alignment were superimposed onto the structure coordinates of the Cα atoms of the corresponding structural conserved regions (SCRs) from the template channel structure (PDB ID: 3JYC). This generated the secondary structure and relative positions of the defined structural elements in the chosen residues of the ROMK1 model. Junctions between secondary structural elements were regularized individually by energy minimization to give a reasonable geometry. Hydrophobic/hydrophilic interactions between residue side chains were observed from the model to provide the required structural–functional interpretation. All calculations and structure manipulations were performed using the Discover/Insight II molecular simulation and modeling program (Accelrys, San Diego, CA; release 950) on Silicon Graphics Octane/SSE and O2/R12000 workstations and an O-300 server.

Molecular dynamics simulations

MD simulations were performed using InsightII 2005 and the Chemistry at Harvard Molecular Mechanics (CHARMM) special forcefield c27b4 as previously described [9]. The system is in an appropriate environment with the TIP3P water model. Additional counterions were added so that the overall net charge on the system was zero. The final system consisted of ~100,000 atoms. During this equilibration process, the water molecules and ions were free to move. All simulations were performed following previously described procedures [9]. The MD simulations were performed only in implicit membranes not real biomembranes. The parameters employed to set up the simulation used Berendsen coupling to maintain a constant temperature of 300 K and a constant pressure of 1 bar. Van der Waal's interactions were modeled using a 6–12 (or Lennard Jones, LJ) potential with a cutoff value of 12 Å. The particle-mesh Ewald method was used to identify long-range electrostatic interactions with a cutoff of 12 Å. The

linear constraint solver algorithm was used to constrain the covalent bonds.

Molecular biology

Site-directed mutagenesis was performed using a commercial mutagenesis kit (Stratagene, La Jolla, CA) and confirmed by nucleotide sequencing as described [9, 15]. Capped mRNAs of the wild-type and mutant channels were transcribed in vitro using T7 RNA polymerase (Ambion; http://www.ambion.com) [9, 11, 15].

Oocytes preparation and injection

Female *Xenopus laevis* frogs were anesthetized briefly by immersion in 0.1% 3-aminobenzoic acid ethyl ester, and a few lobes of the ovaries were removed after a small abdominal incision. The incision was sutured and the frogs were allowed to revive after surgery [9, 16]. The oocytes were incubated for 90 min at room temperature (23–25°C) with 2 mg/ml collagenase (Type I, Sigma, St. Louis, MO) in OR_2 solution consisting of (in mM) 82 NaCl, 2 KCl, 1 $MgCl_2$, and 5 HEPES, pH 7.4, to remove the follicular layer. After ten washes with OR_2 solution, oocytes at Dumont stage V–VI were selected and injected with 30 ng mRNA, then incubated at 18°C in ND96 solution consisting of (in mM) 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES, pH 7.5, supplemented with 100 mg/l penicillin-streptomycin and 10 mg/ml geneticin. Channel activity was assessed 3 days post-injection.

Giant patch-clamp recording

Xenopus oocytes were injected with wild-type or mutant ROMK1 mRNA and giant patch-clamp recording was performed as described previously [9–11, 15]. The pipette (extracellular) solution contained (in mM) 100 KCl, 2 $CaCl_2$, and 5 HEPES, pH 7.4, while the bath (cytoplasmic) solution contained either 100 KCl, 5 HEPES, 5 EGTA, and 1 $MgCl_2$ (pH 7.4) (Mg^{2+} solution) or 100 KCl, 5 HEPES, 5 EDTA, 4 NaF, 3 Na_3VO_4, and 10 $Na_4P_2O_7$ (FVPP solution) as indicated for each experiment. Inward K^+ currents (at a −60 mV holding potential at 23–25 °C) were recorded on a chart recorder using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) and the recorder strips were scanned and analyzed on a computer. All the data were expressed as the mean±standard error of the mean.

$$\sigma_M = \frac{\sigma}{\sqrt{N}} \quad (1)$$

where σ is the standard deviation of the original distribution, and N is the sample size (the number of scores each mean is based upon). Statistical analysis was performed using analysis of variance (ANOVA) followed by Tukey-Kramer post hoc test when significance was reached in the ANOVA. Differences were considered significant at $P<0.05$.

Drug treatment and administration

Each vial of anti-PIP_2 mAb stock (PreSeptive Biosystems, Framingham, MA) was reconstituted in 0.5 ml distilled water and diluted 40:1 under experimental solutions (to yield a final concentration of 40 nM) [15]. PMA (Sigma) and GF109203X (Sigma) were dissolved in dimethylsulfoxide. In the experiments on the role of PKC in regulating the pH_i sensitivity of ROMK1 channels, the injected oocytes were pre-incubated for 5 min with PMA (300 nM) [2] and GF109203X (10 μM) [17, 18] in ND96 solution before inside-out patch clamp recordings.

Results

Structural analysis of ROMK1 channel phosphorylation

To better understand the regulation of pH_i in ROMK1 mediated by PKC from a structural perspective, we employed a combination of homology modeling and MD simulations. Figure 1a shows a comparison of the sequences of the ROMK1 channel (GenBank accession number P48048) and the closely related Kir2.2 channels (PDB ID: 3JYC). The Kir2.2 channel shares approximately 50% sequence identity with ROMK1 channels. Residues 40–368 in the ROMK1 channel sequence were aligned with residues 43–370 of the Kir2.2 sequence for homology modeling. Calculated sequence identities for residues in the N-terminal (40–76 in ROMK1 and 43–69 in Kir2.2), TM1 (77–120 in ROMK1 and 70–122 in Kir2.2), pore (121–153 in ROMK1 and 123–155 in Kir2.2), TM2 (154–175 in ROMK1 and 156–177 in Kir2.2) and C-terminal (176–368 in ROMK1 and 178–370 in Kir2.2) domains are 40.5%, 38.1%, 53.4%, 61%, and 37.7%, respectively, with similarities of 68.5%, 65.6%, 81.7%, 86% and 69.5%, respectively. Figure 1b shows the homology model of the ROMK1 channel based on the crystal structure of the Kir2.2 channel, which is more suitable than the KirBac1.1 channel. The important motifs, such as pore helices and selective filter, are located at pore domains, and the glycine hinges are located at the end of TM2. The TM domains of the tetrameric ROMK1 channel structure comprise TM1 and TM2 in each subunit (Fig. 1b). Several crucial structural features in the intracellular domains are highlighted and the residue numbers indicated. For example, the PKC phosphorylation site, Thr-193, is located in the C-terminal domain and juxtaposed to the PIP_2 binding site residues Arg-188, Arg-217, and Lys-218 (Fig. 2a).

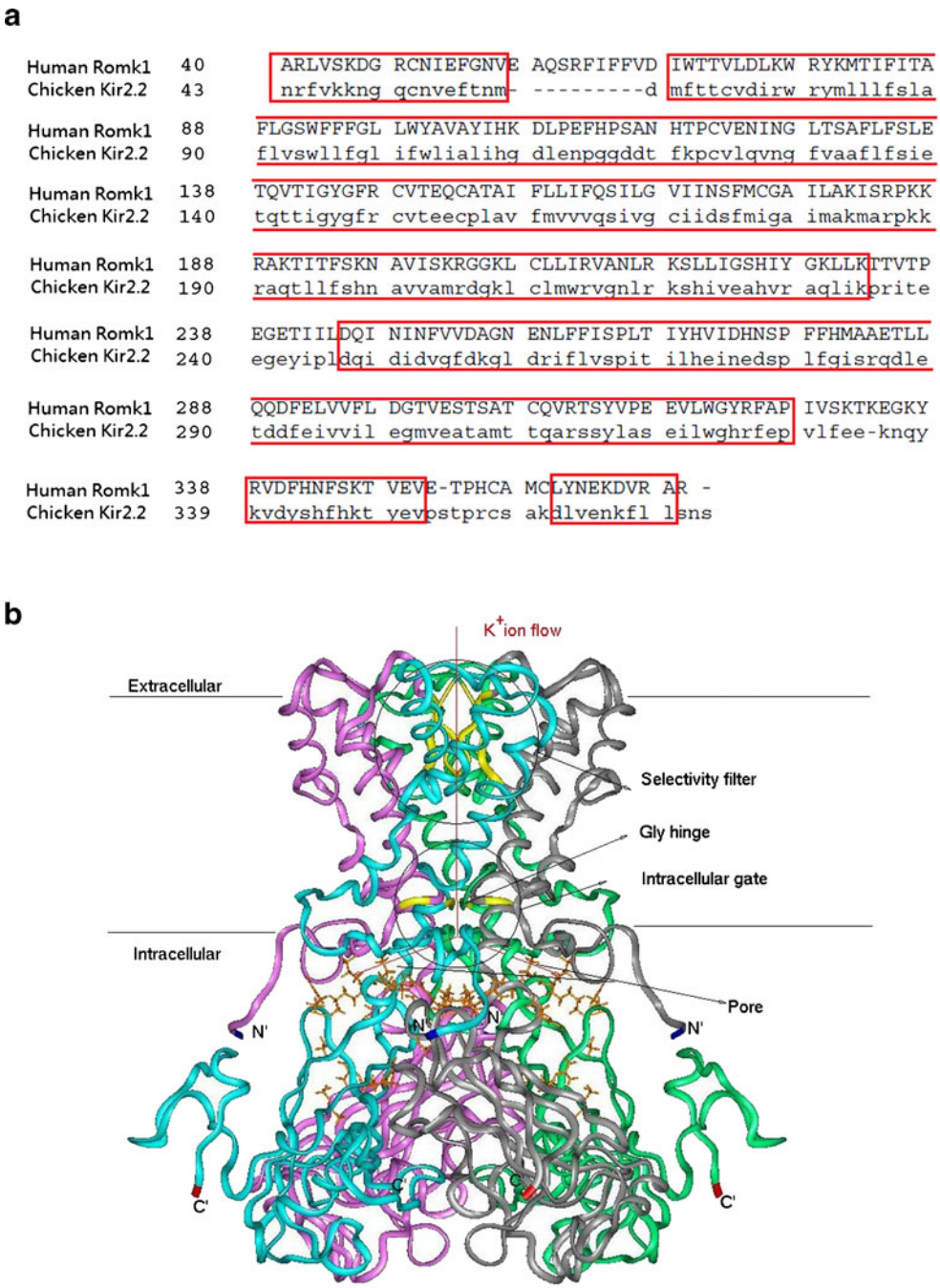

Fig. 1 Structural details of ROMK1 channels involved in PKC-mediated phosphorylation and the PIP_2-channel interaction on pH_i gating. **a** Sequence alignment of KirBac1.1 (PDB code: 3JYC) and ROMK1 (Swiss-prot ID: P48048). Residues 40–368 in the ROMK1 sequence were aligned with residues 43–370 in the Kir2.2 sequence. Structural conserved regions (SCRs) are *boxed in red*. The coordinates of the Cα atoms in template proteins (Kir2.2) were used to build up the ROMK1 channel model framework (Cα atom trace). Gaps between two SCRs were filled with loops generated from a loop database. **b** Overall structure of the ROMK1 tetrameric model. The main chains of the four subunits are represented with ribbons in different colors, the N- and C-terminus being marked in *blue* and *red*, respectively. Important motifs, such as two membrane-spanning α-helices, the selectivity filter, glycine hinge, intracellular gate, and the channel pore, and the direction of potassium flow are indicated with *arrows*. The side-chains of crucial basic residues discussed in the text are shown as *orange sticks*

MD simulation of ROMK-1 with and without PKC phosphorylation

To further understand the mechanism of ROMK1 channels involved in PKC-mediated phosphorylation and the PIP_2-channel interaction on pH_i gating, we used MD simulation to investigate the structural details of the Thr-193 neighborhood before and after phosphorylation. The MD simulation showed that PKC-mediated phosphorylation induced a change in the total root mean square deviation (RMSD) and total energy of ROMK1 channels. This conformation could reflect a relatively more stable transition state (Fig. 3a). The range for the distance between R188 and T193 was 12–15 Å before PKC phosphorylation, and that between R188 and T193 was 2–5 Å after PKC phosphorylation (Figs. 2, 3b). After phosphorylation, the results shown in Fig. 3c,d indicate a stable conformation at the new distance of residues Arg-188 and Arg-217 to the putative membrane. After phosphorylation, distance to the putative membrane for Arg-188 increases from 12.5 Å to 22–25 Å (Fig. 3d). In comparison, the distance to the putative membrane for Arg-217 is consistent at 13–14 Å

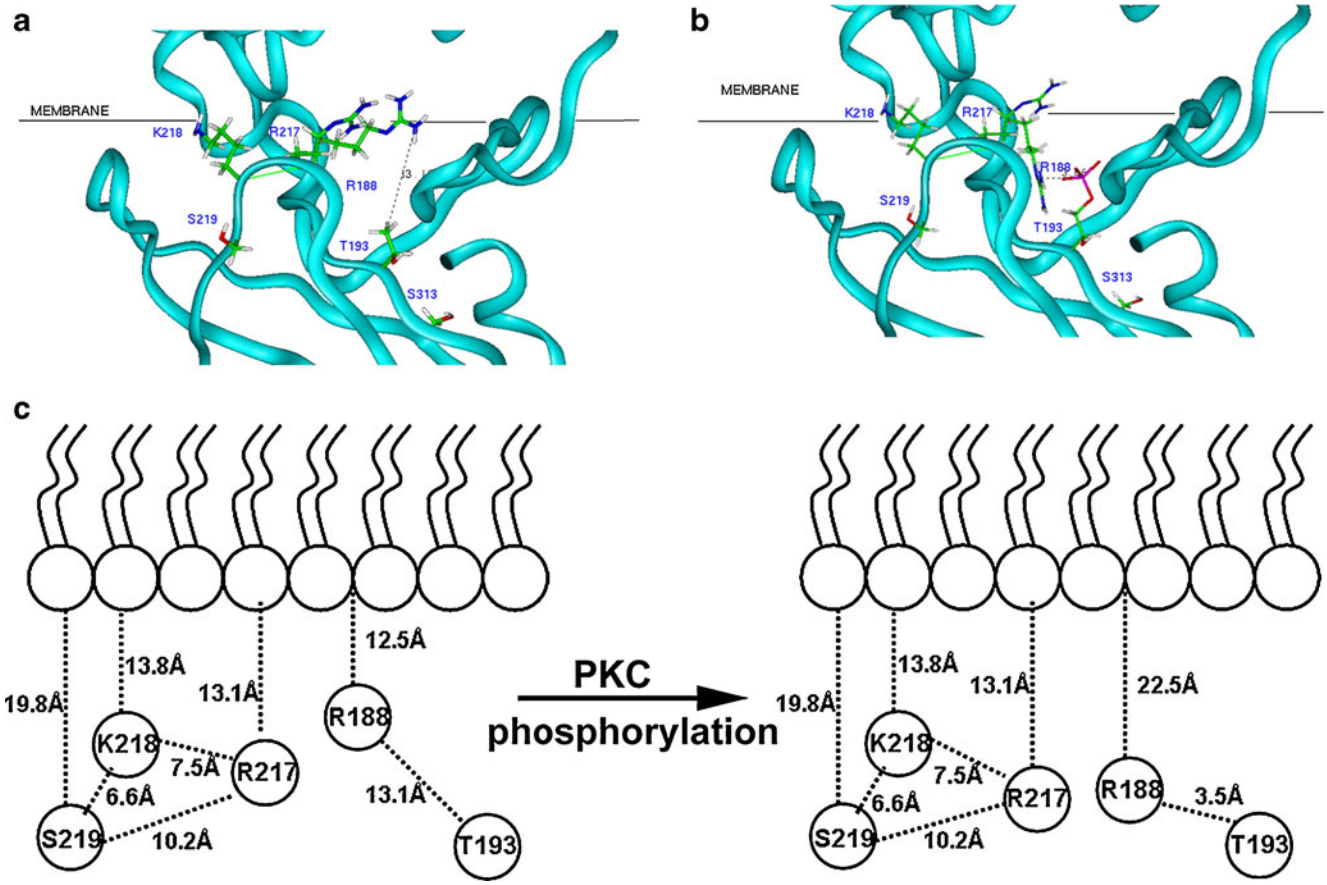

Fig. 2 Structural details of ROMK1 channels involved in PKC-mediated phosphorylation and the PIP_2-channel interaction on pHi gating. **a**, **b** Main structure depicted as *cyan ribbon*. Side chains of all crucial residues possibly involved in PIP_2 binding and PKC-mediated phosphorylation are illustrated with *sticks* in colors according to their atom types. It is interesting to note that a critical PIP_2 binding site, R188, is located near the cytoplasmic face of membrane, and two PIP_2 binding residues (such as R217 and K218) lie approximately on the same plane. The PKA-mediated phosphorylation sites S219 or S313, as well as a PKC-mediated phosphorylation site T193 are also presented. Structural details of ROMK1 channels after MD simulation with PKC-mediated phosphorylated Thr-193. **c** Diagram showing the distances of R188, T193, R217, K218, and S219 from the putative membrane obtained by MD simulations

(Fig. 3c). The results indicate that PKC-mediated phosphorylation of T193 might disrupt PIP_2-channel interaction via a charge–charge interaction between T193 and R188 (Fig. 2, Table 1). The results also suggest that PKC phosphorylation affects the sensitivity of the channels to pH_i via a PIP_2-dependent mechanism.

PKC-mediated phosphorylation mediates the pH_i gating of ROMK1 channels

ROMK1 channels were expressed in *Xenopus* oocytes and the K^+ currents recorded in on-cell, giant patches, which were then excised into Mg^{2+}-free bath solution containing a mixture of phosphatase inhibitors, fluoride, vanadate, and pyrophosphate (FVPP solution). This solution prevents run-down of the ROMK1 current, probably by inhibiting both Mg^{2+}-dependent protein phosphatases and lipid phosphatases, thus slowing channel dephosphorylation and membrane PIP_2 depletion [9, 11, 15, 19]. The activity of ROMK1 channels expressed in *Xenopus* oocytes was measured after incubation with PMA (300 nM)—a specific and potent PKC activator—for 5 min. As shown in Fig. 4a, subsequent acidification inhibited current flow in a steep, pH-dependent manner. The effective pK_a for inhibition of ROMK1 channels by intracellular acidification was shifted from 6.85±0.01 to 7.01±0.01(mean±SE, n=8; Fig. 4b,d). The pH_i sensitivity of ROMK1 channels was increased by PMA. Pre-treating the oocytes with the selective PKC inhibitor, GF109203X (10 μM), for 5 min decreased the effective pK_a for inhibition of ROMK1 channels by intracellular acidification (6.54±0.03, mean±SE, n=4; Fig. 4c,d). The results suggest that the pH_i sensitivity of ROMK1 channels can be regulated by PKC.

To identify the PKC phosphorylation site, the amino acid sequences of ROMK1 channels were aligned. Numerous putative PKC phosphorylation sites fitting the consensus sequence R(K)-$X_{0\text{-}2}$-T/S-$X_{0\text{-}2}$-R(K) found in previous reports [5, 20] were identified in ROMK1 channels. We

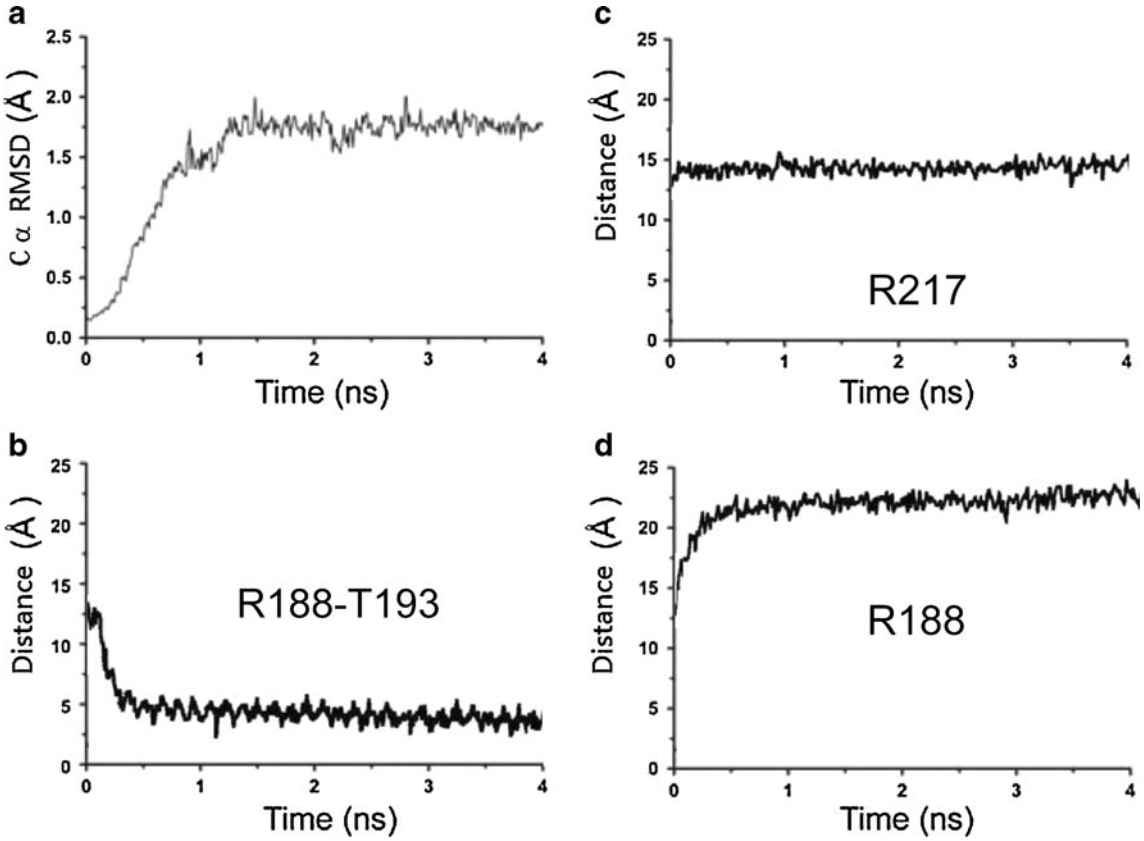

Fig. 3 MD simulation of ROMK1 channels. **a** Plot of the Cα root mean square deviation (RMSD) for all residues of the ROMK1 tetramer versus simulation time. The overall RMSD for the 4 ns simulation was 1.8 Å. The plot demonstrates the structural stability of the model over the entire 4,000 ps simulation run. **b** The range of the distance between R188 and T193 was 12–15 Å before PKC phosphorylation, and 2–5 Å after PKC phosphorylation. **c**, **d** The distance from the residues Arg-188 and Arg-217 to the putative membrane calculated over the simulation. After phosphorylation, the distance to the putative membrane for Arg-188 increases from 12.5 Å to 22–25 Å (**d**). In comparison, the distance to the putative membrane for Arg-217 is consistent, at 13–14 Å (**c**)

performed a systematic screening of these predicted sites in ROMK1 channels by single mutations of serines or threonines to alanine residues.

We tested three mutant channels with a single mutation representing each group and showed that the effective pK_a for T191A, T193A and S201A mutants was shifted in the acidic direction. (6.74±0.01 n=8, 6.58±0.01 n=8 and 6.77±0.01 n=7, respectively; Fig. 5a–d). This suggests that Thr193 is involved in regulation of the pH_i sensitivity of ROMK1 channels via PKC-induced phosphorylation.

PKC-mediated phosphorylation directly regulates pH_i sensitivity of ROMK1 channels

One common mechanism whereby PKC regulates protein function is by direct phosphorylation of target proteins [2]. We investigated whether PKC-mediated phosphorylation directly regulates the pH_i sensitivity of ROMK1 channels. Pre-treatment of oocytes with the PKC activator PMA (300 nM) for 5 min did not change the effective pK_a for inhibition of ROMK1 channels (the effective pK_a for T193A being 6.62±0.01 n=6; Fig. 6a) by intracellular acidification compared with non-treatment control (Fig. 6b).

To further confirm the hypothesis that PKC-mediated phosphorylation directly regulates pH_i sensitivity of ROMK1 channels, we pretreated oocytes expressing T193A mutated channels with the selective PKC inhibitor, GF109203X (10 μM) for 5 min. The effective pK_a for T193A (6.6± 0.02 n=6; Fig. 6c) was indistinguishable with that of non-treated mutated channels (Fig. 6d). These results indicate that PKC phosphorylation directly affects ROMK1 channels, thus regulating the sensitivity of the channels to pH_i.

Phosphorylation mediates the activity of ion channels by a variety of mechanisms, for example, inducing conformational changes and/or adding negative charges [11, 18, 21]. Mutation of the PKA target site Thr-193 to aspartate, which mimics the negative charge carried by a phosphate group bound to a serine shifted the effective pK_a for inhibition of ROMK1 channels by intracellular acidification comparing with T193A mutated channels (Fig. 7b); the effective pK_a for

Table 1 Comparison of the distance and energy between residues at the phosphorylation site before and after molecular dynamic (MD) simulations

	Distance between residues (Å)	Distance to the putative membrane (Å)[a]	Total energy (kcal/mol)	Energy change ($\triangle E=E_{after}-E_{before}$) (kcal/mol)[b]
Model containing phosphate group before MD simulations[c]	T193-R188: 13.1	R188: 12.5 R217: 13.1	592.13	
Model containing phosphate group after MD simulations	T193-R188: 4.5	R188: 22.3 R217: 13.6	562.33	−29.80
	T193-R188: 8.1	R188: 20.1 R217: 13.8	581.24	−12.87
	T193-R188: 4.2	R188: 22.4 R217: 13.6	535.23	−56.90
	T193-R188: 4.6	R188: 22.1 R217: 13.6	562.35	−29.78
	T193-R188: 3.9	R188: 22.8 R217: 13.6	528.16	−63.97
	T193-R188: 3.5	R188: 22.5 R217: 13.1	511.22	−80.91

[a] As described in Fig. 5b, the distance indicated here was measured between the residue tips, and between the residue tips and the Cα atom of the aromatic belt (W69, W77, Y79, and W92) of the putative membrane

[b] E_{after} represents the total energy of the model containing the phosphate group before MD simulations. E_{before} represents the total energy of the model containing the phosphate group after MD simulations

[c] The phosphate group was manually added into the system to allow the simulation to be performed

T193D was 7.42±0.01 n=6; Fig. 7a. These results indicate that PKC-mediated phosphorylation could in turn mediate pH_i sensitivity of ROMK1 channels via charge–charge interactions.

Effects on PKC phosphorylation of PIP_2-channel interactions

Previous reports have shown that reduction of membrane PIP_2 content contributes to the inhibition of ROMK1 channels by PKC [2]. The channel-PIP_2 interaction may act like a switch that controls pH_i inhibition of ROMK1 [10]. We next wanted to explore whether the PIP_2 that is involved in ROMK1 channels via PKC phosphorylation affects the sensitivity of the channels to pH_i. Figure 8a shows representative tracings of wild-type ROMK1 channels and those mutants inhibited by anti-PIP_2 antibodies. At pH_i 7.4, the effect of PMA on ROMK1 channel inhibition by anti-PIP_2 antibodies was decreased as compared with the wild-type. The $t_{1/2}$ of inhibition by anti-PIP_2 antibodies was 75±3 s. Thus, it appears that PKC diminishes the PIP_2-Romk1 channel interaction. However, the T193A mutant showed decreased sensitivity to inhibition by anti-PIP_2 antibodies. The $t_{1/2}$ of inhibition by anti-PIP_2 antibodies was 160±2 s (Fig. 8b). The observations indicate the same pH_i sensitivity as for PKC mutants. These results indicate that the sensitivity to pH_i of PKC phosphorylation of ROMK1 channels is related to PIP_2.

Regulation of the ROMK1 channels via inward rectification of K^+ channels by PIP_2 involves direct binding of PIP_2 to a region of the C terminus that includes Arg-188 [15]. We found that the PIP_2-binding site mutant R188Q increases the sensitivity of these channels to intracellular protons [10]. To further investigate PIP_2 involvement in ROMK1 channels in terms of PKC phosphorylation affecting the sensitivity of the channels to pH_i, a T193A/R188Q double mutant was constructed. The effective pK_a values for mutant T193A/R188Q was 7.77±0.01, n=4, Fig. 8c,d). These results confirm that a PIP_2-channel interaction regulates the pH_i sensitivity of ROMK1, and that PKC phosphorylation affects the sensitivity of the channels to pH_i via a PIP_2-dependent mechanism.

Discussion

The activity of ROMK1 channels is mediated by PKC-mediated phosphorylation [2, 5, 18, 22] and intracellular pH [8–12], but the molecular mechanisms underlying the regulation of pH_i sensitivity by this mechanism remain unknown. PIP_2 is critical for opening of ROMK1 channels [15, 23], as well as the regulation of ROMK1 by other pathways such as PKA [9, 11], and intracellular pH [10] interacts with PIP_2 regulation of the channel. The present study demonstrates that PKC, activated by PMA, increases the sensitivity of ROMK1 channels to intracellular protons

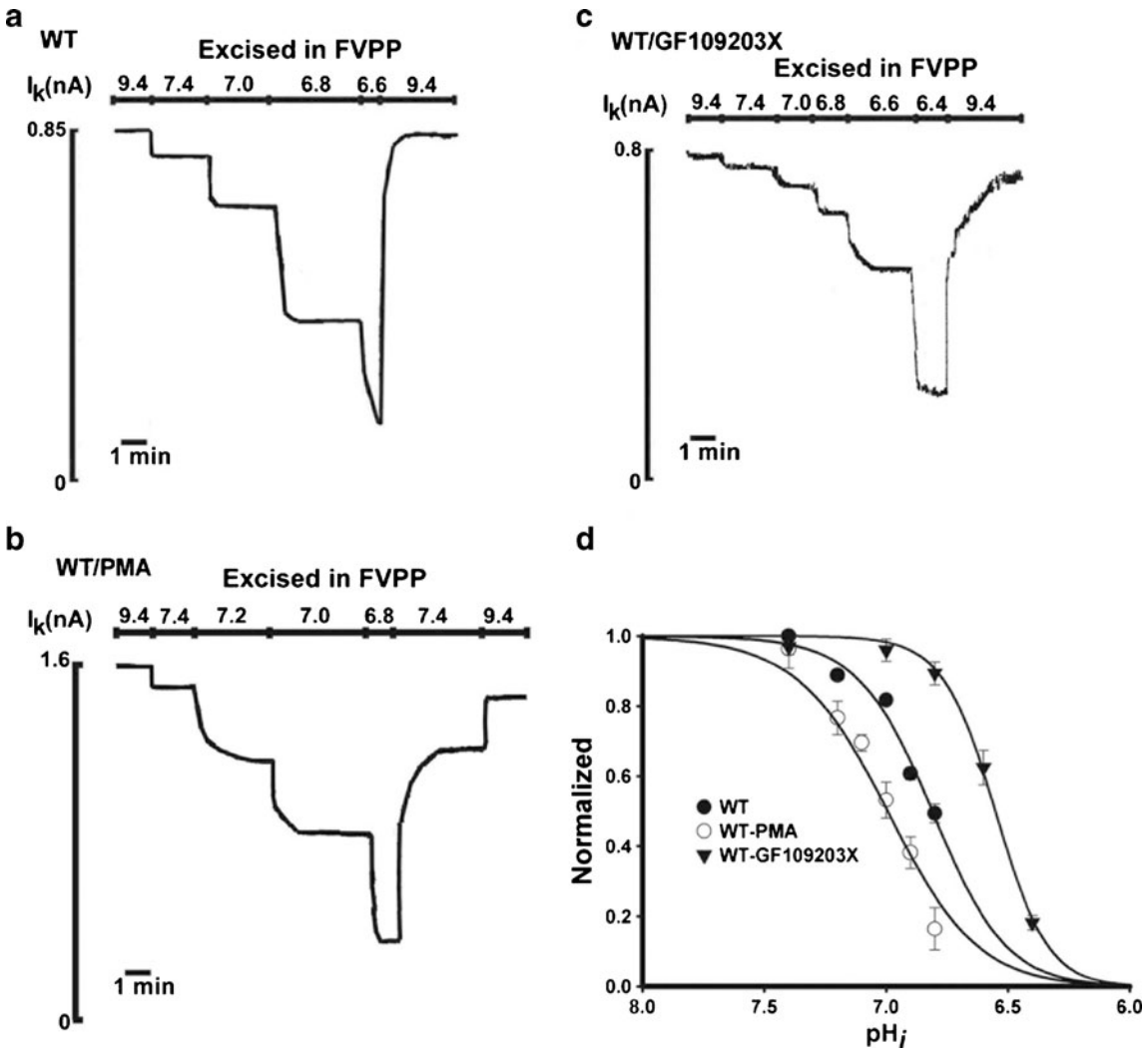

Fig. 4 Effects of PKC-mediated phosphorylation on pH_i gating in ROMK1 channels. ROMK1 channels were expressed in *Xenopus* oocytes and the K^+ currents (I_k) recorded in on-cell, giant patches, which were then excised into FVPP solution. Voltage pulses were applied from −100 mV to +100 mV at 25 mV increments. The holding potential was −60 mV. **a** The excised patches of ROMK1 channel current titrated to the intracellular pH (pH_i) value (from 9.4 to 6.6), currents were inhibited by progressively more acidic pH_i. After inhibition, membrane patches were alkalinized to pH_i 9.4 to assess the level of remaining currents. Experiments with 90% of the total inhibitory currents at the end of an experiment were not used for analysis. **b** Pre-treatment with PKC activator, PMA (300 nM), for 5 min; the acidification resulted in a steep pH-dependent inhibition with a effective acidic dissociation constant (pK_a) of 7.01±0.01 (n=8, P<0.05). **c** Pre-treatment with PKC inhibitor, GF109203X (10 μM), for 5 min; the acidification caused a pH-dependent inhibition with a pK_a of 6.54±0.03 (n=4). **d** Comparison of the pH_i dependent inhibition of ROMK1 channels and PKA-mediated phosphorylation of ROMK1 channels. The relative currents [%; normalized to the maximal currents (I_{max}) at pH_i 9.4] at different pH_i values were fitted to the Hill equation

and reduces the channel-PIP_2 interaction (Figs. 4, 8). Meanwhile, PKC-mediated phosphorylation directly affects ROMK1 channels to regulate the sensitivity of the channels to pH_i (Fig. 6). Furthermore, we found that mutation of PKC-induced phosphorylation sites (T193A) has different effects on the interaction of channel-PIP_2 and pH_i sensitivity, respectively (Figs. 5, 8), suggesting T193 is the main PKC-induced phosphorylation site required for regulation of pH_i sensitivity of ROMK1 channels. Taken together, these results suggest that PKC-mediated phosphorylation regulates the activity of ROMK1 channels to intracellular protons through destroying the channel-PIP_2 interaction (Figs. 2, 3).

The mechanism of pH_i gating has been studied most widely in the ROMK1 channel, which is found in the apical membrane of renal tubular epithelia, where it secretes excess K^+ into the urine [3, 24, 25]. This K^+ transport pathway is regulated by intracellular pH as well as by the luminal K^+ concentration (K^+-dependent inactivation) [26, 27], and both regulatory mechanisms are essential for the renal control of K^+ homeostasis. The sensitivity of ROMK

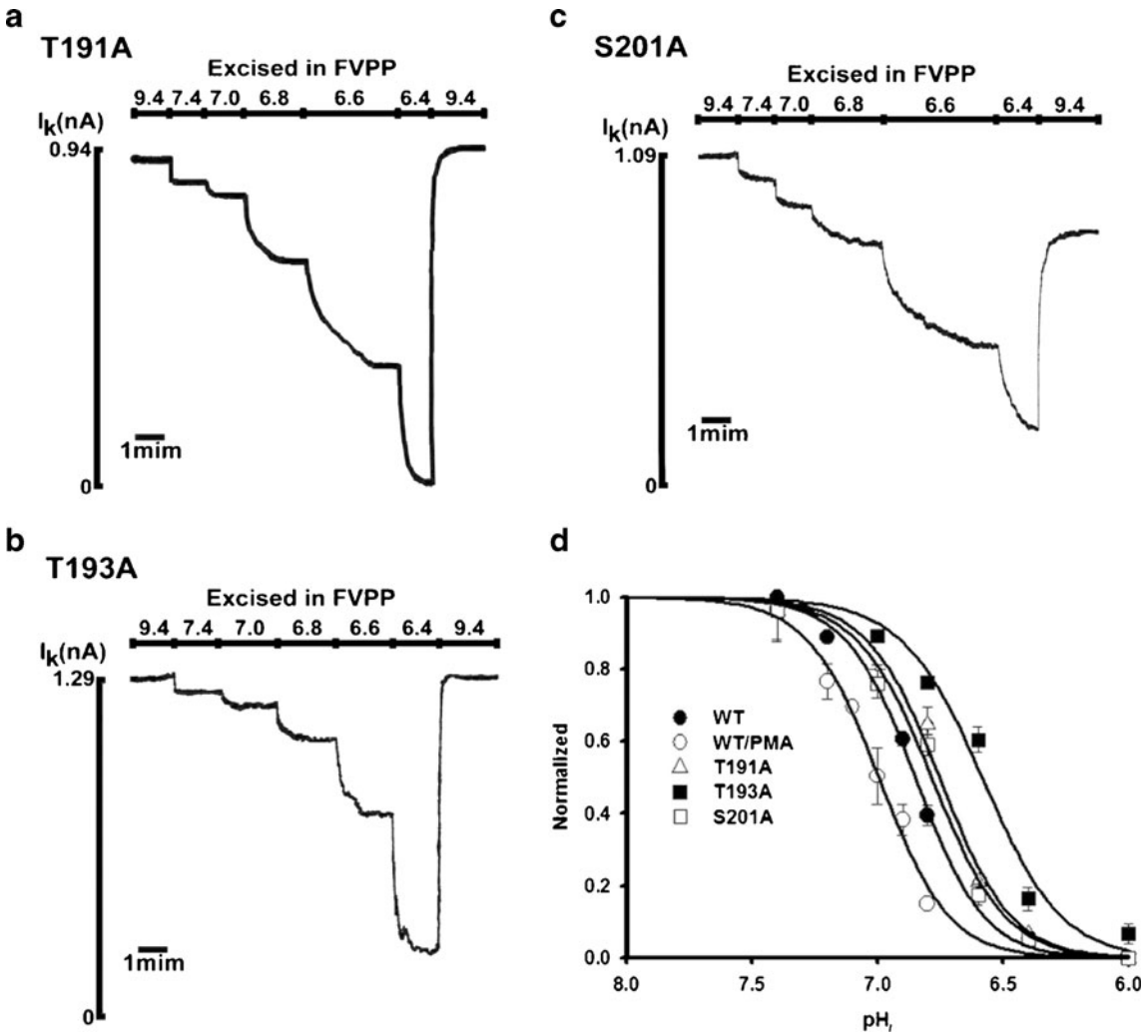

Fig. 5 Effects of mutations at putative PKC phosphorylation sites in ROMK1 channels on pH_i gating. **a** pK_a for T191A mutant channels = 6.74±0.01 (n=8). **b** pK_a for T193A mutant channels = 6.60±0.01 (n=8). **c** pK_a for S201A mutant channels = 6.77±0.01 (n=7). **d** pK_a for T234A mutant channels = 7.19±0.01 (n=8). *F*, Comparison of the pH_i response curves for the wild-type and PKC-mutated ROMK1 channels

channels to cytosolic protons provides a major mechanism for regulation of channel activity, and a number of other ROMK channel regulators [e.g., PKA phosphorylation and PIP_2] [9, 28] also function by modifying pH sensitivity. This is highlighted by the fact that inherited mutations in ROMK1 that interfere with this pH_i sensing mechanism result in the hypokalaemic disorder known as type II Bartter's syndrome [3, 9, 25, 29], suggesting that the regulation of ROMK1 channels by intracellular H^+ underlies many important aspects of physiology as well as pathophysiology.

PKC is found in almost all cell types as an effector of various signal transduction pathways and has been demonstrated to regulate ion channels. PKC plays an important role in water and solute absorption in the proximal tubule (PT) and potassium secretion in the CCDs [30]. In addition, several studies have implicated PKC-mediated phosphorylation in regulation of intracellular pH. Elevation of lysosomal pH_i, dependent on activation of PKC, is a property of signaling in the macrophage lysosomal secretory response [31]. The elevation of pH_i in rat pinealocytes has been implicated in PKC isozyme translocation and norepinephrine stimulation [32]. Therefore, PKC phosphorylation is involved in modulation of pH_i in fibroblasts involved in cell–cell contact interactions [33].

PKC modifies the regulation of intracellular pH through the activation of the Na^+-H^+ exchanger, and additionally alters myofibrillar Ca^{2+} sensitivity [34]. PKC has been reported to antagonize PKA activation of ROMK1 channels in CCDs [2, 14]. We have elucidated some of the molecular mechanisms underlying PKA-mediated phosphorylation in regulating the pH_i of ROMK1 channels [9]. In the present study, we have presented evidence for the pH_i gating of ROMK1 channels by PKC-mediated phosphorylation. Furthermore, we show that Thr-193 is the phosphorylation site on PKC that regulates the pH_i sensitivity of ROMK1 channels (Fig. 5).

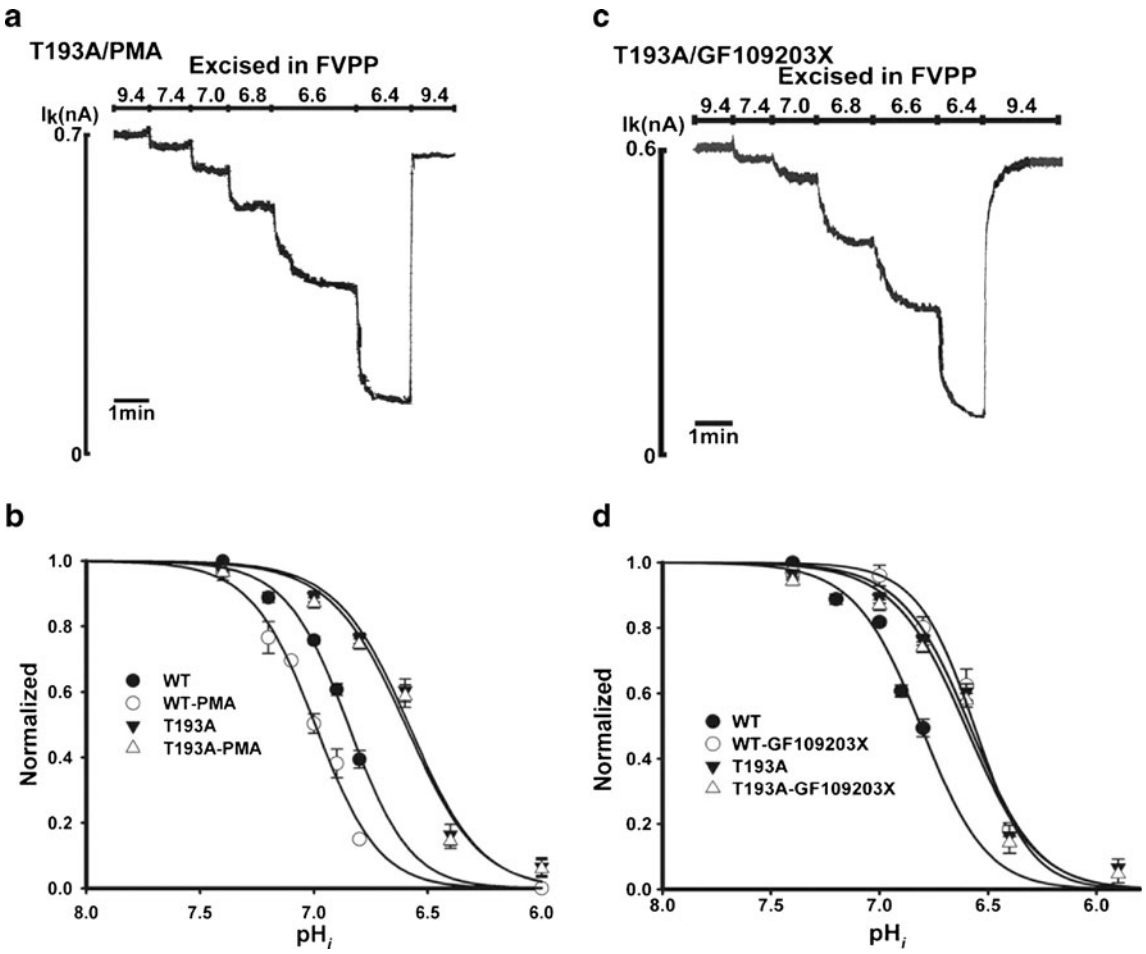

Fig. 6 PKC-mediated phosphorylation directly regulates pH_i sensitivity of ROMK1 channels. **a** Effective pK_a for T193A mutant channels = 6.62±0.01 (n=6) in the presence of PMA (300 nM). **b** Comparison of the pH_i response curves for the wild-type and T193A channels in presence and absence of PMA. **c** Effective pK_a for T193A mutant channels = 6.6±0.02 n=6 in the presence of GF109203X (10 μM). **d** Comparison of the pH_i response curves for the wild-type and T193A channels in presence or absence of GF109203X. The effective pK_a for T234A mutant channels was 7.21±0.01 (n=6) in the presence of PMA

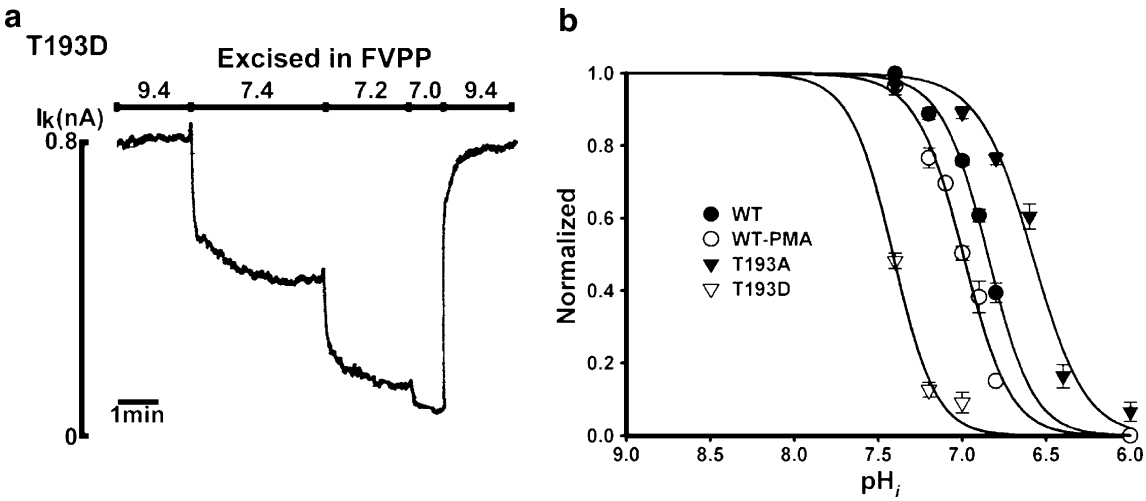

Fig. 7 Effects of mutation of T193 on pH_i gating. **a** pK_a for T193D mutant channels = 7.42±0.01 (n=6). **b** Comparison of pH_i response curves for the wild-type and T193 mutated ROMK1 channels

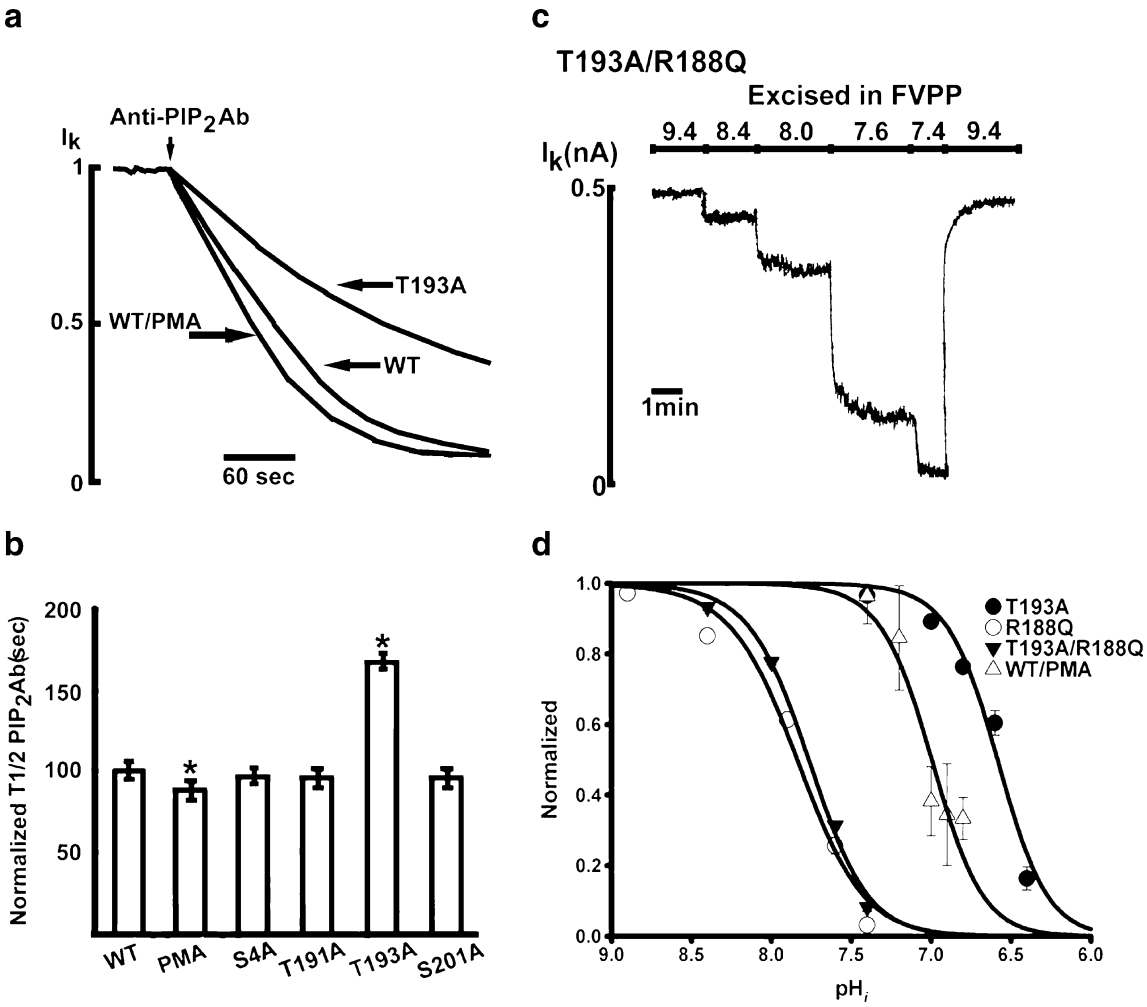

Fig. 8 Effects of PKC-mediated phosphorylation and the PIP_2-channel interaction on pH_i gating. **a** At pH_i 7.4, the wild-type ROMK1 (WT) channel was inhibited by application of anti-PIP_2 antibodies significantly slower than PKC-phosphorylated channels (PMA), but faster than T193A channels ($t_{1/2}$=100±3 s for WT, 75±3 s for PMA and 160±2 s for T193A, n=4, P<0.05). **b** Half-time ($t_{1/2}$) for maximal inhibition by anti-PIP_2 antibody (40 nM) for the wild-type (WT) and the mutant channels of ROMK1. Mean±SEM, n=4 for each group. * indicates P<0.05 by ANOVA. **c** pK_a for T193A/R188Q double mutant channels = 7.77±0.01 (n=4). **d** Comparison of the pH_i response curves for the mutated ROMK1 channels

These results suggest that inhibition of ROMK1 processes mediated by PKC phosphorylation is probably of importance in regulating pH_i.

The MD simulations were performed not only in implicit membrane but also using the CHARMM special forcefield c27b4 as previously described [9]. Analysis of the results of MD simulations gave us a working hypothesis. We focused on the biologically interesting conformational changes induced by PKC-mediated phosphorylation (Figs. 1–3) and re-examined these with electrophysiological experiments (Figs. 4–8). Although continuum electrostatic models present only a simplified picture of the complex interactions of biological molecules and lipid bilayers, the results of this study also suggest that the generalized Born-solvent-accessible surface area (GBSA) model with implicit membrane approximation will be a useful method for structural modeling of membrane protein complexes [35]. We can use such fast and convenient computational methods for modeling membrane protein molecules and to provide working hypotheses to re-examine by functional experiments. Therefore, we will discuss not only what we observed in MD simulations, but also the meaning of our results in terms of the biophysics of electrophysiological phenomena. The following provides a compact and careful discussion of the simulation results.

Our results demonstrate that PKC-mediated phosphorylation acts to regulate ROMK1 channel activity to intracellular protons by destroying the channel-PIP_2 interaction. Others have also reported a relationship between PIP_2 and PKC-mediated channel regulation. When PKC is activated by PMA, membrane levels of PIP_2 decrease and PIP_2-dependent renal epithelial sodium channel (ENaC) activity

is reduced [36]. Nasuhoglu et al. [37] reported that PMA and diacylglycerol decrease membrane PIP_2 content in guinea pig ventricles and in a mouse CCD cell line. Because PIP_2 is essential for maintaining ROMK1 channel activity [11, 15], a decrease in PIP_2 sensitivity is expected to inhibit channel activity. We suggest that disruption of the channel-PIP_2 interaction observed in the R188, R217 and K218 mutants caused an alkaline shift in pH_i sensitivity [10, 23]. Previously, we found that PKA-mediated phosphorylation and PIP_2 sequence regulates the pH_i sensitivity of ROMK1 channels [9]. In this study, we found that activation of PKC with PMA (300 nM) increases the sensitivity of ROMK1 channels to intracellular protons and reduces channel-PIP_2 interactions (Figs. 4, 8), and that mutation of the PKC phosphorylation site T193A induces changes in the interaction of channel-PIP_2 and pH_i sensitivity (Figs. 5, 8). The effective pK_a values for mutants T193A/R188Q, the PIP_2-binding site mutant, shifted to more alkaline values compared with T193A mutants (Fig. 8). Our MD simulation data (Table 1, Fig. 3c) shows that PIP_2 binding free energy increases in T193 mutated (T193A) or unphosphorylated ROMK1 channels. Thus, channel-PIP_2 interactions may work as a switch that controls pH_i-induced inhibition of ROMK1 channels. Destroying the channel-PIP_2 interaction increases the pH_i sensitivity of ROMK1 (Figs. 7, 8).

It has been reported recently that serine residue 201 is another PKC phosphorylation site that is essential for the expression of ROMK1 at the cell surface [5]. Our data suggest another role for PKC in the regulation of ROMK1 involving the pH_i sensitivity. It is conceivable that a variety of PKC isoforms expressed in the CCD have different effects on the modulation of ROMK channels. PKC-ε and -ζ are two major PKC isoforms found in rabbit CCD [38]. This view has also been supported by experiments in which isolated rabbit CCDs and cultured rabbit CCD cells have been used to demonstrate the presence of PKC-α, -ε, and –ζ in the CCD [39]. Thus, an area of further study would be to investigate the effect of PKC in regulation of pH_i sensitivity of ROMK1 channels via different PKC isoforms.

Conclusions

Recent studies suggest that the activation of PKC by PMA inhibits ROMK1 channels via a phosphatidylinositol-4,5-bisphosphate (PIP_2)-dependent mechanism [2]. Moreover, we have found that PIP_2-channel interactions influence the regulation of ROMK1 channels by intracellular protons [10], and suggested a sequence regulatory mechanism for the pH_i gating of ROMK1 channels by PKA-mediated phosphorylation [9]. The molecular mechanism of PKC-mediated phosphorylation in regulating the pH_i sensitivity of ROMK1 channels is probably via a PIP_2-dependent mechanism. Here, we investigated whether PKC-mediated phosphorylation is involved in regulating the pH_i sensitivity of ROMK1 channels and/or PIP_2. Our results allowed us to conclude that PKC phosphorylation at T193 plays an important role in regulating the sensitivity of ROMK1 channels to pH_i, which is also associated with effects on PIP_2.

In conclusion, ROMK1 channels are regulated by multiple signaling pathways, including PKC, PKA, protein tyrosine kinase and pH_i [2, 40]. The pH_i-gating of ROMK1 channels is believed to work as a physiological feedback mechanism that prevents excessive loss of K^+ during metabolic acidosis when K^+ levels in the serum, and consequently in the primary urine, become elevated. Our findings provide novel mechanistic insights into the role of PKC-mediated phosphorylation in regulation of ROMK1 channels to pH_i sensitivity. Moreover, the regulation of pH_i-gating of ROMK1 channels by PKC-mediated phosphorylation functions via a PIP_2-dependent mechanism.

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References

1. Sugimoto Y, Namba T, Shigemoto R, Negishi M, Ichikawa A, Narumiya S (1994) Distinct cellular localization of mRNAs for three subtypes of prostaglandin E receptor in kidney. Am J Physiol 266:F823–F828
2. Zeng WZ, Li XJ, Hilgemann DW, Huang CL (2003) Protein kinase C inhibits ROMK1 channel activity via a phosphatidylinositol 4,5-bisphosphate-dependent mechanism. J Biol Chem 278:16852–16856. doi:10.1074/jbc.M300619200
3. Hebert SC, Desir G, Giebisch G, Wang W (2005) Molecular diversity and regulation of renal potassium channels. Physiol Rev 85:319–371. doi:10.1152/physrev.00051.2003
4. Cantone A, Yang X, Yan Q, Giebisch G, Hebert SC, Wang T (2008) Mouse model of type II Bartter's syndrome. I. Upregulation of thiazide-sensitive Na-Cl cotransport activity. Am J Physiol Renal Physiol 294:F1366–F1372. doi:10.1152/ajprenal.00608.2007
5. Lin D, Sterling H, Lerea KM, Giebisch G, Wang WH (2002) Protein kinase C (PKC)-induced phosphorylation of ROMK1 is essential for the surface expression of ROMK1 channels. J Biol Chem 277:44278–44284. doi:10.1074/jbc.M203702200
6. Sterling H, Lin DH, Chen YJ, Wei Y, Wang ZJ, Lai J, Wang WH (2004) PKC expression is regulated by dietary K intake and mediates internalization of SK channels in the CCD. Am J Physiol Renal Physiol 286:F1072–F1078. doi:10.1152/ajprenal.00425.2003
7. Wei Y, Zavilowitz B, Satlin LM, Wang WH (2007) Angiotensin II inhibits the ROMK-like small conductance K channel in renal cortical collecting duct during dietary potassium restriction. J Biol Chem 282:6455–6462. doi:10.1074/jbc.M607477200

8. Choe H, Zhou H, Palmer LG, Sackin H (1997) A conserved cytoplasmic region of ROMK modulates pH sensitivity, conductance, and gating. Am J Physiol 273:F516–F529
9. Lee CH, Huang PT, Lou KL, Liou HH (2008) Functional and structural characterization of PKA-mediated pHi gating of ROMK1 channels. J Mol Graph Model 27:332–341. doi:10.1016/j.jmgm.2008.06.001
10. Leung YM, Zeng WZ, Liou HH, Solaro CR, Huang CL (2000) Phosphatidylinositol 4,5-bisphosphate and intracellular pH regulate the ROMK1 potassium channel via separate but interrelated mechanisms. J Biol Chem 275:10182–10189
11. Liou HH, Zhou SS, Huang CL (1999) Regulation of ROMK1 channel by protein kinase A via a phosphatidylinositol 4,5-bisphosphate-dependent mechanism. Proc Natl Acad Sci USA 96:5820–5825
12. McNicholas CM, MacGregor GG, Islas LD, Yang Y, Hebert SC, Giebisch G (1998) pH-dependent modulation of the cloned renal K+channel, ROMK. Am J Physiol 275:F972–F981
13. Leng Q, MacGregor GG, Dong K, Giebisch G, Hebert SC (2006) Subunit-subunit interactions are critical for proton sensitivity of ROMK: evidence in support of an intermolecular gating mechanism. Proc Natl Acad Sci USA 103:1982–1987. doi:10.1073/pnas.0510610103
14. Wang WH, Giebisch G (1991) Dual modulation of renal ATP-sensitive K+channel by protein kinases A and C. Proc Natl Acad Sci USA 88:9722–9725
15. Huang CL, Feng S, Hilgemann DW (1998) Direct activation of inward rectifier potassium channels by PIP2 and its stabilization by Gbetagamma. Nature 391:803–806. doi:10.1038/35882
16. Proks P, Capener CE, Jones P, Ashcroft FM (2001) Mutations within the P-loop of Kir6.2 modulate the intraburst kinetics of the ATP-sensitive potassium channel. J Gen Physiol 118:341–353
17. Minami K, Gereau RWT, Minami M, Heinemann SF, Harris RA (1998) Effects of ethanol and anesthetics on type 1 and 5 metabotropic glutamate receptors expressed in *Xenopus laevis* oocytes. Mol Pharmacol 53:148–156
18. Lee CH, Lee CY, Tsai TS, Liou HH (2008) PKA-mediated phosphorylation is a novel mechanism for levetiracetam, an antiepileptic drug, activating ROMK1 channels. Biochem Pharmacol 76:225–235. doi:10.1016/j.bcp.2008.04.012
19. Hilgemann DW, Ball R (1996) Regulation of cardiac Na+, Ca2+ exchange and KATP potassium channels by PIP2. Science 273:956–959
20. Ho K, Nichols CG, Lederer WJ, Lytton J, Vassilev PM, Kanazirska MV, Hebert SC (1993) Cloning and expression of an inwardly rectifying ATP-regulated potassium channel. Nature 362:31–38. doi:10.1038/362031a0
21. Lee CH, Tsai TS, Liou HH (2008) Gabapentin activates ROMK1 channels by a protein kinase A (PKA)-dependent mechanism. Br J Pharmacol 154:216–225. doi:10.1038/bjp.2008.73
22. Macica CM, Yang Y, Lerea K, Hebert SC, Wang W (1998) Role of the NH2 terminus of the cloned renal K+channel, ROMK1, in arachidonic acid-mediated inhibition. Am J Physiol 274:F175–F181
23. Zeng WZ, Liou HH, Krishna UM, Falck JR, Huang CL (2002) Structural determinants and specificities for ROMK1-phosphoinositide interaction. Am J Physiol Renal Physiol 282:F826–F834. doi:10.1152/ajprenal.00300.2001
24. Wang WH (2006) Regulation of ROMK (Kir1.1) channels: new mechanisms and aspects. Am J Physiol Renal Physiol 290:F14–F19. doi:10.1152/ajprenal.00093.2005
25. Rapedius M, Fowler PW, Shang L, Sansom MS, Tucker SJ, Baukrowitz T (2007) H bonding at the helix-bundle crossing controls gating in Kir potassium channels. Neuron 55:602–614. doi:10.1016/j.neuron.2007.07.026
26. Rapedius M, Haider S, Browne KF, Shang L, Sansom MS, Baukrowitz T, Tucker SJ (2006) Structural and functional analysis of the putative pH sensor in the Kir1.1 (ROMK) potassium channel. EMBO Rep 7:611–616. doi:10.1038/sj.embor.7400678
27. Sackin H, Nanazashvili M, Li H, Palmer LG, Walters DE (2007) External K activation of Kir1.1 depends on the pH gate. Biophys J 93:L14–L16. doi:10.1529/biophysj.107.110122
28. Levitan IB (1994) Modulation of ion channels by protein phosphorylation and dephosphorylation. Annu Rev Physiol 56:193–212. doi:10.1146/annurev.ph.56.030194.001205
29. Welling PA, Ho K (2009) A comprehensive guide to the ROMK potassium channel: form and function in health and disease. Am J Physiol Renal Physiol 297:F849–F863. doi:10.1152/ajprenal.00181.2009
30. Zhang W, Zitron E, Bloehs R, Muller-Krebs S, Scholz E, Zeier M, Katus H, Karle C, Schwenger V (2008) Dual regulation of renal Kir7.1 potassium channels by protein Kinase A and protein Kinase C. Biochem Biophys Res Commun 377:981–986. doi:10.1016/j.bbrc.2008.10.110
31. Tapper H, Sundler R (1995) Protein kinase C and intracellular pH regulate zymosan-induced lysosomal enzyme secretion in macrophages. J Leukoc Biol 58:485–494
32. Ho AK, Ling A, Chik CL (2000) Intracellular pH on translocation of protein kinase C isozymes in rat pinealocytes. J Neurochem 75:1845–1851
33. Galkina SI, Sud'ina GF, Dergacheva GB, Margolis LB (1995) Regulation of intracellular pH by cell-cell adhesive interactions. FEBS Lett 374:17–20
34. Takeishi Y, Ping P, Bolli R, Kirkpatrick DL, Hoit BD, Walsh RA (2000) Transgenic overexpression of constitutively active protein kinase C epsilon causes concentric cardiac hypertrophy. Circ Res 86:1218–1223
35. Spassov VZ, Yan L, Szalma S (2002) Introducing an implicit membrane in generalized born/solvent accessibility continuum solvent. J Phys Chem B 106:8726–8738
36. Ma HP, Eaton DC (2005) Acute regulation of epithelial sodium channel by anionic phospholipids. J Am Soc Nephrol 16:3182–3187. doi:10.1681/ASN.2005040434
37. Nasuhoglu C, Feng S, Mao Y, Shammat I, Yamamato M, Earnest S, Lemmon M, Hilgemann DW (2002) Modulation of cardiac PIP2 by cardioactive hormones and other physiologically relevant interventions. Am J Physiol Cell Physiol 283:C223–C234. doi:10.1152/ajpcell.00486.2001
38. DeCoy DL, Snapper JR, Breyer MD (1995) Anti sense DNA down-regulates proteins kinase C-epsilon and enhances vasopressin-stimulated Na+absorption in rabbit cortical collecting duct. J Clin Invest 95:2749–2756. doi:10.1172/JCI117978
39. Wilborn TW, Schafer JA (1996) Differential expression of PKC isoforms in fresh and cultured rabbit CCD. Am J Physiol 270:F766–F775
40. Giebisch G, Wang W (2000) Renal tubule potassium channels: function, regulation and structure. Acta Physiol Scand 170:153–173