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Protein Kinase C (PKC)-induced Phosphorylation of ROMK1 Is Essential for the Surface Expression of ROMK1 Channels*

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Abstract

We carried out *in vitro* phosphorylation assays to determine whether ROMK1 is a substrate of protein kinase C (PKC) and used the two-electrode voltage clamp method to investigate the role of serine residues 4, 183, and 201, the three putative PKC phosphorylation sites, in the regulation of ROMK1 channel activity. Incubation of the purified His-tagged ROMK1 protein with PKC and radiolabeled ATP resulted in ^{32}P incorporation into ROMK1 detected by autoradiography. Moreover, the *in vitro* phosphorylation study of three synthesized peptides corresponding to amino acids 1–16, 174–189, and 196–211 of ROMK1 revealed that serine residues 4 and 201 of ROMK1 were the two main PKC phosphorylation sites. In contrast, ^{32}P incorporation of peptide 174–189 was absent. *In vitro* phosphorylation studies with ROMK1 mutants, R1S4/201A, R1S4/183A, and R1S183/201A, demonstrated that the phosphorylation levels of R1S4/201A were significantly lower than those of the other two mutants. Also, the Ba^{2+}-sensitive K^+ current in oocytes injected with green fluorescent protein (GFP)-R1S4/201A was only 5% of that in oocytes injected with wild type GFP-ROMK1. In contrast, the K^+ current in oocytes injected with GFP-ROMK1 mutants containing either serine residue 4 or 201 was similar to those injected with wild type ROMK1. Confocal microscope imaging shows that the surface expression of the K^+ channels was significantly diminished in oocytes injected with R1S4/201A and completely absent in oocytes injected with R1S4/183/201A. Furthermore, the biotin labeling technique confirmed that the membrane fraction of ROMK channels was almost absent in HEK293 cells transfected with either R1S4/201A or R1S4/183/201A. However, when serine residues 4 and 201 were mutated to aspartate, the K^+ currents and the surface expression were completely restored. Finally, addition of calphostin C in the incubation medium significantly decreased the K^+ current in comparison with that under control conditions. Biotin labeling technique further indicated that inhibition of PKC decreases the surface ROMK1 expression in human embryonic kidney (HEK) cells transfected with ROMK1. We conclude that ROMK1 is a substrate of PKC and that serine residues 4 and 201 are the two main PKC phosphorylation sites that are essential for the expression of ROMK1 in the cell surface.

ROMK channel is an inwardly rectifying K^+ channel with two transmembrane segments (1,2). ROMK channels are located in the apical membrane of the thick ascending limb and the cortical collecting duct (CCD)[1] (3,4). ROMK channels in the thick ascending limb

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mediate K^+ recycling across the apical membrane and are involved in K^+ secretion in the CCD (2,5). Defective ROMK channels have been shown to cause abnormal salt transport characterized by a significant salt waste (6). There are three ROMK isoforms in the kidney: ROMK1 is located only in the CCD, ROMK3 is expressed only in the thick ascending limb, and ROMK2 is present in both nephron segments (7). ROMK1 has three putative serine phosphorylation sites, serine residues 4, 183, and 201, whereas ROMK2 as well as ROMK3 have only two putative serine PKC phosphorylation sites located in the C terminus (1). Stimulation of PKC has been shown to inhibit the activity of the small conductance K^+ (SK) channel in the CCD (8,9). Since ROMK1 is believed to be an important component of the native SK channel, it is conceivable that ROMK1 can also be regulated by PKC. Indeed, we have shown previously that addition of exogenous PKC catalytic subunits inhibited the activity of ROMK1 in excised patches in *Xenopus* oocytes (10). However, there is no direct evidence that ROMK1 can be phosphorylated by PKC. Moreover, it is also not clear whether all three putative serine PKC phosphorylation sites can be equally modified by PKC. Finally, it has not yet been explored whether PKC has an effect on the ROMK1 channel other than inhibiting the K^+ channel in the cell membrane. The aims of the present study are to determine whether PKC can phosphorylate ROMK1 and to explore the role of PKC-induced phosphorylation of ROMK1 in the modulation of K^+ channel activity.

Materials and Methods

Generation of ROMK1 Mutants

We used site-directed mutagenesis to generate the following ROMK1 mutants: R1S4/201A, R1S4/183A, R1S183A, R1S201A, and R1S4/201D. The ROMK1 mutants, R1S183/201A, R1S4A, and R1S4/183/201A, were kindly provided by Dr. Hebert's laboratory, Yale University. Each mutation was confirmed by nucleotide sequencing.

Purification of the His-tagged ROMK Channels

ROMK1 and mutants (except for R1S4/201D) were subcloned into a pBADHisB vector using restriction enzyme sites, *Xho*I and *Eco*RI, and the constructs were transformed to *Escherichia coli* Top10 stain (Invitrogen). The expression was induced by 0.02% arabinose at 37 °C by vigorously shaking for 2 h. The His-tagged ROMK channel proteins were purified as described previously (11), and their purity was determined by Coomassie Blue staining. Western blot analysis using a ROMK1 antibody (Alamone Laboratories, Jerusalem, Israel) was conducted to confirm that the purified proteins were ROMK channels.

Peptide Synthesis

Three peptides corresponding to amino acid sequences 1–16 (MGASERSVFRVLIRAL), 174–189 (MCGAILAKISRPKKRA), and 196–211 (KNAVISKRGGKLCLLI) of ROMK1 were synthesized by Sigma-Genosys. As negative controls, peptides containing a serine-to-alanine mutation at residues corresponding to serine 4, 183, and 201 of ROMK1 (residues indicated in bold) were also made and are referred to as peptide 1–16(S4A), 174–189(S183A), and 196– 211(S201A), respectively.

In Vitro Phosphorylation

In vitro phosphorylation was prepared as described previously (11). Briefly, 10 μl of purified His-tagged ROMK1 proteins (0.12 μg) or peptides (1 μg) and 25 ng of PKCα (Upstate

[1]The abbreviations used are: CCD, cortical collecting duct; PKC, protein kinase C; HEK, human embryonic kidney; GFP, green fluorescent protein; EGFP, enhanced GFP; MOPS, 4-morpholinepropanesulfonic acid; PTK, protein-tyrosine kinase; NHS, *N*-hydroxysulfosuccinimide.

Biotechnology, Lake Placid, NY) were incubated at 30 °C for 15 min in the presence of 20 mM MOPS (pH 7.2), 15 mM $MgCl_2$, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM $CaCl_2$, 0.5 mg/ml phosphatidylserine, 0.05 mg/ml diglycerides, and 100 μM [^{32}P]ATP (400 cpm/pmol). The reactions were stopped by adding 3× SDS sample buffer, and the mixture was boiled for 5 min. The peptides or proteins were resolved by SDS-polyacrylamide gel electrophoresis using 20 or 10% gel, respectively. The gels were stained by Coomassie Blue R-250, destained, and subjected to autoradiography using Kodak AR film at −80 °C. The density of the band was determined using Alpha DigiDoc 1000 (Alpha Innotech Corp., San Leandro, CA).

Preparation of Xenopus Oocytes

Female *Xenopus laevis* were obtained from NASCO (Fort Atkinson, WI), and oocytes were isolated as described previously (12). In brief, the follicular layer of oocytes was removed under a dissecting microscope with two watchmaker forceps. After dissection, the oocytes were incubated overnight at 19 °C in a solution containing 66% Dulbecco's modified Eagle's medium/F12 medium with freshly added 2.5 mM sodium pyruvate and 50 μg/ml gentamycin. Viable oocytes were selected and microinjected with cRNA containing either GFP-ROMK1 or GFP-ROMK1 mutants (25 ng). In some experiments, oocytes were injected with ROMK1 without GFP tag (5 ng/egg). Previous studies demonstrated that the biophysical properties of GFP-tagged ROMK1 are similar to those of ROMK1 (13,14). The oocytes were incubated at 19 °C in a 66% Dulbecco's modified Eagle's medium/F12 medium, and experiments were performed 2–3 days after injection. To study the effect of PKC on ROMK1 expression, 50% of the eggs after injection were selected for incubation in medium containing 200 nM calphostin C.

Two-electrode Voltage Clamp

A Warner oocyte clamp OC-725C was used to measure the whole cell K^+ current. Voltage and current microelectrodes were filled with 1 M KCl and had a resistances of less than 2 megaohms. Series resistance of the pipette was compensated, and currents were recorded on a chart recorder (Gould TA240). To correct for leak currents, 2 mM Ba^{2+} was used to determine the Ba^{2+}-sensitive K^+ current.

Fluorescence Localization of ROMK1

The membrane expression of ROMK1 was examined 48 h after injection by laser scanning confocal microscopy. Three sections of each oocyte membrane were recorded, and the signal was averaged for each egg. Oocytes were imaged using a Bio-Rad MRC1000 confocal microscope. GFP fluorescence was excited at 488 nM with an argon laser beam and viewed with an inverted Olympus microscope equipped with a ×20 dry lens. XY scans were obtained at approximately the midsection of each egg. All images were acquired, processed, and printed with identical parameters.

Preparation of cRNA for Oocyte Injection

The preparation of cRNA encoding GFP-ROMK1 and ROMK1 mutants has been described previously (12). The GFP-ROMK1 cDNA construct was prepared as follows: Full-length ROMK1 cDNA was made from PCDNA3.1/ROMK1 by PCR using the sense primer of TTGTAGGTGGAAGGATCCTGCTACATCTGGGTGTCG and the antisense primer of TGGGCCTAAAAGAATTCAGCTGCTGTGCACGACAAC. The 1.2-kb cDNA digested with EcoRI and BamHI was cloned into PEGFPC vector (Clontech Laboratories) cut with the same restriction enzymes. The sequence of the GFP-ROMK1 construct was confirmed by sequencing (W. M. Keck Biotechnology Resource Laboratory, Yale University, New

Haven, CT). To make cRNA coding for GFP-ROMK1, cDNA of the fusion protein was subcloned into pSport vector and transcribed *in vitro* from the T7 promoter.

Biotinylation, Immunoprecipitation, and Western Blot Analysis

HEK293 cells were transfected with GFP-ROMK1 or mutants followed the method described previously (14). ROMK channels in the cell membrane were quantitated by labeling the HEK293 cells with cell-impermeant sulfo-NHS-biotin (Pierce). Following biotinylation, the cells were washed two times with PBS and trypsinized with trypsin-EDTA. They were pelleted by centrifugation for 5 min at 10,000 rpm, washed two times with PBS, and lysed with cold radioimmune precipitation buffer (1× PBS, 1% Igepal CA-630, 0.1% SDS, 0.5% deoxycholate) supplemented with 1 mM sodium molybdate, 1 mM sodium fluoride, 1 μM phenylmethylsulfonyl fluoride, and 100 μl of protease inhibitor mixture (Sigma) per ml of lysis buffer. After clarification, total protein concentrations was determined with the Bio-Rad protein assay kit, and aliquots of lysates containing equal amounts of protein were immunoprecipitated overnight with 1 μg of a monoclonal antibody of GFP (Clontech) and 20 μl of protein A/G agarose (Santa Cruz Biotechnology, Santa Cruz, CA). After centrifuging and washing two times with PBS, proteins were resolved by electrophoresis on 10% SDS gel and transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked with 5% milk in Tris-buffered saline, and the biotin-labeled GFP-ROMK1 proteins were detected using NeutrAvidin horseradish peroxidase (Pierce). Changes in biotin-labeled surface ROMK1 proteins were normalized with the corresponding total ROMK1 protein detected with ROMK antibody (Alamone Laboratories, Jerusalem, Israel). The density of the band was determined using Alpha DigiDoc 1000 (Alpha Innotech, San Leandro, CA).

Immunocytochemistry

COS7 cell line (ATCC) was transiently transfected with pEGFP-ROMK1, R1S4/201A, R1S4/201D, and R1S4/183/201A using the method described previously (14). The expression of ROMK1 and ROMK1 mutants was examined by confocal microscope. Also, the cells transfected with R1S4/201A and R1S4/183/201A were fixed with pure methanol for 10 min in −20 °C. The slide containing the ROMK mutant-transfected cells was washed twice with PBS and blocked with a PBS buffer composed of 2% goat serum, 1% bovine serum albumin, and 0.4% Triton X-100 for 1 h. The cells were then stained with a polyclonal antibody of calnexin (1:100) (Stressgen) overnight at 4 °C. The slide was washed with 0.4% Triton X-100 in PBS for 30 min. The confocal microscope was used to examine the localization of ROMK channels and calnexin.

Experimental Solution and Statistics

The bath solution for the two-electrode voltage clamp was composed of 150 mM KCl, 2.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 1 mM EGTA, 5 mM HEPES (pH 7.4). Calphostin C was purchased from Sigma and added directly to the bath to reach the final concentration. The results obtained from experiments with calphostin C were compared with those without PKC inhibitor. We present data as mean ± S.E. The Student's *t* test was used to determine the significance.

Results

His-tagged ROMK1 was expressed in *E. coli*, purified, and used as an *in vitro* substrate for PKC. We confirmed the previous observation that Coomassie Blue staining can identify a 50-kDa protein, which is also recognized by ROMK antibody (11). Fig. 1A is a typical Coomassie Blue staining showing the presence of a 50-kDa protein purified from *E. coli* transformed with constructs containing ROMK1 mutants including R1S4A, R1S183A,

R1S201A, R1S183/201A, R1S4/201A, and R1S4/183A. Western blot analysis further demonstrated that those 50-kDa proteins can be detected by a ROMK antibody (Fig. 1B). To assess whether ROMK1 is a substrate for PKC, the purified ROMK1 proteins were incubated with PKCα in the presence of [^{32}P]ATP. Fig. 2 is a typical autoradiograph showing that PKC phosphorylated two proteins, an 81- and a 50-kDa protein. The 81-kDa protein is most likely autophosphorylated PKC and is phosphorylated even in the absence of ROMK1. The 50-kDa protein corresponds to ROMK1, and its phosphorylation was not detected if ROMK1 was omitted for the assay. This result suggests that ROMK1 is an *in vitro* PKC substrate. From analysis of the amino acid sequence of ROMK1, it contains three putative PKC phosphorylation sites, serine residues 4, 183, and 201.

To determine whether PKC can phosphorylate each of these serine residues, we carried out *in vitro* phosphorylation using three synthesized peptides that correspond to the amino acid sequences 1–16, 174–189, and 196–211 of ROMK1. PKC preferentially phosphorylated peptides 1–16 and 196–211 (Fig. 3A). In contrast, PKC failed to phosphorylate peptide 174–189. The PKC-induced phosphorylation of peptides 1–16 and 196–211 was specific for serine residues 4 and 201 because ^{32}P incorporation fell by 90% (n = 3) (Fig. 3, B and C) when alanine was replaced with serine residue 4 (1–16(S4A)) or 201 (196–211(S201A)).

The finding that peptide 174–189 could not be phosphorylated by PKC suggests that serine residue 183 of ROMK1 may not be a major phosphorylation site for PKC. This possibility was further explored by using purified mutant ROMK1 proteins, R1S183/201A, R1S4/201A, and R1 S4/183A, in which only one serine residue (4, 183, or 201) was kept intact. The purified ROMK1 mutants were incubated with PKC and [^{32}P]ATP and resolved by electrophoresis. After exposure of the gel to an x-ray film, the autoradiographs revealed that [^{32}P] was significantly incorporated into R1S183/201A and R1S4/183A, whereas [^{32}P] incorporation of R1S4/201A was only 20 ± 5% (n = 7) of that incorporated into either R1S183/201A or R1S4/183A (Fig. 4A). Fig. 4B demonstrates that PKC-induced phosphorylation of R1S183/201A is approximately half of that observed with wild type ROMK1. The same results were observed when R1S4/183A was used as an *in vitro* substrate for PKC (data not shown). This is consistent with the notion that PKC appears to preferentially target both serine residues 4 and 201.

After observing that serine residues 4 and 201 may be two main sites for the PKC-induced phosphorylation, we examined the role of serine residues 4 and 201 in the regulation of channel activity. The two-electrode voltage clamp technique was used to study the K^+ current in oocytes injected with ROMK1, R1S4A, R1S183/201A, R1S183A, R1S4/201A, R1S201A, and R1S4/183A. Fig. 5 shows that the Ba^{2+}-sensitive K^+ current of oocytes injected with wild type GFP-ROMK1 was 6.5 ± 0.9 μA (n = 25 from five frogs), a value not significantly different from that in oocytes injected with GFP-ROMK1 mutants, R1S4A (6.3 ± 1.4 μA, n = 20), R1S183/201A (5.7 ± 0.2 μA, n = 21), R1S183A (7.2 ± 0.5 μA, n = 18), R1S201A (5.9 ± 0.2 μA, n = 18)), and R1S4/183A (5.5 ± 0.2 μA, n = 21). In contrast, the Ba^{2+}-sensitive K^+ current in oocytes injected with GFP-R1S4/201A was only 0.5 ± 0.1 μA (n = 67, seven frogs), and no K^+ current was detected in oocytes injected with R1S4/183/201A (n = 30, four frogs). This suggests that serine residues 4 and 201 are absolutely required for ROMK1 activity. To further determine whether a decrease in K^+ currents in oocytes injected with R1S4/201A resulted from lack of membrane expression of ROMK1 or the expression of silent K^+ channels, we used confocal microscopy to examine the surface expression in oocytes injected with GFP-ROMK1 and GFP-ROMK1 mutants. From inspection of Fig. 6, it is apparent that fluorescence intensity, an index of ROMK1 expression, in oocytes injected with R1S4/201A was only 5 ± 1% of that in oocytes injected with GFP-ROMK1. In contrast, a strong fluorescence image was observed in oocytes injected with R1S4A, R1S183A, R1S201A, R1S4/183A, and R1S183/201A. No

fluorescence signal can be detected from oocytes injected with R1S4/183/201A (data not shown). These observations demonstrate that either serine residue 4 or 201 is required for the expression of ROMK1 channels in the cell membrane.

This conclusion is also supported by experiments in which the biotin labeling technique was used to examine the role of putative PKC phosphorylation sites, serine residues 4 and 201, in the regulation of the membrane expression of ROMK1 channels (Fig. 7). The GFP-ROMK1, R1S4/201A, and R1S4/183/201A were transfected in HEK293 cells. The ROMK1/mutants expressed in the cell membrane were labeled with biotin at 4 °C. The ROMK1/mutant channels were harvested by immunoprecipitation of the cell lysate with GFP antibody. The total ROMK1/mutant channels and the biotin-labeled K^+ channels were detected with ROMK antibody and neutravidin, respectively. Fig. 7A shows that although the total R1S4/201A and R1S4/183/201A channels were almost the same as ROMK1 (*bottom panel*), the biotin-labeled R1S4/201A and R1S4/183/201A were only 6 ± 1% and 4 ± 1% (n = 5) of wild type ROMK1, respectively.

The notion that PKC-induced phosphorylation is required for the ROMK1 expression in the cell surface is further supported by experiments in which mutation of both serine residues 4 and 201 to aspartate restored the K^+ currents. Fig. 5 summarizes results in which the K^+ current was measured in oocytes injected with GFP-R1S4/201D. It is apparent that the K^+ current increased slightly by 30 ± 10%, from 6.5 to 8.5 ± 1.5 μA (n = 42, four frogs), in comparison with currents measured in oocytes injected with GFP-ROMK1. Moreover, confocal imaging showed strong surface expression of oocytes injected with R1S4/201D (Fig. 6). This suggests that mutation of serine residues 4 and 201 to the negative charged amino acid mimics the effect of PKC-induced phosphorylation and facilitates the membrane expression of ROMK1. This notion is also supported by experiments in which the biotin labeling technique was used to examine the expression of R1S4/201D in HEK293 cells. Fig. 7B is a typical Western blot showing the surface expression of R1S4/201D in HEK293 cells. The *top panel* shows the biotin-labeled surface R1S4/201D and ROMK1, whereas the *bottom panel* demonstrates the total ROMK1 and R1S4/201D. Clearly, mutation of serine residues 4 and 201 to negative charged amino acid has slightly increased the biotin-labeled fraction of R1S4/201D (120 ± 19% of the ROMK1 control, n = 4).

After establishing that serine residues 4 and 201 play a key role in the regulation of ROMK1 membrane expression, we extended our study to examine whether the lack of membrane expression of R1S4/201A and R1S4/183/201A resulted from no translation of the ROMK mutants or from a defective membrane delivery. GFP-ROMK1 and mutants were transiently expressed in COS7 cells, and confocal microscopy was used to determine the location of ROMK channels. Fig. 8 is a typical confocal image showing that surface expression of ROMK channels is observed in COS7 cells transfected with GFP-ROMK1, whereas R1S4/201A and R1S4/183/201A are mainly located in the perinuclear region. In contrast, the expression of R1S4/201D is the same as that of wild type ROMK1. We also carried out immunocytochemistry with an antibody of calnexin to determine whether R1S4/201A and R1S4/183/201A are colocalized with calnexin. Since calnexin is a chaperon protein in the endoplasmic reticulum (15), it has been used as an endoplasmic reticulum marker. Fig. 9 is a representative cell image demonstrating colocalization between the R1S4/201A or R1S4/183/201A and the endoplasmic reticulum marker. This suggests that mutation of serine residues 4 and 201 may interfere with the ROMK channel export.

To further examine the role of PKC in the regulation of ROMK1 expression, we also investigated the effect of calphostin C, a PKC inhibitor, on ROMK1 current. Fig. 10 summarizes results from experiments in which calphostin C was added to the incubation medium immediately after oocytes were injected with either GFP-ROMK1 or ROMK1 (n =

44, five frogs). It is apparent that 200 nM calphostin C significantly decreased K^+ currents by 61 ± 9% (n = 44), in comparison with K^+ currents measured in oocytes incubated in medium without PKC inhibitor. The effect of calphostin C is specifically related to the inhibition of PKC-induced phosphorylation of ROMK1 because calphostin C had no significant effect on K^+ current in oocytes injected with R1S4/201D. To determine whether the decrease in K^+ current induced by inhibition of PKC results from reducing the surface expression of ROMK1 or diminished ROMK1 translation, we examined the surface ROMK1 and the total ROMK1 expression in HEK293 cells in the presence or absence of calphostin C (Fig. 11A). The surface fraction of ROMK1 was labeled with biotin (*top panel*), and the total ROMK1 channels were detected with ROMK antibody (*bottom panel*). Inhibition of PKC with 200 nM calphostin C did not affect the translation of ROMK1 as evidenced by the finding that equal amounts of ROMK1 were expressed in the presence and absence of calphostin C. However, calphostin C significantly decreased the biotin-labeled ROMK1 by 65 ± 7% (n = 5). In contrast, neither total R1S4/201D nor the biotin-labeled ROMK1 mutant expression was significantly affected by inhibition of PKC in HEK293 cells transfected with R1S4/201D (109 ± 15% of the control value, n = 4) (Fig. 11B). This indicates that calphostin C-induced decrease in ROMK1 current results from the inhibition of ROMK1 export to the cell membrane.

Discussion

In the present study, we have demonstrated that PKC can phosphorylate ROMK1 channels *in vitro* and that the PKC-induced phosphorylation of ROMK1 plays a key role in the regulation of the surface expression of ROMK1. ROMK1 is mainly located in the apical membrane of the CCD and is a major contributor to the apical K^+ conductance (2). Although it is not clear whether additional subunits, such as cystic fibrosis transmembrane conductance regulator (CFTR) or sulfonylurea receptor, are required to form a native SK channel in the CCD (16–19), it is well established that ROMK1 is the key component of the SK channel (2,20,21), which plays an important role in K^+ secretion in the CCD under physiological conditions (2,22). However, it is possible that K^+ channels other than ROMK1 may also be involved in the regulation of K^+ secretion, such as Ca^{2+}-activated large conductance K^+ channels (23,24) and voltage-gated K^+ channels (25). It has been demonstrated that an increase in tubule flow rate stimulates the activity of the Ca^{2+}-activated large conducance K^+ channels (26). However, it is generally believed that ROMK1 is the main K^+ channel responsible for K^+ secretion under physiological conditions.

K^+ secretion in principal cells requires two steps: K^+ enters the cell across the basolateral membrane via Na,K-ATPase, and K^+ then leaves the cell across the apical membrane through ROMK1 (5). To maintain a constant intracellular K^+ concentration during K^+ secretion, the turnover rate of the Na,K-ATPase must be in concert with the activity of the apical SK channels. We have demonstrated previously that PKC plays a key role in linking the function of the Na,K-ATPase to the activity of the apical SK channels: an inhibition of the Na,K-ATPase leads to an increase in the intracellular Ca^{2+} and activation of Ca^{2+}-dependent PKC, which in turns down-regulates the SK channels (9). This suggests that PKC-induced phosphorylation is an important mechanism for regulating the ROMK-like SK channels in the CCD.

The mechanism by which PKC-induced phosphorylation regulates ROMK1 and other ROMK isoforms is not completely understood. Our previous investigation demonstrated that addition of exogenous PKC decreased ROMK1 activity in inside-out patches. This suggests that PKC-mediated phosphorylation of ROMK1 can inhibit channel activity (10). In the present investigation, we have provided three lines of evidence to suggest that PKC can also have a stimulatory effect on ROMK channels because PKC is involved in the regulation of

ROMK1 export to the cell membrane. First, mutation of the two putative PKC phosphorylation sites of ROMK1 (serine residues 4 and 201) to alanine almost completely abolished K^+ currents. Second, mutation of serine residues 4 and 201 to the negatively charged aspartate mimicked the PKC-induced phosphorylation and restored K^+ currents in oocytes injected with R1S4/201D. Third, inhibition of PKC significantly diminished the K^+ current in oocytes injected with ROMK1 and the biotin-labeled surface ROMK1 channels in HEK293 cells. The reason that calphostin C did not completely abolish K^+ current in oocytes injected with ROMK1 may be due to the fact that calphostin C did not completely block PKC activity. Alternatively, there is a calphostin C-insensitive PKC isoform that modulates the phosphorylation of ROMK1.

Take together, our data suggest a dual role of PKC in the regulation of ROMK1: 1) PKC is involved in the ROMK1 export to the cell membrane and 2) PKC can decrease the activity of ROMK1 existing in the cell membrane. It is conceivable that a variety of PKC isoforms expressed in the CCD have different effects on the modulation of ROMK channels. PKC isoforms located in the perinuclear region may be involved in the phosphorylation of newly synthesized ROMK channels, and the PKC-mediated phosphorylation of ROMK channels is essential for ROMK export to the cell membrane. On the other hand, once ROMK channels have been delivered to the cell membrane, stimulation of PKC inhibits channel activity. However, the observation that mutation of serine residues 4 and 201 to the negatively charged aspartate did not decrease K^+ currents in oocytes injected with R1S4/201D suggests that phosphorylation of serine 4 and 201 alone may not be sufficient to block the activity of ROMK1. It is possible that the PKC-induced inhibition of ROMK1 channels may require the involvement of a protein other than ROMK1. This protein may also be a substrate for PKC and required for the inhibitory effect of PKC on ROMK1. In this regard, it has been reported recently that the PKC-induced phosphorylation may decrease the sensitivity of ROMK2 to PIP2 (27). Because PIP2 is essential for maintaining the ROMK channel activity (28,29), a decrease in PIP2 sensitivity is expected to inhibit the channel activity. One possible interpretation is that stimulation of PKC not only phosphorylates ROMK channels but also an unidentified protein, which decreases the affinity of ROMK channels to PIP2 or PIP2 concentrations. Further experiments are needed to explore the mechanism by which PKC inhibits ROMK1.

ROMK1 has three putative serine phosphorylation sites, serine residues 4, 183, and 201 (1). Three lines of evidence indicate that serine residues 4 and 201 are the two major phosphorylation sites of PKC. First, strong ^{32}P incorporation is observed in peptides containing amino acids 1–16 and 196–211 of ROMK1. In contrast, ^{32}P incorporation into the peptide corresponding to amino acids 174–189 of ROMK1 was almost absent. Second, mutation of serine residues corresponding to serine 4 of ROMK1 in peptide 1–16 and serine 201 of ROMK1 in peptide 196–211 almost completely abolished the PKC-induced phosphorylation. This suggests that PKC specifically phosphorylates serine 4 and 201 of ROMK1. Third, PKC-induced phosphorylation of R1S4/201A was only a modest 20% of those observed with R1S4/183A and R1S183/201A. Therefore, our data strongly indicate that PKC primarily phosphorylates ROMK1 on serine residues 4 and 201 and, to a much lesser extent, on serine residue 183. Also, our present study suggests that phosphorylation of either serine residue 4 or 201 is essential for facilitating ROMK1 export to the cell membrane. First, mutation of serine residues 4 and 201 to alanine almost completely abolished the K^+ current and ROMK1 channel expression. Second, mutation of serine residues 4, 183, and 201 to alanine completely blocked the ROMK1 expression. Third, the K^+ current and ROMK1 expression in oocytes injected with R1S183/201A, R1S4/183A, R1S4A, R1S183A, and R1S201A were similar to those with wild type ROMK1. This indicates that if either serine residue 4 or 201 is intact, the expression of ROMK1 appears to be normal.

The mechanism by which PKC-induced phosphorylation regulates the ROMK1 export is not clear. The observation that inhibition of PKC decreased the expression level of ROMK1 suggests that PKC-induced phosphorylation is required for the export of ROMK1. We cannot completely exclude the possibility that the effect of calphostin C on ROMK1 channel expression results from inhibition of a pathway that is required for ROMK1 trafficking. However, two lines of evidence strongly suggest that the effect of calphostin C on ROMK1 channel activity results from inhibition of ROMK1 phosphorylation: 1) inhibition of PKC had no effect on K^+ currents in oocytes injected with R1S4/201D and 2) calphostin C reduced the biotin-labeled ROMK1 but had no effect on the surface expression of R1S4/201D in HEK293 cells. The physiological significance of the stimulatory effect of PKC on ROMK channels is not clear. An increase in dietary K^+ intake has been shown to increase the apical ROMK-like SK channel in the rat CCD (30). It would be interesting to determine whether PKC is involved in mediating the effect of high dietary K^+ intake on ROMK channels. We need further experiments to explore the mechanism of PKC-mediated ROMK1 trafficking and the physiological significance.

We have demonstrated previously that protein-tyrosine kinase (PTK) and protein tyrosine phosphatase are involved in ROMK1 trafficking (13,14,31) and have now provided evidence that PKC is also required for the ROMK1 export. It is possible that PKC and PTK regulate two different pools of ROMK channels. We speculate that stimulation of PTK activity increases internalization of ROMK1 from cell membrane to endosome, whereas stimulation of protein tyrosine phosphatase activity enhances the insertion of ROMK1 from endosome to the cell membrane. In contrast, PKC-induced phosphorylation of ROMK channels may be essential for the export of *de novo* synthesized ROMK channels. However, it is also conceivable that PKC may also be involved in mediating the effect of stimulation of PTK. A tyrosine phosphorylation has been reported to activate atypical PKC, which in turns stimulates the glucose transport in rat adipocytes (32). Also, PKC has been shown to be a downstream signaling of insulin and to stimulate the exocytosis of glucose transport such as GLUT4 (32,33). We need additional experiments to explore the possibility that PKC may be a downstream signaling of stimulation of PTK in the CCD.

We conclude that ROMK channels are a substrate of PKC and that serine residues 4 and 201 of ROMK1 are the two major phosphorylation sites of PKC. Serine residues 4 and 201 play an important role in the regulation of ROMK1 export to the cell membrane.

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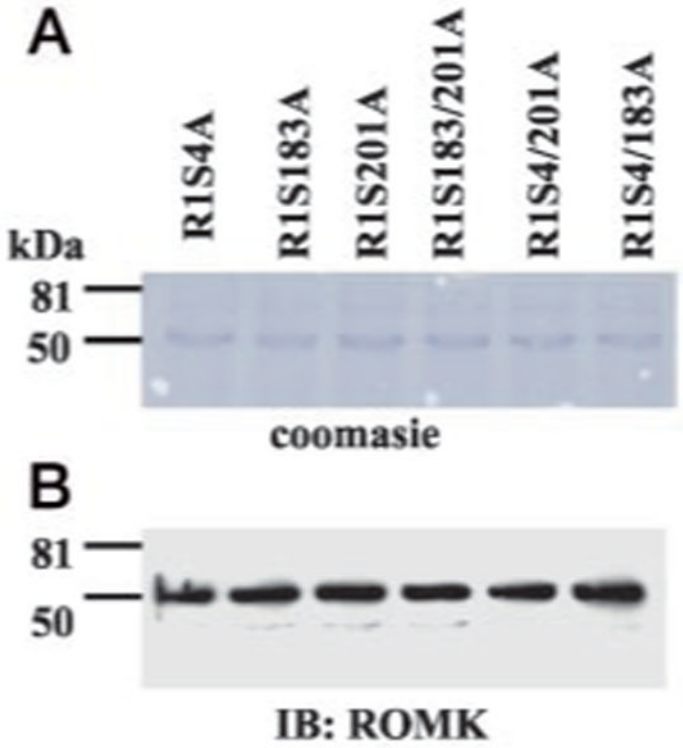

Fig. 1.

A, a Coomassie Blue staining shows that a 50-kDa protein was purified from *E. coli* transformed with ROMK1 mutants (R1S4A, R1S183A, R1S201A, R1S183/201A, R1S4/201A, and R1S4/183A). *B*, a Western blot analysis with ROMK antibody. *IB*, immunoblot.

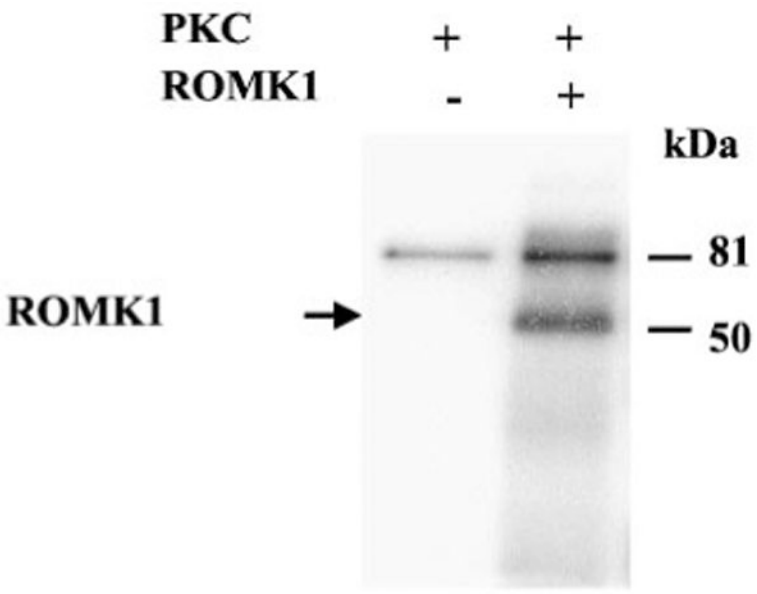

Fig. 2. An autoradiograph showing that radiolabeled ATP was incorporated into an 81- and a 50-kDa protein, respectively

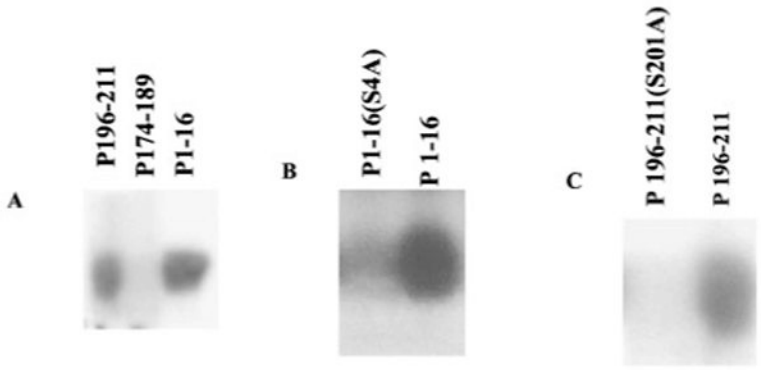

Fig. 3. Autoradiograph showing the PKC-induced phosphorylation of three synthesized peptides

A, an autoradiograph illustrating the results from an *in vitro* phosphorylation assay of three peptides corresponding to amino acid sequences of ROMK1, 196–211, 174–189, and 1–16, respectively. *B*, *in vitro* phosphorylation assay with peptides 1–16 and 1–16(S4A), which has the same sequence as that of 1–16 except that serine residue 4 was mutated to alanine. *C*, *in vitro* phosphorylation assay with peptides 196–211 and 196–211(S201A), which has the same sequence as that of peptide 196–211 except that serine residue 201 was mutated to alanine.

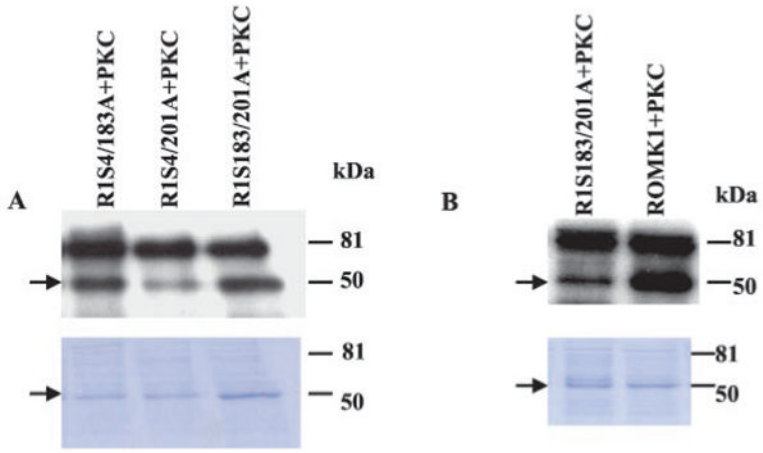

Fig. 4. Autoradiograph demonstrating the PKC-mediated phosphorylation of ROMK1 R154/183A, R154/201A, and R15183/201A

In *A*, the *top panel* of the figure shows the result from an *in vitro* PKC phosphorylation of ROMK1 mutants, R1S4/183A, R1S4/201A and R1S183/201A. The *bottom panel* of the figure is a Coomassie Blue staining demonstrating the amount of proteins which were used to normalize data. The arrow indicates the position of ROMK1. In *B*, the *top panel* of the figure shows the result from an *in vitro* phosphorylation of ROMK1 and R1S183/201A. The *bottom* of the figure is a Coomassie Blue staining.

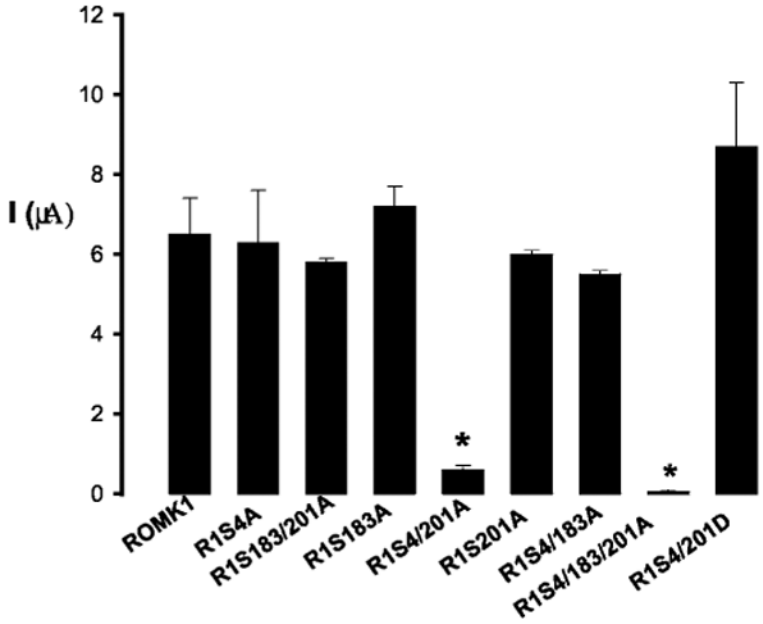

Fig. 5. The Ba^{2+}-sensitive K^+ currents measured with two-electrode voltage clamp in oocytes injected with GFP-ROMK1 and its mutants, respectively

Each egg was injected with 25 ng of cRNA encoding ROMK1 or mutants.

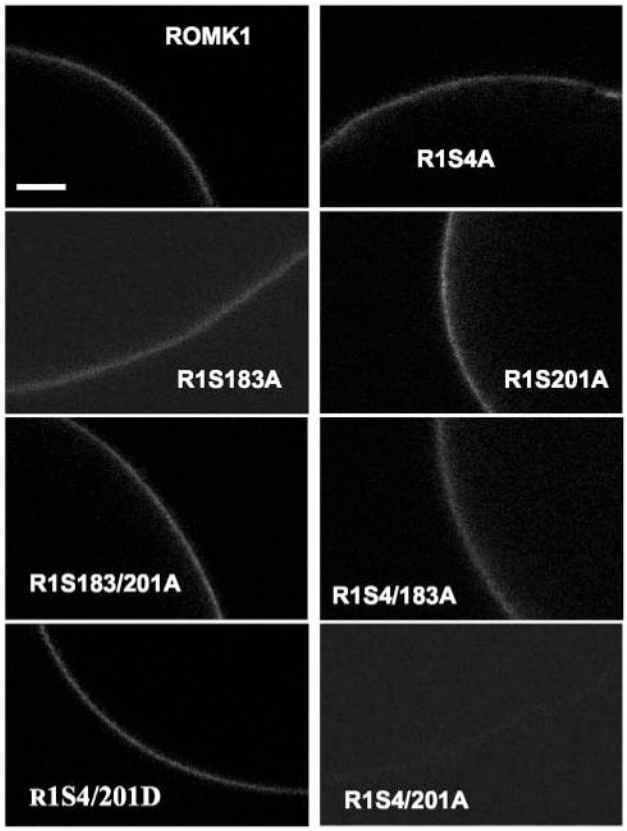

Fig. 6. Confocal microscope images demonstrating the surface expression of ROMK1 or mutants in oocytes injected with GFP-ROMK1 and ROMK1 mutants, respectively

The *bar* represents 100 μm.

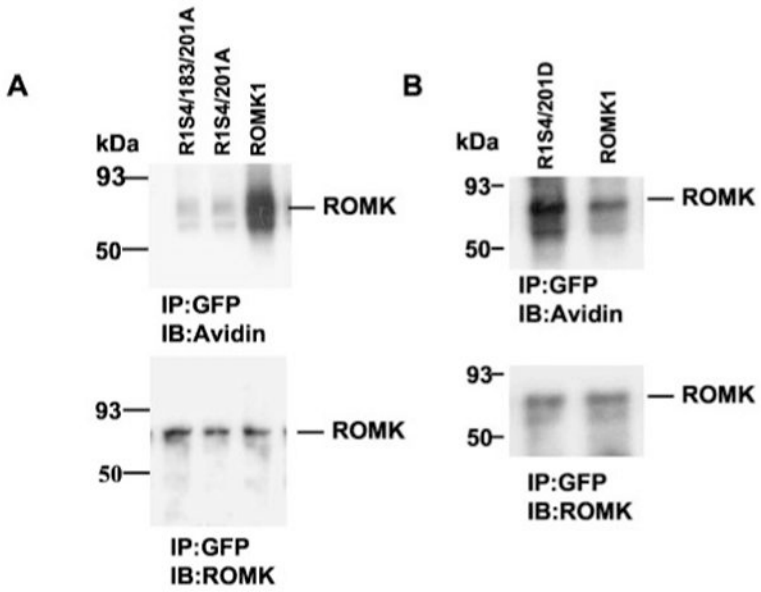

Fig. 7. Western blot showing membrane expression of ROMK1, R1S4/201A and R1S4/183/201A (*A*), and R1S4/201D (*B*)

The *top panel* demonstrates the biotin-labeled ROMK1 or mutants, and the *bottom panel* shows the total ROMK1 or mutant content in HEK293 cells. The ROMK1 position is indicated in the figure by a *short bar*. *IB*, immunoblot. *IP*, immunoprecipitation.

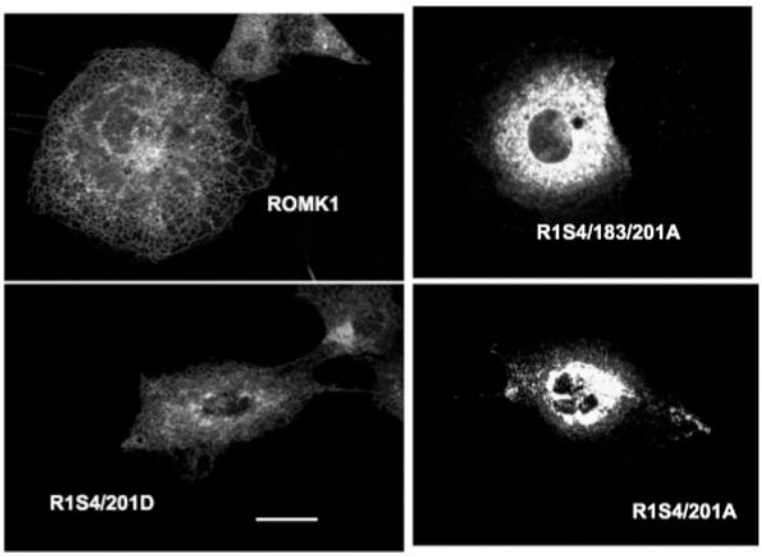

Fig. 8. Confocal microscope images show the expression of ROMK1 and ROMK1 mutants in COS7 cells

Bar represents 25 μm.

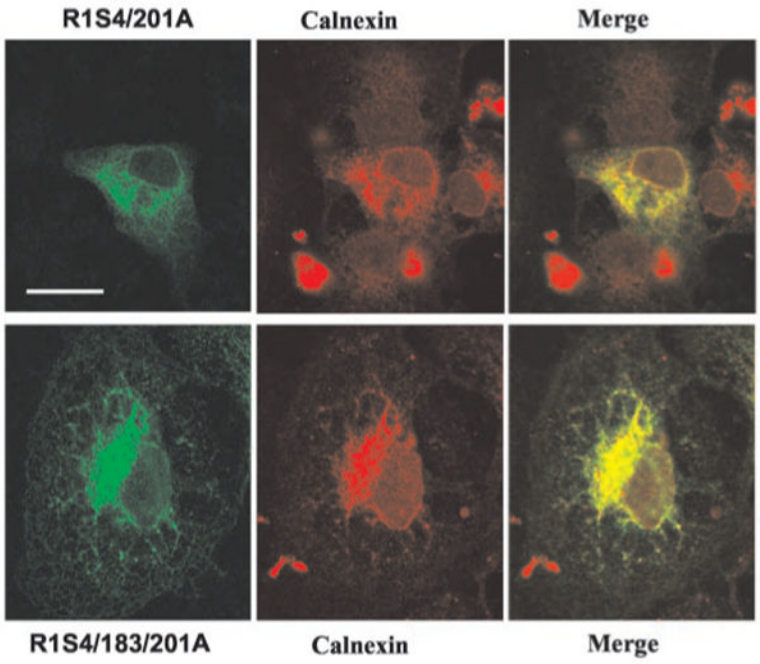

Fig. 9. Confocal microscope images demonstrate the location of R1S4/201A (*green*), R1S4/183/201A (*green*), and calnexin (*red*)

GFP-R1S4/201A and GFP-R1S4/183/201A were transiently transfected to COS7 cells. The cells were fixed with methanol 2 days after transfection, and the immunocytochemical study was carried out using antibodies of calnexin (1:100).

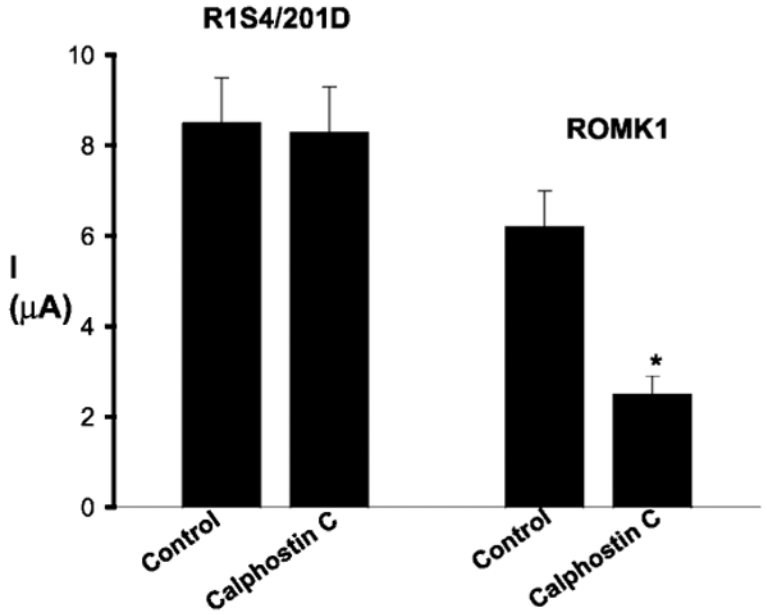

Fig. 10. The effect of calphostin C (200 nM) on K^+ current in oocytes injected with GFP-ROMK1 and GFP tagged R1S4/201D

After injection, oocytes were divided into control and experimental groups in which oocytes were incubated in a medium containing 200 nM calphostin C. K^+ currents were measured 48–72 h after injection. The *asterisk* indicates that the data are significantly different from the control.

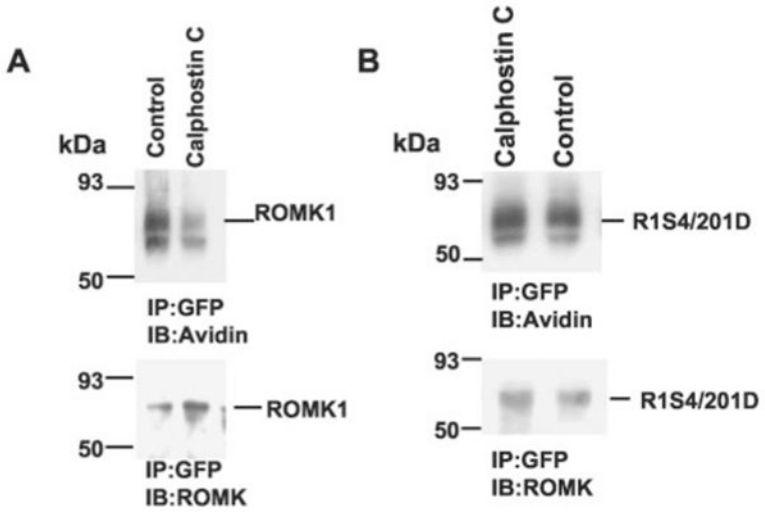

Fig. 11. Western blot shows the effect of calphostin C on the biotin-labeled ROMK1 (*A*) and R1S4/201D (*B*)

HEK293 cells were transfected with GFP-ROMK1 or GFP-R1S4/201D in the presence or absence of calphostin C (200 n_M). The surface K^+ channels were labeled with biotin 48 h after transfection. The cell lysates were immunoprecipitated with GFP, and the biotin-labeled ROMK1/mutant was detected with avidin. The expression of ROMK1 or mutant in the cell membrane was normalized by comparison with its corresponding total ROMK channels. *IB*, immunoblot. *IP*, immunoprecipitation.