Published in final edited form as:
Am J Physiol Renal Physiol. 2002 October ; 283(4): F671–F677. doi:10.1152/ajprenal.00160.2002.

K depletion increases protein tyrosine kinase-mediated phosphorylation of ROMK

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Abstract

We purified Histagged ROMK1 and carried out in vitro phosphorylation assays with ^{32}P-radiolabeled ATP to determine whether ROMK1 protein is a substrate for PTK. Addition of active c-Src and [^{32}P]ATP to the purified ROMK1 protein resulted in the phosphorylation of the ROMK1 protein. However, c-Src did not phosphorylate R1Y337A in which tyrosine residue 337 was mutated to alanine. Furthermore, phosphopeptide mapping identified two phosphopeptides from the trypsin-digested ROMK1 protein. In contrast, no phosphorylated peptide has been found in the trypsin-digested R1Y337A protein. This suggested that two phosphorylated peptides might contain the same tyrosine residue. Also, addition of c-Src and [^{32}P]ATP phosphorylated the synthesized peptide corresponding to amino acid sequence 333–362 of the COOH terminus of ROMK1. We then examined the effect of dietary K intake on the tyrosine-phosphorylated ROMK level. Although the ROMK channels pulled down by immunoprecipitation with ROMK antibody were the same from rats on a K-deficient diet or on a high-K diet, more ROMK channels were phosphorylated by PTK in rats on a K-deficient diet than those on a high-K diet. We conclude that ROMK1 can be phosphorylated by PTK and that tyrosine residue 337 is the key site for the phosphorylation. Also, the tyrosine phosphorylation of ROMK is modulated by dietary K intake. This strongly suggests that PTK is an important member of the aldosterone-independent signal transduction pathway for regulating renal K secretion.

Keywords

renal potassium secretion; hypokalemia; dietary potassium intake; protein tyrosine phosphatase; cortical collecting duct

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The small-conductance K (SK) channel plays an important role in regulating renal K secretion, because the SK channels provide the major conductive pathway for K across the apical membrane of the cortical collecting duct (CCD) (5). Several factors, such as

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aldosterone and vasopressin, have been shown to regulate renal K secretion (3,19,29). In addition, dietary K intake is involved in regulating renal K secretion; a high-K (HK) intake increases, whereas a low-K intake suppresses, renal K secretion (13,14,21).

We have previously demonstrated that PTK plays a key role in mediating the effect of dietary K intake on the SK channel in the CCD (24,27). We have speculated that a low-K intake increases phosphorylation of the SK channels by PTK and accordingly decreases channel activity in the CCD. In contrast, an HK intake decreases the PTK-induced tyrosine phosphorylation or enhances dephosphorylation of the SK channels and increases channel activity in the CCD (26). Moreover, a decrease in channel activity after stimulation of PTK is most likely mediated by enhancing the endocytosis of the SK channels, because high concentrations of sucrose abolished the effect of stimulating PTK (26). This notion was further supported by experiments in which stimulating PTK decreased the membrane density of ROMK1 channels, which are generally believed to be closely related to native SK channels (12,23). Also, our previous experiments strongly suggested that tyrosine residue 337 in the COOH terminus of ROMK1 was the key site for the effect of PTK, because the inhibitory effect of PTK on channel activity was absent in the oocytes injected with an ROMK1 mutant, R1Y337A, in which the tyrosine residue was mutated into alanine. However, there was no direct evidence to prove that PTK can phosphorylate ROMK1. Furthermore, the notion that dietary K intake regulates the tyrosine phosphorylation level of ROMK channels has not yet been explored directly. Therefore, the first purpose of the present study is to determine whether ROMK1 is a substrate for the PTK and whether tyrosine residue 337 is important for the PTK-induced phosphorylation. The second purpose is to examine whether dietary K intake changes the phosphorylation level of ROMK channels.

METHODS

Tissue preparation

Pathogen-free Sprague-Dawley rats (50–60 g, either sex) were purchased from Taconic (Germantown, NY). After 1 wk of recovery from shipping-related stress, the animals were fed with either an HK diet (10%, wt/wt) or a K-deficient (KD) diet (<0.001%; Harlan Teklad, Madison, WI) for 7 days before use. The weight of rats was 100–110 g before use. The reason for using young rats is that previous patch-clamp experiments were performed in rats of the same age. The rats were killed by cervical dislocation, and the kidneys were immediately removed. Five to six rats were used for each set of experiments to investigate tyrosine phosphorylation of ROMK. The renal cortex and outer medulla were separated under a dissecting microscope and were suspended in RIPA solution (1:8 ratio, wt/vol) containing 50 mM Tris·HCl (pH=7.4), 10 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 0.1% SDS, 1 mM sodium molybdate, 1 mM para-nitrophenylphosphate, and 1 mM EDTA. For every 125-mg tissue sample, we added a 25-μl cocktail of protease and phosphatase inhibitors containing aprotinin (1 μg/ml), leupeptin (1 μg/ml), pepstatin A (1 μg/ml), Na_3VO_4 (1.5 mM), and sodium fluoride (1 mM). The samples were left on ice for 15 min and homogenized with a mortar and pestle. The protein concentrations were measured twice with the Pierce BSA protein assay. The difference from two measurements was <5%. The homogenized tissue sample was incubated in the presence of DNAse (5 μg/ml) and rabbit IgG serum at 4°C for 60 min. The mixture was then centrifuged at 3,000 rpm for 10 min at 4°C, and the resultant supernatant was collected. Moreover, after performing electrophoresis, we stained the gel with 0.25% Coomassie blue R-250 to confirm that an equal amount of proteins was loaded. The intensity of Coomassie blue staining is determined by using the Alpha DigiDoc 1000 digital imaging system (Alpha Innotech, San Leandro, CA).

Immunoprecipitation and Western blot analysis

The protein samples (500 μg) harvested from kidneys of animals on an HK or KD diet were used for immunoprecipitation experiments. The antibodies for either ROMK (Alomone, Jerusalem, Israel) or 4G10 (Upstate Biotechnology, Lake Placid, NY) were added to the renal samples with a ratio of 4 μg/l mg protein, and the mixture was gently rotated at 4°C overnight, followed by incubation with 25 μl protein A/G plus agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for an additional 2 h at 4°C. The tube containing the mixture was centrifuged at 3,000 rpm, and the agarose bead pellet was mixed with 25 μl 2× SDS sample buffer containing 4% SDS, 100 mM Tris·HCl (pH 6.8), 20% glycerol, 200 mM DTT, and 0.2% bromophenol blue. After boiling the sample for 5 min, we loaded the supernatant onto 10% SDS-polyacrylamide gels to separate the proteins by electrophoresis and transferred them to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk (1% BSA) in Trisbuffered saline, rinsed, and washed with 0.05% Tween 20-Tris-buffered saline buffer. ROMK antibody and 4G10 were diluted at 1:300 and 1:1,000, respectively. We used enhanced chemiluminescence (Amersham Pharmacia Biotech) to detect the protein bands, and the intensity of the corresponding band was determined with densitometry. Each Western blot analysis was repeated at least twice.

DNA construction and protein purification

ROMK1 or the ROMK1 mutant R1Y337A, in which tyrosine 337 was mutated with alanine, was ligated into a pBADHisB vector using restriction enzyme sites, *Xho*I, and *Eco*RI. After confirmation that the full length of $(His)_6$ROMK1 sequence was in frame, the plasmid containing the inserts was used for transformation of *Escherichia coli* Top 10 strain. The positive colonies were incubated at 37°C with vigorous shaking and induced with 0.02% arabinose for 2 h at 37°C in a shaker. The fusion protein was purified according to the protocol provided by Invitrogen (Xpress Protein Purification System). Briefly, the cells harvested from 50 ml culture media were centrifuged at 5,000 rpm for 5 min. The resultant pellet was resuspended in 10 ml native binding buffer (20 mM phosphate, 500 mM NaCl, pH 7.8). The tube containing the sample was left on ice for 15 min in the presence of 100 μg/ml lysozyme (Sigma). The sample was subjected to freezing in liquid nitrogen and thawing at 37°C at least three times, followed by centrifuging at 3,000 rpm for 15 min. The resultant supernatant was added to the columns for elution, and the columns were prepared according to the protocol provided by the manufacturer (Invitrogen). The His-tagged ROMK1 proteins were harvested by sequentially applying 5 ml elution buffer containing 50, 200, 350, and 500 mM imidazole (pH 6.0), respectively. The purity of the eluted His-tagged ROMK protein was determined by Coomassie blue staining of SDS gels, and ROMK1 was detected by Western blot analysis using a ROMK antibody (Fig. 1).

Phosphorylation analysis

For in vitro phosphorylation study, 20 μl of the purified His-tagged ROMK1 or R1Y337A and 1 μl (2 U) of the active c-Src (Upstate Biotechnology) were incubated at 30°C for 30 min in the presence of [^{32}P]ATP (10 μCi) dissolved in 8.3 μl reaction buffer, which was composed of (in mM) 25 Tris·HCl (pH 7.4), 10 $MgCl_2$, 0.5 EGTA, 0.5 DTT, and 2 $MnCl_2$ as well as 62.5 μM Na_3VO_4 and 7.5 μM ATP (20 counts·min^{-1}·fmol^{-1}). The reactions were stopped by adding 3× SDS sample buffer, and the mixture was boiled for 5 min. The proteins were resolved by a 10% SDS gel and stained by Coomassie blue R-250. After the gel was destained and dried, the radioactivity was detected by overnight exposure at −80°C. The same method was used for in vitro phosphorylation of the peptide corresponding to amino acid sequence 333–362 in the COOH terminus of ROMK1 (KEGKYRVNFHNFGKTVEVETPHCAMCLYNE), except that the peptide was separated by a 20% SDS gel. The peptide was synthesized at the laboratory of P. Welling.

Phosphopeptide mapping

Two-dimensional tryptic phosphopeptide map analysis was carried out by the method described by Kazlauskas and Cooper (9). Recovered samples were treated with L-1-tosylamido-2-phenylethylchloromethyl ketone-treated trypsin at a final concentration of 0.4 mg/ml for 12 h at 37°C. Trypsin-digested samples were resolved in two dimensions by using cellulose plates. Briefly, the sample was resolved by electrophoresis in the first dimension using 15% glacial acetic acid and 5% formic acid and by chromatography in the second dimension using isobutyric acid/pyridine/ acetic acid/H_2O in the ratio of 62.5:4.8:2.9:28. The plates were dried and the phosphopeptides were identified by using a Storm 860 PhosphorImager (Molecular Dynamics).

Statistics

Data are shown as means ± SE. We used paired Student's *t*-tests to determine the significance of the difference between the control and experimental groups. Statistical significance was taken as $P < 0.05$.

RESULTS

To determine whether ROMK1 is a substrate for PTK, we used the purified recombinant ROMK1 protein to carry out in vitro phosphorylation studies. Figure 2*A* is a representative gel analysis from 10 such experiments in which ^{32}P-label incorporation into ROMK1 protein was assessed in the presence or absence of c-Src. From an inspection of Fig. 2*A*, it is apparent that c-Src phosphorylated two proteins, a 60- and a 45- to 46-kDa protein, in the presence of ^{32}P-radiolabeled ATP. The 45- to 46-kDa protein was ROMK1 because it was absent in the experiment in which ROMK1 was not added (Fig. 2, *A* and *B*). The 60-kDa protein was most likely the autophosphorylated c-Src because the 60-kDa protein band cannot be detected in the absence of c-Src (Fig. 2*A*). Moreover, the phosphorylation level of c-Src was diminished in the absence of ROMK1. This is possibly because the addition of the substrate, such as ROMK1, stimulates the autophosphorylation of c-Src. Figure 2*A* also demonstrates that ^{32}P-label incorporation into ROMK1 protein was c-Src dependent, because no phosphorylated ROMK1 could be detected in the absence of c-Src. A faint band at ~80 kDa can also be detected in *lane 1* (Fig. 2*A*). Because the band is absent in *lane 2*, in which ROMK was not loaded, we speculated that the band may be a modified ROMK1 channel.

Analyzing the amino acid sequence of ROMK1 revealed that tyrosine residue 337 in the COOH terminus was a putative PTK phosphorylation site (7). This possibility was examined by experiments in which in vitro phosphorylation of a synthesized peptide corresponding to the amino acid sequence 333–362 in the COOH terminus of ROMK1 was carried out to determine whether the peptide is a substrate for c-Src. Figure 3*A* is a typical result from three experiments showing that c-Src phosphorylated the peptide. After establishing that c-Src can phosphorylate ROMK1 and the peptide containing tyrosine residue 337, we extended our study by examining whether c-Src can phosphorylate the ROMK1 mutant R1Y337A, in which tyrosine residue 337 was mutated into alanine. Figure 3*B* is a representative gel from seven such experiments, demonstrating tyrosine phosphorylation levels of both ROMK1 and R1Y337A in the presence of c-Src and [^{32}P]ATP. Clearly, R1337A phosphorylation by c- Src was abolished, although the same amount of protein of ROMK1 and R1Y337A was loaded (Fig. 3*B*, *bottom*). This strongly suggests that tyrosine residue 337 is the major site for c-Src-mediated phosphorylation.

Two-dimensional phosphopeptide mapping studies also indicated that tyrosine residue 337 is critical for tyrosine phosphorylation of ROMK1. From an inspection of Fig. 4*A*, it is

apparent that two major phosphopeptides can be observed after trypsin digestion of ROMK1. In contrast, the phosphorylation of both peptides is absent using peptides digested from R1Y337A (n = 4; Fig. 4*B*). This suggests that the two phosphorylated peptides may contain the same tyrosine residue 337 or that phosphorylation of tyrosine residue 337 is essential for the phosphorylation of the second tyrosine residue.

After establishing that ROMK1 can be phosphorylated by c-Src and that tyrosine residue 337 is a critical site for the c-Src-mediated phosphorylation of ROMK1, we examined the effect of dietary K intake on the tyrosine phosphorylation of ROMK. Figure 5*A* is a Western blot showing the protein level of the tyrosine-phosphorylated ROMK in the renal cortex and outer medulla in rats on a KD or HK diet after immunoprecipitation with 4G10, an antibody that recognizes protein-phosphotyrosine. Apparently, the ROMK protein that is phosphorylated by PTK increased by 80 ± 5% in the renal cortex and 150 ± 20% (n = 6 rats) in the outer medulla in rats on a KD diet compared with those on an HK diet. In contrast, the Western blot shows that the expression level of ROMK was the same between rats on KD and HK diets (Fig. 5*B*). Moreover, addition of either 100 μM phosphotyrosine or 100 μM phenyl phosphate, which has been shown to interact with the 4G10 antibody (8), significantly decreased the level of the tyrosine-phosphorylated ROMK (65 ± 9%). This suggests that 4G10 specifically pulls down proteinphosphotyrosine or phosphotyrosine (data not shown).

The notion that a low-K intake increases the population of ROMK channels that are phosphorylated by PTK is also supported by experiments in which ROMK channels were immunoprecipitated with ROMK antibody and followed by immunobloting with 4G10. From an inspection of Fig. 6, *A* and *B*, it is apparent that although the same amount of ROMK was pulled down by immunoprecipitation (Fig. 6*B*), phosphorylation levels increased by 92 ± 20% (n = 5) in the renal cortex and by 85 ± 13% (n = 7 rats) in the outer medulla of rats on a KD diet compared with those on an HK diet.

DISCUSSION

The SK channel plays an important role in K secretion in the CCD and K recycling in the thick ascending limb (TAL) (4,5,10,17,18,22). Although it is possible that subunits, such as cystic fibrosis transmembrane conductance regulators or sulphonylurea receptors (11,16), are required for forming the native SK channels, it is generally accepted that the ROMK channel is the key component of the SK channels in the CCD and TAL (23). It has been well established that dietary K intake regulates K secretion in the CCD; an HK intake stimulates, whereas a low-K intake decreases, K secretion in the CCD (24,27). The mechanism by which a low-K intake reduces K secretion includes decreasing the number of functional SK channels in the cell membrane. We have previously reported that PTK is a key mediator in the effect of low-K intake on the apical SK channels, because an increase in PTK expression is closely related to a decrease in channel activity during K restriction (24,27).

The notion that PTK and protein tyrosine phosphatase are involved in regulating ROMK1 has also been confirmed in experiments in which inhibiting protein tyrosine phosphatase decreased, whereas inhibiting c-Src increased, channel activity in oocytes injected with c-Src and ROMK1. Moreover, the finding that the effect of stimulating c-Src was absent in the oocytes injected with R1Y337A suggests that tyrosine residue 337 is essential for the effect of PTK on ROMK1 channels (12). We speculated that PTK can directly phosphorylate ROMK1 and that tyrosine residue 337 of ROMK1 is the key site for PTK-mediated phosphorylation. This was supported by two lines of evidence: *1*) c-Src phosphorylated the peptide corresponding to the amino acid sequence between 333 and 362 of the COOH terminus of ROMK1; and *2*) phosphopeptide mapping detected two phosphorylated peptide

segments in ROMK1, whereas no phosphopeptide could be identified in R1Y337A. Although we cannot completely exclude the possibility that PTK may phosphorylate a tyrosine residue other than 337, the finding that no phosphorylated peptide segment was detected in the trypsin-digested R1Y337A suggested that two phosphorylated peptide segments obtained from trypsin-digested ROMK1 may contain the same tyrosine residue 337. Alternatively, phosphorylating tyrosine residue 337 is essential for c-Src phosphorylating the second tyrosine residue.

A large body of evidence indicates that PTK plays an important role in regulating activity of a variety of ion channels (2,15,20,25,28). PTK has been shown to inhibit delayed-rectifier K channels (20), Kv1.3 (a voltage-gated K channel) (2), inwardly rectifying Kir2.1 (28), and Ca^{2+}-activated large-conductance K channels (15) by direct phosphorylation. Although we have demonstrated that ROMK1 is the substrate for PTK, the previous finding that addition of exogenous cSrc did not inhibit channel activity in inside-out patches excludes the possibility that phosphorylation of ROMK1 channels can directly inhibit channel activity (12). However, the observation that PTK inhibitor increases, whereas protein tyrosine phosphatase inhibitor decreases, activity of ROMK1 channel in oocytes injected with cSrc and ROMK1 strongly suggested that tyrosine phosphorylation and dephosphorylation of ROMK1 play an important role in modulating activity of ROMK1.

A previous study has also demonstrated that a low-K diet increases expression and activity of PTK, such as c-Src and c-Yes (27). Moreover, the stimulatory effect of herbimycin A, an inhibitor of PTK, on activity of the ROMK-like SK channel in the CCD was significantly higher in rats on a KD diet for >5 days than those on a normal diet. Furthermore, the effect of herbimycin A on channel activity was almost absent in the CCD harvested from rats on an HK diet (27). From those observations, we speculated that the population of the ROMK-like channels that are phosphorylated by PTK should be significantly higher in the CCD harvested from rats on a KD diet than those on an HK diet (27). This hypothesis is supported by the present observation that the number of ROMK channels phosphorylated by PTK was significantly higher in the renal cortex and outer medulla from rats on a KD diet than those on an HK diet. This increase possibly results from an upregulation of PTK concentration including c-Src and c-Yes (27). However, further experiments are needed to determine which type of PTK is mainly responsible for phosphorylation of the ROMK-like K channel in vivo.

Three ROMK isoforms, ROMK1, ROMK2, and ROMK3, have been identified in the kidney (1,7). It has been demonstrated that ROMK1 is mainly located in the CCD, whereas ROMK3 is exclusively distributed in the TAL. ROMK2 is located in the TAL and the CCD (1). Although the present investigation did not examine whether PTK can phosphorylate ROMK2 and ROMK3, it is conceivable that ROMK2 and ROMK3 are also substrates for PTK because the putative PTK phosphorylation site is present in the COOH terminus of ROMK2 and ROMK3. Also, we have observed that a low-dietary K intake increases c-Src expression in the renal outer medulla, in which >90% of the tubule population is mTAL. Therefore, it is conceivable that tyrosine phosphorylation of ROMK2 and ROMK3 also increased in the kidneys harvested from rats on a KD diet. Thus it is highly possible that the phosphorylated ROMK channels shown in Fig. 5 and Fig. 6 included all three ROMK isoforms. However, the mechanism by which PTK regulates ROMK2 and ROMK3 may be different from that regulating ROMK1. This conclusion is on the basis of the observation that addition of herbimycin A increased activity of the ROMK-like SK channels only in the CCD but not in the TAL harvested from rats on a KD diet (6). This suggests that tyrosine phosphorylation of ROMK2 and ROMK3 is not sufficient to initiate the endocytosis of ROMK2 and ROMK3. In contrast, tyrosine phosphorylation of ROMK1 can initiate the

internalization of ROMK1. We need further experiments to examine how PTK-induced tyrosine phosphorylation of ROMK2 and ROMK3 regulates channel activity.

We conclude that ROMK1 is a substrate for PTK and that tyrosine residue 337 is a critical site for PTK phosphorylation. Moreover, a low-K intake increases, whereas an HK intake decreases, the tyrosine phosphorylation of ROMK channels. Our data strongly suggest that PTK is an important member of the aldosterone- independent signal transduction pathway for regulating renal K secretion.

Acknowledgments

We thank Melody Steinberg for editing the manuscript.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-47402 and DK-54983.

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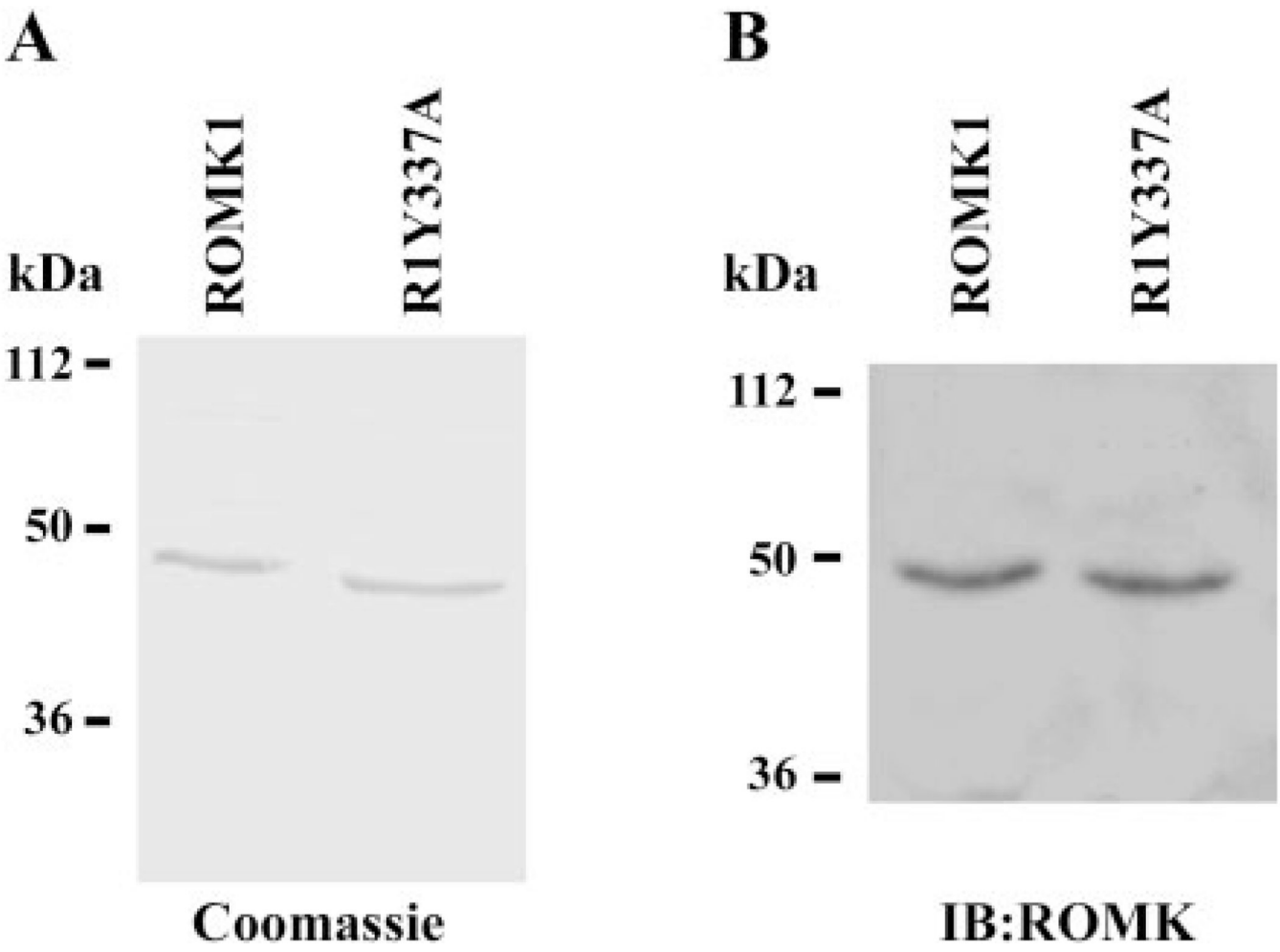

Fig. 1.

A: Coomassie blue staining detects a single 45- to 46-kDa band protein harvested and purified from *Escherichia coli* transfected with pBADHisB vector containing ROMK1 or the ROMK1 mutant R1S337A. *B*: Western blot demonstrates that the ROMK antibody recognizes the 45- to 46-kDa protein purified from *E. coli* transfected with the His-tagged ROMK1 and R1S337A.

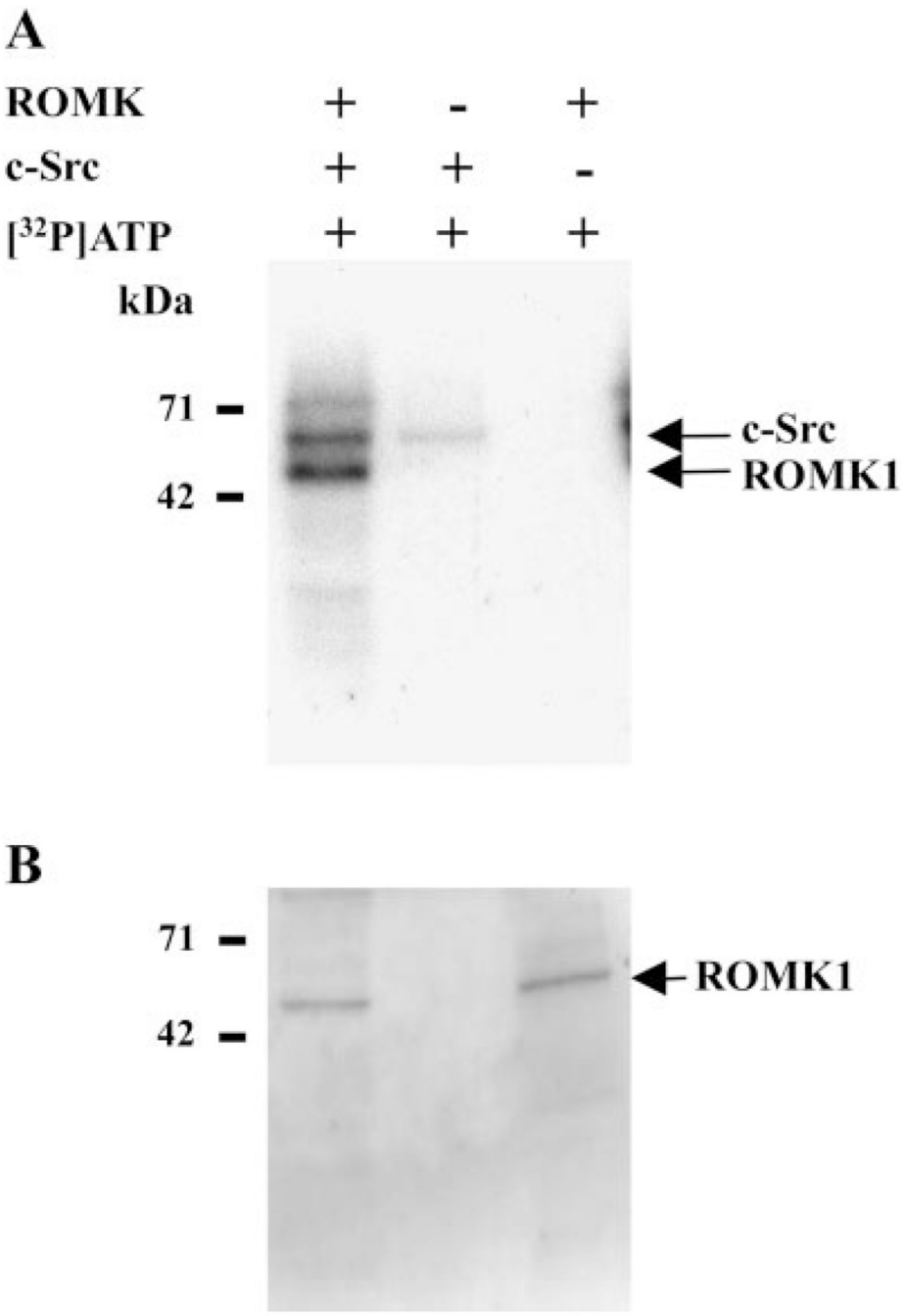

Fig. 2.

A: in vitro phosphorylation of the purified ROMK1 protein. The His-tagged ROMK1 was purified from *E. coli* transfected with pBADHisB vector containing ROMK1 DNA. *Lane 1*: purified ROMK1 (15 μg) was incubated with [^{32}P]ATP and 2 U c-Src. ROMK1 was absent in *lane 2*, whereas c-Src was absent in *lane 3*. *B*: Coomassie blue staining showing ROMK1 bands.

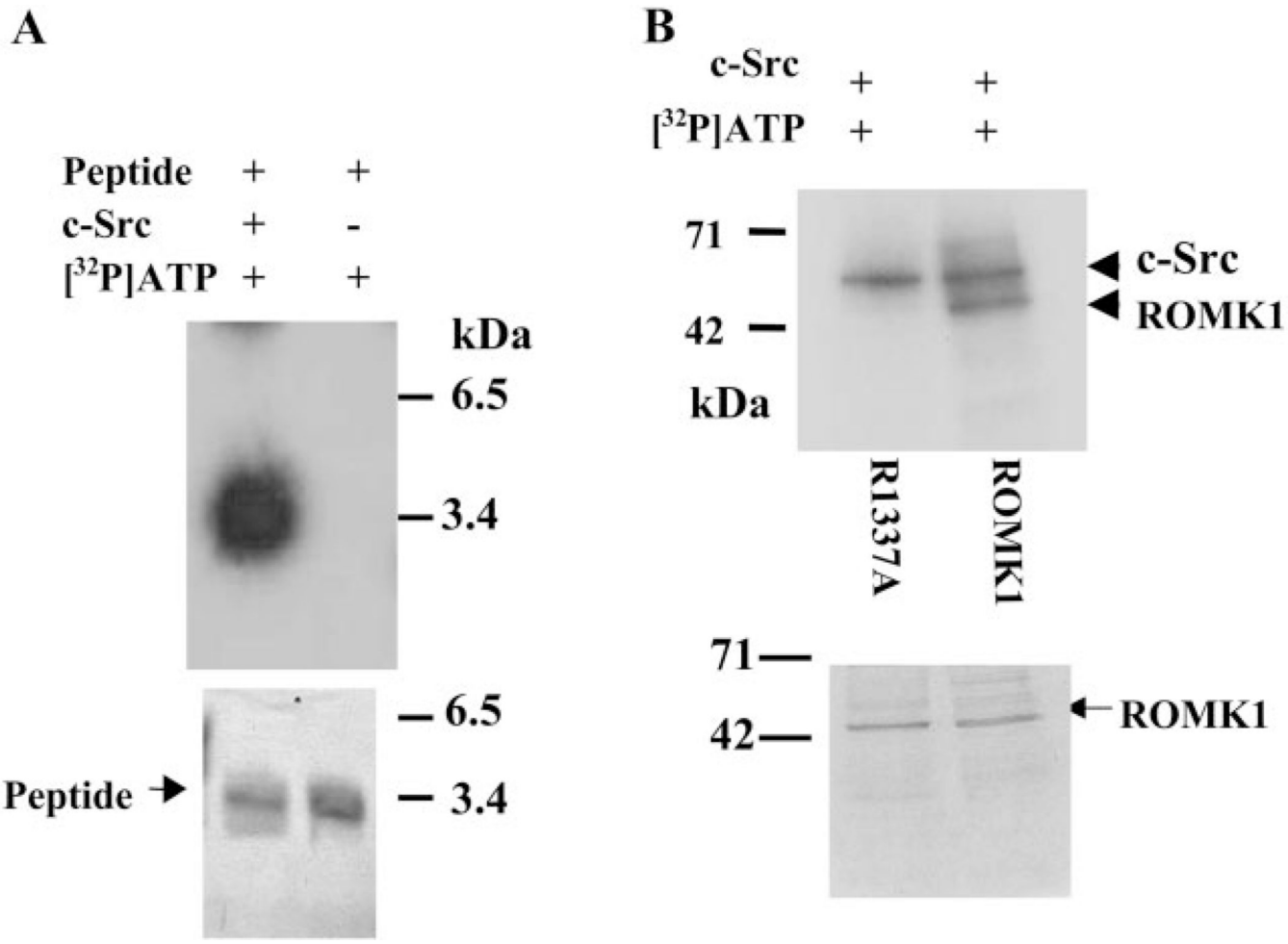

Fig. 3.

A: in vitro phosphorylation of the purified peptide corresponding amino acid sequence 333–362 in the COOH terminus of ROMK1. *Lane 1*: peptide was incubated with [^{32}P]ATP (10 μCi) and 2 U c-Src. *Lane 2*: peptide was incubated with [^{32}P]ATP in the absence of c-Src. *Bottom*: Coomassie blue staining of the peptide load. *B*: in vitro phosphorylation of the purified His-tagged ROMK1 protein and the Histagged ROMK mutant protein (R1S337A). The Histagged ROMK1 and His-tagged R1Y337A were purified from *E. coli* transfected with pBADHisB vector containing ROMK1 or R1Y337A DNA, respectively. Purified R1S337A (15 μg; *lane 1*) and ROMK1 (15 μg; *lane 2*) were incubated with [^{32}P]ATP and 2 U c-Src. *Bottom*: Coomassie blue staining demonstrating the ROMK1/mutant bands.

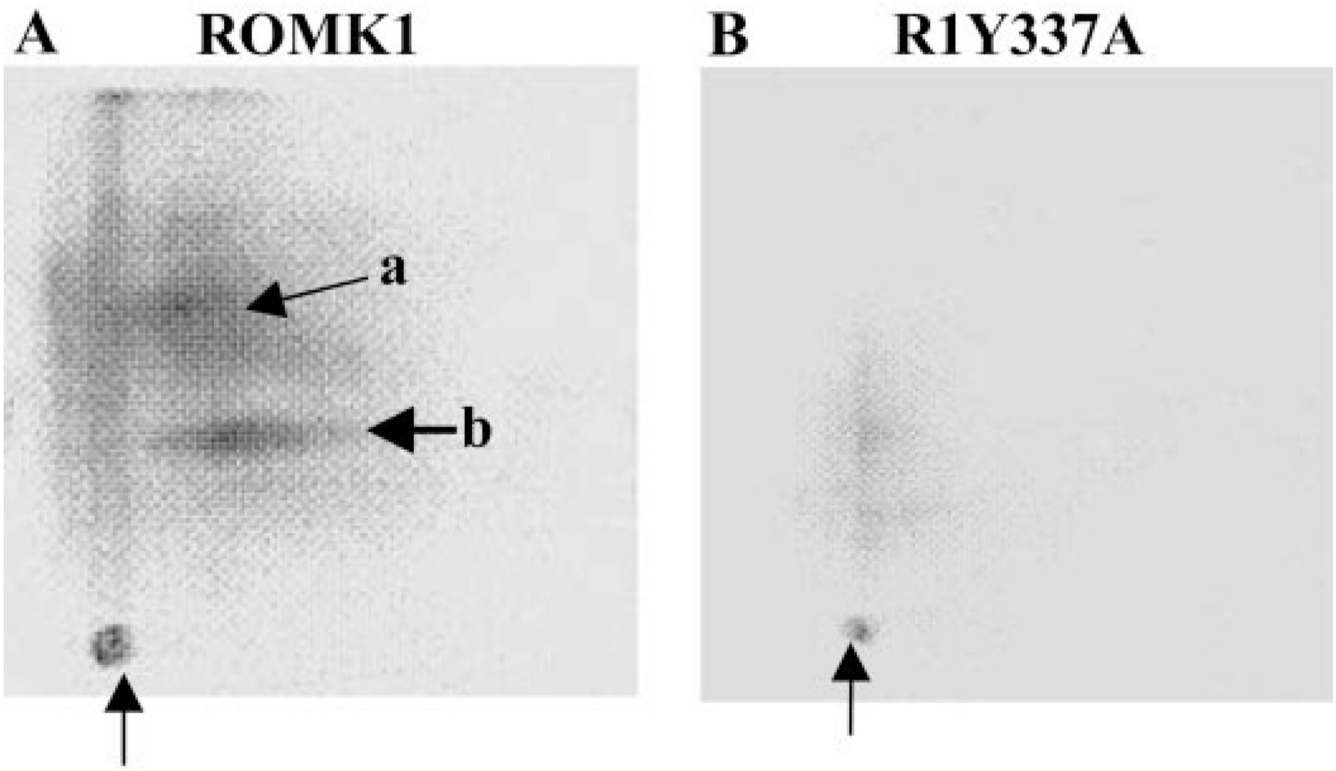

Fig. 4.

Two-dimensional tryptic phosphopeptide mapping of ROMK1 (*A*) and R1Y337A (*B*). His-tagged ROMK1 and His-tagged R1Y337A were harvested from *E. coli* and purified by imidazole columns. After in vitro phosphorylation by c-Src in the presence of [^{32}P]ATP, the proteins were gel purified and digested with trypsin. The tryptic peptides were resolved by electrophoresis and chromatography in two dimensions. Arrows, origin; a and b, tyrosine-phosphorylated peptides.

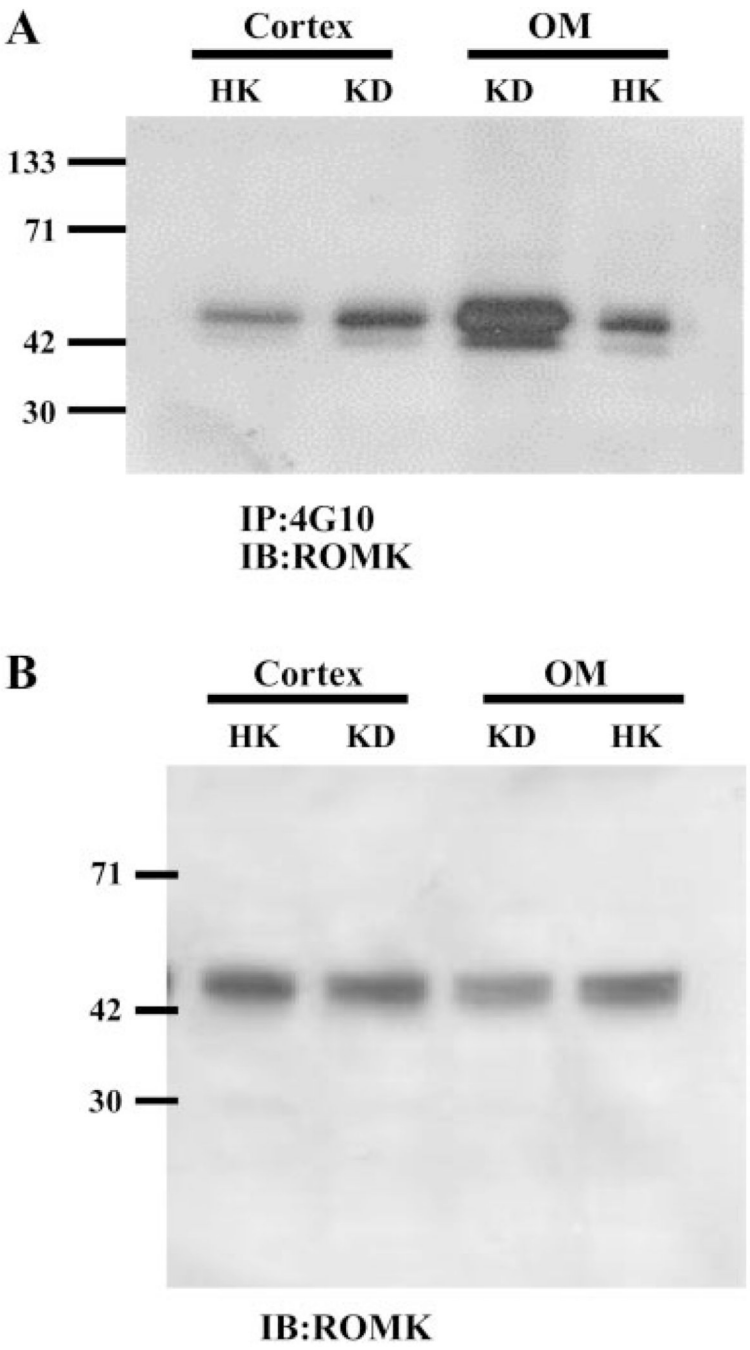

Fig. 5.

A: Western blot shows the level of tyrosine-phosphorylated ROMK in the renal cortex and outer medulla (OM) from rats on K-deficient (KD) and high-K (HK) diets after immunoprecipitation (IP) of ROMK channels with 4G10, an antibody that reacts with phosphotyrosine. *B*: ROMK expression in the renal cortex and in the renal outer medulla from rats on HK and KD diets. The protein concentration used for Western blot analysis of ROMK (*B*) was 100 μg. IB, immunoblot.

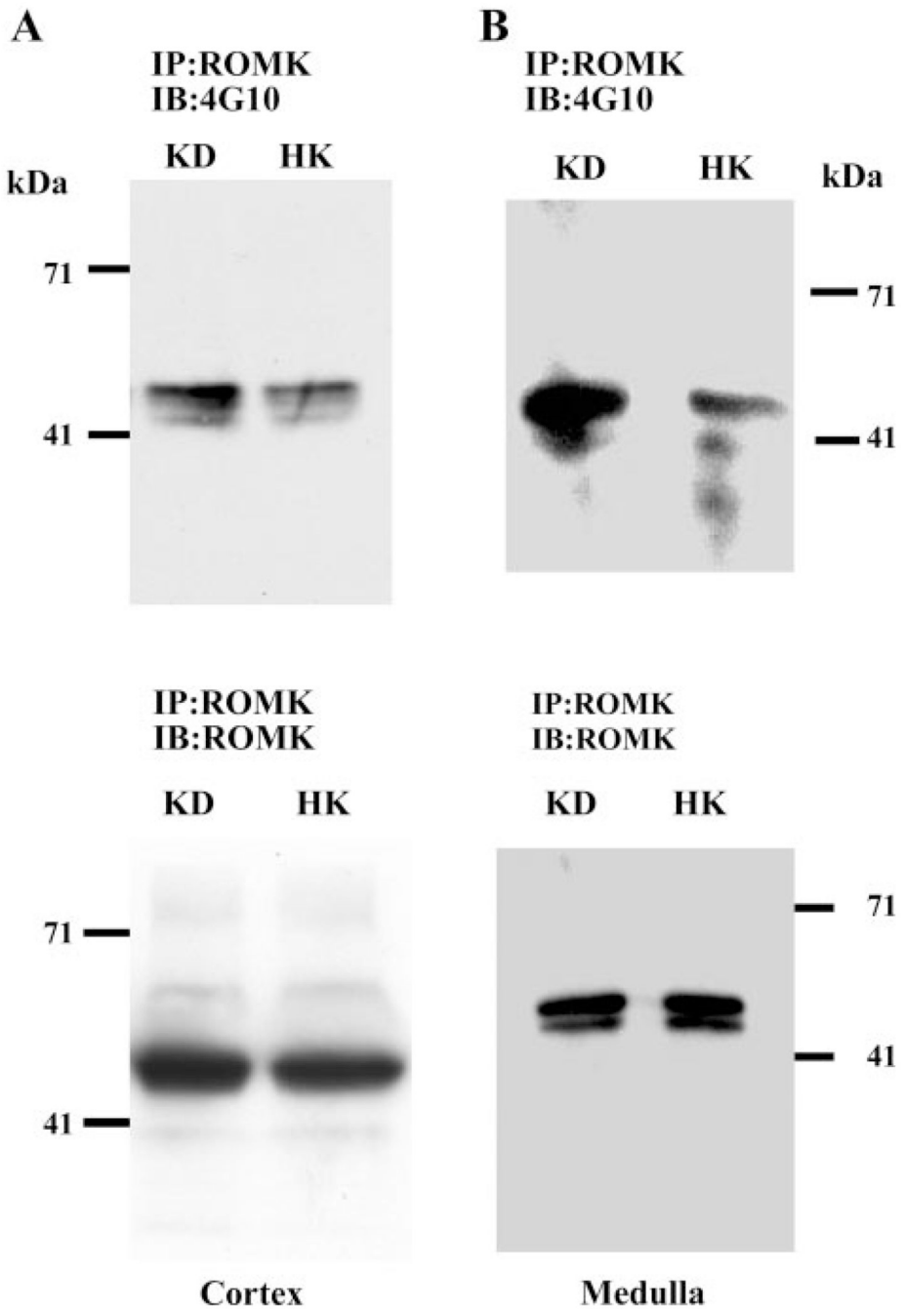

Fig. 6.

Immunoprecipitation of ROMK channels with ROMK antibody and detection of tyrosine-phosphorylated ROMK channels with 4G10 in the renal cortex (*A*) and renal outer medulla (*B*) from rats on KD and HK diets.