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Protein tyrosine kinase is expressed and regulates ROMK1 location in the cortical collecting duct

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Abstract

We previously demonstrated that dietary K intake regulates the expression of Src family PTK, which plays an important role in controlling the expression of ROMK1 in plasma membrane (Wei Y, Bloom P, Lin D-H, Gu RM, and Wang WH. *Am J Physiol Renal Physiol* 281: F206–F212, 2001). In the present study, we used the immunofluorescence staining technique to demonstrate the presence of c-Src, a member of Src family PTK, in the thick ascending limb (TAL) and collecting duct. Confocal microscopy shows that c-Src is highly expressed in the renal cortex and outer medulla. Moreover, c-Src and ROMK are coexpressed in the same nephron segment. Also, the positive staining of c-Src is visible in tubules stained with Tamm-Horsfall glycoprotein or aquaporin-2. This suggests that c-Src is present in the TAL, cortical collecting duct (CCD), and outer medullary collecting duct (OMCD). To study the role of PTK in the regulation of ROMK membrane expression in the TAL and CCD, we carried out immunocytochemical staining with ROMK antibody in the CCD or TAL from rats on either a high-K (HK) or K-deficient (KD) diet. A sharp membrane staining of ROMK can be observed in the TAL from rats on both HK and KD diets. However, a clear plasma membrane staining can be observed only in the CCD from rats on a HK diet but not from those on a KD diet. Treatment of the CCD from rats on a HK diet with phenylarsine oxide (PAO) decreases the positive staining in the plasma/subapical membrane and increases the ROMK staining in the intracellular compartment. However, PAO treatment did not significantly alter the staining pattern of ROMK in the TAL. Moreover, the biotinylation technique has also confirmed that neither herbimycin A nor PAO has significantly changed the biotin-labeled ROMK2 in HEK293 cells transfected with ROMK2 and c-Src. We conclude that c-Src is expressed in the TAL, CCD, and OMCD and that stimulation of PTK increases the ROMK channels in the intracellular compartment but decreases them in the apical/subapical membrane in the CCD.

Keywords

potassium secretion; endocytosis; exocytosis; dietary potassium intake; c-Src

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Dietary K Intake is an important factor regulating renal K secretion: a high-K (HK) intake stimulates, whereas a low-K intake decreases, renal K secretion (4,10,11). However, the mechanism by which dietary K intake regulates K secretion is not completely understood. An increase in K intake has been shown to raise plasma aldosterone (4) and stimulate Na absorption in the cortical collecting duct (CCD). As a consequence, the driving force for K secretion increases sharply and K secretion is stimulated. Moreover, it has also been reported that a HK intake can increase K secretion by an aldosterone-independent mechanism (16). We recently demonstrated that a HK diet decreases, whereas a low-K diet increases, the expression of c-Src, a nonreceptor type of PTK, in the renal cortex and outer medulla (28). Moreover, inhibition of PTK significantly augments the activity of ROMK-like small-conductance K (SK) channels in the CCD harvested from rats on a K-deficient diet (26,28). In contrast, inhibition of PTP decreases the activity of the SK channels in the CCD obtained from rats on a HK diet (27). This suggests that PTK plays a key role in mediating the effect of dietary K intake on K secretion. We previously demonstrated that ROMK1 is a substrate of PTK and that a high dietary K intake decreases, whereas a low dietary K intake increases, the level of the tyrosine-phosphorylated ROMK (8). Therefore, it is conceivable that PTK must be colocalized with ROMK channels to phosphorylate ROMK channels. Because c-Src is an ubiquitously expressed Src-family PTK, the first goal of the present study is to examine whether c-Src is coexpressed with ROMK.

Also, our previous studies with confocal microscopy and the biotinylation technique demonstrated that the increase in ROMK1 channel activity induced by inhibition of PTK results from enhancing exocytosis (14). In contrast, inhibition of PTP facilitates endocytosis (23). However, these experiments were carried out in oocytes or cultured cells; the results have not been confirmed in native tissue. Therefore, the second goal of the present study is to determine whether PTK is involved in the regulation of the distribution of ROMK channels in the CCD.

METHODS

Tissue preparations

We used Sprague-Dawley rats (6 wk, either sex; Taconic Farms, Germantown, NY) for experiments. One week after arriving, rats were kept on different K diets (Harlan Teklad, Madison, WI): K-deficient (KD; <0.01%), HK (10% wt/wt), and normal-K (NK; 0.7–1%) diets for 1 wk before use. Rats were anesthetized with isoflurane, and the abdomen was cut open for perfusion of kidneys with 50 ml of PBS containing heparin (40 U/ml) followed by 200 ml of 4% paraformaldehyde. After perfusion, the kidneys were removed and subjected to postfixation with 4% paraformaldehyde for 12 h. The kidneys were dehydrated and cut into 8- to 10-μm slices by a Leica1900 cryostat (Leica). The tissue slices were dried at 42°C for 1 h.

We followed the methods described previously to isolate CCDs and thick ascending limbs (TALs) (26). The tubules were placed on a thin cover glass coated with polylysine. The CCD or TAL harvested from rats on a HK diet was incubated in a medium containing 1 μM phenylarsine oxide (PAO) at 37°C for 30 min. After incubation, the tubules were fixed with 4% paraformaldehyde.

Immunocytochemical staining

The slides were washed with 1× PBS for 15 min and permeablized with 0.4% Triton dissolved in 1× PBS buffer containing 1% BSA and 0.1% lysine (pH 7.4) for 15 min. Kidney slices were blocked with 2% goat serum for 1 h at room temperature and then incubated with ROMK antibody (Alomone, Jerusalem, Israel), c-Src monoclonal antibody

(Transduction Lab), aquaporin-2 (AQP2) antibody (Alomone), or Tamm-Horsfall glycoprotein (THP) antibody (ICN, Pharmaceutical, Aurora, OH) for 12 h at 4°C. Slides were thoroughly washed with 1× PBS followed by incubation in second antibody mixtures in 0.4% Triton dissolved in 1× PBS for 2 h at room temperature.

For staining the CCD or TAL, the tubules were washed with 1× PBS for 5 min, fixed with 4% paraformaldehyde in PBS (pH 7.4) for 10 min at room temperature, and permeablized with 0.4% Triton dissolved in 1× PBS containing 1% BSA and 0.1% lysine (pH 7.4) for 1 h at room temperature. Nonspecific staining was blocked by incubation of slides with 2% goat serum for 12 h at 4°C, and the tubules were then incubated with ROMK antibody 1:100 in 1× PBS buffer containing 1% BSA, 0.1% lysine, 0.4% Triton (pH 7.4) for 12 h at 4°C. The slide was washed thoroughly with 0.4% Triton in 1× PBS (pH 7.4) for 30 min and followed by addition of secondary antibody in 1× PBS buffer containing 0.4% Triton (pH 7.4) for 2 h at 4°C. After a complete wash with 1× PBS for 40 min, the tubule slides were observed with a confocal microscope (Bio-Rad MRC 1000 system).

The fluorescence intensity of the ROMK staining was measured with Scion Image software (Scion, Frederick, MD). The program allows us to measure not only the intensity but also the size of the area. Typically, four different sites in the cell membrane or subapical membrane and at least four intracellular sites with the same area were selected for the measurement of fluorescence intensity, and the mean value was calculated from these measurements. Four to six cells in the middle section of each tubule were selected for the measurement, and the reading from ROMK-negative cells served as background. We selected only the middle section for analysis because the cell quality at both ends was low. The low quality of cells at two ends is the result of either dissection or mechanical stretch induced by adhesion. The size of the area selected for the analysis contains ~15–20 cells. From these 15 to 20 cells, only 4 to 6 cells could be chosen for measurement of fluorescence intensity. The criteria used for the selection are that *1*) cells should have a clear luminal membrane and *2*) they have positive ROMK staining (plasma membrane or intracellular). Therefore, we basically selected almost every available cell for the measurement. Because each experiment has different settings regarding gain and contrast, data are presented as the ratio calculated from the fluorescence intensity between the plasma membrane and intracellular compartment. This allows us to compare the changes in the fluorescence intensity in different tubules.

Biotinylation, immunoprecipitation, and Western blot analysis

HEK293 cells were transfected with green fluorescence protein (GFP)-ROMK1 or ROMK2 and c-Src followed by the method described previously (23). Two days after transfection, the cells were treated with 1 μM PAO, 5 μM herbimycin A, or vehicle for 10 min. ROMK channels in the cell membrane were quantitated by labeling the HEK293 cells with cell-impermeant Sulpho-NHS-biotin (Pierce). After biotinylation, the cells were washed 2× with PBS and trypsinized with trypsin-EDTA. They were pelleted by centrifugation for 5 min at 10,000 rpm, washed 2× with PBS, and lysed with cold RIPA 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 cocktail (Sigma) per milliliter of lysis buffer. After clarification, total protein concentrations were determined with the Bio-Rad (Bio-Rad Laboratories, Richmond, CA) protein assay kit, and aliquots of lysates containing equal amounts of protein were immunoprecipitated overnight with 1 μg of monoclonal antibody of GFP (Clontech) and 20 μl of protein A/G agarose (Santa Cruz Biotechnology, Santa Cruz, CA). After centrifuging and being washed 2× with PBS, proteins were resolved by electrophoresis on 10% SDS gel and transferred to PVDF membranes (Bio-Rad). The membranes were blocked with 5% milk in TBS and the biotin-labeled GFP-ROMK proteins were detected using Neutravidin horseradish peroxidase

(Pierce). Changes in biotin-labeled surface ROMK proteins were normalized with the corresponding total ROMK protein detected with ROMK antibody. The density of the band was determined using Alpha DigiDoc 1000 (Alpha Innotech, San Leandro, CA). The results obtained from experiments with either herbimycin A or PAO treatment were compared with the control group.

We present data as means ± SE and one-way ANOVA test was used to determine the significance between data obtained from tubules of high K-adapted animals and PAO-treated tubules or those on a K-deficient diet.

RESULTS

The ROMK antibody used in the present study has been well characterized by Mennitt et al. (13) and has also been used in our previous study (23). The antibody for c-Src has been used for Western blot analysis in our previous experiments (26). To further validate the usage of ROMK antibody, we carried out immunocytochemical staining of kidney from ROMK(+/+) and ROMK(−/−) mice. Figure 1 is a confocal image demonstrating that positive ROMK staining is clearly visible in the kidney from ROMK(+/+) mice (Fig. 1*A*) but is completely absent in ROMK(−/−) mice (Fig. 1*B*). Figure 1*C* shows a magnified image of the outer medulla from ROMK(+/+) mice, whereas Fig. 1*D* is a corresponding section from ROMK(−/−) mice. It is apparent that ROMK staining is present in the medullary TAL (mTAL) but is completely absent in the mTAL in ROMK(−/−) mice. Thus the results confirm that the positive fluorescence staining with Alomone antibody is related to ROMK. We also carried out Western blot analysis using HEK293 cells transfected with GFP-ROMK1. We confirmed the previous finding (23) that ROMK antibody detects a 71-kDa protein that is also recognized by GFP antibody (data not shown).

After characterizing ROMK antibody, we examined whether c-Src is expressed in ROMK-positive tubules. Figure 2 shows a typical immunocytochemical staining of c-Src and ROMK in the renal cortex (*A*) and outer medulla (*B*) obtained from rats on a NK diet. We confirmed the previous report that ROMK is located in the CCD, TAL, and connecting tubules (7,13,21,29). Moreover, tubules expressing ROMK channels are also positive for c-Src staining to a variable extent. Figure 2*C* is an amplified section from Fig. 2, *A* and *B* (indicated by an arrow), showing the colocalization of ROMK and c-Src in the renal cortex (*left*) and outer medulla (*right*). However, some ROMK-positive tubules (possible TAL) did not have detectable c-Src staining (upper *right* corner of Fig. 2*B*).

Figure 3 is a magnified immunocytochemical staining of c-Src and ROMK in the renal cortex (*A*) and outer medulla (*B*). From inspection of Fig. 3, it is clear that c-Src is highly expressed in cells in which ROMK staining is also positive. Figure 3, *C* and *D*, demonstrates that c-Src and ROMK are expressed in the TAL and CCD. In addition, c-Src staining is also observed in tubules with no ROMK staining. However, no efforts were made to identify the origin of these tubules because the goal of the present study is to establish that c-Src is expressed in the TAL, CCD, and OMCD.

To further confirm that c-Src is expressed in the TAL, we carried out double immunocytochemical staining with c-Src and THP, a marker of the TAL. Figure 4 shows the confocal image of the double staining in the renal cortex (*A*) and outer medulla (*B*). It is apparent that c-Src is positive in the THP-positive tubules. Figure 4, *C* and *D*, has magnified images showing that c-Src is expressed in the cortical TAL (*C*) and mTAL (*D*). We also employed AQP2 as a marker of the CCD and connecting tubule to confirm that c-Src is expressed in the CCD and connecting tubule. Figure 5 is a confocal image showing that c-Src is expressed in the AQP2-positive tubules in both renal cortex (*A*) and outer medulla (*B*).

Figure 6 is a magnified confocal image demonstrating that c-Src is highly expressed in principal cells in the CCD (*A*) or medullary collecting duct (*B*).

After establishing that c-Src is expressed in the TAL and CCD, we examined the effect of PTK in the regulation of ROMK channel distribution. We previously demonstrated that PAO decreases the ROMK-like channel activity in the CCD (26). Because the effect of PAO can be blocked by either 20% sucrose-containing bath solution or concanavalin A treatment, it has been speculated that the effect of PAO is the result of stimulation of internalization (27). This hypothesis was examined by testing the effect of PAO on ROMK distribution in the isolated CCD by immunofluorescence staining. The CCD from rats on a HK diet was isolated and incubated in a solution containing 1 μM PAO to inhibit the PTP or in control media for 30 min. After incubation, both the control and PAO-treated CCDs were fixed and processed on the same slide. Figure 7 shows a representative confocal image from 12 tubules (4 rats) demonstrating ROMK staining in the untreated CCD (control) and in the PAO-treated CCD from a rat on a HK diet. Under control conditions, a sharp membrane staining with ROMK antibody is clearly visible (Fig. 7, *A* and *B*). We selected four to six cells from each CCD, measured the relative fluorescence intensity in the plasma membrane/subapical membrane and intracellular compartment, and calculated the ratio between the plasma membrane and intracellular compartment. Results summarized in Fig. 8 show that the ratio was 2.7 ± 0.3 (n = 55 cells). In contrast, treatment of the CCD with PAO reduced the ratio of fluorescence intensity between the plasma membrane and intracellular compartment to 0.3 ± 0.1 (n = 51 cells). Figure 7, *D* and *E*, shows that the most significant ROMK staining is present in the perinuclear region, and no sharp membrane fluorescence signal can be observed in the PAO-treated CCD. The effect of PAO on ROMK location is reversible because washout restored the plasma membrane location of ROMK. Figure 7*G* is a typical confocal image of the CCD from a rat on a HK diet (n = 4 tubules from 2 rats) that was treated with PAO for 30 min and followed by incubation in a PAO-free media for 30–45 min. Although some intracellular ROMK staining remained, a clear sharp plasma membrane staining of ROMK antibody is visible (Fig. 7, *G* and *H*). We cannot exclude the possibility that the apparent apical membrane ROMK staining is actually a subapical membrane staining. However, the fact that no significant ROMK staining in the apical or subapical membrane is visible in every CCD treated with PAO strongly indicates that stimulation of PTK facilitates the internalization of ROMK.

Also, we examined the effect of PAO on ROMK distribution in the TAL. Figure 9 shows the ROMK staining in the TAL from rats on a HK diet. ROMK staining in the TAL shows considerable variation: 28 of 95 cells (10 tubules from 4 rats) did not show ROMK staining and the heterogeneous ROMK staining has also been reported by other investigators (3,29). However, a sharp ROMK staining in the plasma membrane can be clearly observed in both the control TAL (Fig. 9, *A* and *B*) and the PAO-treated tubule (Fig. 9, *D* and *E*). Moreover, PAO treatment did not increase the intensity of intracellular ROMK staining because the ratio between the plasma membrane and intracellular compartment remained unchanged (control 1.9 ± 0.2, n = 63 cells; PAO 1.8 ± 0.2, n = 60 cells; Fig. 8). This suggests that the response of ROMK channels to inhibiting PTP in the TAL is different from that in the CCD. This finding is consistent with the patch-clamp experiments in which PAO did not decrease the activity of the ROMK-like SK channels in the TAL (27).

Inhibition of PTP is expected to enhance the activity of PTK, which can facilitate the tyrosine phosphorylation of ROMK1 and endocytosis. Because PTK expression and activity are higher in the kidney from rats on a low-K diet than those on a control diet, it is expected that the ROMK staining in the plasma membrane should be low in the CCD from K-restricted rats. This hypothesis was tested by conducting immunostaining of the CCD from rats on a low-K diet. Figure 10 demonstrates the ROMK staining in the CCD from rats on a

KD diet (*A* and *B*). It is apparent that a significant ROMK staining is present in the perinuclear region, and the ratio of fluorescence intensity between the plasma membrane and the intracellular compartment was 0.2 ± 0.1 (n = 50 cells). This ratio is very similar to that observed in the PAO-treated CCD from rats on a HK diet. In contrast, a clear membrane staining of ROMK can be observed in the apical membrane of the TAL in rats on a KD diet (Fig. 10, *E* and *F*), although the intensity of ROMK staining in the plasma membrane is slightly decreased. The ratio of fluorescence intensity between the plasma membrane and intracellular compartment is 1.35 ± 0.3 (n = 60 cells from 10 tubules). Thus this further suggests that the response of apical K channels to PTK in the TAL is different from that in the CCD.

Because ROMK1 is expressed only in the CCD, whereas ROMK2 is present in the TAL (1), it is possible that the regulation of ROMK2 by PTK and PTP is different from that of ROMK1. This hypothesis is tested by examining the effects of PAO and herbimycin A on ROMK location in HEK293 cells transfected with ROMK1 or ROMK2 and c-Src. Figure 11 is a Western blot demonstrating the effect of inhibiting PTP or PTK on the biotin-labeled ROMK1 and ROMK2. It is apparent that neither PAO nor herbimycin A has a significant effect on ROMK2 channel distribution in HEK293 cells transfected with GFP-ROMK2 and c-Src (n = 4). In contrast, PAO treatment decreases by 60 ± 8% (n = 4), whereas herbimycin A treatment increases by 90 ± 8% the biotin-labeled ROMK1. To exclude the possibility that biotin labeled the intracellular ROMK, we carried out the experiments in which c-Src was used as the internal control. The cells were transfected with ROMK and c-Src. After biotin labeling, cells were lysed and immunoprecipitated with c-Src antibody. The expression of c-Src was confirmed by Western blot and no biotin-labeled c-Src was identified (data not shown).

DISCUSSION

The ROMK channel is an inwardly rectifying K channel (6) and plays a key role in K recycling in the TAL and K secretion in the CCD (25). Genetic studies demonstrated that a defective gene product encoding ROMK causes Bartter's disease (22). Although it is possible that other subunits such as CFTR or sulfonylurea agent receptor are required to form the native SK channel (2,20), it is firmly established that ROMK is a key component of the native SK channel because no channel activity was found in ROMK-knockout mice (9).

Our previous experiments showed that PTK plays an important role in mediating the effect of low-K intake on the ROMK channel: *1*) tyrosine phosphorylation of ROMK is enhanced in the K-restricted animals (7); *2*) inhibition of PTK increased the ROMK-like SK channel activity in the CCD (26). Therefore, it is suggested that PTK should be present in the ROMK-positive cells. This speculation is now confirmed by the finding that c-Src is expressed in the TAL, connecting tubules, CCD, and OMCD. Moreover, because the expression of Src-family PTK is regulated by dietary K intake (28), this further supports the role of Src-family PTK in the regulation of ROMK channels by dietary K intake.

It is well documented that dietary K intake plays an important role in the regulation of K secretion: a HK intake increases, whereas a low-K intake decreases, K secretion (4,10,11,17). The effect of K intake on K secretion is partially achieved by regulating the number of apical ROMK channels in the CCD: a HK intake increases, whereas a low-K intake decreases, the number of ROMK channels in the CCD. The patch-clamp experiments demonstrated that the number of the ROMK-like SK channels in the CCD doubled in the CCD from rats on a HK diet for 24 h (19). However, we and other investigators did not observe a significant decrease in channel number in the CCD from rats on a low-K diet compared with those observed from the control rats (18,28). It is possible that the channel

activity under control conditions is underestimated because the experiments were performed in the nonperfused and split-open CCDs. Therefore, factors that regulate the activity of apical K channels in vivo may be inactivated in the patch-clamp experiments. For instance, it has been demonstrated that an increase in luminal Na entry stimulates the basolateral Na-K-ATPase, which leads to an increase in the apical K conductance by a "cross-talk" mechanism (15). However, such a cross-talk mechanism may not be active in the split-open CCDs. Therefore, it is possible that the channel activity in the CCD from rats on a low-K diet is actually lower than those observed in an animal on a NK diet. However, because channel open probability is still close to 90% in the CCD from rats on a KD diet (Wang W-H, unpublished observations), it is unlikely that a decrease in channel activity is the result of a lowered channel open probability. Therefore, the decrease in channel activity must result from a reduction of total ROMK protein or the number of functional ROMK channels in the cell membrane.

Our previous observation that the total protein concentration of ROMK in the renal cortex and outer medulla is similar in the rats on a KD diet to those on a HK diet excludes the possibility that a low level of ROMK expression is responsible for decreasing ROMK channel number in the CCD from rats on a KD diet (28). Mennitt et al. (12) further demonstrated that the ROMK expression in the cell membrane fraction was lower in the kidney from rats on a KD diet than those observed in kidneys from rats on a NK or HK diet. Therefore, this suggests that the internalization of ROMK channels could be a possible mechanism by which low-K intake decreases the ROMK channel number in the plasma membrane in the CCD. This notion is supported by the present finding that ROMK staining was mainly located in the perinuclear region in the CCD from rats on a KD diet. In contrast, a sharp membrane staining of ROMK has been observed in the CCD from rats on a HK diet. Therefore, it is conceivable that a low dietary K intake stimulates the internalization of ROMK1 in the CCD, whereas a HK intake stimulates the insertion.

Three lines of evidence indicate that the endocytosis of ROMK1 is controlled by tyrosine phosphorylation of ROMK1. First, inhibition of PTP increased the tyrosine-phosphorylated ROMK1 and decreased the number of ROMK1 in cell surface in HEK293 cells transfected with ROMK1 and c-Src (23). Second, inhibition of PTK decreased the tyrosine-phosphorylated ROMK1 and increased the number of ROMK1 in the cell membrane (24). Third, mutation of tyrosine residue 337 to alanine abolished the effects of PAO and herbimycin A on ROMK1 trafficking (23,24). However, these experiments were performed in HEK293 cells transfected with ROMK1 and c-Src, and the hypothesis is still required to be tested in the native tissue. In the present study, we provide the evidence that PTK plays a key role in the regulation of ROMK1 trafficking. Stimulation of PTK activity by inhibiting PTP diminished the membrane location of ROMK1 and increased the distribution of ROMK1 in the intracellular compartment in the CCD from rats on a HK diet.

ROMK1 is mainly located in the CCD, whereas ROMK2 and ROMK3 are expressed in the TAL (1). Although ROMK2 also contains a tyrosine phosphorylation site in the COOH terminus, we reported that inhibition of PTP had no effect on the activity of ROMK-like SK channels in the TAL (5), suggesting that tyrosine phosphorylation of ROMK2 alone may not be sufficient to induce endocytosis. This speculation is further confirmed by the present finding that neither herbimycin A nor PAO has an effect on the surface ROMK2 in the HEK293 cells transfected with ROMK2 and c-Src. Also, a sharp membrane staining of ROMK can be observed in the TALs from animals on a HK or a KD diet. This contrasts with results obtained from the CCD in which no sharp membrane staining of ROMK was observed in the CCD from rats on a KD diet. This suggests that the mechanism by which dietary K intake regulates ROMK2/3 is different from that for ROMK1. This notion is also supported by the observation that inhibition of PTP did not significantly change the

fluorescence intensity of ROMK staining in the plasma membrane in the TAL but decreased the ROMK staining intensity in the plasma membrane in the CCD from rats on a HK diet. Previous studies demonstrated that c-Src is highly expressed in the TAL and regulated by dietary K intake (28). Although stimulation of PTK does not increase the endocytosis of apical K channels in the TAL, PTK is most likely still to play an important role in the regulation of apical K channels in the TAL. This view is supported by the previous finding that the stimulation of PTK decreased the 70-pS K channel activity (5). Moreover, it is possible that ROMK2/3 could be involved in forming the 70-pS K channel because no 70-pS K channel can be found in ROMK(−/−) mice (9). Thus it is possible that the mechanism by which PTK regulates ROMK2/3 in the TAL is to change the channel open probability rather than endocytosis. We need further experiments to examine this speculation.

We conclude that c-Src is expressed in the TAL, CCD, and OMCD and that stimulation of PTK activity increases the number of ROMK in the intracellular compartment and decreases ROMK channels in the apical or subapical membrane.

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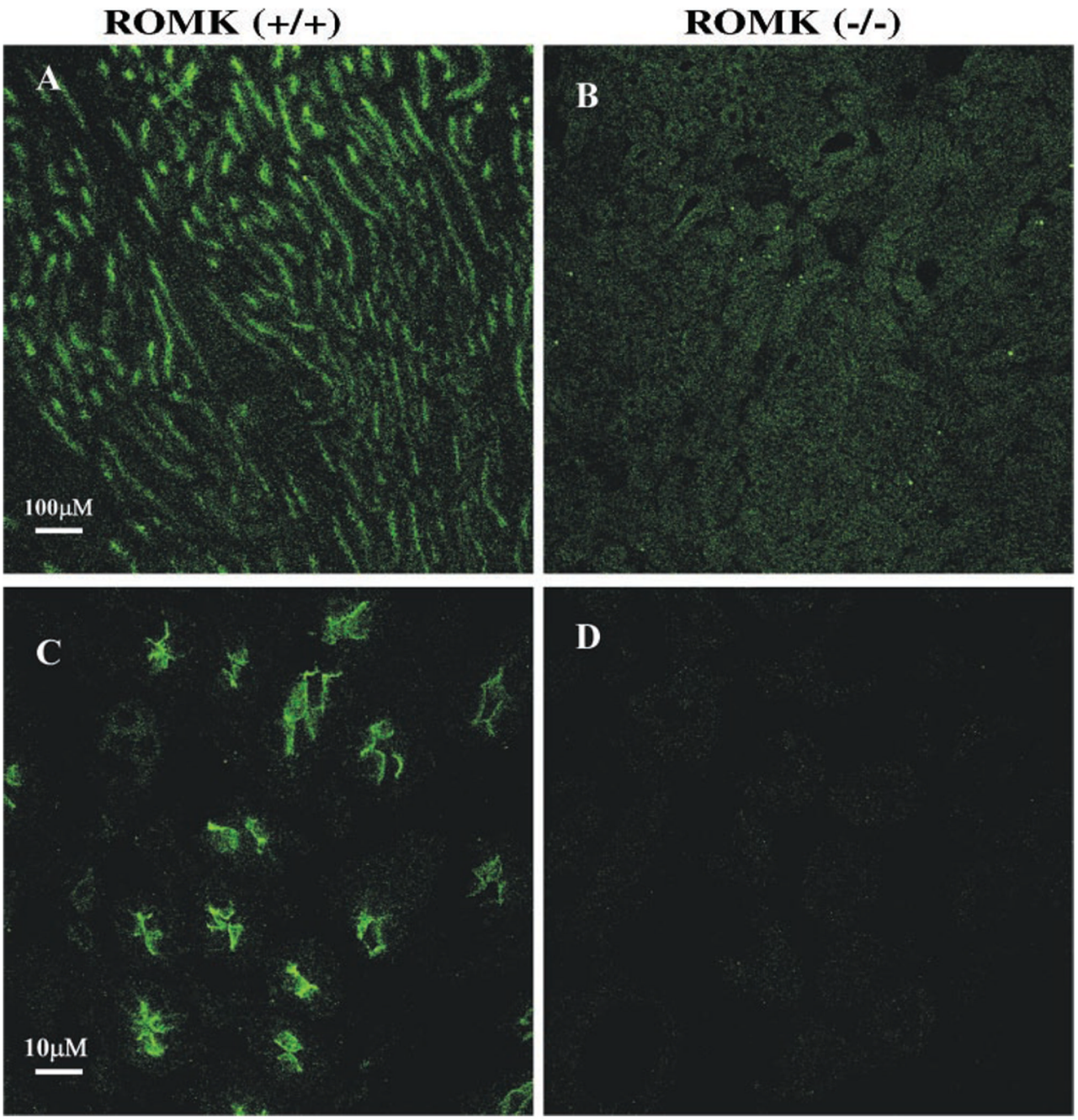

Fig. 1.

Confocal images with low magnification showing ROMK staining in the kidney from a ROMK(+/+) mouse (*A*) and ROMK(−/−) mouse (*B*). A magnified image demonstrating the ROMK staining in the outer medulla from a ROMK(+/+) mouse (*C*) and ROMK(−/−) mouse (*D*) is also shown.

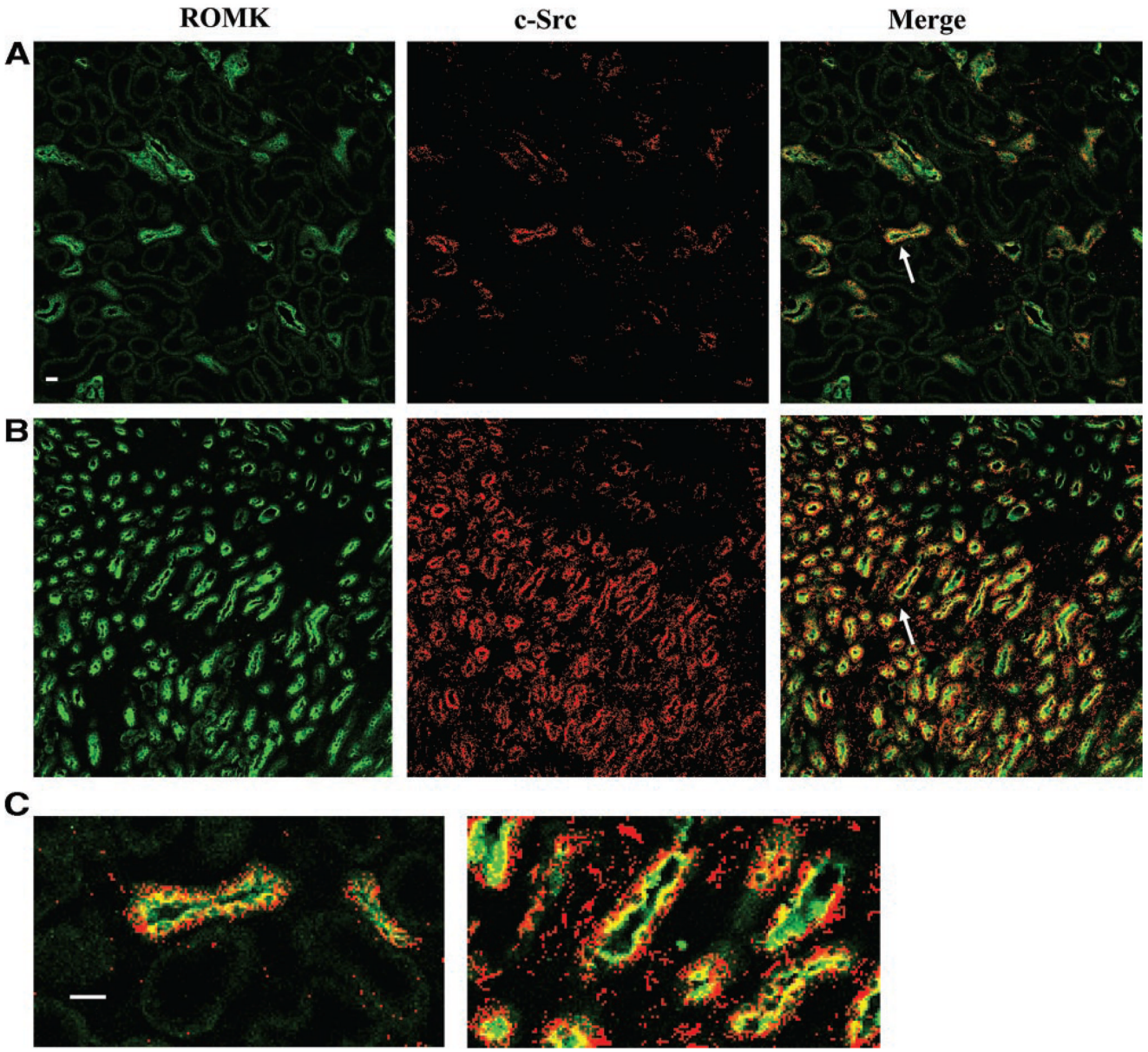

Fig. 2.

Confocal images showing the colocalization of ROMK (green) and c-Src (red) in the renal cortex (*A*) and outer medullar (*B*) from rats on a normal-K diet. *C*: magnified confocal image showing the double staining of ROMK and c-Src from a tubule indicated by an arrow in Fig. 2*A*, *left* and Fig. 2*B*, *right*. Bars = 10 μM.

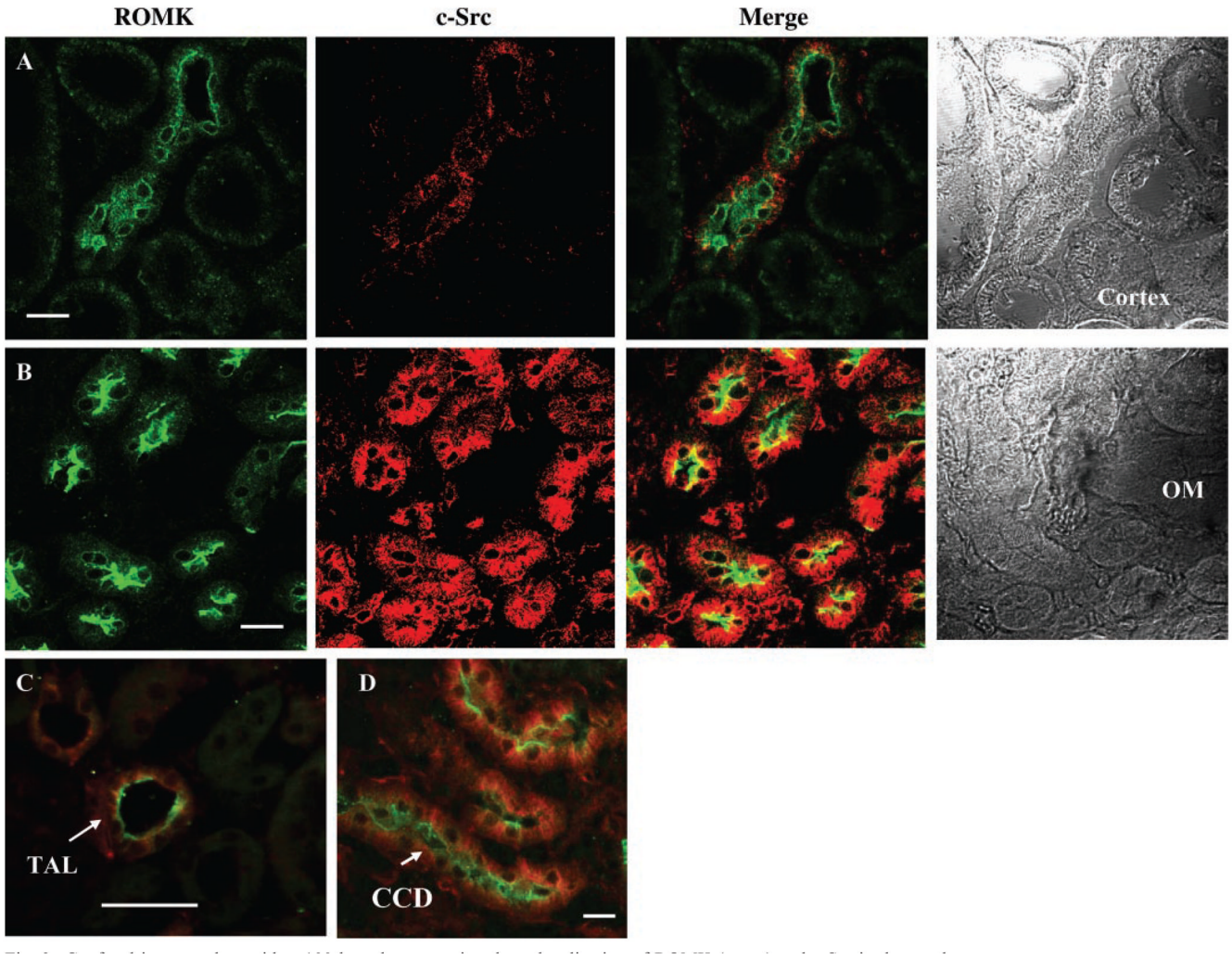

Fig. 3.

Confocal images taken with ×100 lens demonstrating the colocalization of ROMK (green) and c-Src in the renal cortex (*A*) and outer medulla (OM; *B*) from rats on a normal-K diet. Confocal images demonstrating colocalization of ROMK and c-Src in the thick ascending limb (TAL; *C*) and the cortical collecting duct (CCD; *D*). Bars = 10 μM.

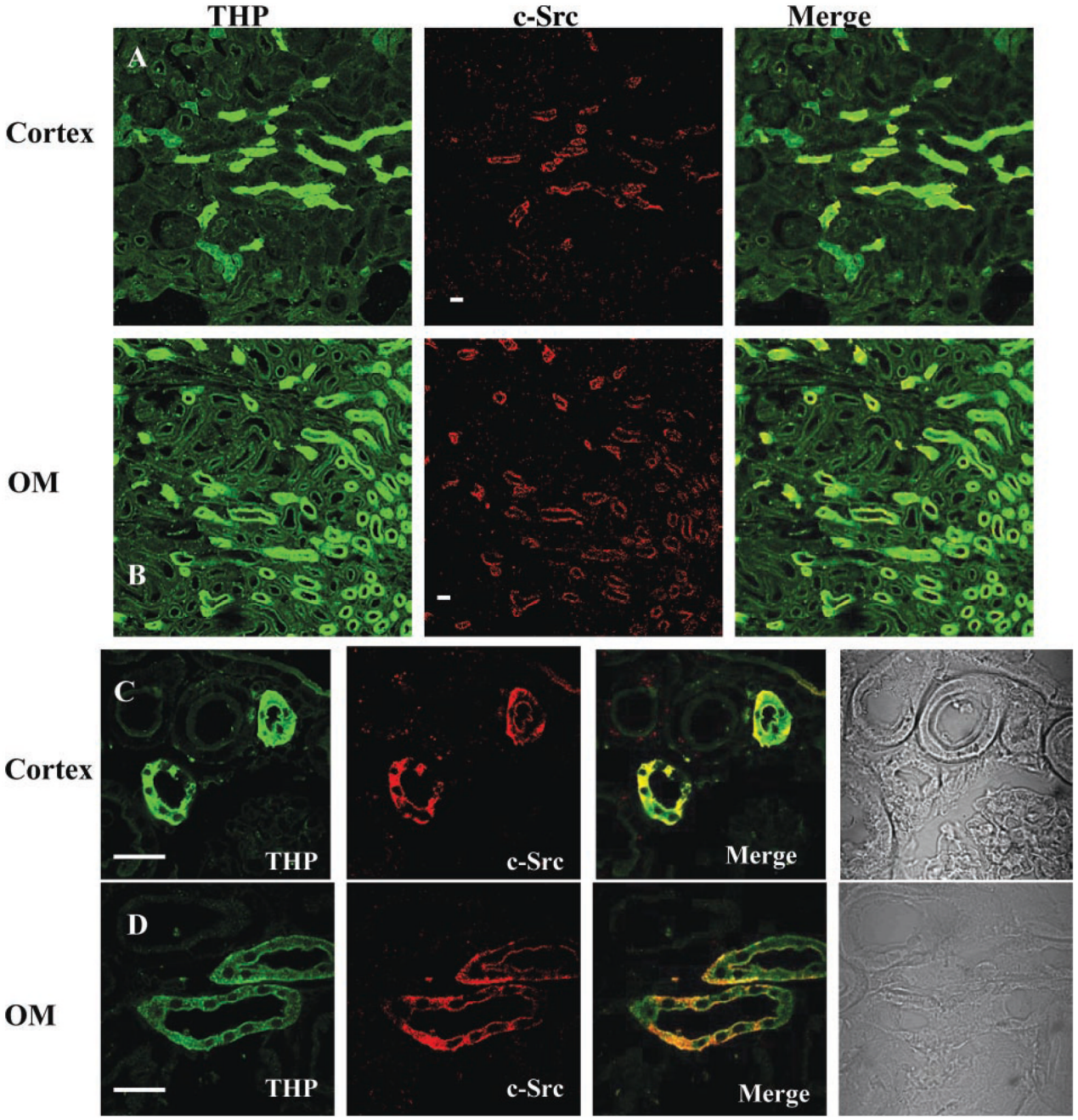

Fig. 4.

Confocal images showing the colocalization of Tamm-Horsfall protein (THP; green) and c-Src (red) in the renal cortex (*A*) and OM (*B*) from rats on a normal-K diet. A magnified confocal image showing the double staining of THP and c-Src in the renal cortex (*C*) and OM (*D*) is also shown. Bars = 10 μM.

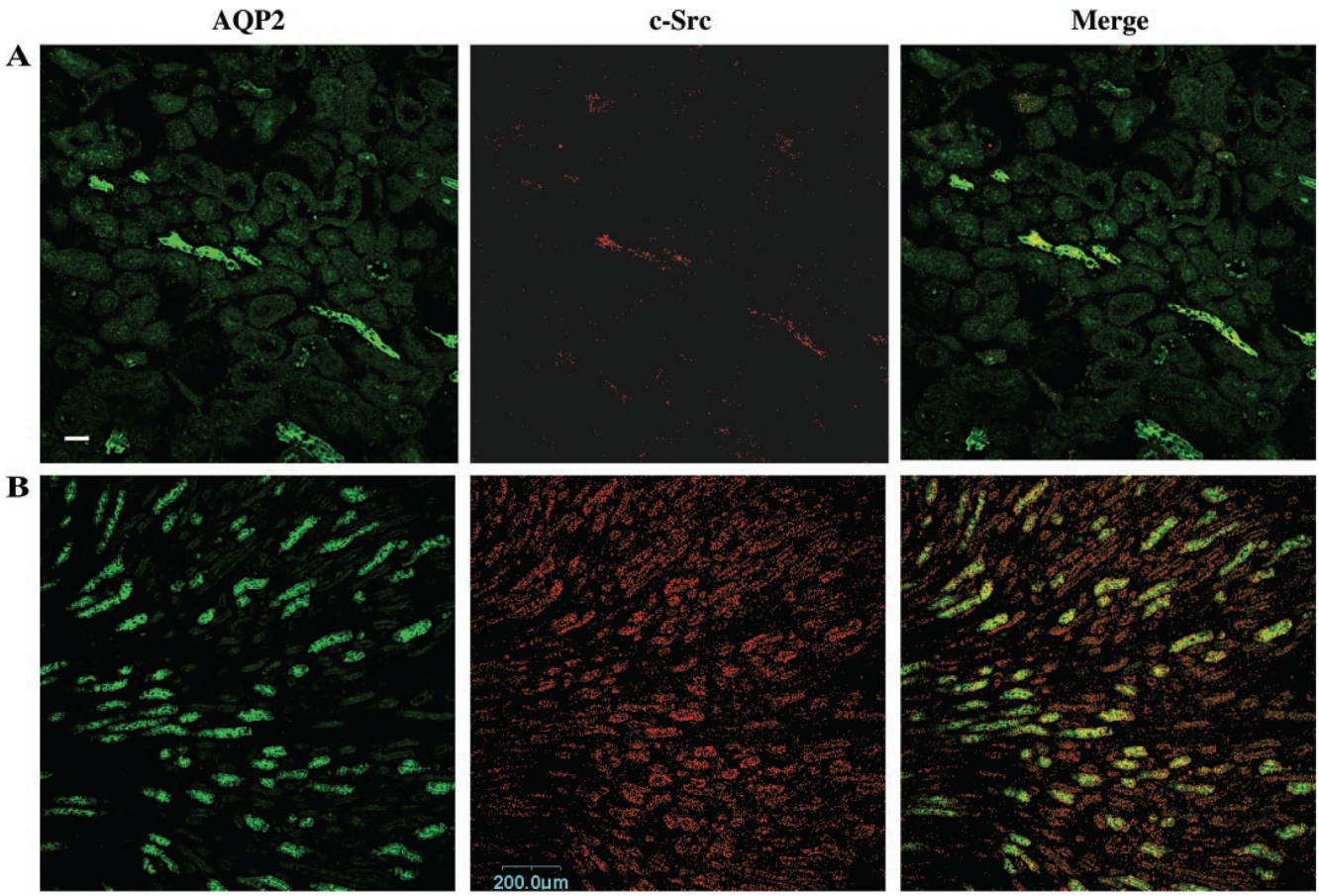

Fig. 5.
Confocal images showing the colocalization of aquaporin-2 (AQP2; green) and c-Src (red) in the renal cortex (*A*) and OM (*B*) from rats on a normal-K diet. Bar = 10 μM or as indicated.

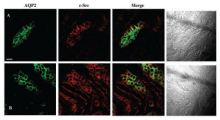

Fig. 6.
Magnified confocal image (×100) showing the double staining of AQP2 and c-Src in the renal cortex (*A*) and OM (*B*). Bar = 10 μM.

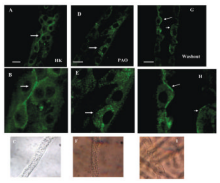

Fig. 7.
Immunofluorescence staining of ROMK in the CCD from the rat on a high-K (HK) diet in the absence (*A*) or presence of 1 μM phenylarsine oxide (PAO; *D*) or washout of PAO (*G*). Bars = 10 μm. *B*, *E*, and *H*: magnified images from corresponding cells indicated by arrows in *A*, *D*, and *G*, respectively. *C*, *F*, and *I*: plain images of the corresponding CCD (same tubule).

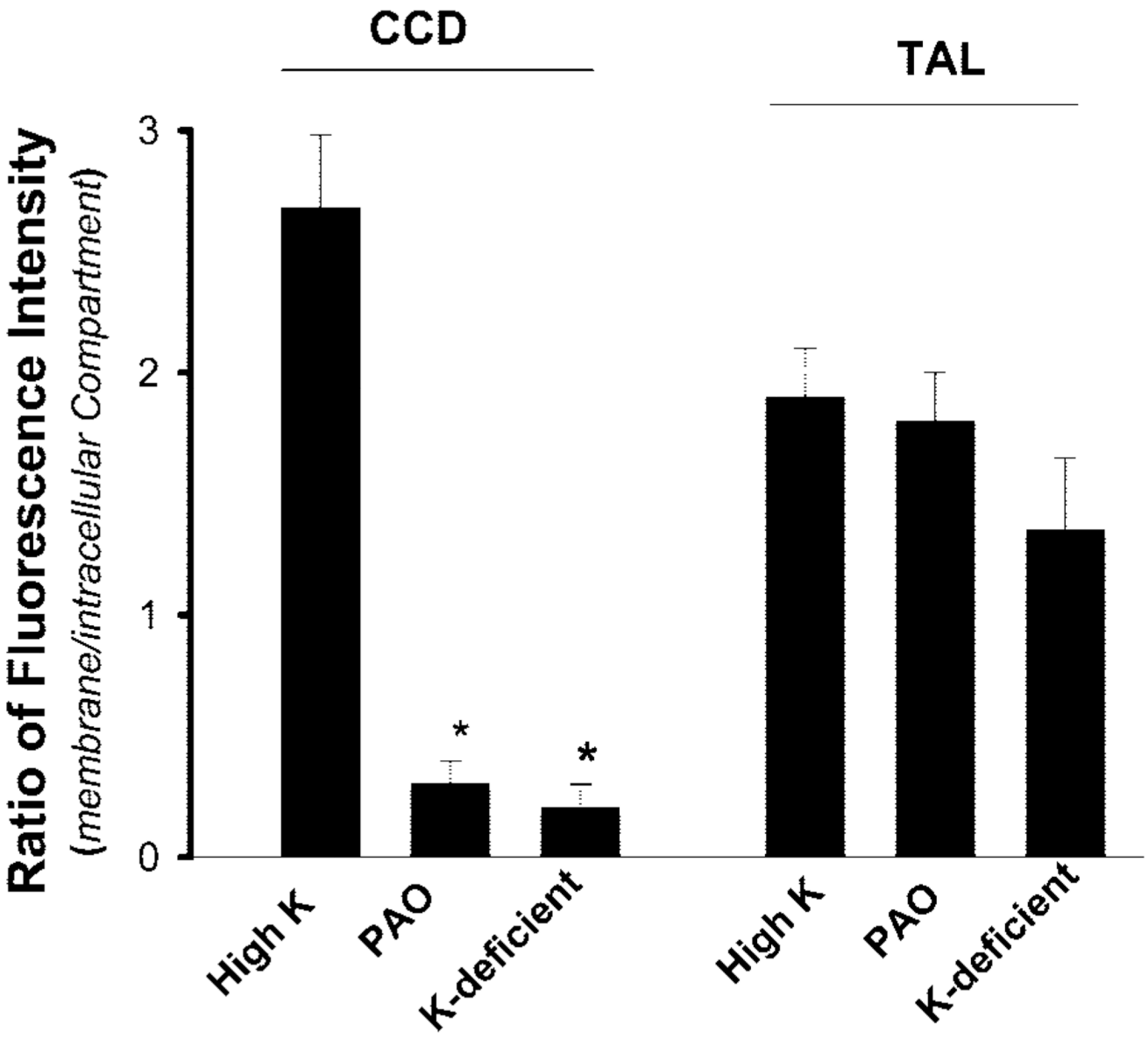

Fig. 8.

Relative ratio of fluorescence intensity between plasma membrane and intracellular compartment in the CCD and TAL. Number of cells measured for fluorescence intensity ranges from 50 to 60 cells. *Difference between HK and PAO-treated group or between HK and low-K group is significant ($P < 0.001$, 1-way ANOVA test).

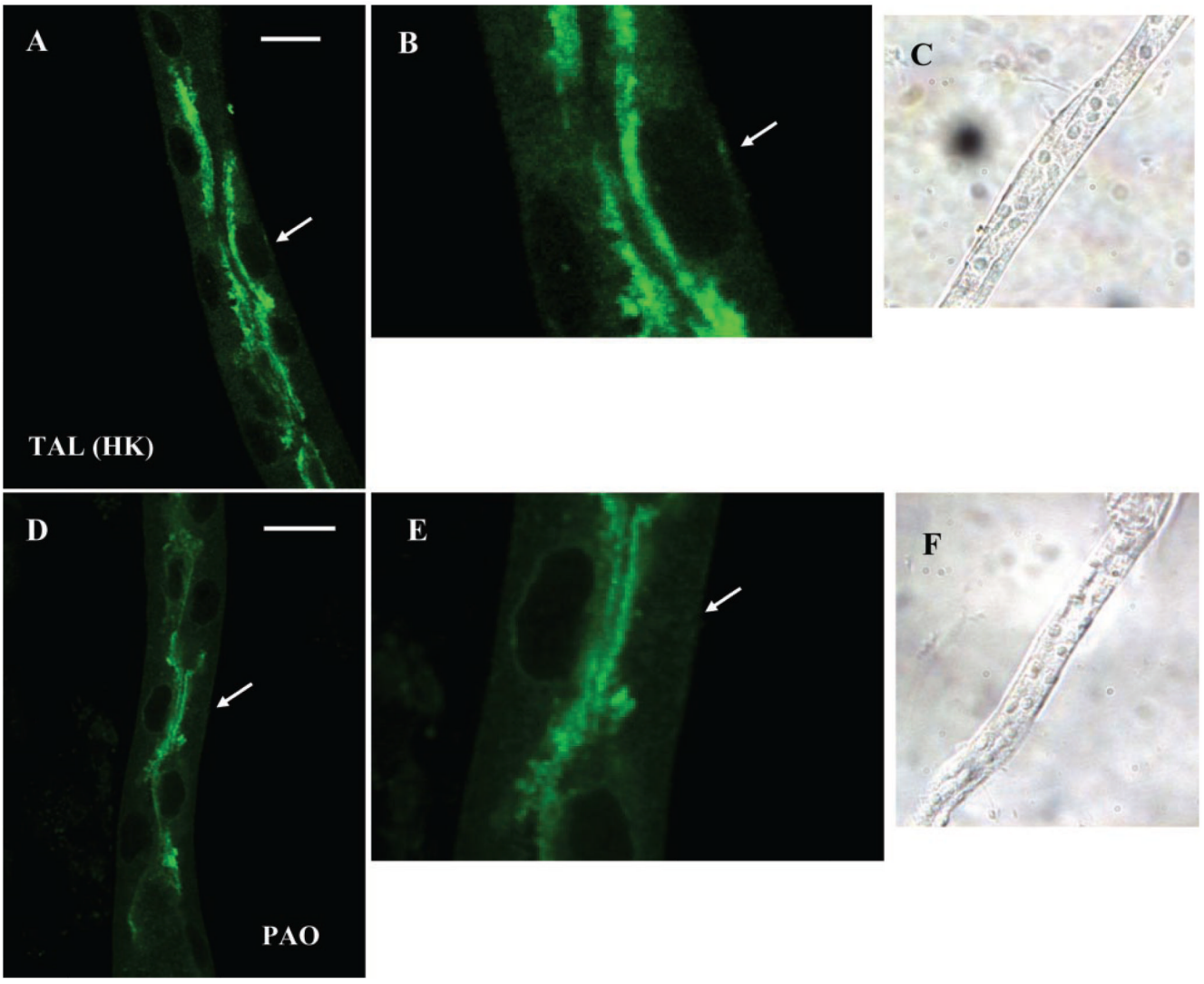

Fig. 9.

Immunocytochemical staining of ROMK in the TAL from the rat on a HK diet in the absence (*A*) or presence of PAO (*D*). Bars = 10 μm. *B* and *E*: enlarged images from the corresponding cells indicated by arrows in *A* and *D*, respectively. *C* and *F*: plain images of TALs shown in *A* and *D* (same tubule).

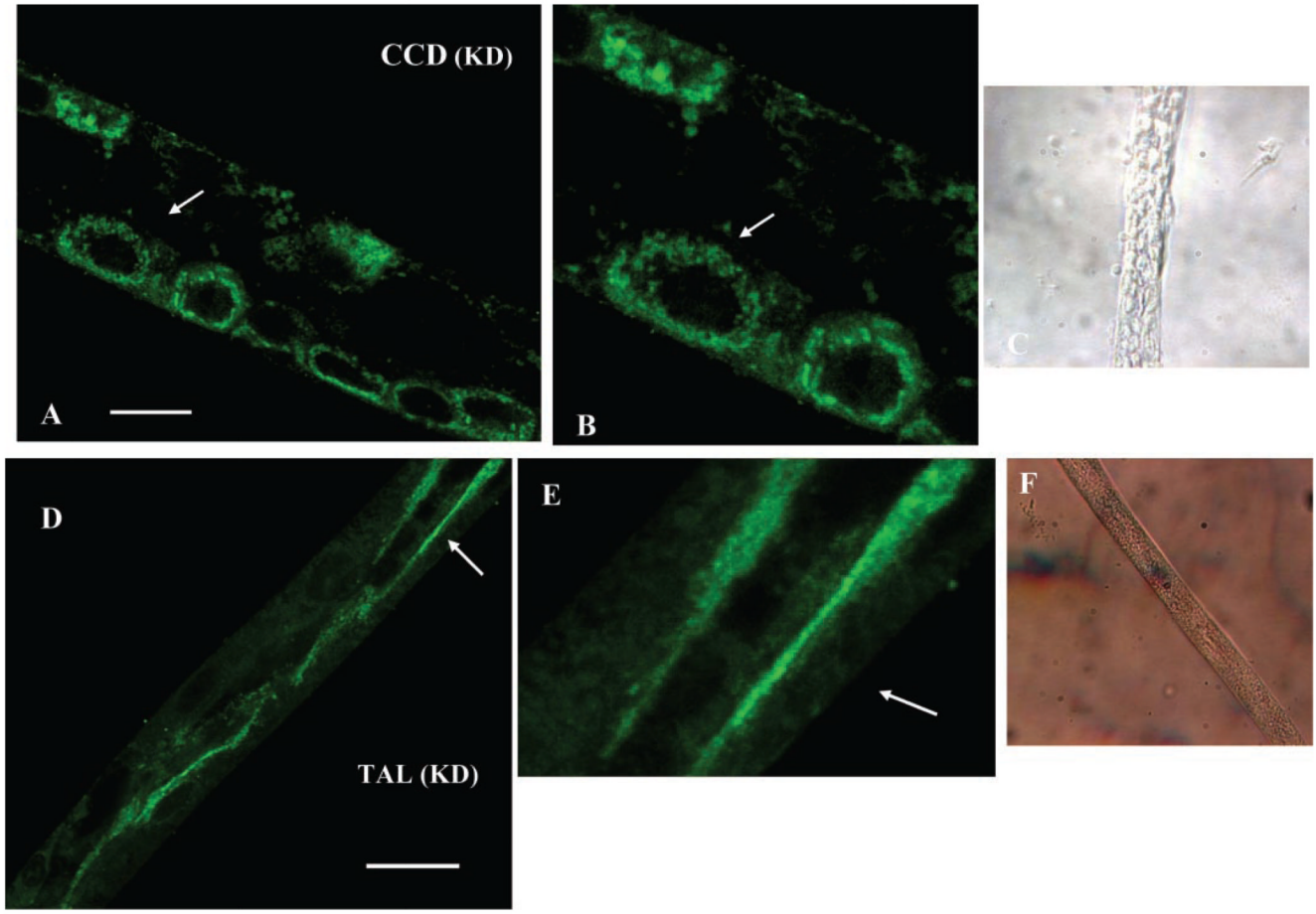

Fig. 10.

Immunocytochemical staining of ROMK in the CCD (*A*) and TAL (*D*) from rats on a K-deficient (KD) diet. *B* and *E*: confocal images magnified from the corresponding cells indicated by arrows in *A* and *D*, respectively. *C* and *F*: plain images of the corresponding tubule (same tubule).

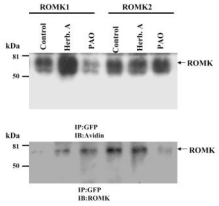

Fig. 11.

Western blot analysis showing the effect of herbimycin A (Herb. A) and PAO treatment on the surface localization of ROMK1 and ROMK2. HEK293 cells transfected with either GFP-ROMK1 or GFPROMK2 and c-Src were treated with herbimycin A, PAO, or vehicle for 10 min. The ROMK channels were harvested by immunoprecipitation (IP) of the cell lysate with GFP antibody. *Top*: surface-localized ROMK was detected by biotin labeling followed by Western blot analysis with Neutravidin. *Bottom*: total ROMK is recognized by ROMK antibody. The variation of total ROMK amount in each experiment has been taken into the consideration for the normalization data by comparing the level of ROMK in the membrane to the total ROMK protein. The biotin-labeled ROMK is indicated by an arrow.