Evidence for endocytosis of ROMK potassium channel via clathrin-coated vesicles

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Departments of 1Medicine and 2Pediatrics, University of Texas Southwestern Medical Center, Dallas, Texas 75390-8856; 2Department of Biomedical Science, University of Sheffield, Sheffield S102TN, United Kingdom; and 4Department of Physiology, University of Maryland School of Medicine, Baltimore, Maryland 21201

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Zeng, Wei-Zhong, Victor Babich, Bernardo Ortega, Raymond Quigley, Stanley J. White, Paul A. Welling, and Chou-Long Huang. Evidence for endocytosis of ROMK potassium channel via clathrin-coated vesicles. Am J Physiol Renal Physiol 283: F630–F639, 2002. First published April 16, 2002; 10.1152/ajprenal.00378.2001.—ROMK channels are present in the cortical collecting ducts of kidney and are responsible for K+ secretion in this nephron segment. Recent studies suggest that endocytosis of ROMK channels is important for regulation of K+ secretion in cortical collecting ducts. We investigated the molecular mechanisms for endocytosis of ROMK channels expressed in Xenopus laevis oocytes and cultured Madin-Darby canine kidney cells. When plasma membrane insertion of newly synthesized channel proteins was blocked by incubation with brefeldin A, ROMK currents decreased with a half-time of ~6 h. Coexpression with the Lys44→Ala dominant-negative mutant dynamin, but not wild-type dynamin, reduced the rate of reduction of ROMK in the presence of brefeldin A. Mutation of Asn371 to Ile in the putative NPXY internalization motif of ROMK1 abolished the effect of the Lys44→Ala dynamin mutant on endocytosis of the channel. Coimmunoprecipitation study and confocal fluorescent imaging revealed that ROMK channels associated with clathrin coat proteins in Madin-Darby canine kidney cells. These results provide compelling evidence for endocytosis of ROMK channels via clathrin-coated vesicles.

THE PHYSIOLOGICAL ROLE of the low-conductance K+ channels in apical membranes of principal cells of the cortical collecting ducts (CCDs) in the regulation of K+ secretion is well established (43). Secretion of K+ in this nephron segment is mediated by active transport of K+ into the cell through basolateral Na+-K+-ATPase and followed by passive movement of K+ into the tubular fluid through the apical K+ channels. cDNAs for ROMK1 and its isoforms ROMK2 and ROMK3 have been isolated (4, 15, 44). On the basis of the distribution of mRNA and proteins and biophysical characterization, it is known that the cDNAs for ROMK encode the low-conductance K+ channels in the apical membranes of the thick ascending limb of Henle’s loop and CCD (11, 12).

As the final common pathway for K+ secretion in CCDs, ROMK channels are regulated by dietary K+ intake (43). To maintain K+ homeostasis, the ability of the kidney to secrete K+ increases when dietary intake increases. This response, called K+ adaptation, is associated with an increase in the number of active channels in rat CCDs. Studies using cell-attached patch-clamp recording have found that the number of active K+ channels in the apical membranes of CCDs increases by a factor of 3–4 when animals are fed a high-K+ diet for 1–2 wk (29). The increase in the density of active channels because of high K+ intake is not associated with an increase in the mRNA for ROMK in isolated rat CCDs (10). These results raise the possibility that ROMK channels in the apical membranes of CCD undergo active trafficking, and the increase in channel density in K+-adapted animals may be due to alterations in these processes.

Recently, Wang et al. (40) reported that Src kinase activity in rat kidney cortex is inversely correlated with dietary K+ intake in these animals. Low dietary K+ intake increases the activity of Src kinase, whereas high dietary K+ intake decreases the activity. Application of tyrosine kinase inhibitors and tyrosine phosphatase inhibitors increases and decreases, respectively, the number of channels in cell-attached recordings of CCDs isolated from these animals as well as in oocytes coexpressing ROMK1 and recombinant Src kinase (24, 41). The effects of these tyrosine kinase and phosphatase inhibitors on channel activity are prevented by pretreating the cells with inhibitors of endocytosis (24, 41). These results suggest that alteration of endocytosis of K+ channels is important for physiological regulation of the K+ channels in CCDs by dietary K+ intake.

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There are multiple pathways for endocytosis in mammalian cells, including phagocytosis, internalization via caveolae, and clathrin-dependent endocytosis (17, 23, 25). Phagocytosis (or “cell eating”) occurs only in specialized cells. Caveolae are small microdomains of plasma membranes that are enriched in cholesterol and glycosphingolipids. Some glycosphatidylinositol-anchored proteins and receptors of the seven-transmembrane-domain family are endocytosed via caveolae. Endocytosis via clathrin-coated vesicles is a well-characterized form of pinocytosis and is a common mechanism for retrieval of plasma membrane proteins (17, 23, 25).

The clathrin coat contains two oligomeric proteins, clathrin, and clathrin adaptor protein (AP) complexes (37). Clathrin is composed of three light (∼33 kDa) and three heavy (∼180 kDa) chains that form a three-legged structure called triskelion. The AP complexes are heterotetrameric. Thus far, three adaptor complexes, AP-1, AP-2, and AP-3, have been found (18). The localization and function of the three AP complexes are different. AP-2 complexes are localized to the coated pits and are involved in plasma membrane endocytosis. Assembly of clathrin is not sufficient to drive vesicle budding. Budding of CCVs from plasma membranes requires dynamins, members of the ~100-kDa GTPase protein family (34). The role of dynamins in clathrin-dependent endocytosis from the plasma membrane is well established. Mutations of dynamin that interfere with GTP binding and hydrolysis [such as Lys44→Ala (K44A)] severely inhibit CCV-mediated endocytosis from plasma membranes (8). In the present study, we sought to investigate the role of CCVs in endocytosis of ROMK.

MATERIALS AND METHODS

Materials. All chemicals are from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Restriction endonucleases are from New England Biolabs, GIBCO-BRL, or Roche-Mannheim. Brefeldin A (BFA) stocks (in ethanol; 15 mM) were purchased from the pSPORT plasmid (15). Site-directed mutagenesis of the pSPORT plasmid (15). Site-directed mutagenesis of WT and K44A mutant rat dynamin II were originally constructed in pCMV-5 vector (2). An EcoRI-XbaI fragment was excised from the original pCMV-dynamin plasmid and subcloned into pBluescript SK vector doubly digested with EcoRI and XbaI. mCAP cRNAs for WT and K44A dynamins were transcribed in vitro using T7 RNA polymerase after linearization of pBluescript-dynamin plasmid DNAs with XbaI restriction endonuclease. Construction of the NH2-terminal enhanced green fluorescent protein (EGFP)-tagged ROMK2 in pEGFP-C2 vector (Clontech), pEGFP-ROMK2, has been described (28).

Immunostaining and laser scanning confocal imaging. Madin-Darby canine kidney (MDCK) cell lines stably transfected with pEGFP-C2 empty vector or pEGFP-ROMK2 were grown in DMEM/F-12 media (1:1) with 10% fetal calf serum and selected with geneticin at 0.3 mg/ml as previously described (28). Cells were subcultured at 1:10 dilution twice a week. For immunostaining by monoclonal antibody against clathrin heavy chain, cells were grown on cover glasses to ~50% confluence at 37°C and further incubated at 25°C for 24–48 h. Surface expression of ROMK in MDCK cells is low at 37°C (6, 28). Growing cells at 25°C increases the stability and surface expression of the channel (6, 28). MDCK cells (on cover glasses) were fixed in 4% formalin in PBS and permeabilized by 0.1% Triton X-100. After blocking for the non-specific staining by 5% BSA, cells were incubated sequentially with mouse monoclonal anti-clathrin heavy chain antibody (1:200 dilution; ICN Biochemicals), rabbit anti-mouse IgG (1:100) and rhodamine-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Fluorescent images were visualized through a Zeiss ×100 objective lens with a Zeiss LSM410 microscope. To detect the fluorescence of the green fluorescent protein (GFP), samples were excited with a 643 Kr/Ar ion laser at 488 nm. Fluorescent emissions were passed through a 510/540 band-pass filter. To detect fluorescence raised by rhodamine, samples were excited at 568 nm and emissions were passed through a 590-nm-long pass filter.

Coimmunoprecipitation of ROMK2 with clathrin coat proteins. MDCK cells stably transfected with ROMK2 (cultured as above) were lysed in a radioimmunoprecipitation assay buffer containing (in mM) 150 NaCl, 10 HEPES (pH 7.5), 1 EGTA, 1 DTT, 1 pheylmethylsulfonyl fluoride, as well as 1% (vol/vol) Nonidet P-40, 1% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) SDS, 10 μM leupeptin, and 1 μM pepstatin at 4°C for 1 h. Lysates were centrifuged in a microfuge at 15,000 rpm at 4°C for 30 min. Supernatants were incubated with either monoclonal antibody against α-adaptin (1:30 dilution; Transduction Lab) or control mouse IgG for 2 h and then precipitated by protein G-Sepharose. The immunoprecipitates were separated by 10% SDS-PAGE and detected in immunoblot analysis by using monoclonal antibodies against α-adaptin and against clathrin heavy chain and a polyclonal antibody against ROMK1.

Two-electrode voltage-clamp recording. X. laevis oocytes were prepared as previously described (16, 19, 20). Oocytes were injected with cRNA for ROMK1, cRNA for WT or dominant-negative dynamin, and/or cRNA for syntaxin, as indicated. Current-voltage (I-V) relationships (∼100 to +100 mV, in 25-mV steps) were measured in oocytes at −23°C by a two-electrode voltage clamp (TEVC) using an OC-725C oocyte clamp amplifier (Warner Instruments, Hamden, CT), pCLAMP6 software, and Digidata 1200A digitizer (Axon Instruments, Foster City, CA). The resistance of current and voltage microelectrodes (filled with 3 M KCl solution) was 1–2 MΩ. The bath solution contained (in mM) 96 KCl, 1 MgCl2, 1 CaCl2, and 5 HEPES (pH 7.5 by KOH).

Whole-cell patch-clamp and single-channel patch-clamp recording. For patch-clamp recording, MDCK cells were grown to ~50% confluence at 37°C and further incubated at 25°C for 24–48 h. Cells were dissociated by limited trypsin treatment and transferred to a recording chamber. For ruptured whole-cell recording of MDCK cells, patch-clamp pipettes (pulled from borosilicate glass, Warner Instruments) were filled with solutions containing (in mM) 140 KCl, 1
MgCl₂, 2 Na₂ATP, 1 EGTA, and 5 HEPES (pH 7.4 titrated with KOH). Both solutions contained (in mM) 140 KCl, 1 MgCl₂, 1 CaCl₂, 5 HEPES (pH 7.4 with KOH), and 5 glucose. For cell-attached single-channel recording (in MDCK cells and in oocytes), pipette solutions were (in mM) 100 KCl, 1 MgCl₂, 2 CaCl₂, and 5 HEPES (pH 7.4 titrated with KOH). Pipette tip resistance ranged from 3 to 5 MΩ. Currents (whole cell or single channel) were recorded with an Axopatch 200B patch-clamp amplifier (Axon Instruments). Single-channel currents were low-pass filtered at 1 kHz using an eight-pole Bessel filter, sampled every 0.1 ms (10 kHz) with a Digidata-1200A interface, and stored directly onto a computer hard disk (100 GB) using pCLAMP7 software (19). Data were transferred to CD for long-term storage. For analysis, event list files were generated using the Fetchan program and analyzed for open probability (Pₒ) and amplitude histogram using pCLAMP7 pSTAT (version 6.0.5, Axon Instruments). Pₒ was analyzed on segments of continuous recording from patches that contained only one active channel during the lifetime of the recording. Pₒ was determined using the criteria of threshold crossing of currents (50%) (19).

RESULTS

Endocytosis of ROMK1 in oocytes. X. laevis oocytes possess the machinery for endocytosis via CCVs and have been used for studying CCV-mediated endocytosis of many ion channels, including the epithelial Na⁺ channels (ENaCs) and the CFTR Cl⁻ channels (5, 38). I-V relationships were recorded in oocytes (~5 oocytes/time point) every 24 h postinjection of cRNA to monitor plasma membrane expression of ROMK1 channels. After the maximal steady-state expression of ROMK1 was reached (~72–96 h), BFA was added. BFA is a fungal metabolite that inhibits transport of the newly synthesized channel proteins to plasma membranes by blocking anterograde trafficking of vesicles from the endoplasmic reticulum to the Golgi complex (38, 42). BFA has been shown to be active in X. laevis oocytes and inhibits protein trafficking in these cells (26). BFA does not affect CCV-mediated endocytosis in plasma membranes (42). I-V relationships were again recorded from oocytes at 3, 6, 12, 24, and 48 h after addition of BFA (+BFA, Fig. 1A). After 3- or 24-h incubation with BFA, some of the oocytes were transferred to BFA-free solutions for further incubation (washout, Fig. 1A). Another group of oocytes received vehicle (ethanol) instead of BFA from the beginning and served as time controls (–BFA, Fig. 1A).

As shown in Fig. 1A, whole-cell inward K⁺ currents (Iₒ; measured at −100 mV) decreased to the level of background currents during 24 h of incubation with BFA. Measured currents showed the characteristic weak inward rectification in I-V relationships and were sensitive to Ba²⁺ (not shown). ROMK1 currents recovered if oocytes were transferred to BFA-free solutions after incubation in BFA, suggesting that the effect of BFA was not toxic. The half-time for reduction in currents in the presence of BFA was 5.7 ± 2.2 h (n = 6). For comparison, ENaC currents decay with a half-time of 3.6 h in oocytes in the presence of BFA (38). Cell-attached single-channel recording was performed in oocytes before addition of BFA (Control, Fig. 1B) and in oocytes after incubation in BFA for 6 h (Fig. 1B). As shown, neither single-channel conductance nor Pₒ of the channels were affected by BFA (Pₒ; 0.91 ± 0.08 at −100 mV and conductance 36 ± 2.3 pS from 0 to −100 mV, n = 5, for control vs. 0.90 ± 0.14 and conductance 35 ± 1.8 pS, n = 8, for BFA-treated cells; Fig. 1B). These results suggest that the progressive reduction of ROMK currents in BFA is not due to an effect of BFA on the opening of the channels but rather is a result of a decrease in the number of functional channels in the plasma membrane. This decrease in the number of functional ROMK channels in the plasma membrane may be due to endocytosis or may be due to degradation and/or sequestration of channels by other membrane proteins. The role of endocytosis will be examined here.

Inhibition of ROMK1 currents by syntaxin 5. As an independent confirmation of the results from oocytes incubated with BFA, we used syntaxin 5 overexpression as an additional tool to inhibit transport of the newly synthesized ROMK1 to plasma membranes.

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Syntaxins are a family of membrane proteins that are involved in the trafficking of membrane vesicles (39). Syntaxin 5 is expressed in the Golgi complex and plays an important role in regulating vesicle transport between endoplasmic reticulum (ER) and the Golgi (9). Overexpression of syntaxin 5 disrupts the stoichiometric interactions among the endogenous transport-associated regulatory proteins and thus inhibits ER to Golgi transport (33, 35).

ROMK1 currents in plasma membranes of oocytes were monitored using TEVC. Seventy-two hours later, oocytes were injected with either cRNA for syntaxin 5 (3 ng, ○) or water (●). I-V relationships were further recorded over 48 h. Ba²⁺-insensitive currents were subtracted from the total K⁺ currents (at −100 mV) to give $I_k$ (μA) as shown. A: experimental paradigm as in A except that various concentrations of cRNA for syntaxin 5 or for syntaxin 3 were injected at time 72 h. Maximal ROMK1 currents (at −100 mV) before injection of syntaxin cRNA were presented as 100% of control. Currents after 24 h of injection of syntaxin cRNA were compared with the maximal ROMK1 currents (% control). Values are means ± SE.

Fig. 2. Effect of coexpression of syntaxin 5 on ROMK1 channels in oocytes. A: oocytes were injected (at time 0) with cRNA for ROMK1 (5 ng), and I-V relationships were recorded by TEVC. Seventy-two hours later, oocytes were injected with either cRNA for syntaxin 5 (3 ng, ○) or water (●). I-V relationships were further recorded over 48 h. Ba²⁺-insensitive currents were subtracted from the total K⁺ currents (at −100 mV) to give $I_k$ (μA) as shown. B: experimental paradigm as in A except that various concentrations of cRNA for syntaxin 5 or for syntaxin 3 were injected at time 72 h. Maximal ROMK1 currents (at −100 mV) before injection of syntaxin cRNA were presented as 100% of control. Currents after 24 h of injection of syntaxin cRNA were compared with the maximal ROMK1 currents (% control). Values are means ± SE.

Fig. 3. Effect of coexpression of Lys44→Ala (K44A) dominant-negative mutant dynamin on ROMK1 channels. A: $I_k$ (μA) were measured by TEVC from oocytes coexpressing ROMK1 (5 ng cRNA) and K44A mutant dynamin (cRNA 15 ng) or coexpressing ROMK1 (5 ng cRNA) and wild-type (WT) dynamin (15 ng cRNA) for 72 h after injection of cRNA had higher currents ($I_k$ 54 ± 8 μA at −100 mV) compared with those coexpressing ROMK1 and WT dynamin (8 ± 1 μA). Furthermore, the reduction in ROMK1 currents over 24-h incubation of BFA was markedly inhibited in oocytes coexpressing ROMK1 and K44A mutant dynamin (Fig. 3B). The half-time for reduction in cur-

due to a reduction in the number of active channels. Figure 2B (● and solid line) shows the dose-dependent effect of various concentrations of cRNA for syntaxin 5 injected after maximal expression of ROMK1 currents in plasma membranes. The amount of cRNA required for inhibition of ROMK1 currents (1–3 ng) was comparable to the concentrations of syntaxin cRNA found to regulate other channels in oocytes (3, 27, 31, 36). In contrast to syntaxin 5, syntaxin 3 is expressed in plasma membranes and regulates vesicle trafficking at this site (21, 22, 32). As expected from its non-Golgi location, syntaxin 3 (injected after maximal expression of ROMK1) did not cause a reduction in ROMK1 currents (○ and dotted line, Fig. 2B).

Inhibition of endocytosis of ROMK in oocytes by coexpression with a dominant-negative dynamin. The K44A mutation of dynamin impairs GTP binding and hydrolysis. Overexpression of this K44A dominant-negative mutant dynamin inhibits budding of CCVs and endocytosis from plasma membranes but does not inhibit budding of vesicles from the trans-Golgi network (TGN) (1, 8).

The role of CCVs in endocytosis of ROMK1 was examined using K44A mutant rat dynamin II (14). Oocytes were injected with cRNA for ROMK1 combined with cRNA for either WT or K44A mutant dynamin. Seventy-two hours after injection of cRNAs, I-V relationships were recorded in oocytes by TEVC. Afterward, oocytes received either BFA (5 μM) or vehicle (ethanol), and I-V relationships were recorded every 6 h for an additional 24 h. As shown in Fig. 3A, oocytes coexpressing ROMK1 and K44A mutant dynamin (Fig. 3A) for 72 h after injection of cRNA had higher currents ($I_k$ 54 ± 8 μA at −100 mV) compared with those coexpressing ROMK1 and WT dynamin (8 ± 1 μA). Furthermore, the reduction in ROMK1 currents over 24-h incubation of BFA was markedly inhibited in oocytes coexpressing ROMK1 and K44A mutant dynamin (Fig. 3B).

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rents in oocytes treated with BFA was 5.5 ± 3.2 and 18.3 ± 3.7 h (n = 4; P < 0.05, unpaired Student's t-test) for oocytes coexpressing WT and dominant-negative dynamin, respectively. The relatively smaller reduction in ROMK currents observed in oocytes coexpressing K44A dynamin mutant may be caused by dynamin-independent internalization and/or degradation pathways (17, 23, 25). As was reported for ENaC in oocytes (38), baseline endocytosis of ROMK1 was not affected by overexpression of WT dynamin (compare the reduction in currents in BFA for ROMK1 + WT dynamin in Fig. 3B with that for ROMK1 alone in Fig. 1A). These results suggest that endocytosis of ROMK1 in oocytes is, at least partly, mediated by dynamin-dependent CCVs.

Internalization signal for endocytosis via CCVs. The COOH-terminal tail of ROMK1 (amino acids 373–378) contains the sequence YDNPNF, which is highly homologous to the (Y/F)(D/E)NPXY internalization motif of many membrane proteins that are internalized via CCVs (23), such as the LDL receptor, β-amyloid precursor protein, and mannose receptor (Fig. 4A). To test the importance of this region for endocytosis of ROMK1, Asn375 was mutated to Ile, and the effect on ROMK1 currents in oocytes was examined. We found that mutation of Asn375, but not of Asn377, abolished the ability of the K44A dynamin mutant to enhance ROMK currents (Fig. 4B; also compare with Fig. 3A). Moreover, mutation of Asn375, but not Asn377, abolished the decay of ROMK1 in the presence of BFA for 6 h (Fig. 4C, also compare with Fig. 1A).

Endocytosis of ROMK2 in MDCK cells. To examine endocytosis of ROMK in a mammalian cell system, we used an MDCK cell line stably transfected with a GFP-tagged ROMK2. Fusion of the GFP NH2 terminus to the ROMK peptide does not affect trafficking and function of the channel (28). Stable transfection avoids potential artifacts of overexpression of proteins commonly observed with transient transfection. As reported previously (28), GFP was distributed throughout the cytoplasm and nuclei and GFP-tagged ROMK2 was targeted to the plasma membrane of MDCK cells (top left and bottom left, respectively, Fig. 5A). Functional channel activity on the cell surface was confirmed by patch-clamp recording. Whole-cell inwardly rectifying K+ currents were recorded from cells ex-
pressing GFP-ROMK2 (Fig. 5, A and B). Currents recorded from cells expressing GFP alone (Fig. 5, A and B) were not significantly different from background currents in the control untransfected cells (not shown). The single-channel kinetics and $P_o$ of GFP-ROMK2 in MDCK cells (Fig. 5C and data not shown) were not significantly different from channels expressed in oocytes (19). When exocytotic insertion of ROMK was blocked by BFA, whole-cell inwardly rectifying $K^+$ currents in MDCK cells decreased by 56% over 6 h (open bars, Fig. 5D). Currents did not change significantly if cells were incubated with vehicle (ethanol; shaded bars, Fig. 5D). Incubation with BFA did not affect single-channel conductance or $P_o$ of GFP-ROMK2 in MDCK cells (not shown).

Localization of ROMK in CCVs. To provide direct evidence for endocytosis of ROMK via CCVs, we examined the subcellular distribution of GFP-ROMK2 in MDCK cells. The distribution of CCVs was examined by labeling with a monoclonal antibody against clathrin heavy chain. Because of nonspecific interactions of antibodies with the filter membrane, it was not feasible in these studies to grow cells in Transwells for obtaining optimal confocal images along the z-axis, as shown in Fig. 5A. Nevertheless, as shown in the image sectioned at the $xy$-plane (left, Fig. 6A), GFP-ROMK2 in MDCK cells was evidently distributed to the plasma membrane. Additionally, GFP-ROMK2 was present intracellularly in a punctate pattern, suggesting localization to the intracellular organelles. The staining by anti-clathrin heavy chain antibody was membranous as well as punctate intracellularly (middle, Fig. 6A). This distribution is consistent with the pattern of distribution of CCVs (1). Partial colocalization of GFP-ROMK2 with CCVs was evident from the merged image of distribution of GFP-ROMK2 and clathrin heavy chain (yellow, right, Fig. 6A). The localization of ROMK to the plasma membrane-associated CCVs suggests endocytosis of the channel at the plasma membrane. Besides endocytosis of plasma membrane proteins, CCVs are also involved in transport of proteins from TGN to lysosomes (37). ROMK channels expressed in MDCK cells are labile (6, 28). A significant fraction of channel protein is degraded before being targeted to

Fig. 5. Endocytosis of ROMK in Madin-Darby canine kidney (MDCK) cells. A, left: confocal fluorescent images in MDCK cells stably transfected with green fluorescent protein (GFP; top) or with GFP-tagged ROMK2 (bottom). Confocal images (section along $zx$-plane) were from individual subconfluent cells grown on a permeable support. GFP-ROMK2 was predominantly targeted to the plasma membrane. In contrast, GFP was diffusely present in cytoplasm and nuclei. Right: $I_k$ recorded from dissociated MDCK cells (see MATERIALS AND METHODS for preparation of cells) stably transfected with GFP (top) or with GFP-tagged ROMK2 (bottom). Inwardly rectifying $K^+$ currents were observed in cells transfected with GFP-ROMK2 but not in cells transfected with GFP. The currents were inhibited by 5 mM Ba$^{2+}$ in the bath (not shown). B: $I-V$ relationships of $I_k$ as recorded in A. $V_m$, membrane potential. C: cell-attached single-channel recording of the GFP-ROMK2 channel in MDCK cells. Recording of a single channel in a patch at $-100 \text{ mV}$ is shown. A portion of the recording is also shown at an expanded time scale. D: effect of BFA on whole-cell ROMK currents. MDCK cells were recorded before and 6 h after incubation with BFA (open bars) or with vehicle (ethanol, shaded bars). N.S., statistically not significant by unpaired Student’s $t$-test. Values are means ± SE ($n = 10$) of $I_k$ (pA) at $-100 \text{ mV}$. 

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the surface membrane (6, 28). The colocalization of ROMK with CCVs in the perinuclear region likely represents proteins in the synthetic pathway or the pathway for degradation. As reported previously (28) and also shown in Fig. 5A, GFP was distributed in cytoplasm and nuclei (left, Fig. 6B). For comparison, GFP was not localized to the plasma membrane-associated CCVs (right, Fig. 6B). A small amount of GFP was found in the perinuclear CCVs, probably representing proteins in the synthetic and/or degradation pathway.

Coimmunoprecipitation of ROMK with clathrin coat proteins. We further investigated endocytosis of ROMK via CCVs using coimmunoprecipitation. Besides endocytosis of plasma membrane proteins, CCVs are also involved in transport of proteins from TGN to lysosomes (37). CCV-mediated endocytosis at the plasma membrane involves AP2 (containing α-adaptin subunit), whereas transport of proteins between TGN and lysosomes involves AP1 (containing γ-adaptin). To examine association of ROMK with plasma membrane-associated CCVs, we used antibody against α-adaptin for immunoprecipitation. As shown in Fig. 7, antibody

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**Fig. 6.** Confocal fluorescent images of MDCK cells. **A**: MDCK cells stably transfected with GFP-ROMK2. Cells were stained by a monoclonal antibody against clathrin heavy chain, followed by rabbit anti-mouse IgG and by rhodamine-conjugated goat anti-rabbit IgG. **B**: MDCK cells stably transfected with GFP. Cells were stained as described in A. Images were visualized with a laser scanning fluorescent microscope with the excitation wavelength selected for GFP (left) or for rhodamine (middle). Right: merged images.

**Fig. 7.** Coimmunoprecipitation of ROMK2 with clathrin coat proteins. Solubilized membrane fractions from MDCK cells stably expressing ROMK2 were immunoprecipitated by monoclonal antibodies against α-adaptin or control mouse IgG. Immunoprecipitates were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted sequentially using antibodies against α-adaptin, clathrin heavy chain, and ROMK. IP Ab, immunoprecipitate antibody; none, no antibody added.
against α-adaptin, but not control IgG, coimmunoprecipitated clathrin heavy chain and ROMK from MDCK cells expressing ROMK2.

**DISCUSSION**

Several studies have suggested that ROMK channels undergo endocytosis in the plasma membrane of X. laevis oocytes and rat CCDs (24, 40, 41). The purpose of this study was to examine the kinetics and the role of CCVs in endocytosis of ROMK channels. The abundance of ROMK proteins in plasma membranes in the steady state represents the balance between exocytotic insertion of the newly synthesized proteins and retrieval of the proteins. Using BFA (or overexpression of syntaxin 5) to block the anterograde transport of the newly synthesized proteins to plasma membranes, we found that ROMK1 channels undergo endocytosis in plasma membranes of oocytes with a half-time of ~6 h by electrophysiological recording. Electrophysiological recording was chosen for these studies because it is highly quantitative and selective for active channels that are present in the plasma membranes only. Additionally, it allows us to compare our results with the published literature on regulation of the K⁺ channel by changes in dietary K⁺ intake [which mainly examines density of active channels using electrophysiological recordings (10, 24, 29, 30, 40, 41); see below]. ROMK channels are expressed in renal tubular epithelial cells (12). To know whether the endocytosis we observed also occurs in renal epithelial cells, we further examined endocytosis of the channel using a MDCK cell line stably expressing ROMK2. We found that endocytosis of ROMK2 also occurs in these cells with a time course similar to the endocytosis of ROMK1 in oocytes. ROMK2 lacks the first 19 amino acids of ROMK1 and is otherwise identical to ROMK1 (12). The similar rate of endocytosis of ROMK1 and ROMK2 is also consistent with the finding of an NPYX internalization motif in the COOH terminus of both ROMK1 and ROMK2.

There are multiple pathways for endocytosis (17). Endocytosis of ENaC and CFTR both involve clathrin-coated pits (5, 38). We therefore examined whether CCVs are involved in endocytosis of the ROMK channels. Dynamins are a ~100-kDa GTPase protein family that play an important role in endocytosis mediated via CCVs (1, 8). The role of dynamin-dependent CCVs in endocytosis of ROMK in oocytes is demonstrated by inhibition of endocytosis because of coexpression of the channels with the dominant-negative dynamin mutants. Dynamin has also been reported to be involved in internalization of caveolae (13). Direct demonstration of ROMK in CCVs will provide further confirmation of endocytosis of ROMK via CCVs. We attempted to examine the localization of ROMK in CCVs in oocytes using immunofluorescent staining. However, specific staining of CCVs in X. laevis oocytes was not feasible using available commercial antibodies. Localization of ROMK with plasma membrane-associated CCVs, nevertheless, is evident in MDCK cells by using immunofluorescent colocalization and coimmunoprecipitation.

The cytoplasmic domains of many membrane proteins that are internalized via CCV-mediated endocytosis contain specific sequence information that facilitates localization to coated pits (23). Several types of internalization signals have been found (23). One of these is the NPYX motif, which contains consensus amino acid sequence asparagine, proline, any amino acids, and tyrosine, respectively (7, 23). In this study, we also reported that the COOH terminus of ROMK1 contains an NPYX internalization motif. These results, together with the results of dominant-negative dynamin and the results of association of ROMK with CCVs, provide strong evidence that ROMK channels are regulated by CCV-mediated endocytosis. The possibility that ROMK may also be regulated by other internalization pathways, however, cannot be excluded.

Endocytosis via CCVs in plasma membranes has multiple roles. These roles include maintenance of cellular homeostasis by recovering protein components that are inserted into the plasma membrane by ongoing secretory activity, uptake of molecules from the extracellular space, and regulation of the number of membrane proteins in physiological and pathophysiological conditions (17). Several lines of evidence suggest that regulation of the apical membrane channel density by dietary K⁺ intake may occur by means of alterations of endocytosis of the channels (24, 40, 41). High K⁺ intake may decrease CCV-mediated endocytosis of the channels in the apical membrane and thus lead to the increase in the density of the channels. A recent study reported that the increase in the density of active K⁺ channels in rat CCDs occurs within 6 h (essentially 1 meal) of a high-K⁺ diet (30). We found that the half-time for reduction in ROMK currents in oocytes in the presence of BFA is ~6 h. This value may represent an underestimation of the rate of endocytosis of ROMK in plasma membranes if significant amounts of the endocytosed channels are recycled back to the plasma membranes. On the other hand, it may be an overestimation if significant pools of inactive channels not readily available for endocytosis are present in the plasma membranes. Nevertheless, the time course of endocytosis of ROMK measured by electrophysiological recording in our studies is in agreement with the time course of changes in the density of active K⁺ channels in CCDs associated with alterations of dietary K⁺ intake. The similar time course supports the hypothesis that variations of dietary K⁺ intake regulate K⁺ channel density by altering CCV-mediated endocytosis of the channels.

What might the signaling mechanism(s) be that links the changes in dietary K⁺ intake to the control of channel density by means of alterations of endocytosis of the channels? Studies by Wang and colleagues (24, 40, 41) suggest that changes in dietary K⁺ intake affect the activity of the Src tyrosine kinase and the related tyrosine phosphatases, which in turn alters the rate of endocytosis and/or exocytosis of the channels. Having
found that the mechanism of the endocytosis of ROMK is via CCVs and determined the kinetics of endocytosis, one can examine in future studies how tyrosine kinases and/or phosphatases regulate the CCV-mediated endocytosis of the channels.

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