Protein kinase C inhibits caveolae-mediated endocytosis of TRPV5

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Cha S-K, Wu T, Huang C-L. Protein kinase C inhibits caveolae-mediated endocytosis of TRPV5. Am J Physiol Renal Physiol 294: F1212–F1221, 2008. First published February 27, 2008; doi:10.1152/ajprenal.00007.2008.—Transient receptor potential vanilloid 5 (TRPV5) constitutes the apical entry pathway for transepithelial Ca2+ reabsorption in kidney. Many hormones alter renal Ca2+ reabsorption at least partly by regulating TRPV5. The mechanism for acute regulation of TRPV5 by phospholipase C-coupled hormones is largely unknown. Here, we found that protein kinase C (PKC) activator 1-oleoyl-acetyl-sn-glycerol (OAG) increased TRPV5 current density and surface abundance in cultured cells. The OAG-mediated increase of TRPV5 was prevented by preincubation with specific PKC inhibitors. Coexpression with a dominant-negative dynamin increased the basal TRPV5 current density and prevented the increase by OAG. Knockdown of caveolin-1 by small interference RNA (siRNA) prevented the increase of TRPV5 by OAG. In contrast, knockdown of clathrin heavy chain had no effects. OAG had no effect on TRPV5 expressed in caveolin-1 null cells derived from caveolin-1 knockout mice. Forced expression of recombinant caveolin-1 restored the regulation of TRPV5 by OAG in caveolin-1 knockout cells. Mutations of serine-299 and/or serine-654 of TRPV5 (consensus residues for phosphorylation by PKC) abolished the regulation by OAG. Parathyroid hormone (PTH) increased TRPV5 current density in cells coexpressing TRPV5 and type 1 PTH receptor. The increase caused by PTH was prevented by PKC inhibitor, mutation of serine-299/serine-654, or by knockdown of caveolin-1. Thus, TRPV5 undergoes constitutive caveolae-mediated endocytosis. Activation of PKC increases cell surface abundance of TRPV5 by inhibiting the endocytosis. This mechanism of regulation by PKC may contribute to the acute stimulation of TRPV5 and renal Ca2+ reabsorption by PTH.

calbindin-D28K, and extrusion through the basolateral membrane via Na+/Ca2+ exchangers and Ca2+-ATPases (22). The transient receptor potential vanilloid 5 (TRPV5) channel is localized to the apical membrane of distal convoluted tubules and connecting tubules and functions as a gatekeeper for transepithelial Ca2+ reabsorption in the kidney. Many hormones, including parathyroid hormone (PTH), calcitonin, 1,25(OH)2-vitamin-D3, estrogen, adenosine, arginine vasopressin, prostaglandin E2, regulate renal Ca2+ reabsorption at least partly via TRPV5 (22, 26, 46).

Regulation of TRPV5 by hormones can occur via alteration of gene transcription and protein expression and/or acutely via activation of intracellular signaling pathways such as protein kinase C (PKC) and protein kinase A (PKA) (22). Estrogen and vitamin D increase gene transcription and expression of TRPV5 and related Ca2+ transporters in the distal renal tubules (21, 37). PTH also increases total protein expression of TRPV5 and related Ca2+ proteins, providing a mechanism of long-term regulation of renal Ca2+ reabsorption (21, 48). In addition, PTH can enhance renal Ca2+ reabsorption within 10–15 min of its administration. The mechanism of acute regulation of TRPV5 by PTH remains elusive. Activation of both PKA and PKC has been suggested (2, 3, 12, 23, 27). Activation of PKC may potentially be important for regulation of Ca2+ reabsorption in distal renal tubules by phospholipase C (PLC)-activating hormones besides PTH (21). How activation of PKA and PKC leads to increase in TRPV5 channel activity is not known.

PKC can exert positive or negative regulation on ion transporters and channels. Among members of TRP superfamily of ion channels, PKC downregulates many TRPC channels including TRPC3, 4, 5, 6, 7 (42, 47, 52, 58), whereas it upregulates TRPV1, 5, and 6, and TRPM4 (1, 11, 13, 17, 32, 34, 35, 39, 51, 57). The regulation by PKC occurs via alteration of intrinsic channel properties (such as single channel open probability) and/or of their trafficking. For example, activation of PKC promotes cell surface delivery of TRPV1 channels (32, 57) as well as increasing the single channel open probability (39). Multiple mechanisms may underlie regulation of ion channels by PKC. These include direct phosphorylation of ion channels and indirect effects through intermediate signaling proteins or molecules. For example, activation of PKC causes reduction of PI(4,5)P2 content, which is a critical regulator of many ion channels and transporters (56).

Recently, Gkika et al. (17) reported that serine protease tissue kallikrein stimulates Ca2+ reabsorption via activation of Gαq-PLC-coupled bradykinin type 2 receptor. Activation of PKC by tissue kallikrein via the bradykinin receptor decreases...
membrane retrieval of TRPV5 to increase its surface abundance. The molecular mechanism for internalization for TRPV5 and how PKC activation leads to accumulation of TRPV5 channels at the plasma membrane remains unknown. In the present study, we show that TRPV5 undergoes constitutive caveolin-dependent endocytosis and that activation of PKC inhibits this process causing accumulation of TRPV5 at the cell surface. We further show that inhibition of caveolar endocytosis of TRPV5 via PKC underlies the activation of the channel by PTH.

**METHODS**

**Materials and DNA constructs.** 1-Oleoyl-acetyl-sn-glycerol (OAG), phorbol-12-myristate-13-acetate (PMA), α-PMA, BAPTA-AM, genistein, and carbachol were purchased from Sigma (St. Louis, MO). PP1 and PP2 (4-amino-5-[4-chlorophenyl]-7-[1-buty1] pyrazolo[3,4-d]pyrimidine 1 and 2) were obtained from Biomol Research Laboratory (Plymouth Meeting, PA). PTH and GF109203X were purchased from Eli Lilly (Indianapolis, IN) and Calbiochem (La Jolla, CA), respectively. The rabbit polyclonal anti-GFP antibody and anti-rabbit horseradish peroxidase secondary antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Ez-link-NHS-SS-biotin and streptavidin-agarose beads were obtained from Pierce Biotechnology (Rockford, IL).

FGF-tagged TRPV5 contains coding region of rabbit TRPV5 (55) cloned in-frame to a commercial pEGFP-N3 vector. Human type 3 muscarinic receptor (hM3R), human type 1 PTH receptor (hPTH1R), and caveolin-1 are in pcDNA3 vector. Point mutation of PKC phosphorylation site of TRPV5 was generated by site-directed mutagenesis (QuickChange kit, Stratagene) and confirmed by sequencing. Sense and antisense oligonucleotide for clathrin heavy chain were 5'-UCCAAUUUGAGACCAAUU dT dT and 5'-AAUUGGUCUUC-GAAUUGGA dT dT, respectively. Sense and antisense oligonucleotide for caveolin-1 were 5'-CUCAAACCUCUACGAGUAUU and 5'-UCAUCGUUGAGGUGUUAGUU, respectively.

**Cell culture and transfection.** HEK293 cells were cultured as described (28). Madin-Darby canine kidney (MDCK) cells were cultured in the same condition as HEK293 cells. Wild-type and caveolin-1 null (Cov-1−/−) fibroblasts (gift of R. Anderson, UT Southwestern Medical Center at Dallas) were cultured in low glucose DMEM supplemented with 10% fetal bovine serum, glutamine, penicillin, and streptomycin. Cells were transiently cotransfected with cDNA (0.1 μg per 6-well) for GFP-TRPV5 plus cDNAs for hM3R, PTH1R, caveolin-1 (α-isoform), and/or wild-type or dominant-negative (lysine-44 to alanine) rat dynamin II as indicated in each experiment. Transfection was performed using FuGENE6 reagent (Roche Diagnostics, Indianapolis, IN) per manufacturer’s protocol. In each experiment, the total amount of DNA for transfection was balanced by using empty vectors. Transfected cells were identified for whole cell recording by green fluorescence. Approximately 36–48 h after transfection, cells were dissociated and placed in a chamber for ruptured whole cell recordings. For knockdown by siRNA, oligonucleotides (200 nM each) were mixed with cDNAs for TRPV5 and other indicated constructs for cotransfection.

**Electrophysiological recordings.** TRPV5 currents were recorded using the ruptured whole cell configuration of the patch-clamp technique as described previously (55). For whole cell recordings, the pipette and bath solution contained (in mM) 140 Na-Asp (sodium aspartate), 10 NaCl, 10 EDTA, and 10 HEPES (pH 7.4) and 140 Na-Asp, 10 NaCl, 1 EDTA, and 10 HEPES (pH 7.4), respectively. The resistance of electrodes containing the pipette solution was 1.5–3 MΩ. An Ag/AgCl pellet connected via a 3 M KC1/agar bridge was used to ground bath. The cell membrane capacitance and series resistance were monitored and compensated (>75%) electronically using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Voltage protocol consists of 0 mV membrane holding potential and successive voltages sets (500-ms duration) from −150 to 100 mV in +5-mV increments. Data acquisition was performed using ClampX 9.2 software (Axon Instruments). Currents were low-pass filtered at 2 kHz using 8-pole Bessel filter in the clamp amplifier, sampled every 0.1 ms (10 kHz) with Digitida-1300 interface, and stored directly to a computer hard disk.

**Surface biotinylation assay.** For biotinylation of cell surface TRPV5, cells were washed with ice-cold PBS and incubated with 0.75 ml PBS containing 1.5 mg/ml EZ-link-NHS-SS-biotin for 1 h at 4°C. After being quenched with glycine (100 mM), cells were lysed in a RIPA buffer (150 mM NaCl, 50 mM Tris·HCl, 5 mM EDTA, 1% Triton X-100, 0.5% DOC, and 0.1% SDS) containing protease inhibitor cocktail. Biotinylated proteins were precipitated by streptavidin-agarose beads. Beads were subsequently washed four times with PBS containing 1% Triton X-100. Biotin-labeled proteins were eluted in sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose membranes for Western blotting. TRPV5 proteins on the membrane were detected using a rabbit polyclonal anti-GFP antibody. Biotinylation experiment was performed three times with similar results.

**Data analysis.** Data analysis and curve fitting were performed with the Prism (v3.0) software (GraphPad Software, San Diego, CA). Statistical comparisons between two groups of data were made using two-tailed unpaired t-test. Multiple comparisons were determined using one-way ANOVA followed by Tukey’s multiple comparison tests. P values less than 0.05 and 0.01 were considered significant for single and multiple comparisons, respectively. Data were presented as means ± SE.

**RESULTS**

**PKC activator increases TRPV5 activity.** Many TRP channels including TRPV5 are downstream of PLC, which catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) to produce inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 releases Ca2+ from internal Ca2+ store while DAG activates PKC. We examined the regulation of TRPV5 by DAG using a synthetic OAG. TRPV5 current density was measured in human embryonic kidney (HEK) cells by using ruptured whole cell patch-clamp recordings. Current-voltage (I-V) relationship curves show characteristic inward rectifying currents in TRPV5-transfected cells, but not in mock-transfected cells (Fig. 1, A and B). Incubation of OAG (10 μM for 1 h) augmented TRPV5 current density but had no apparent effects on general properties of whole cell currents (Fig. 1A). OAG had no effects on currents of mock-transfected cells (Fig. 1, A and B). Increase of TRPV5 current density by OAG was concentration dependent; median effective concentration (EC50) and Hill coefficient were estimated at 0.27 μM and 0.81 ± 0.1, respectively (Fig. 1C). Stimulation of TRPV5 by OAG was saturated at 10 μM. We next examined the time course of stimulation of TRPV5 by OAG (10 μM). The stimulation of TRPV5 by OAG was detected after 10-min incubation and reached maximal stimulation at 1 h (Fig. 1D).

In the above and following electrophysiological experiments throughout the paper, TRPV5-expressing cells were incubated with OAG for indicated duration before rupture for whole cell recording. We found that, when applied after rupture of membrane patch into whole cell configuration, OAG did not cause an increase of TRPV5 currents (Fig. 1E). Thus, an intact intracellular milieu is required for the effects by OAG. We next measured the effects of OAG on surface abundance of TRPV5 expressed in HEK293 cells using a biotinylation assay. Surface
TRPV5 was detected only when biotin was added (Fig. 1F). Incubation with OAG increased the steady-state surface abundance of TRPV5 but not TRPV5 in the total cell extracts (Fig. 1F). These results are consistent with the notion that OAG affects membrane trafficking of TRPV5 (see below Figs. 3–6).

Role of PKC and isoforms in the increase of TRPV5 activity. DAG and analogs can directly activate TRP channels independent of activation of PKC (18). The EC50 for DAG activation of PKC is in the sub-μM range. In contrast, ~100 μM DAG is required for activation of ion channels via PKC-independent mechanism. The above finding that OAG activates TRPV5 with an EC50 of 0.27 μM supports the effect is through activation of PKC. Nevertheless, we used PKC inhibitors to confirm the role of PKC activation in the regulation of TRPV5 and to explore the involvement of PKC isoforms. There are multiple PKC isoforms classified into three subfamilies (conventional, novel, and atypical) based on structure, biochemical, and pharmacological properties (8). Conventional PKCs (α, βI, βII, and γ) require Ca2+, phosphatidylserine (PS), and DAG for activity; novel PKCs (δ, ε, η, and θ) require PS and DAG; and atypical PKCs (ζ and λ/ι) require only PS (8).

The bisindolemaleimide compound GF109203X (“GFX”) is a potent PKC inhibitor that inhibits all PKC classes at micromoles and selectively inhibits conventional and novel PKCs at sub-μM concentrations (29, 38). As shown, OAG-mediated TRPV5 activation was blocked by pretreatment of both high (1 μM) and low (0.1 μM) concentration of GFX (Fig. 2A). Both DAG and phorbol ester PMA can activate conventional and novel PKCs but not atypical PKCs (8). We found that TRPV5 current density was increased by PMA but not by α-PMA, an inactive analog of PMA (Fig. 2B). PMA-induced TRPV5 activation was also blocked by GFX. Long-term treatment of PMA downregulates PMA-sensitive PKC isoforms expressed in HEK293 cells (5). HEK293 cells were incubated with 1 μM PMA for 24 h. Downregulation of PMA-sensitive PKCs by long-term incubation abolished OAG-mediated TRPV5 activa-
Activation of conventional (but not novel) PKCs is dependent on intracellular Ca\(^{2+}\) (32). We found that OAG-mediated TRPV5 activation was blocked by pretreatment of a membrane-permeable Ca\(^{2+}\) chelator, BAPTAAM. Together, these results suggest that conventional PKC(s) is (are) involved in the augmentation of TRPV5 activity.

Activation of PKC inhibits caveolin-dependent endocytosis of TRPV5. Recently, Gkika et al. (17) reported that PKC activation increases the number of TRPV5 channels at the plasma membrane by delaying retrieval of TRPV5 channels. However, the molecular mechanism for endocytosis of TRPV5 and how PKC activation leads to delayed endocytosis of TRPV5 channels are not known. We first examined the molecular pathway(s) for endocytosis of TRPV5. Budding and fission of clathrin-coated vesicles (CCVs) and of caveolar vesicles require small GTPase proteins dynamin (7, 25). Coexpression of a dominant-negative dynamin (lysine-44 to alanine [K44A] mutant of dynamin II) inhibits both CCV-mediated and caveolar endocytosis (7, 25). Coexpression with the K44A dominant-negative dynamin II increased basal TRPV5 current density (Fig. 3A), indicating that constitutive dynamin-dependent endocytosis of TRPV5 occurs in the basal state. Moreover, coexpression with dominant-negative dynamin II prevented OAG-mediated TRPV5 activation while wild-type dynamin II had no effects (Fig. 3A). We showed in previous studies the efficiency of expression of K44A dominant-negative dynamin II in HEK cells and its effect on preventing dynamin-dependent endocytosis (20, 28). These results indicate that activation of PKC inhibits dynamin-dependent endocytosis of TRPV5.

Both caveolae-mediated and CCV-mediated endocytosis requires dynamin (7, 25). Next, we examined the role of CCVs vs. caveolae in the endocytosis of TRPV5 by using small interference RNA (siRNA) to knock down endogenous clathrin heavy chain (CHC) or caveolin-1. There are three caveolins encoded by separate genes (6, 36). Caveolin-1 is an essential structural component of caveolae in most cell types. Cells lacking caveolin-1 (except in muscles) do not have caveolae, but may have noncaveolae “lipid rafts” (36). HEK293 cells express caveolin-1 (53). We found that knocking down caveolin-1 (“Cav-1”) increased basal TRPV5 current density and prevented OAG-mediated increase of currents (Fig. 3B). Expression of Cav-1 in HEK cells and knockdown of Cav-1 are evident by Western blot analysis of endogenous Cav-1 (Fig. 3B). In contrast, knocking down CHC using siRNA had no effects on basal TRPV5 current, nor did it affect OAG-mediated increase of currents (Fig. 3B). We previously showed that the siRNA for CHC markedly decreases the abundance of endogenous protein in HEK cells (20, 28). As a positive control, knockdown of CHC blocked the endocytosis of ROMK1, which is mediated by CCV (results not shown and also Refs. 20, 28).

Cells lacking caveolin-1 (derived from Cav-1 gene knockout mice; Cav-1\(^{-/-}\) cells) were used for further examination of the role of caveolin-1 in the endocytosis of TRPV5. OAG increased TRPV5 current density expressed in wild-type mouse...
fibroblasts but not in similarly prepared Cav-1/−/− cells (Fig. 3C). Furthermore, OAG-mediated TRPV5 activation was rescued by forced expression of recombinant caveolin-1 rescued OAG-mediated TRPV5 activation in Cav-1/−/− cells. Empty vector ("Vector") was used for transfection control (n = 5 for each). Western blot shows that Cav-1 expression in wild-type but not Cav-1/−/− fibroblasts and expression of recombinant Cav-1 in Cav-1/−/− cells. E: effect of tyrosine kinases on TRPV5 current density. Concentrations used were vehicle (DMSO, 1:10,000 dilution), OAG (10 μM), genistein (50 μM), PP1 [4-amino-5-[4-chlorophenyl]-7-[r-butyl] pyrazolo[3,4-d]pyrimidine 1; 10 μM], PP2 [4-amino-5-[4-chlorophenyl]-7-[r-butyl] pyrazolo[3,4-d]pyrimidine 2; 10 μM]. Incubation time was 1 h. A–E: data were presented as means ± SE, n = 5 for each; *P < 0.05, **P < 0.01 vs. vehicle.

Phosphorylation of TRPV5 is critical for increase of TRPV5 by PKC. The intracellular domain of TRPV5 contains six potential PKC phosphorylation sites (17). Among these, serine-299 and serine-654 are critical for TRPV5 activation by PKC (17). We examined whether these two serine residues of TRPV5 are important for regulation of caveolar endocytosis of TRPV5 by PKC. As shown in Fig. 4A, mutation of serine-299 and/or serine-654 to alanine completely abolished activation of TRPV5 by OAG. The effect of single mutation (S299A or S654A) was the same as double mutation (S299A/S654A), indicating that both serine residues are essential for the increase of TRPV5 currents by OAG. In contrast to OAG,
genistein increased current density on S299A/S654A double mutant of TRPV5 (Fig. 4B). Thus, mutations of the serine residues prevent the regulation by PKC but do not affect the process of endocytosis of TRPV5 itself.

Hydrolysis of PLC by G protein-coupled receptors produces DAG to activate PKC. We examined the physiological role of PKC in the regulation of TRPV5 by PLC-activating hormone. Cells were cotransfected with TRPV5 and M3R. M3R is coupled to G<sub>q</sub> for activation of PLC and PKC (54). Stimulation of the receptor by carbachol (CCh) increased TRPV5 current density (Fig. 4C). Preincubation with the PKC inhibitor, GFX, completely abolished CCh-mediated TRPV5 activation (Fig. 4C). Furthermore, CCh did not increase current density in S299A/S654A mutant channels (Fig. 4D).

**DISCUSSION**

There are multiple pathways for endocytosis (7). CCVs and caveola are common mechanisms for internalization of resident cell surface proteins (7). Caveola are small flask-shaped invaginations (~60 nm in diameter) of the plasma membrane present in many different cell types, including renal epithelial...
TRPV5 in Cav-1 stimulation of TRPV5 by PTH (each).

presumably due to desensitization of receptors (not shown) (Ref. 45) (n before recording. The effect of PTH diminished with longer incubation, inhibition of endocytosis from cell membrane allows channel

endocytosis via caveolae and insertion from de novo synthesis. We show that activation of PKC causes inhibition of caveolae-mediated endocytosis of TRPV5. Inhibition of endocytosis from cell membrane allows channel

cells (9, 34, 35). The plasma membranes that form caveolae are rich in cholesterol and sphingolipids. Caveolins are structural components of caveolae important for stabilization of the specialized membrane domains (36). There are three caveolins, caveolin-1, -2, and -3, each encoded by a separate gene (6, 36). Caveolin-1 and -2 have a relatively ubiquitous distribution pattern, being coexpressed in most differentiated cell types. However, only caveolin-1 is necessary and sufficient for the formation of caveolae. Expression of caveolin-2 alone is not sufficient to drive caveolae formation in cells lacking caveolin-1. Caveolin-3 is muscle specific. Caveolae were initially thought to be relatively immobile structures. Later studies, however, revealed that caveolae travel to deep intracellular locations and exchange contents with other endocytic pathways including recycling compartments (33). It is now well-established that caveolae, like CCVs, are true endocytic vesicles that play an important role in the regulation of density of cell surface proteins (6, 36, 37).

In the present study, we show that TRPV5 undergoes constitutive endocytosis via caveolae. The steady-state abundance of TRPV5 channels at the cell surface is a balance between endocytosis via caveolae and insertion from de novo synthesized proteins (and from recycling of internalized proteins if recycling occurs). We show that activation of PKC causes inhibition of caveolae-mediated endocytosis of TRPV5. Inhibition of endocytosis from cell membrane allows channel

proteins to accumulate, leading to a new steady state with an increased abundance of TRPV5 at the cell surface. Our results also provide some information on the rate of constitutive endocytosis of TRPV5 via caveolae in the HEK cell expression system. TRPV5 current density is increased by about twofold from the basal level by OAG within ~30 min (Fig. 1C). Assuming the inhibition caused by OAG is complete and occurs immediately, the results suggest that the entire cell surface abundance of TRPV5 undergoes one round of endocytosis in ~30 min. For comparison, cell surface half-life of the epithelial Na⁺ channel ENaC is ~30 min (30). Whether recycling of TRPV5 occurs and, if so, the kinetics and extent of internalized TRPV5 are recycled back to cell surface is not known.

PTH is a principal calcitropic hormone critical for maintenance of calcium homeostasis (14, 22). It is secreted from parathyroid glands in response to a decrease in serum Ca²⁺ levels. PTH raises serum Ca²⁺ by releasing Ca²⁺ from bone, and by increasing intestinal Ca²⁺ absorption and renal Ca²⁺ reabsorption. With respect to the mechanism by which PTH stimulates renal Ca²⁺ reabsorption, PTH increases the expression of renal transcellular Ca²⁺ transport proteins, including TRPV5, calcibindin-D₂K, and the Na⁺/Ca²⁺ exchanger NCX1 (48). These changes of protein expression may contribute to the long-term regulation of Ca²⁺ transport by PTH. PTH also increases renal Ca²⁺ reabsorption acutely (23). Incubation with PTH for 15–30 min increases Ca²⁺ transport in isolated distal renal tubules and in cultured tubular cells, suggesting that the acute effect of PTH is also, at least partly, through transcellular Ca²⁺ transport (2, 3, 12, 23). How PTH regulates the transcellular Ca²⁺ transport acutely and the signaling pathway(s) involved remains unclear. Both activation of PKA and PKC have been proposed for mediating the acute stimulation of renal Ca²⁺ transport by PTH (2, 3, 23, 27). In the present study, we show that, in nonpolarized cultured cells expressing recombinant TRPV5 and PTHR, stimulation by PTH causes inhibition of caveolar endocytosis of TRPV5 via PKC. One limitation of our study is that it is in cultured HEK cells and nonpolarized MDCK cells. Gikka et al. (17) recently reported that TRPV5 undergoes constitutive endocytosis in HEK cells and activation of PKC inhibits endocytosis of TRPV5 by phosphorylation on serine-299 and serine-654 of the channel. Gikka et al. further reported that this regulation by PKC contributes to the stimulation of TRPV5-mediated renal Ca²⁺ reabsorption in vivo by serine protease tissue kallikrein (17). However, the mechanism of endocytosis of TRPV5 and its relevant regulation by PKC are not known. Caveolin-1 is present in the apical membrane of renal epithelial cells (9, 37) where TRPV5 is localized. Our findings that mutations of the same serine-299 and serine-654 residues prevent the inhibition of caveolae-mediated endocytosis of TRPV5 by PKC suggest that the mechanism is relevant to the regulation of TRPV5 by PKC in vivo. Another important finding of our study is that phosphorylation of TRPV5 at serine-299 and serine-654 is not necessary for constitutive endocytosis of the channel. Rather, it is important for the inhibition of caveolar endocytosis of TRPV5 by PKC (Fig. 4). Finally, it should be mentioned that the possibility that other regulatory mechanisms [such as activation by PKA (3) and membrane hyperpolarization by PKC (16)] also contribute to calcium transport in the native distal tubules remains.
During preparation of our manuscript, van de Graaf et al. (49) reported that TRPV5 undergoes constitutive dynamin- and clathrin-dependent endocytosis in HEK and Hela cells. Similar to the report by van de Graaf et al., we found that TRPV5 endocytosis is dynamin dependent. However, in contrast to their findings, we found that knockdown of CHC by siRNA had no effect on endocytosis of TRPV5 and on regulation of TRPV5 by OAG (Fig. 3B) and by PTH (not shown). The effectiveness of knockdown of CHC by siRNA is documented by findings that the siRNA decreases expression of endogenous CHC and prevents clathrin-mediated endocytosis of ROMK (20, 28; results not shown in the present study). Another difference between ours and van de Graaf et al.’s report is the kinetics of endocytosis (∼15–30 min in ours vs. ∼8 h in theirs). Reasons for differences between ours and van de Graaf et al.’s findings are not known at the present time. It is conceivable that TRPV5 undergoes internalization via both caveolae and CCV depending on cell types and abundance of internalization machinery in cell types and cell culture conditions. It should be mentioned that van de Graaf et al. studied an internalization machinery in cell types and cell culture conditions and an EIA award from the American Heart Association (0440019N). C. L. Huang holds the Jacob Lemann Professorship in Calcium Transport, University of Texas Southwestern Medical Center.

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GRANTS

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