WNK4 Kinase Stimulates Caveola-mediated Endocytosis of TRPV5 Amplifying the Dynamic Range of Regulation of the Channel by Protein Kinase C*

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WNK (with-no-lysine [K] kinase-4) is present in the distal nephron of the kidney and plays an important role in the regulation of renal ion transport. The epithelial Ca²⁺ channel TRPV5 (transient receptor potential vanilloid 5) is the gatekeeper of transecellular Ca²⁺ reabsorption in the distal nephron. Previously, we reported that activation of protein kinase C (PKC) increases cell-surface abundance of TRPV5 by inhibiting caveola-mediated endocytosis of the channel. Here, we report that WNK4 decreases cell-surface abundance of TRPV5 by enhancing its endocytosis. Deletion analysis revealed that stimulation of endocytosis of TRPV5 involves amino acids outside the kinase domain of WNK4. We also investigated interplay between WNK4 and PKC regulation of TRPV5. The maximal level of TRPV5 current density stimulated by the PKC activator 1-octoyl-acetyl-sn-glycerol (OAG) is the same with or without WNK4. The relative increase of TRPV5 current stimulated by OAG, however, is greater in the presence of WNK4 compared with that without WNK4 (Δ215% increase versus 60% increase above the level without OAG). Moreover, the rate of increase of TRPV5 by OAG is faster with WNK4 than without WNK4. The enhanced increase of TRPV5 in the presence of WNK4 is also observed when PKC is activated by parathyroid hormones. Thus, WNK4 exerts tonic inhibition of TRPV5 by stimulating caveola-mediated endocytosis. The lower basal TRPV5 level in the presence of WNK4 allows amplification of the stimulation of channel by PKC. This interaction between WNK4 and PKC regulation of TRPV5 may be important for physiological regulation of transecellular Ca²⁺ reabsorption by parathyroid hormones or the tissue kallikrein in vivo.

WNK (With-no-lysine [K]) kinases are large serine-threonine protein kinases characterized by an atypical placement of the catalytic lysine (1). Mammalian WNK kinases consist of four members, named WNK1–4 (1–3). Each WNK kinase is encoded by a separate gene. WNK1–4 are between 1,200- and 2,100-amino acids long. Each contains a ~270-amino acid conserved kinase domain located near the amino terminus (1). In addition, they share an autoinhibitory domain, one to two coiled-coil domains, and multiple PXXP proline-rich motifs for potential protein-protein interaction (1–3). The remaining amino acid sequences of WNK1–4 are not conserved.

Mutations in WNK1 and WNK4 cause the autosomal-dominant disease pseudohypoaldosteronism type 2 (PHA2) (3). PHA2-causing mutations in WNK1 are large deletions within the first intron resulting in an increase in the abundance of transcript. Mutations in WNK4 are nonsense mutations in the coding sequence leading to altered protein function. PHA2 is characterized by hypertension and hyperkalemia (3, 4). Interestingly, patients with PHA2 caused by WNK4 mutations, but not patients with WNK1 mutations, also have abnormally high urinary calcium excretion (hypercalcuria) (5, 6).

WNK kinases are broadly distributed. WNK1 has multiple alternatively spliced isoforms, including an ubiquitous long isoform and a kidney-specific isoform lacking the kinase domain and predominantly expressed in distal tubules of the kidney (7, 8). WNK2 is predominantly expressed in heart, brain, and colon (7); WNK3 and WNK4 are mostly expressed in kidney, heart, and brain (7, 9, 10). In addition, WNK4 is expressed in many other epithelial tissues (10). The distribution of WNK1 and WNK4 in the renal tubules, and that mutations in them cause hypertension and hyperkalemia, suggest that they play important roles in regulating renal ion transport. Indeed, WNK1 and WNK4 have been shown to regulate renal ion transport proteins, including paracellular tight junction proteins claudins; cation-chloride co-transporters NCC, KCI cotransporters, and Na⁺-K⁺-2Cl⁻ cotransporters; epithelial Na⁺ channel; and renal K⁺ channel ROMK (11, 12).

TRPV5 (transient receptor potential vanilloid 5) channel is localized to the apical membrane of distal convoluted tubules and connecting tubules and functions as a gatekeeper for transeellular Ca²⁺ reabsorption in the kidney. Many hormones, including parathyroid hormone (PTH), regulate renal Ca²⁺ reabsorption at least partly via TRPV5 (13, 14). One mechanism for PTH regulation of TRPV5 is by increasing the protein expression of TRPV5, providing a mechanism for long term regulation of renal Ca²⁺ reabsorption (15). In addition, PTH...
can enhance renal Ca\(^{2+}\) reabsorption within 10–15 min of its administration. The mechanism of acute regulation of TRPV5

by PTH involves activation of protein kinase A and PKC (16, 17). Recently, we reported that TRPV5 undergoes constitutive caveolin-dependent endocytosis and that activation of PKC inhibits this process causing accumulation of the channel at the cell surface (18). This inhibition of caveolar endocytosis of TRPV5 via PKC may be one of the mechanisms for acute regulation of the channel by PTH. In the present study, we report that WNK4 stimulates caveola-mediated endocytosis of TRPV5 and thereby increases the range of regulation of the channel by PKC. This interaction between WNK4 and PKC regulation of TRPV5 may be important for physiological regulation of renal Ca\(^{2+}\) reabsorption by PTH or tissue kallikrein in vivo.

**EXPERIMENTAL PROCEDURES**

*DNA Constructs—*GFP-tagged TRPV5 has been described previously (18). cDNA for type 1 parathyroid hormone receptor (PTH1R) and caveolin-1 are in pcDNA3 vector. WNK4 point mutation and deletion constructs have been described (19). Sense and antisense oligonucleotides for clathrin heavy chain, caveolin-1, and intersectin have been described (18, 19).

*Cell Culture and Transfection—*HEK293 cells were cultured as described (18, 19). Wild type and caveolin-1-null (Cav-1\(^{-/-}\)) fibroblasts (gift of Richard Anderson, University of Texas Southwestern Medical Center at Dallas) were cultured in low glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, glutamine, penicillin, and streptomycin. Cells were co-transfected with cDNA (0.1 \(\mu\)g/6 wells) for GFP-TRPV5 plus cDNAs for WNK4, intersectin, type 1 PTH receptor (PTH1R), caveolin-1, and/or wild type or dominant-negative (lysine-44 to alanine) rat dynamin 2 as indicated in each experiment. In each experiment, the total amount of DNA for transfection was balanced by using empty vectors. Transfected cells were identified 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 small interference RNA (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 (18, 19). For ruptured whole-cell recordings, the pipette and bath solution contained 140 mM Na-Asp (sodium aspartate), 10 mM NaCl, 10 mM EDTA, and 10 mM HEPES (pH 7.4) and 140 mM Na-Asp, 10 mM NaCl, 1 mM EDTA and 10 mM HEPES (pH 7.4), respectively. The resistance of electrodes containing the pipette solution was 1.5–3 mehm. An Ag/Ag pellet connected via a 3 M KCl/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 in duration) from −150 mV to 100 mV in +25 mV increments. Data acquisition was performed using ClampX 9.2 software (Axon Instruments). Currents were low pass filtered at 2 kHz using an 8-pole Bessel filter in the clamp amplifier, sampled every 0.1 ms (10 kHz) with Digidata-1300 interface, and stored directly to a computer hard disk. For perforated whole-cell patch clamp recordings, a stock solution of gramicidin was prepared at 50 mg/ml (in dimethyl sulfoxide) and diluted (50 \(\mu\)g/ml final concentration) in a pipette solution containing 145 mM KCl, 10 mM HEPES, and 2 mM EDTA (pH 7.2) before recording.

**Surface Biotinylation Assay—**For biotinylation of cell-surface TRPV5, cells were washed with ice-cold phosphate-buffered saline and incubated with 0.75 ml phosphate-buffered saline containing 1.5 mg/ml EZ-link NHS-SS-biotin for 1 h at 4 °C. After quenching with glycine (100 mM), cell were lysed in a RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, and 0.1% SDS) containing protease inhibitor mixture. Biotinylated proteins were precipitated by streptavidin-agarose beads. Beads were subsequently washed four times with phosphate-buffered saline 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 five times with similar results.

**Data Analysis—**Data analysis was performed with the Prism (version 3.0) software (GraphPad Software, San Diego, CA). Statistical comparisons between two groups of data were made using two-tailed unpaired Student’s *t* test. Multiple comparisons were determined using one-way analysis of variance 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 mean ± S.E.

**RESULTS**

WNK4 Decreases Cell-surface Abundance of TRPV5 Channel—
We examined the effect of WNK4 on the TRPV5 channel using ruptured whole-cell patch clamp recording. Human embryonic kidney (HEK) cells were transfected with TRPV5 with or without WNK4. Whole-cell currents were measured under voltage clamp in voltage steps from −150 to +100 mV. Current density (nA/pF; current (nA) normalized to cell capacitance (pF), indicative of cell size) is shown. As shown in current-voltage (*I–V*) relationship curves (Fig. 1A), cells expressing TRPV5 exhibited characteristic inward-rectifying currents, and coexpression with full-length WNK4 (FL-WNK4) reduced current density by −65% (versus cells coexpressing with empty vector). Background current density in mock-transfected HEK cells is negligible (<100 pA/pF; data not shown). Fig. 1B summarizes the average results of TRPV5 current density measured at −100 mV cotransfected with empty vector or with FL-WNK4. Because WNK kinases regulate several ion transporters by altering their membrane trafficking, we examined the cell-surface expression of TRPV5. Fig. 1C shows validation of cell-surface biotinylation assays; cell-surface abundance of TRPV5 (*TRPV5 Surface*) was detected only in cells transfected with TRPV5 and treated with biotin. Surface biotinylation assays revealed that coexpression with WNK4 (compared with empty
vector) decreased cell-surface abundance of TRPV5 (Fig. 1D, TRPV5-Surface). TRPV5 protein abundance in the lysates (Fig. 1D, TRPV5-Lysates) was not different between WNK4- and vector-transfected cells. Fig. 1E summarizes averaged surface versus lysate TRPV5 abundance (quantified by densitometry) from five different experiments. We have found that HEK cells express endogenous WNK4, which contributes to the regulation of transfected renal outer medullary K\textsuperscript{+}/H\textsuperscript{+} (ROMK) channels (20). Here, we found that knockdown of endogenous WNK4 using siRNA increased TRPV5 current density (Fig. 1F), indicating that endogenous WNK4 contributes to inhibition of TRPV5 channel.

Amino Acids 444–584 of WNK4 Are Important For Regulation of TRPV5—WNK4 is a 1,223-amino acids protein that contains a kinase domain near the N terminus (Fig. 2A). Many PHA2-causing missense mutations of WNK4 occur at a hot spot consisting of amino acids glutamate-alanine-aspartate-glutamine (Fig. 2A, underlined EADQ), frequently referred to as the “acidic motif.” The regulation of the ion channel ROMK by WNK4 does not require kinase activity but rather involves a protein-protein interaction with WNK4 mediated by amino acids outside the kinase domain (19). We have further shown that the acidic motif and the preceding proline-rich region of WNK4 are involved in this regulation of ROMK (19). Here, we examined the region of WNK4 involved in the regulation of TRPV5. TRPV5 was coexpressed with FL-WNK4, a fragment of WNK4 containing amino acids 1–584 (WNK4-(1–584)) or amino acids 1–444 (WNK4-(1–444)). We found that WNK4-(1–584) decreased TRPV5 current density as well as the FL-WNK4, whereas WNK4-(1–444) had no effect on TRPV5 despite similar expression levels (Fig. 2B). Mutations of WNK4 that cause PHA2 disease alter its regulation of ion transport...
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WNK4 Enhances Caveola-mediated Endocytosis of TRPV5—WNK kinases alter cell-surface abundance of ROMK, epithelial Na⁺ channels, and epithelial Na⁺ channels (11, 12). We tested a disease-causing glutamate 559-to-lysine mutation (E559K) and found that this mutation enhanced the ability of WNK4-(1–584) to inhibit the TRPV5 current (Fig. 2C). The level of expression was not different between wild type (WT) and E559K WNK4-(1–584). The effects of WT and E559K WNK4-(1–584) to decrease cell-surface abundance of TRPV5 were confirmed by biotinylation assays (Fig. 2D). WNK4 boosts the role of dynamin-dependent endocytosis of TRPV5 by WNK4-(1–584) mediating endocytosis (18). We examined the role of dynamin-dependent endocytosis in the regulation of TRPV5 by WNK4. Coexpression of a dominant-negative dynamin increased basal TRPV5 current and prevented the WNK4-(1–584)-mediated decrease of TRPV5 current density (Fig. 3A). For comparison, WNK4-(1–584) decreased current density in cells coexpressing wild type dynamin 2 similarly as in Cav-1−/− (Fig. 4A). Forced expression of recombinant caveolin-1, but not of empty vector, in Cav-1−/− restored the regulation of TRPV5 by WNK4 (Fig. 4B). The complete loss of WNK4 on TRPV5 in Cav-1−/− cells, with knockdown of endogenous caveolin-1 or cells coexpressed with dominant-negative dynamin also suggest that WNK4 does not directly affect protein synthesis and/or exocytosis of the TRPV5 channel.

Intersectin Is Important for WNK4 Stimulation of Endocytosis of TRPV5—Intersectin is a multimodular endocytic scaffold protein that contains five Src-homology 3 (SH3) domains (22). Intersectin, through its SH3 domains, interacts with the proline-rich domain of dynamin to recruit it to the active endocytosis site (23, 24). Mutation of the intersectin ortholog in fruit flies results in loss of dynamin in the synaptic endocytosis active zone and defective synaptic transmission (25). We have recently reported that WNK1 and -4 interact with intersectin and that this interaction is critical for WNK1 and -4 stimulation of clathrin-coated vesicle-mediated endocytosis of ROMK (19). Intersectin is also present in caveolae and is believed to play an important role in fission of caveolar vesicles via dynamin similar to that for clathrin-coated vesicles (26, 27). We thus tested proteins, including claudin, ROMK, NCC, and epithelial Na⁺ channels (11, 12). We tested a disease-causing glutamate 559-to-lysine mutation (E559K) and found that this mutation enhanced the ability of WNK4-(1–584) to inhibit the TRPV5 current (Fig. 2C). The level of expression was not different between wild type (WT) and E559K WNK4-(1–584). The effects of WT and E559K WNK4-(1–584) to decrease cell-surface abundance of TRPV5 were confirmed by biotinylation assays (Fig. 2D).
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![Graph A](image1.png)

**FIGURE 3. Role of endocytosis in WNK4 regulation of TRPV5.** A, role of dynamin 2 in WNK4-(1–584) regulation of TRPV5. Cells were co-transfected with TRPV5 and with empty vector, wild type (WT-D2), or dominant-negative dynamin 2 (DN-D2). B, dominant-negative dynamin 2, but not WT dynamin 2, prevents a WNK4-induced decrease in TRPV5 cell-surface abundance. Similar results were observed in two separate experiments. C, effect of knockdown endogenous caveolin-1 by small interference RNA (siRNA Cav-1) compared with control oligonucleotides (Control Oligo). Successful knockdown of Cav-1 was evident by Western blot analysis. In each panel, an asterisk denotes p < 0.01 without versus with WNK4-(1–584) by unpaired two-tailed Student’s t test. The double asterisk denotes p < 0.01 between indicated groups by unpaired two-tailed Student’s t test. NS denotes statistically not significant.

![Graph B](image2.png)

**FIGURE 4. Effect of WNK4 on TRPV5 in caveolin-1-null cells.** A, TRPV5 was coexpressed with WNK4-(1–584) or empty vector in wild type cells (Cav-1+/+); or cells lacking caveolin-1 (Cav-1−/−). B, TRPV5 was coexpressed with WNK4-(1–584) or empty vector in cells lacking caveolin-1 (Cav-1−/−) and coexpressed without (no Caveolin-1) or with caveolin-1 (Caveolin-1). In each panel, an asterisk denotes p < 0.01 without versus with WNK4-(1–584) by unpaired two-tailed Student’s t test.

whether intersectin is involved in the regulation of TRPV5 by WNK4. Knockdown of endogenous intersectin using siRNA prevented WNK4 regulation of TRPV5 (Fig. 5, A and B). Suc-

cessful knockdown of intersectin by the siRNA has been demonstrated previously (data not shown) (19). Control oligonucleotides had no effect on WNK4 regulation of TRPV5. WNK4 interacts with intersectin through its SH3 domains (19). A fragment of intersectin containing only SH3 domains functions as a dominant-negative for WNK4 regulation of endocytosis of ROMK (19). We found that coexpression of the dominant-negative intersectin (Fig. 5, DN-ITSN), but not of the full-length intersectin (Fig. 5, WT-ITSN), prevented WNK4 regulation of TRPV5 (Fig. 5, C and D).

Interplay between WNK4 and PKC Regulation of TRPV5—Activation of PKC increases cell-surface abundance of TRPV5 by inhibiting caveola-mediated endocytosis of the channel (18), suggesting a potentially interesting interaction between WNK4 and PKC regulation of TRPV5. We examined the effect of PKC on TRPV5 with or without coexpression with WNK4-(1–584). Without WNK4-(1–584) (Fig. 6, Vector), activation of PKC by a membrane-permeable diacylglycerol analog (1-oleoyl-acetyl-sn-glycerol, OAG) increased TRPV5 current density as expected (Fig. 6, A and B). The increase in TRPV5 by OAG was prevented by preincubation with a PKC inhibitor (18) (data not shown), confirming that the effect of OAG was through activation of PKC. In the presence of WNK4, the basal TRPV5 current was lower than that without WNK4 (Fig. 6, Vector). OAG increased TRPV5 current in the presence of WNK4 to the same maximal level as seen without WNK4 (Fig. 6, A and B). Thus, the relative increase of TRPV5 current density by OAG (versus before OAG) is markedly amplified by coexpression with WNK4 (Fig. 6C). We have shown that activation of PKC by PTH via the PTH1R also increases TRPV5 by inhibiting caveola-mediated endocytosis (18). Consistent with this idea, we found that PTH stimulation of the PTH1R receptor increased TRPV5 current to the same maximal level with or without WNK4 (Fig. 6, D and E) and that the relative increase of current stimulated by PTH is amplified in the presence of WNK4 (Fig. 6F). The effect of PTH was completely prevented by a PKC inhibitor (see also Ref. 18) (data not shown), confirming its effect through activation of PKC.

The above experiments were performed by incubating cells with OAG for 30 min before ruptured whole-cell recording. These experiments aimed to detect the maximal effect of OAG.
We further examined the OAG-stimulated increase of TRPV5 current in a continuous real-time manner using perforated whole-cell patch clamp recording. Unlike that in the ruptured whole-cell mode, the intracellular milieu and the machinery for endocytosis is preserved in the perforated whole-cell recording. Currents elicited by voltage ramp from −100 to +100 mV over 400 ms were recorded at 10-s intervals from cells expressing TRPV5 with or without WNK4 (Fig. 7A). As shown in Fig. 7B, addition of OAG caused an increase in TRPV5 current in ∼30 s and continued over the period of recording (10 min). The relative increase of TRPV5 currents stimulated by OAG over 10 min is much greater in cells coexpressing WNK4 than with empty vector (Fig. 7B). Without addition of OAG, TRPV5 current remained stable over 10 min (Fig. 7B, Time control). Fig. 7C shows the absolute TRPV5 current amplitude before and 10 min after OAG. As shown, the rate of increase and the dynamic range of regulation by OAG are enhanced in the presence of WNK4.

DISCUSSION

The TRPV5 channel forms the luminal gate of transcellular Ca\(^{2+}\) reabsorption in the distal nephron of the kidney and is a target of regulation of renal Ca\(^{2+}\) transport by pH and hormones (13, 15). The abundance of proteins at the cell surface is a balance between retrieval and insertion of the newly synthesized and recycled proteins. Recently, we reported that TRPV5 undergoes constitutive caveola-mediated endocytosis, and activation of PKC inhibits the endocytosis leading to accumulation of TRPV5 at the cell surface (18). In the present study, we report that WNK4 kinase enhances the endocytosis of TRPV5. As would be expected, an increase in endocytosis decreases the density of cargos at the cell surface, the number of channels internalized per unit time in the steady state should be the same irrespective of the rate of endocytosis. If so, one would expect that the rate of increase caused by decreasing endocytosis be the same with or without WNK4. One possible explanation for our finding may be related to the fact that there are three types of caveolae with different membrane dynamic activities (28). Type 1 caveolae are able to pinch off the plasma membrane forming endocytic vesicles and also travel to the deeper intracellular organelles, such as late endosomes. Type 2 caveolae are more superficially localized and involved in continuous rounds of fission and fusion without traveling deep below the cell surface. Type 3 is relatively uncharacterized. It is conceivable that WNK4 may alter the distribution of TRPV5 in caveolae and/or affect the dynamics of three caveolae differentially. Redistribution to a faster turnover caveola compartment (such as type 2) may allow TRPV5 to reappear at the cell surface faster when endocytosis is inhibited.

WNK4 kinase is abundantly expressed in the distal nephron including the distal convoluted tubule and the cortical connecting tubule, where transcellular Ca\(^{2+}\) reabsorption mediated by TRPV5 occurs (13). Tissue kallikrein is a serine protease produced in the cortical connecting tubule (29). Tissue kallikrein activates bradykinin-2 receptor either directly or indirectly by releasing kinin from kininogen (30, 31). A recent study reports that activation of PKC underlies the stimulation of TRPV5 and renal Ca\(^{2+}\) transport by tissue kallikrein (32). Activation of PKC is also implicated as a molecular mechanism for stimulation of TRPV5 and renal Ca\(^{2+}\) transport by PTH (18). Thus, ourfind-
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FIGURE 6. Interaction between WNK4 and PKC regulation of TRPV5. A and B, TRPV5 was coexpressed with or without WNK4-(1–584) and incubated with or without OAG (10 μM) for 30 min before ruptured whole-cell recording. A shows I-V curves. B shows mean (± S.E.) inward current density at −100 mV of results from A, C, results from panel B are shown as relative current to without OAG. D and E, TRPV5 was coexpressed with PTH1R and with or without WNK4-(1–584) and incubated with or without PTH-(1–34) (50 nM) for 15 min before ruptured whole-cell recording. PTH-(1–34) is an N-terminal fragment of PTH consisting of the first 34 amino acids, which possesses the complete biological activity of the full-length PTH (44). D shows I-V curves. E shows mean (± S.E.) inward current density at −100 mV of results from D, F, results from panel E are shown as relative current to without PTH. In each panel, an asterisk denotes p < 0.01 without versus with OAG (or PTH) by unpaired two-tailed Student’s t test. The double asterisk denotes p < 0.01 between indicated groups by unpaired two-tailed Student’s t test. NS denotes statistically not significant.

FIGURE 7. Interaction between WNK4 and PKC regulation of TRPV5 in perforated whole-cell recording. A, voltage ramp protocol (evoked every 10 s) and configuration for perforated whole-cell recording. Representative ramp current tracings before OAG and 5 min after application of OAG are shown. B, normalized current amplitude (I/I0) (current amplitude normalized to current amplitude at time 0) of currents at 0 and 10 min after OAG in cells coexpressed without (vector) or with WNK4-(1–584) (WNK4). In time control, no OAG was added. Data points (circles and lines) are mean ± S.E. (n = 6 each) of currents at −100 mV. C, absolute current amplitude at time 0 and 10 min after OAG in cells coexpressed without (vector) or with WNK4-(1–584) (WNK4).

WNK4 interacts with SH3 domains of intersectin through a set of multiple proline-rich motifs residing between amino acids 545 and 558 (19). Intersectin, in turn, recruits dynamin and other endocytic accessory proteins to participate in the formation of clathrin-coated vesicles and the separation of vesicles from the plasma membrane. Fission of caveolar vesicles also requires dynamin (36). The role of intersectin in the fission of caveolar vesicles and its importance in caveola-mediated endocytosis has been demonstrated (26, 27). Our present study shows that intersectin is necessary for WNK4 stimulation of endocytosis of TRPV5 via caveolae. Moreover, the region of WNK4 involved in the endocytosis of TRPV5 is the same as that in the endocytosis of ROMK. Thus, WNK4 regulates both clathrin-mediated and caveola-mediated endocytosis by the same mechanism involving an interaction with intersectin. Our present results are in contrast to those by Jiang et al. (37, 38) reporting that WNK4 increases surface expression of TRPV5 and that it occurs by enhancing the secretory pathway. The precise reason(s) for these differences is unknown but may be related to differences in the experimental system employed (mammalian cells in our study versus Xenopus oocytes in their study (37, 38).

Patients with PHA2 with WNK4 mutations develop hypercalciuria (5). Studies using an expression system and in animals and humans, have provided compelling evidence that mutations of WNK4 that cause PHA2 increase the activity of NCC, causing Na+ retention and hypertension (39–42). Inhibition of NCC by thiazide diuretics or loss-of-function mutations of the
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NCC gene causes hypocalciuria (43). Thus, hypercalciuria in patients with WNK4 mutations is probably, at least in part, due to the increase in the activity of NCC. In support of this idea, inhibition of NCC by thiazide diuretics ameliorates hypercalciuria in patients or animal models of PHA2 with WNK4 mutations (5, 41, 42). However, patents with PHA2 typically develop hypercalciuria before the onset of hypertension (5). Moreover, transgenic mice overexpressing a bacterial artificial chromosome carrying the mouse NCC gene do not show hypercalciuria despite Na$^{+}$ retention and hypertension,3 suggesting that other factors may also be involved. We and others (19, 34) have shown that disease-causing mutant WNK4s exert a greater inhibition of ROMK than wild type WNK4. These results raise the possibility that WNK4 regulation of TRPV5 may also be contributory to hypercalciuria in PHA2. Further studies of TRPV5 expression in PHA2 patients and/or animal models are required.

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