WNK kinases influence TRPV4 channel function and localization

Yi Fu, Arohan Subramanya, David Rozansky, and David M. Cohen

1Division of Nephrology and Hypertension, Department of Medicine, 2Division of Nephrology, Department of Pediatrics, Oregon Health and Science University, and 3Portland Veterans Affairs Medical Center, Portland, Oregon

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The WNK kinases influence TRPV4 channel function and localization. The transient receptor potential (TRP) family, is gated by hypotonicity. Kinases of the WNK family influence expression and function of the thiazide-sensitive Na+/Cl− cotransporter, and monogenic human hypertension has been linked to mutations in the gene coding for WNK4. Along with TRPV4, WNK isoforms are highly expressed in the distal nephron. We show here that coexpression of WNK4 downregulates TRPV4 function in human embryonic kidney (HEK-293) cells and that this effect is mediated via decreased cell surface expression of TRPV4; total abundance of TRPV4 in whole cell lysates is unaffected. The effect of the related kinase WNK1 on TRPV4 function and surface expression was similar to that of WNK4. Disease-causing point mutations in WNK4 abrogate, but do not eliminate, the inhibitory effect on TRPV4 function. In contrast to wild-type WNK4, a kinase-dead WNK4 point mutant failed to influence TRPV4 trafficking; however, deletion of the entire WNK4 kinase domain did not blunt the effect of WNK4 on localization of TRPV4. Deletion of the extreme COOH-terminal putative coiled-coil domain of WNK4 abolished its effect. In immunoprecipitation experiments, we were unable to detect direct interaction between TRPV4 and either WNK kinase. In aggregate, these data indicate that TRPV4 is functionally regulated by WNK family kinases at the level of cell surface expression. Because TRPV4 and WNK kinases are coexpressed in the distal nephron in vivo and because there is a tendency toward hypercalcemia in TRPV4−/− mice, we speculate that this pathway may impact systemic Ca2+ balance. In addition, because WNK kinases and TRPV4 are activated by anisotonicity, they may comprise elements of an osmosensing or osmotically responsive signal transduction cascade in the distal nephron.

hypertension; calcium balance; hypotonicity; osmoregulation

THE TRANSIENT RECEPTOR POTENTIAL (TRP) CHANNELS REPRESENT A LARGE FAMILY OF CATION CHANNELS REGULATED BY DIVERSE AFFERENT INPUTS; A SUBSET OF TRP CHANNELS RESPOND TO ENVIRONMENTAL STIMULI SUCH AS TEMPERATURE AND TONICITY (6). ALTHOUGH TRPV4 [ALSO KNOWN AS VR-OAC (18), OTRPC4 (28), VRL-2 (10), AND TRP12 (37)] HAS BEEN DESCRIBED IN OTHER CONTEXTS, ITS FUNCTIONAL ROLE WAS ESTABLISHED WHEN IT WAS IDENTIFIED AS THE MAMMALIAN HOMOLOG OF THE CAENORHABDITIS ELEGANS OSMORESENSE PROTEIN OSMO9 (18, 20, 28). ON THE BASIS OF ITS EXPRESSION IN THE BLOOD-BRAIN BARRIER-DEFICIENT OSMORESENSING NUCLEI OF THE HYPOTHALAMUS (18), IN CONJUNCTION WITH THE ABNORMAL WATER METABOLISM EXHIBITED BY MICE HARBORING TARGETED DELETIONS OF THE TRPV4 GENE (19, 24), THE CHANNEL IS BELIEVED TO BE INSTRUMENTAL IN PHYSIOLOGICAL OR PATHOPHYSIOLOGICAL REGULATION OF SYSTEMIC WATER BALANCE. HOWEVER, IN ADDITION TO A ROLE IN SYSTEMIC OSMOREGRELATION, TRPV4 ALSO PLAYS A ROLE IN OSMOREGRELATION AT THE CELLULAR LEVEL. IN HUMAN AIRWAY EPITHELIAL CELLS (3) AND CHINESE HAMSTER OVARY CELLS (4), TRPV4 EXPRESSION WAS ESSENTIAL FOR THE REGULATORY VOLUME DECREASE THAT FOLLOWED HYPTONIC CELL SWELLING. TRPV4 ALSO LIKELY PLAYS A DIRECT ROLE IN KIDNEY PHYSIOLOGY. TRPV4 mRNA IS EXPRESSED MOST ABUNDANTLY IN KIDNEY, PRIMARILY IN KIDNEY CORTEX (10, 18, 28, 37). TRPV4 PROTEIN IS ABUNDANTLY EXPRESSED ALONG THE NEPHRON DISTAL TO THE JUNCTION OF THE DESCENDING AND ASCENDING THIN LIMB OF HENLE’S LOOP, WITH THE SOLE EXCEPTION OF THE CELLS OF THE MACULA DENSATA (29); THIS DISTRIBUTION IS CONSISTENT WITH A ROLE IN LOCAL OSMOSENSING (7).

THE WNK FAMILY OF KINASES IS DESCRIBED BY XU ET AL. (38) IN A SCREEN FOR NOVEL MITOGEN-ACTIVATED PROTEIN (MAP)/EXTRACELLULAR SIGNAL-REGULATED PROTEIN KINASE (ERK) KINASE (MEK) FAMILY MEMBERS IN RAT BRAIN. INTEREST IN THESE PUTATIVE KINASES INCREASED WHEN MUTATIONS IN THE WNK1 AND WNK4 GENES WERE CAUSALLY LINKED TO SEVERE HYPERTENSION (35) IN A SUBSET OF PATIENTS DIAGNOSED WITH FAMILIAL HYPERCALCEMIA AND HYPERTENSION (ALSO KNOWN AS GORDON’S SYNDROME OR PSEUDOHYPOALDOSTERONISM TYPE II). BECAUSE OF THE CLINICAL AND BIOCHEMICAL ABNORMALITIES ASSOCIATED WITH THIS DISEASE, ABERRANT REGULATION OF THE THIAZIDE-SENSITIVE Na+/Cl− COTRANSporter OF THE DISTAL CONVOLUTED TUBULE WAS SUSPECTED. YANG ET AL. (43) AND WILSON ET AL. (36) SUBSEQUENTLY SHOWED THAT WNK4 DOWNREGULATES EXPRESSION OF THE THIAZIDE-SENSITIVE Na+/Cl− COTRANSporter, AND SOME, BUT NOT ALL, DISEASE-CAUSING POINT MUTANTS DID NOT. IN CONTRAST, WNK1 EXHIBIT ED NO EFFECT WITH RESPECT TO THIAZIDE-SENSITIVE Na+/Cl− COTRANSporter FUNCTION; HOWEVER, THE KINASE BLUNTED THE INHIBITORY EFFECT OF WNK4 (43). BIOCHEMICALLY, WNK KINASES ARE ACTIVATED BY A VARIETY OF STIMULI, INCLUDING HYPERTONICITY AND HYPOTONICITY (17, 38). BECAUSE WNK KINASES AND TRPV4 ARE OSMOTICALLY RESPONSIVE AND EXPRESSED IN THE DISTAL NEPHRON (35, 41), AMONG OTHER SITES, WE SPECULATED THAT WNKs MAY PARTICIPATE IN THE REGULATION OF THIS CATION CHANNEL.

METHODS

CELL SURFACE BIOTINYLATION AND IMMUNOBLOTTING. PROTEIN EXTRACTS (20 µG) WERE USED FOR IMMUNOBLOTTING WITH POLYCLONAL RABBIT ANTI-TRPV4 (41) AT 1:1,000 DILUTION AS PREVIOUSLY DESCRIBED (41). THE SECONDARY ANTIBODY WAS GOAT ANTI-RABBIT HORSESHIRE PEROXIDASE AT 1:4,000 DILUTION, AND VISUALIZATION WAS VIA CHEMILUMINESCENCE PLUS REAGENT (PERKINELMER LIFE SCIENCE). FOR ANTI-V5 IMMUNOBLOTTING, PRIMARY ANTIBODY DILUTION OF 1:5,000 WAS USED; SECONDARY ANTIBODY WAS GOAT ANTI-MOUSE HORSESHIRE PEROXIDASE AT 1:6,000 DILUTION. FOR CELL SURFACE BIOTINYLATION 48 H AFTER TRANSIENT TRANSECTION, HUMAN EMBRYONIC KIDNEY (HEK-293) CELL MONOlayers WERE WASHED THREE TIMES WITH ICE-COLD PBS, INCUBATED WITH 0.5 MG/Ml SULFO-NHS-LC-BIOTIN.

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(Pierce Biotechnology, Rockford, IL) for 30 min at 4°C, quenched by incubation with 100 mM glycine in ice-cold PBS for 30 min at 4°C, and then washed three times with ice-cold PBS. Monolayers were lysed in lysis buffer [125 mM NaCl, 50 mM Tris (pH 7.5), 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 µg/mL leupeptin, 1 µg/mL aprotilin, 1 µg/mL pepstatin A, 25 mM β-glycerophosphate, and 2 mM sodium pyrophosphate] for 30 min at 4°C. The protein concentrations were determined by the Bradford method (Bio-Rad). ImmunoPure streptavidin beads (40 µL; Pierce Biotechnology) were added to ~3 µg of biotinylated protein, and the mixture was incubated at 4°C for 4 h. The beads were washed five times with ice-cold PBS and eluted with 1× SDS sample buffer. The eluted proteins were immunoblotted with anti-V5 antibody as described above.

**Transient transfection and fura 2 ratiometry.** HEK-293 cells were transiently transfected with Lipofectamine PLUS (Life Technologies) in accordance with the manufacturer’s directions using 15 µL of PLUS reagent, 30 µL of Lipofectamine, and 10 µg of plasmid DNA reagent per 100-mm dish of cells. After 48 h, the cells were harvested, washed, and resuspended in 10 ml of Hank’s balanced salt solution [HBSS: 130 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl2, 1.18 mM MgSO4, 5 mM glucose, and 15 mM HEPES (pH 7.5)] supplemented with 2 µM fura-2 AM and 100 µL of 2% Pluronic F-127 (20% stock solution in DMSO; Molecular Probes, Eugene, OR) per 100-mm dish and then incubated for 45 min at 37°C. The cells were pelleted at 1,000 g for 5 min at 25°C, resuspended with 1–2 ml of HBSS to achieve final concentration of ~2–8 × 10^5 cells/ml, and maintained on ice for 30 min. The suspended fura-2 loaded cells (50 µL) were assayed for intracellular Ca^{2+} concentration in a cuvette filled with prewarmed (37°C) HBSS (in the presence or absence of extracellular Ca^{2+}) under constant gentle stirring (2-ml final volume) as previously reported (41). Fluorescent emission was monitored at 510 nm and 525 nm wavelengths with an excitation at 340 and 380 nm using a fluorescence spectrophotometer (model F-2500, Hitachi Instruments, Naperville, IL). Calibration of the fura 2 signal was performed as previously described (27) using a fura-2 Ca^{2+} dissociation constant of 224 nM (11). For each experiment, data from three separate cuvettes of treated cells were averaged, and experiments were repeated at least three times. Murine WNK4 and rat WNK1 cDNAs were the kind gifts of Dr. David Ellison and Dr. Chao-Ling Yang, and Dr. Melanie Cobb, respectively. Rat WNK1 protein (NP_446246) diverges from mouse (NP_941992) and human (NP_732993–888219), this corresponds precisely to the absence of exons 792–1038 of the canonical human WNK1 sequence. After alignment with the human WNK1 gene (NC_000012; nucleotides 732993–888219), this corresponds precisely to the absence of exons 11 and 12; alternative splicing of these exons in mRNA from rat kidney has been described elsewhere (25). Nonetheless, all full-length rat WNK1 protein sequences returned via National Center for Biotechnology Information protein search (http://www.ncbi.nlm.nih.gov/) similarly lack these two exons (Q9JJH7, NP_446246, and AAPF72458). Most in vitro and cell culture studies have used this identical clone originally isolated by Xu et al. (38).

Mutated amino acid residues in murine WNK4 differ from their human counterparts on the basis of numbering of the conceptual translation products from NM_175579 for mouse and NM_032387 for human. The human disease-causing WNK4 mutants hWNK4E562K and hWNK4K565E correspond to mouse mWNK4E559K and mWNK4K562E, respectively, in the present study. Putative kinase-dead murine WNK4 is mWNK4D318A. These point mutants, as well as kinase-dead WNK1K233M and WNK1S382A, were generated via site-directed mutagenesis (QuikChange, Stratagene) in accordance with the manufacturer’s directions. The SMART online resource (http://smart.embl-heidelberg.de/) was used for determination of conserved motifs in rat WNK1 and murine WNK4. Three putative coiled-coil domains were detected in rat WNK1 using the Cotls2 program [based on the algorithm of Lupas et al. (21)] at residues 194–217, 563–597, and 1814–1841. No coiled-coil domains were detected in murine WNK4, although there was substantial sequence homology between the isoforms at the first and third putative coiled-coil domains.

**Image processing and statistical analysis.** For quantitation of autoradiograms, films were scanned (Canon LiDE80) and data were reduced using ImageJ (http://rsb.info.nih.gov/ij/; National Institutes of Health) and Excel (Microsoft). For all depicted scans of enhanced chemiluminescence exposures of immunoblots, contrast was improved by decreasing the maximum input level from 255 to ~175 (Adobe PhotoShop CS) to mimic the true appearance of the exposed film. In Fig. 3A, some intervening lanes were digitally removed to preserve consistency with Fig. 3, B and C; all lanes in Fig. 3A are from the same exposure of the same autoradiogram. Where data are shown, all experiments were performed a minimum of three times. Values are means ± SE (Excel, Microsoft), and where indicated, the number of independent experiments is shown. Where multiple comparisons were performed, statistical significance was attributed using the Student’s t-test [for correlated samples using raw data or for independent samples using normalized data (VassarStats; http://faculty.vassar.edu/lowry/VassarStats.html)], in accordance with the false discovery rate procedure, where P_i < d^*_i (where P_i is significance level associated with comparison i and d^*_i is critical significance level); this latter approach is used to test the validity, in parallel, of more than one null hypothesis.

**RESULTS**

HEK-293 cells stably transfected with the toxicity-responsive cation channel TRPV4 exhibit robust Ca^{2+} entry on exposure to a hypotonic milieu (18, 28). This effect is dependent on the presence of extracellular Ca^{2+} and is absent in untransfected or vector-transfected HEK-293 cells (41). For the present series of investigations, we adopted a transient (rather than stable) transfection strategy, which is less subject to confounding effects attributable to over- or underrepresentation of high-expressing clones. HEK-293 cells transiently transfected with TRPV4 exhibited a robust time-dependent response to hypotonicity (Fig. 1A) or the TRPV4 activator 4α-phorbol 12,13-didecanoate (4α-PDD; Fig. 1D), and this effect was abolished in the absence of extracellular Ca^{2+}. Vector-transfected (Fig. 1, B and E) and untransfected (Fig. 1, C and F) HEK-293 cells exhibited a much more modest, but reproducible, increment in intracellular Ca^{2+} in response to both activators of TRPV4, and these effects were similarly dependent on the presence of extracellular Ca^{2+}. By immunoblot analysis, we were unable to detect TRPV4 in vector-transfected or untransfected HEK-293 cells, although expression was abundant in the TRPV4 transfectants (Fig. 1G).

We used this transient transfection strategy to assess the effect of WNK kinases on TRPV4 function. Coexpression of TRPV4 and WNK4 abrogated the Ca^{2+} entry response of TRPV4 to hypotonicity and the TRPV4 activator 4α-PDD (Fig. 2, A and B). For rigorous quantitation, comparisons were made using the increment in intracellular Ca^{2+} (as assessed via fura 2 ratiometry) at a time of maximal stimulus responsiveness (i.e., at 75 s of treatment). The pooled data (Fig. 2, C and D) show that the inhibitory effect of WNK4 was substantial and highly statistically significant.

Heterologously expressed WNK1 is reportedly neutral with respect to function of known transport proteins (43). We compared the effect of WNK1 with that of WNK4. Unexpectedly, WNK1 was virtually as effective as WNK4 at inhibiting the function of TRPV4 in transient transfection in the context...
of TRPV4 activation by hypotonic stress (Fig. 2, A and B) and by 4α-PDD (Fig. 2, B and D). Because the conceptual translation of the rat WNK1 cDNA diverges considerably from that of the reported human and murine clones (see METHODS) and the present studies employed a TRPV4 cDNA of human origin, additional experiments were performed such that the effect of rat WNK1 vis-à-vis murine and human TRPV4 could be assessed in parallel; the effect of rat WNK1 was equivalent, regardless of the species of origin of TRPV4 (data not shown).

In some model systems, WNK1 may influence the effect of WNK4 (17, 44). Therefore, we tested the effect of coexpression of WNK1 and WNK4 with TRPV4. There was no potentiation of the inhibitory response with respect to hypotonicity (Fig. 2, A and C) or 4α-PDD (Fig. 2, B and D) when both kinases were transfected in combination; however, we could not completely exclude a modest antagonistic effect of WNK1 on the WNK4 effect.

We speculated that WNK4 may influence trafficking of TRPV4 to the plasma membrane, as was demonstrated for several Cl– transport proteins. HEK-293 cells were transiently transfected with TRPV4, in the presence or absence of cotransfection with WNK4 or WNK1. Transfectants were then subjected to cell surface biotinylation, and biotinylated proteins were isolated with avidin-agarose beads. WNK4 and WNK1 downregulated TRPV4 cell surface expression to a degree commensurate with the effect of these kinases on agonist-dependent Ca2+ entry (Fig. 3, A and B). Consistent with the intracellular Ca2+ data, there was neither abrogation nor po-
tentiation when WNK1 and WNK4 kinases were expressed in concert (Fig. 3, A and B). Expression of either WNK kinase alone, or in combination, failed to influence total expression of TRPV4 as determined via anti-TRPV4 immunoblotting of whole cell lysates prepared from the transient transfectants (Fig. 3, A and C). Therefore, the WNK effect with respect to TRPV4 function appeared to operate primarily at the level of cell surface localization of the channel.

We next sought to establish the dependence of the WNK4-inducible downregulation of TRPV4 activity on WNK4 kinase activity. A point mutation abolishing WNK4 activity (kinase-dead D318A-WNK4) had been previously described. Heterologous expression of this mutant was used to demonstrate that WNK4 kinase activity was required for WNK4-dependent inhibition of thiazide-sensitive Na+/H+ cotransporter (36, 43) but not WNK4-dependent inhibition of ROMK activity (16). On cotransfection with TRPV4, this “kinase-dead” WNK4 was inert with respect to TRPV4 function, in marked contrast to wild-type WNK4. This effect was evident whether TRPV4 was activated by hypotonicity (Fig. 4A) or 4α-PDD (Fig. 4B). These data suggested that kinase activity was necessary for the WNK4 effect.

Point mutations in WNK4 are causative for the severe form of hereditary hypertension seen in familial hyperkalemia and hypertension, an effect attributed to hypofunctioning of the mutants (35). We therefore tested the ability of two of these disease-causing point mutant forms of WNK4 to inhibit TRPV4 function. We used site-directed mutagenesis to create point mutants in murine WNK4 corresponding to the mutations in the human kindreds (35) (see METHODS). In the setting of hypotonic activation of TRPV4, mutant forms of WNK4 (WNK4E559K or WNK4Q562E variant), when cotransfected with TRPV4, were significantly less effective than wild-type WNK4 at inhibiting TRPV4 function (Fig. 4A). Both mutants, however, retained some efficacy, in that they decreased TRPV4-dependent Ca2+ entry relative to empty vector alone. A similar trend was observed when 4α-PDD served as the activator of TRPV4 (Fig. 4B), although statistical significance was not achieved using this stimulus. In general, the effect of these point mutants was intermediate between the effect of wild-type WNK4 and empty vector control. We conclude that intrinsic kinase activity of WNK4 is potentially instrumental in its downmodulatory effect vis-à-vis TRPV4 function and that disease-causing point mutants of WNK4, in general, are less effective in this respect than wild-type WNK4. Consistent with this model, cell surface expression of TRPV4 was not downregulated by the kinase-dead WNK4, and the effect of the
disease-causing WNK4 mutants was diminished with respect to wild-type WNK4 (data not shown; n = 2).

WNK1 exhibits intrinsic kinase activity (38). To establish a role for this function in the WNK1 effect on TRPV4 localization, we employed two strategies to disrupt WNK1 kinase activity. Lys233 is believed to be the catalytic lysine for WNK1; mutation of this residue to Met resulted in near-total loss of WNK1 kinase activity (38). Ser382 of WNK1 was expected to be phosphorylated on the basis of sequence similarity with other well-studied protein kinases (e.g., MAP kinases). When Ser382 was mutated to Ala, the resultant protein exhibited negligible kinase activity (39). We tested the effect of these WNK1 point mutants on TRPV4-dependent Ca\(^{2+}\) entry. In general, the effect of these mutants was intermediate between those of empty vector control and wild-type WNK1 (Fig. 5, A and B). In the TRPV4 response to hypotonicity, both kinase-dead mutants significantly decreased Ca\(^{2+}\) entry relative to empty vector control (Fig. 5A). The effect of WNK1K233M did not differ from that of wild-type WNK1; however, WNK1S382A was less effective than wild-type WNK1 at suppressing Ca\(^{2+}\) entry in response to hypotonic stress (Fig. 5A). Although a similar trend was observed in the cell response to 4\(\alpha\)-PDD, in this setting the effect of the kinase-deficient mutants did not differ dramatically from that of wild-type WNK1 (Fig. 5B). We conclude that intrinsic kinase activity of WNK1 likely plays a modest role, at best, in the regulation of TRPV4 localization.

We speculated that WNK4 and/or WNK1 may exert an effect on TRPV4 localization via a direct interaction with the channel. We employed a series of reciprocal coimmunoprecipitation strategies but were unable to demonstrate a direct interaction between either kinase and TRPV4 (data not shown).

We next sought to define the WNK4 domains responsible for downregulation of TRPV4 expression and function. Mouse WNK4 has a kinase domain spanning approximately residues 171–429 [as analyzed via the SMART website (http://smart.embl-heidelberg.de/); Fig. 6A]. Immediately downstream of the kinase domain is an “autoinhibitory domain,” which is reasonably well conserved between WNK1 and WNK4. WNK1 has a number of coiled-coil domains potentially mediating protein-protein interactions (38). It has been suggested that WNK4 has a number of corresponding domains (33). However, although there is significant homology between WNK1 and WNK4 at two of these putative domains (corresponding to residues 147–167 and residues 1111–1138 in murine WNK4), we were unable to detect coiled-coil domains when the Coils2 program was used to screen WNK4 directly.

WNK4 deletion mutants were designed to eliminate the first putative coiled-coil domain and nearly all the kinase domain (\(\Delta 29–366\)-mWNK4), the autoinhibitory domain and second putative coiled-coil domain (\(\Delta 366–794\)-mWNK4), the COOH terminus, including the third putative coiled-coil domain (\(\Delta 677–1211\)-mWNK4), and the extreme COOH terminus containing only the third coiled-coil domain and adjacent residues (\(\Delta 1086–1138\)-mWNK4; Fig. 4A). The NH2-terminal WNK4 mutants \(\Delta 29–366\)-mWNK4 and \(\Delta 366–794\)-mWNK4 exhibited preservation of the inhibitory effect vis-à-vis TRPV4 activity in response to hypotonicity (Fig. 6, B and D) or 4\(\alpha\)-PDD (Fig. 6, C and E), even in the absence of an intact kinase domain. The COOH-terminal deletion mutant \(\Delta 677–1211\)-mWNK4 and the extreme COOH-terminal mutant \(\Delta 1086–1211\)-mWNK4 were essentially devoid of inhibitory
effect (Fig. 6, B–E). These data establish the essential nature of the WNK4 extreme COOH terminus for effecting the downregulation of TRPV4 function and, in contrast to our observations using a kinase-dead WNK4 mutant, suggested the dispensability of the kinase domain.

We attempted to establish in preliminary fashion the domain of TRPV4 conferring downregulation in response to WNK4. Human TRPV4 (isoform α or 1) is an 871-amino acid protein with a canonical hexahelical voltage-gated ion channel domain comprising residues H11014–717, as assessed via the DAS Transmembrane Prediction Server (8) (http://www.sbc.su.se/~miklos/DAS/; Fig. 7A). TRPV4 also has as many as six ankyrin binding domains (Fig. 7A), which are believed to be instrumental in protein–protein interaction. Without disturbing the membrane-spanning domains (which would eliminate the cell surface expression we wished to quantitate), we generated a series of deletion mutants of the TRPV4 NH2 and COOH termini (Fig. 7A). Four of five mutants were abundantly expressed on transient transfection when whole cell lysates were immunoblotted for TRPV4 (Fig. 7B); in all four of these cases, the mutant TRPV4 trafficked to the plasma membrane in appreciable amounts, as assessed via cell surface biotinylation (Fig. 7B). In all but one of these highly expressed mutants, downregulation in response to WNK4 cotransfection was preserved (Fig. 7B). Specifically, the WNK4A–147-TRPV4 mutant, although abundantly expressed, was devoid of inhibitory activity, whereas the disease-causing point mutants WNK4E559K and WNK4Q562E exhibited markedly reduced inhibitory effect (A). This pattern was also evident in the setting of 4α-PDD stimulation of TRPV4 (B), although not all differences achieved statistical significance.

Fig. 4. Disease-causing point mutants of WNK4 partially or completely block the effect of WNK4 on TRPV4 activity. HEK-293 cells were transiently transfected with TRPV4 in conjunction with expression plasmid: vector alone, wild-type WNK4, kinase-dead WNK4D318, or disease-causing mutants WNK4E559K and WNK4Q562E. Transfectants were then subjected to TRPV4 activators: hypotonicity (A) and 4α-PDD (B). Vertical axis is increment in intracellular Ca2+ (as assessed via fura 2 ratiometry) at 75 s of treatment, normalized to TRPV4-transfected cells in the absence of cotransfected WNK kinase (Vector). Coexpression of wild-type WNK4 abrogated the effect of hypotonicity and 4α-PDD. In the case of hypotonicity, the kinase-dead WNK4 mutant WNK4D318 was devoid of inhibitory activity, whereas the disease-causing point mutants WNK4E559K and WNK4Q562E exhibited markedly reduced inhibitory effect (A). This pattern was also evident in the setting of 4α-PDD stimulation of TRPV4 (B), although not all differences achieved statistical significance.

Fig. 5. Downregulation of TRPV4-dependent Ca2+ entry is preserved in kinase-dead WNK1 mutants. HEK-293 cells were transiently transfected with TRPV4 in conjunction with expression plasmid: vector alone, wild-type WNK1, kinase-dead WNK1K233M, or kinase-dead WNK1S382A. Transfectants were then subjected to TRPV4 activators: hypotonicity (A) and 4α-PDD (B). Vertical axis is the increment in intracellular Ca2+ (as assessed via fura 2 ratiometry) at 75 s of treatment, normalized to TRPV4 alone (Vector). Coexpression of wild-type WNK1 abrogated the effect of hypotonicity and 4α-PDD on TRPV4-dependent Ca2+ entry. In response to both treatments, both kinase-dead WNK1 mutants blunted TRPV4-dependent Ca2+ entry; only one of the kinase-dead mutants (WNK1S382A) in response to a single stimulus (hypotonicity) was significantly less effective than wild-type WNK1.
dantly expressed, was not downregulated by WNK4 coexpression (Fig. 7, B and C). (Data for the Δ157–299-TRPV4 mutant could not be obtained because of the negligible level of expression of the mutant and are absent from Fig. 7C.) These data suggested that a feature of the TRPV4 NH₂ terminus separate from the ankyrin binding domains was required for the WNK4 effect. Interestingly, and somewhat paradoxically, deletion of a slightly larger domain (in the Δ2–147-TRPV4 mutant) did not abrogate WNK4 responsiveness (Fig. 7, B and C). In an effort to reconcile this discrepancy, we generated two more deletion mutants of TRPV4: Δ189–467-TRPV4, absent all six of the putative ankyrin domains, and Δ1–189-TRPV4, absent the NH₂ terminus upstream of the ankyrin domains (Fig. 7A). Both mutants were subject to downregulation by WNK4 coexpression (data not shown; n = 2). In aggregate, these data supported a role for a unique configuration of the NH₂ terminus (present in the Δ2–157-TRPV4 mutant but absent from the Δ2–147-TRPV4 mutant) and/or the membrane-spanning region (by the process of elimination). Because of
the gross nature of these deletion mutants and the anticipated effect on channel function, Ca\(^{2+}\) entry data in response to TRPV4 were not sought.

**DISCUSSION**

In light of their responsiveness to anisotonicity (17, 38) and their coexpression with the tonicity-responsive TRPV4 in the distal nephron (35, 41), we hypothesized that WNK kinases would influence function or localization of this channel. In the present study, we note that coexpression of WNK4 or WNK1 with TRPV4 results in marked downregulation of trafficking of the latter to the plasma membrane (without influencing overall abundance of the channel). Correspondingly, we observed a decrease in TRPV4 responsiveness to hypotonicity or the TRPV4 activator 4\(^{-}\)PDD.

WNK4 has been characterized primarily as an inhibitor of Cl\(^{-}\) transport. On coexpression in a heterologous model system, WNK4 downregulates expression of the thiazide-sensitive Na\(^{+}\)-Cl\(^{-}\) cotransporter (36, 43), as well as the Na\(^{+}\)-K\(^{+}\)2Cl\(^{-}\) cotransporter and the apical Cl\(^{-}\)/HCO\(_{3}\)\(^{-}\) exchanger (14). With respect to the effect of WNK4 on paracellular Cl\(^{-}\) transport, conflicting data have emerged. One group observed that WNK4 had no effect on paracellular Cl\(^{-}\) transport (42), whereas others noted an increase in activity (15). In addition to Cl\(^{-}\) transport, activity of the K\(^{+}\) channel ROMK is also decreased by coexpression of WNK4 (16).

Disease-causing mutants of human WNK4 include a series of individual missense mutations affecting a small cluster of polar, and primarily positively charged, residues (E559K, D561A, and Q562E). In the present study, mutations in murine WNK4 corresponding to these disease-causing mutations in human WNK4 exhibited a partial loss of inhibitory effect on a given TRPV4 mutant is depicted by a value of unity (dashed line). Only 1 deletion mutant, Δ157–299-TRPV4, was resistant to downregulation by WNK4 (B and C). Closely related Δ2–157-TRPV4 exhibited preserved downregulation by WNK4. Two additional mutants were generated that lacked all putative ankyrin binding domains (Δ189–467-TRPV4) or the entire NH\(_{2}\) terminus upstream of these domains (Δ1–189-TRPV4; A); neither deletion blocked WNK4-dependent downregulation of TRPV4 cell surface expression (not shown).
identical clone for their studies, we do not believe this is the basis for our contrasting observations.

Conflicting data emerge with respect to the requirement for intrinsic kinase activity of WNKS in downregulating TRPV4. Specifically, WNK4 mutated for a key residue within the kinase domain (D318) resulting in a kinase-dead phenotype was less effective than wild-type WNK4 at blocking TRPV4 function. In contrast, deletion of nearly the entire kinase domain (including this key residue) failed to abolish the WNK4 effect. Kinase activity has been a variable requirement for WNK4 effect on channel and transporter activity. For example, inhibition of the thiazide-sensitive Na\(^{+}\)-Cl\(^{-}\) cotransporter by WNK4 expression was prevented if the kinase-dead WNK4 mutant was used (36, 43). However, the WNK4 effect on ROMK appeared to be independent of WNK4 kinase activity, because it was observed even with the kinase-dead (D318A) WNK4 mutant (16). It is conceivable that this charged residue, essential for kinase activity, serves an additional role in WNK4 function. This possibility is further supported by the seemingly modest role of intrinsic kinase activity in the ability of WNK1 to downregulate TRPV4 (Fig. 5).

After initial uncertainty, a role for TRPV4 in central osmoregulation in mammals appears secure (19, 24). The contribution of the abundant renal expression of TRPV4 to this process remains unresolved (18, 28, 29). The extent to which the abnormal salt and water metabolism in mice harboring TRPV4 targeted deletions is reflective of hypothalamic vs. renal inactivation of this channel has not been explored. Renal TRPV4 is restricted to tubule segments lacking constitutive apical water permeability (i.e., sites distal to the genu of the loop of Henle) and localizes to the basolateral membrane in these sites (29). We speculate that TRPV4 may function as a distal nephron sensor of interstitial solute and water and, indirectly, of transcellular resorption via the proximal nephron (7). In the distal nephron, TRPV4 is coexpressed with WNK1 and WNK4 (35, 41). Osmotically responsive WNK kinases may influence expression and function of TRPV4 in this tissue.

The syndrome of familial hyperkalemia and hypertension is causally linked to overexpression of WNK1 or to inactivating mutations in WNK4 (35). Patients with WNK1 mutations exhibit normocalciuria (2), whereas those with mutations in WNK4 manifest hypercalciuria (23). Mayan and colleagues, who correctly predicted constitutive overactivity of the thiazide-sensitive Na\(^{+}\)-Cl\(^{-}\) cotransporter in this syndrome before the elucidation of the molecular phenotype (23), recently proposed that mutant WNK4 causes hypercalciuria by influencing a Ca\(^{2+}\) channel or transporter (22). A WNK4 and TRPV4 functional interaction may be perturbed in the setting of WNK4 mutation.

It is important to emphasize that TRPV4 is not the only candidate Ca\(^{2+}\) channel in this part of the nephron. Although the role of TRPV5 and TRPV6 in regulation of urinary Ca\(^{2+}\) excretion has appropriately received much attention (12), TRPV4 potentially participates in this process as well. As described above, TRPV4 is highly expressed along the distal nephron (29). In addition, there was a trend toward lower plasma Ca\(^{2+}\) levels in TRPV4\(^{-/-}\) mice than in TRPV4\(^{+/+}\) controls [8.8 ± 0.3 vs. 9.8 ± 0.4 (SE) mg/dl, n = 10 (24)]; however, these data did not reach statistical significance in this important, but relatively small, study. Therefore, TRPV4 may influence systemic, as well as local, Ca\(^{2+}\) balance; both may be perturbed by altered WNK abundance or function. An effect of WNK4 on TRPV5 function has been described in preliminary fashion by Peng and colleagues (26); however, inconsistent with the aberrant Ca\(^{2+}\) balance accompanying familial hyperkalemia and hypertension, disease-causing WNK4 mutants were indistinguishable from wild-type WNK4 in vitro assays of TRPV5 function. Therefore, it is possible that mutant WNK4-induced aberrant regulation of TRPV4 or TRPV6 or another Ca\(^{2+}\) transport protein is operative in this disease.

The molecular mechanism through which WNKS influence TRPV4 targeting and function is unclear. WNK1 may function as a MAP kinase kinase kinase kinase (i.e., a “MAP4K”) in the ERK5 pathway; its downstream effects are sensitive to an inhibitor of MEK5 (40). Interestingly, very similar to TRPV4, the function of ERK5 and its upstream activators is regulated by anisotonicity (1, 32), suggesting a physiological signaling “module” encompassing WNKS and TRPV4. WNKS also serve as substrates for the kinase Akt/protein kinase B (13, 31), which is itself an effector of the anisotonicity-responsive phosphatidylinositol 3-kinase pathway (30, 45). Because intrinsic kinase activity is not an absolute requirement for the WNK effect on TRPV4, it is conceivable that these large kinases also act via a scaffolding mechanism, perhaps transiently bridging TRPV4 and another effector. Alternatively, WNK activation may indirectly liberate a small molecule intermediate that influences TRPV4 trafficking; this latter model is consistent with our inability to demonstrate a direct interaction between WNK kinases and TRPV4.

WNKS are believed to influence trafficking of clathrin-coated endocytic vesicles in a dynamin-dependent fashion (16). An analogous, although biochemically distinct, mechanism of rapid, agonist-dependent shuttling to and from the cell membrane has recently been described for a TRP channel. Specifically, growth factor-dependent insertion of TRPC5 appears to require phosphatidylinositol 3-kinase activity and the Rho family GTPase Rac1 (5). As suggested above, phosphatidylinositol 3-kinase may function upstream of WNK activation, biochemically linking these two mechanisms.

In summary, these data support a role for WNK kinases in regulating the subcellular localization of TRPV4. Inasmuch as WNK kinases and TRPV4 are responsive to changes in ambient tonicity, it is tempting to speculate that they jointly participate in an osmosensing or osmotically responsive signal transduction pathway in the distal nephron or elsewhere.

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REFERENCES


