WNK3, a kinase related to genes mutated in hereditary hypertension with hyperkalaemia, regulates the K^+ channel ROMK1 (Kir1.1)

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The serine–threonine kinase WNK3 modulates Cl^- transport into and out of cells through its regulation of *SLC12A* cation–Cl^- cotransporters, implicating it as (one of) the long-sought Cl^-/volume-sensitive kinase(s). Integrators in homeostatic systems regulate structurally diverse but functionally coupled elements. For example, the related kinase WNK4 regulates the Na^+–Cl^- cotransporter (NCC), paracellular Cl^- flux, and the K^+ channel ROMK1 (Kir1.1) to maintain renal NaCl and K^+ homeostasis; mutations in *PRKWNK4*, encoding WNK4, cause a Mendelian disease featuring hypertension and hyperkalaemia. It is known that WNK3 is expressed in the nephron's distal convoluted tubule (DCT) and stimulates NCC activity. Here, we show that WNK3 is also expressed in cortical and outer medullary collecting duct principal cells. Accordingly, we tested WNK3's effect on the mediators of NaCl and K^+ handling in these nephron segments – the epithelial sodium channel (ENaC), paracellular Cl^- flux, and ROMK1 – using established model systems. WNK3 did not alter paracellular Cl^- flux in tetracycline-responsive MDCK II cells, nor affect amiloride-sensitive currents when coexpressed with ENaC in *Xenopus laevis* oocytes. However, additional coexpression studies in oocytes revealed WNK3 inhibited the renal-specific K^+ channel ROMK1 activity greater than 5.5-fold ($P < 0.0001$) by altering its plasmalemmal surface expression; WNK3 did not affect ROMK1's conductance or open/closed probability. In contrast, WNK3 had no effect on the activity of the cardiac long-QT syndrome K^+ channel KCNQ1/KCNE1 when coexpressed in oocytes. Inhibition of ROMK1 is independent of WNK3's catalytic activity and is mediated by WNK3's carboxyl terminus – a mechanism distinct from its known kinase-dependent activation of NCC. A kinase-inactivating point mutation or a missense mutation homologous to one in WNK4 that causes disease produced a gain-of-function effect, enhancing WNK3's inhibition of ROMK1 greater than 2.5-fold relative to wild-type kinase ($P < 0.0001$). The magnitude and specificity of WNK3's effects at both NCC and ROMK1, its coexpression with its targets in the distal nephron, and the established *in vivo* effect of WNK4 at these same targets provide evidence that WNK3's action is physiologically relevant. WNK3 is probably a component of one of the mechanisms that determines the balance between renal NaCl reabsorption and K^+ secretion.

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The kidney regulates the volume and ionic composition of the intravascular fluid, which are necessary for proper tissue perfusion and neuromuscular excitability, respectively. In the kidney, the distal nephron, composed of the distal convoluted tubule (DCT), connecting tubule (CNT), and collecting duct (CD), determines whole-body NaCl and K^+ balance. NaCl reabsorption is mediated by the electroneutral Na^+–Cl^- cotransporter NCC (encoded by *SLC12A3*) in the DCT and early CNT, and electrogenic Na^+ reabsorption in late CNT and CD principal cells via the epithelial Na^+ channel ENaC (encoded by *SCNN1A*,

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SCNN1B and *SCNN1G*). Na^+ reabsorption by ENaC creates a lumen-negative electrical potential difference that provides the driving force for K^+ secretion via the ATP-sensitive renal outer medullary K^+ channel ROMK1 (or Kir1.1; encoded by *KCNJ1*); this lumen-negative potential difference also drives the reabsorption of Cl^- along the paracellular pathway. Aldosterone is the primary regulator of *both* renal NaCl reabsorption (via its regulation of NCC and ENaC) and K^+ secretion (via its effect on ROMK1). Accordingly, the secretion of aldosterone is stimulated by low intravascular volume or hyperkalaemia (high serum K^+ levels), promoting increased renal NaCl reabsorption and K^+ secretion. However, *independent* regulation of these NaCl *versus* K^+ transport pathways is required to maintain overall homeostasis in response to various physiological perturbations when one transport mode is desired over the other (Seldin & Giebisch, 2000).

Insights into the molecular mechanisms underlying the kidney's ability to maintain NaCl and K^+ homeostasis have been obtained in part by studying rare Mendelian diseases that show derangements in renal NaCl and K^+ handling as their primary phenotypes (Lifton *et al.* 2001). Recent genetic analysis identified WNK1 and WNK4 (encoded by *PRKWNK1* and *PRKWNK4*, respectively) as kinases with the properties of integrative regulators of several renal ion transport pathways (Wilson *et al.* 2001). A unique substitution of cysteine for lysine at a highly conserved residue in the catalytic domain characterizes the WNK kinase family (hence WNK, *w*ith *n*o lysine (= *K*)) (Xu *et al.* 2000; Min *et al.* 2004). Only four WNK kinases exist in the human genome (Wilson *et al.* 2001; Verissimo & Jordan, 2001); each shares significant homology in the serine–threonine kinase domain, an autoinhibitory domain, two putative coiled-coil domains, and a short acidic domain. Mutations in *PRKWNK4*, encoding WNK4, cause pseudohypoaldosteronism type II (PHAII), an autosomal dominant disease featuring hypertension and hyperkalaemia due to a coupled increase in renal NaCl reabsorption and deficiency in renal K^+ secretion (Wilson *et al.* 2001). Physiological analysis has shown that wild-type WNK4, via catalytic and non-catalytic mechanisms, inhibits NCC and ROMK1, and activates paracellular Cl^- flux (Wilson *et al.* 2003; Yang *et al.* 2003; Kahle *et al.* 2003, 2004*a*,*b*; Yamauchi *et al.* 2004; Golbang *et al.* 2005). PHAII-causing missense WNK4 mutations cluster within a 10 aa acidic domain conserved among all WNKs; these mutations have sharply divergent effects on these downstream targets. PHAII-causing mutations eliminate inhibition of NCC and augment paracellular Cl^- conductance, while simultaneously enhancing inhibition of ROMK1. Together, these effects help to explain the increase in NaCl reabsorption and the decrease in K^+ secretion seen in affected PHAII patients (Wilson *et al.* 2003; Yang *et al.* 2003; Kahle *et al.* 2003, 2004*a*,*b*; Yamauchi *et al.* 2004; Golbang *et al.* 2005). Intronic deletions that increase expression of WNK1 also cause PHAII (Wilson *et al.* 2001). Recent evidence has shown that WNK1 can reverse WNK4's inhibition of NCC (Yang *et al.* 2003) and increase the activity of ENaC (Naray-Fejes-Toth *et al.* 2004; Xu *et al.* 2005) in oocytes and mammalian cells.

We recently demonstrated that WNK3, a novel WNK kinase family member, localizes to ion-transporting epithelia and GABAergic neurones (Rinehart *et al.* 2005; Kahle *et al.* 2005). WNK3 stimulates *SLC12A* cotransporters that drive Cl^- influx (NCC and the Na^+–K^+–$2Cl^-$ cotransporters), but inhibits *SLC12A* cotransporters that drive Cl^- efflux (K^+–Cl^- cotransporters KCC1 and KCC2) (Rinehart *et al.* 2005; Kahle *et al.* 2005). Silencing WNK3's kinase activity reverses its effect at each of these targets. WNK3's ability to regulate multiple ion transporters that are coexpressed and coordinately function in the same cells (e.g. NKCC1 and KCC1 in epithelial and red blood cells, NKCC1 and KCC2 in GABAergic neurones) suggests it is a key integrator of these transport systems (Rinehart *et al.* 2005; Kahle *et al.* 2005). In this report, we show that WNK3 is expressed in CD principal cells and specifically regulates the kidney-specific K^+ channel ROMK1 in *Xenopus laevis* oocytes. These data, along with the known action of WNK3 on the renal-specific NCC, suggest WNK3 is a component of a regulatory signalling pathway that determines the balance between renal NaCl reabsorption and K^+ secretion.

Methods

Antibodies

Antibodies used were: anti-WNK3 (Rinehart *et al.* 2005; Kahle *et al.* 2005), anti-ZO-1 (Choate *et al.* 2003), anti-aquaporin-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and affinity-purified secondary antibodies conjugated to the CY2 (green) and CY3 (red) fluors (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA).

Immunolocalization

Animal studies are in accordance with the humane practices of animal care established by the Yale Animal Care and Use Committee. Mice were killed by cervical dislocation and excised kidneys were prepared and sectioned as described (Choate *et al.* 2003). Slides were stained with primary and secondary antibodies, and visualized by immunofluorescence microscopy as described (Choate *et al.* 2003). Results were consistent in three different mice.

cDNA constructs *pSPORT–ENaC-α*, *pSPORT–ENaC-β*, and *pSPORT–ENaC-γ* have been previously described

(Shimkets *et al.* 1997). WNK3 (*pGH19–WNK3*), kinase-inactive WNK3 (*pGH19–WNK3 kin–*), and a PHAII-like WNK3 mutant (*pGH19–WNK3 PHAII*) have been previously described (Rinehart *et al.* 2005; Kahle *et al.* 2005). *pSPORT–eGFP–ROMK1* was previously published (Kahle *et al.* 2003). WNK3 was subdivided into the kinase domain (KD, bases 1–1251) and the regulatory domain (RD, bases 1252–5229). Each piece was subcloned into *pGH19* with 5′-*Eco*RI and 3′-*Xho*1 restriction sites.

Paracellular studies

A cDNA encoding full-length human WNK3 (Rinehart *et al.* 2005; Kahle *et al.* 2005), tagged at the carboxyl terminus with haemagglutinin (HA), was subcloned into the *pTRE2hyg* expression vector (BD Biosciences Clontech, Palo Alto, CA, USA) and transfected into MDCK II tet-off cells (Clontech). Clones showing absence of WNK3 expression in the presence of doxycycline and robust expression following the elimination of doxycycline were chosen for further study. Current–voltage (*I–V*) relationships were acquired by voltage-clamping monolayers between −50 and +50 mV, and transepithelial resistance (TER) was calculated from Ohm's law. Dilution potential measurements were obtained as described (Kahle *et al.* 2004*b*). The ion permeability ratio (P_{Cl}/P_{Na}) for the monolayer was calculated from the dilution potential by using the Goldman-Hodgkin-Katz equation. The absolute permeabilities of Na^+ (P_{Na}) and Cl^- (P_{Cl}) were calculated by using a simplified Kimizuka-Koketsu equation. Transepithelial resistance (TER) and dilution potential measurements were performed in triplicate on each of three different wild-type WNK3 clonal cell lines in at least three independent experiments; results from independent clones showed no significant differences and were treated as independent measures. TER and dilution potentials are shown as raw data from a representative experiment. All data are expressed as means ± S.E.M., and statistical comparisons between groups are made by paired Student's *t* test (induced *versus* uninduced).

Functional assays with ENaC

Oocytes were injected with the cRNA of *ENaC* α, β, and γ subunits alone or together with wild-type *WNK3* cRNA, incubated for 2–3 days, and amiloride-sensitive whole-cell Na^+ currents were measured by two-electrode voltage clamp as previously described (Shimkets *et al.* 1997). To prevent Na^+ loading and therefore prolong oocyte viability, oocytes were kept in incubation solution containing 1 μM amiloride prior to experiments. ENaC currents were calculated as the difference in whole-cell current before and after the addition of 10 μM amiloride to the bathing solution at a holding membrane potential of 100 mV. Results are expressed as the mean ± S.E.M. and statistical comparisons between groups were made by a paired *t* test. Data of a representative experiment are shown. Two separate experiments using oocytes from different frogs yielded similar results.

Functional assays with ROMK1

Oocytes were injected with *ROMK1* cRNA alone or together with wild-type or mutant *WNK3* cRNAs, incubated for 2–3 days, and Ba^{2+}-sensitive whole-cell K^+ currents were measured by two-electrode voltage clamp as previously described (Kahle *et al.* 2003). All reported K^+ currents refer to Ba^{2+}-sensitive currents. For *I–V* plots, K^+ currents at each voltage represent the mean ± S.E.M. of at least seven oocytes for each group from a representative experiment. For statistical comparisons of K^+ currents between groups of oocytes, we analysed K^+ currents at +40 mV using two-tailed Student's *t* tests; these comparisons included data from three independent experiments from different frogs. In each experiment, > seven oocytes were analysed for each group.

Functional assays with KCNQ1/KCNE1

Oocytes were injected with *KCNQ1* cRNA alone or together with *KCNE1* and/or *WNK3* cRNAs, incubated for 2 days, and whole-cell K^+ currents were measured by two-electrode voltage clamp as previously described (Kahle *et al.* 2003). For *I–V* plots, K^+ currents at each voltage represent the mean ± S.E.M. of at least seven oocytes for each group from a representative experiment. For statistical comparisons of K^+ currents between groups of oocytes, we analysed K^+ currents at +40 mV using two-tailed Student's *t* tests; these comparisons included data from three independent experiments from different frogs. In each independent experiment, > seven oocytes were analysed for each injected group.

Membrane surface expression studies

Oocytes were injected with enhanced green fluorescent protein (eGFP)-tagged *ROMK1* cRNA alone or together with wild-type or mutant *WNK3* cRNAs, incubated for 2 days, and membrane surface expression of eGFP–ROMK1 was assayed by laser-scanning confocal microscopy as previously described and validated (O'Connell *et al.* 2005). Total membrane fluorescence intensity was calculated for each imaged oocyte using SigmaScan Pro software (Systat Software Inc., Point Richmond, CA, USA) as previously described (Wilson *et al.* 2003). Four total experiments were performed; > 15 oocytes were injected per experimental group, and each experiment used oocytes from a different frog. A

representative experiment is shown. The significance of differences between groups was assessed by two-tailed Student's *t* test.

Cell-attached patch-clamp analysis

After injection, oocytes were immersed in a hyperosmotic solution for 1–2 min (mM: 200 *N*-methyl-D-glucamine, 2 KCl, 1 $MgCl_2$, 10 EGTA, 10 Hepes, adjusted to pH 7.4 with HCl) and vitelline membranes were removed using forceps. Electrodes were pulled from borosilicate glass capillaries (Sutter Instrument Co., Novato, CA, USA) on a Narishige PP-83 (Japan) and polished (Narishige microforge, MF-83, Japan). Electrodes had a tip resistance of 8–9 MΩ when filled with pipette solution (mM: 150 KCl, 1.0 $MgCl_2$, 1.0 $CaCl_2$, 5 Mes/Tris, adjusted to pH 7.4 with either Mes or Tris buffer). Mg^{2+}-free bath solutions were used in all experiments and perfused by a multibarrel quick-exchange solution system (Model SF-77B, Warner, Hamden, CT, USA). Bath solutions contained (mM)

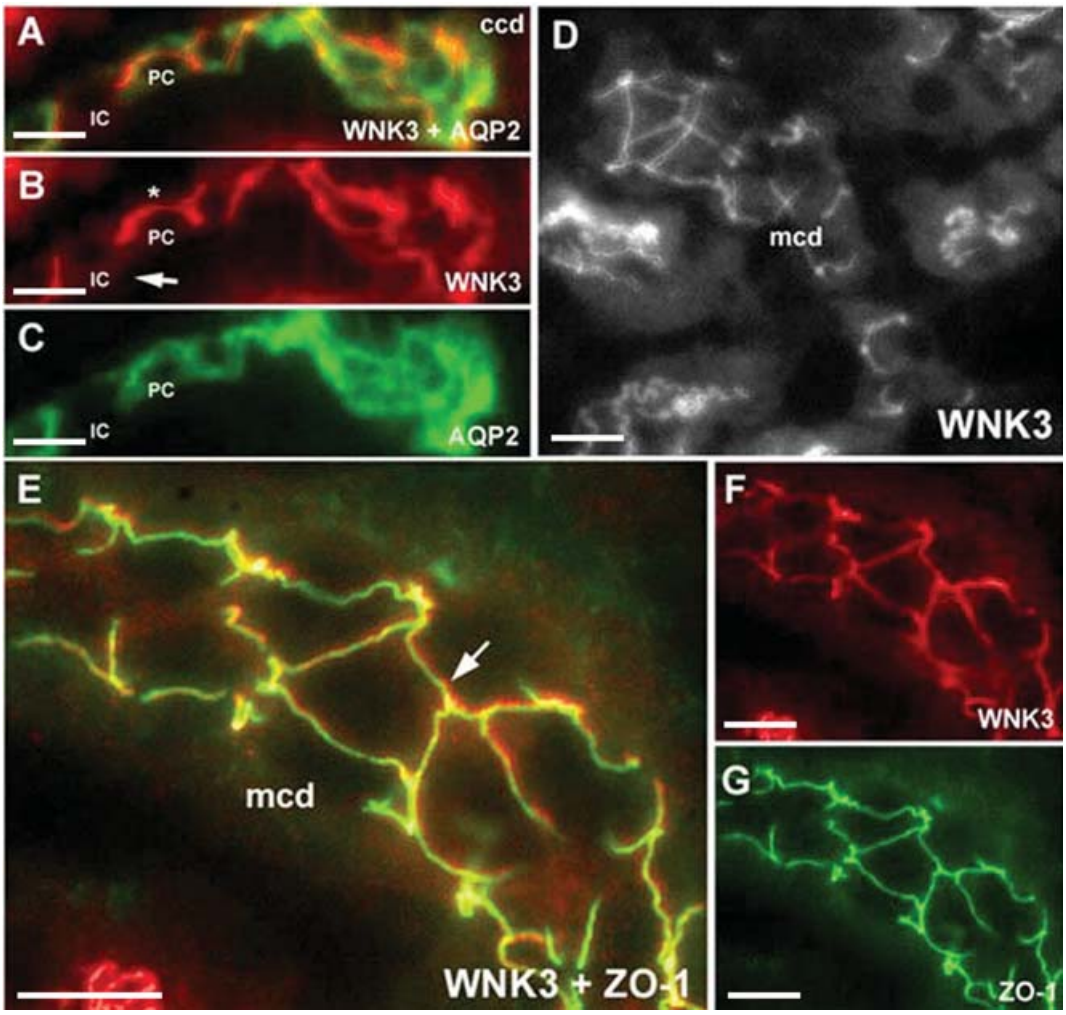
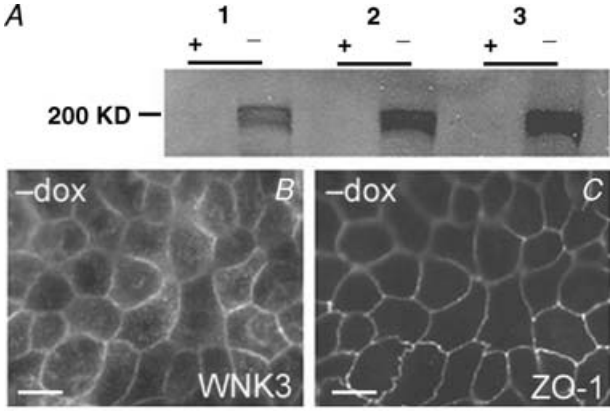


Figure 1. WNK3 expression in cortical (CCD) and outer medullary (OMCD) collecting duct principal cells
Mouse frozen tissue sections of the kidney cortex and medulla were stained with anti-WNK3 antibody, followed by fluorescent second antibodies, and visualized with immunofluorescence light microscopy as described in Methods. *A*, WNK3 expression in the cortical collecting duct (CCD), determined by costaining cortical kidney sections with anti-WNK3 (red) and anti-aquaporin-2 (AQP2), a CD marker (green). Note the presence of WNK3 in AQP2-staining principal cells, but little WNK3 staining in non-AQP2-staining intercalated cells. *B*, same as *A*, WNK3 staining alone. *C*, same as *A*, AQP2 staining alone. *D*, WNK3 staining in the outer medullary collecting duct (OMCD), identified by coexpression of WNK3 in AQP2-staining tubules in the outer medulla. Only WNK3 staining is shown. *E*, WNK3 (red) and ZO-1 (green) immunostaining in the OMCD. Tubule segments were determined by costaining experiments of adjacent sections with anti-WNK3 and anti-AQP2. WNK3 staining overlaps with ZO-1, a marker of tight junctions. Similar results were seen in the CCD. Scale bars, 10 μm in all panels. These data reveal that WNK3 is expressed at intercellular junctions in principal cells in the CCD and OMCD. *F*, same as *E*, WNK3 staining alone. *G*, same as *E*, ZO-1 staining alone.

150 KCl, 2 EDTA, 10 Mes/Tris, and were adjusted to pH 7.4 with Mes or Tris. Single channel currents were recorded in the cell-attached configuration by an EPC-7 amplifier (Heka Elektronik, Lambrecht/Pfalz, Germany) and passed through an 8-pole Bessel filter at 1000 Hz (Warner Instruments, Hamden, CT, USA). Data were acquired via a DigiData 1200 Series digital-to-analog converter (Axon Instruments, Union City, CA, USA), driven by commercial software (pCLAMP Clampex 8.20, Axon Instruments). Currents were analysed off-line with Clampfit 8.20 (Axon Instruments), Fetchan 6.04 (Axon Instruments), and GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA, USA).



D

	MDCK II-WNK3 (+ dox)	MDCK II-WNK3 (- dox)	t-test
TER (Ohms•cm²)	95.86 ± 1.95	94.15 ± 1.49	.61
G_T (mS/cm²)	10.58 ± .22	10.73 ± .21	.48
V_T (mV)	-5.65 ± .28	-5.10 ± .32	.21
P_{Cl}/P_{Na}	.50 ± .02	.54 ± .02	.19
P_{Na} (10^{-6} cm/s)	12.94 ± .23	12.78 ± .15	.67
P_{Cl} (10^{-6} cm/s)	6.48 ± .27	6.92 ± .32	.29

Figure 2. WNK3 does not alter paracellular ion transport
A, regulated expression of WNK3 in subtype II Madin-Darby canine kidney (MDCK) II tet-off cell lines. MDCK II cell clones showing doxycycline-regulated expression of HA-tagged WNK3 were produced as described in Methods. Three independent MDCK II-WNK3 cell clones (1, 2, and 3) were cultured in the presence (+) or absence (–) of doxycycline. Samples were fractionated by SDS-PAGE, followed by immunoblotting for WNK3 with anti-HA antibody. HA-tagged WNK3 migrates as an ∼200-kDa species. *B*, localization of WNK3 in MDCK II cells. Cells were cultured in the presence or absence of doxycycline (Dox), stained for WNK3 with anti-HA antibody, and visualized by immunofluorescence microscopy. Scale bars, 10 μm. WNK3 expression is low in the presence of doxycycline (not shown), but is induced in its absence to points of cell-cell contact in MDCK II cells. *C*, staining of same section as in panel *B* with anti-ZO-1, a marker of the tight junction. *D*, summary of the effect of WNK3 on paracellular ion conductance and selectivity. Mean ± S.E.M. of three separate WNK3-overexpressing MDCK II cell clones, each measurement made on triplicate wells. TER: transepithelial resistance. G_T: conductance. V_T: dilution potential. P_{Cl}/P_{Na} (the ratio of absolute permeabilities for Cl^- and Na^+) were calculated using the constant field equation. P_{Na} or P_{Cl} were calculated using the Kimizuka-Koketsu equation. See Methods for details. These data present no evidence that WNK3 affects paracellular ion transport, in contrast to WNK4, in the same model system (Yamauchi *et al.* 2004; Kahle *et al.* 2004*b*).

Results

WNK3 expression in cortical and outer medullary collecting duct (CD) principal cells

Previous work reported that WNK3 is expressed along the length of the nephron (Rinehart *et al.* 2005). To explore the localization of WNK3 in the CD in more detail, we co-stained mouse kidney sections with a specific anti-WNK3 antibody together with an antibody that serves as a marker of the cortical (CCD) and medullary (OMCD) CD. WNK3 is expressed in both of these tubule segments (Fig. 1). In the CCD and OMCD, WNK3 expression is greater in principal cells than in intercalated cells, as demonstrated by more intense WNK3 immunostaining in cells that express AQP2 (a principal cell marker) *versus* non-AQP2-expressing cells (i.e. intercalated cells; Fig. 1*A*–*D*). In both CCD principal cells and OMCD cells, WNK3 predominantly localizes to intercellular junctions, demonstrated by co-staining experiments with an antibody against zona-occludens-1 (ZO-1), a tight-junction marker (Fig. 1*E*–*G*). Because WNK3 is expressed in CD principal cells, and other WNK kinases regulate distal nephron NaCl and K^+ transport pathways, we investigated whether WNK3 regulates ENaC, the paracellular pathway,

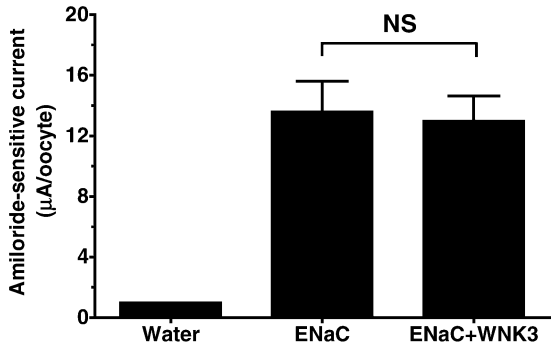


Figure 3. WNK3 does not regulate the activity of the Na^+ channel ENaC
Xenopus laevis oocytes were injected with the cRNA of ENaC α, β, and γ subunits alone or together with wild-type *WNK3* cRNA, incubated for 2–3 days, and amiloride-sensitive whole-cell Na^+ currents were measured by two-electrode voltage clamp. Co-expression of the three ENaC subunits without WNK3 gave a significant amiloride-sensitive Na^+ current; this current was unaffected by co-expression of WNK3. Results are expressed as means ± S.E.M. and statistical comparisons between groups were made by a paired *t* test. Raw data from a representative experiment are shown. These data demonstrate that WNK3 does not affect ENaC activity, in contrast to WNK1 in the same model system (Xu *et al.* 2005).

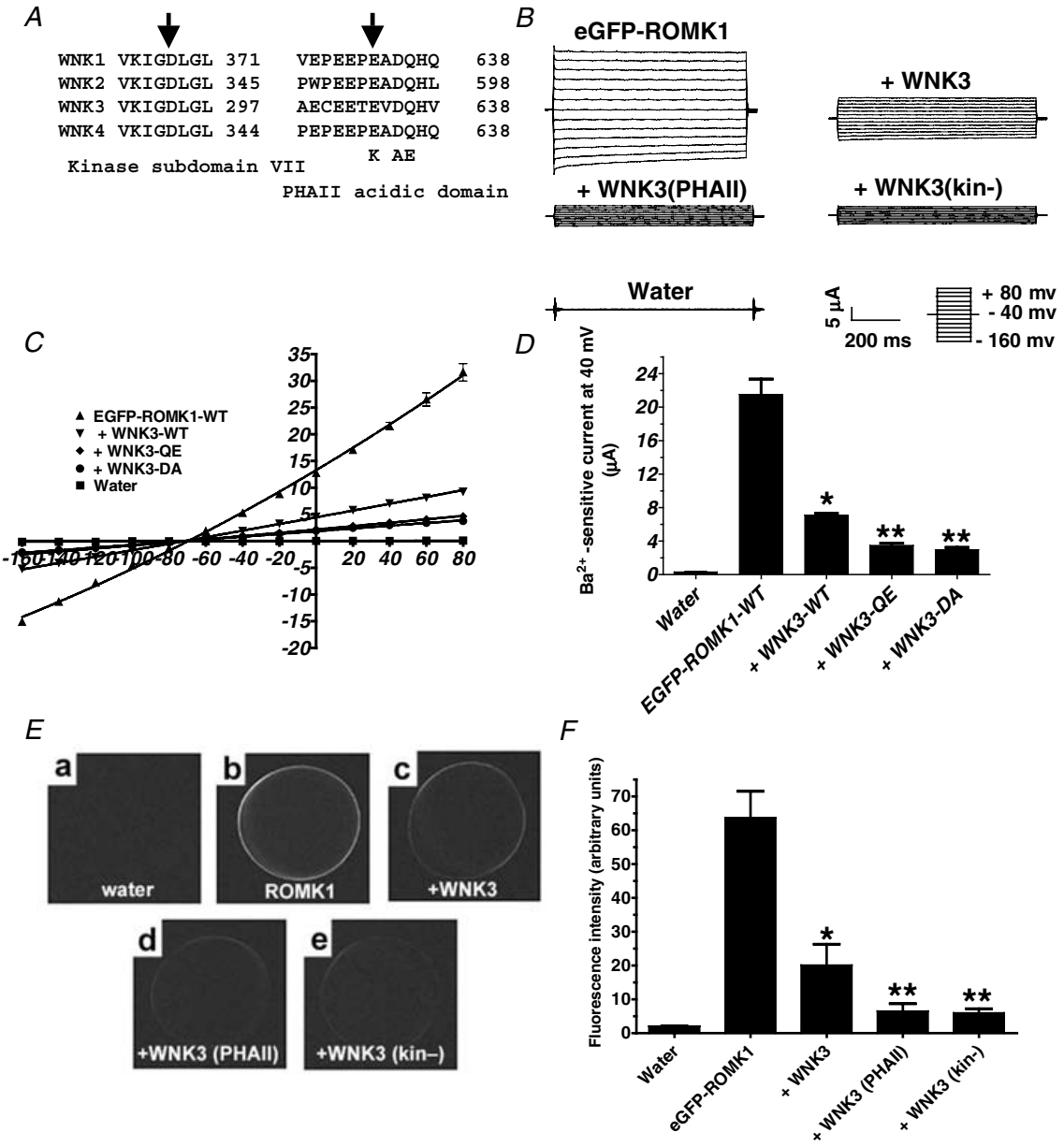


Figure 4. WNK3 regulates the K^+ channel ROMK1 by altering its plasmalemmal expression in a non-catalytic mechanism

A, important conserved domains in the WNK kinase family include the serine–threonine kinase domain (subdomain VII with the residue mutated to alanine to make WNK3 catalytically inactive is shown with an arrow), and the short acidic amino acid domain in which pseudohypoaldosteronism type II (PHAII) mutations in WNK4 cluster (arrow indicates the residue of WNK3 mutated to make the WNK3 PHAII-like mutant). *B*, Ba^{2+}-sensitive whole-cell K^+ currents were recorded from *X. laevis* oocytes expressing eGFP (enhanced green fluorescent protein)-tagged ROMK1 alone, eGFP–ROMK1 plus wild-type WNK3, eGFP–ROMK1 plus PHAII-like mutant WNK3, and eGFP–ROMK1 plus kinas-inactive WNK3 (kin–) (see Methods), and a water-injected control. *C*, current–voltage (*I–V*) relationships, showing the mean ± S.E.M. of the K^+ currents for each experimental group of oocytes as indicated. *D*, comparison of Ba^{2+}-sensitive K^+ currents at +40 mV in the presence or absence of wild-type, PHAII-like mutant WNK3 (PHAII), or kinase-inactive WNK3 (kin–). Results are expressed as the mean ± S.E.M. and statistical comparisons between groups were made by a paired *t* test. Raw data of a representative experiment are shown. *E*, the indicated proteins were expressed in oocytes, and membrane expression of eGFP–ROMK1 was quantified by confocal microscopy. Representative confocal images of a water-injected oocyte (*a*), and oocytes expressing eGFP-ROMK1 in the absence (*b*) or presence (*c*) of wild-type WNK3 are shown. Oocytes expressing eGFP-ROMK1 in the presence of PHAII-like mutant WNK3 (PHAII) (*d*) or kinase-inactive WNK3 (kin–) (*e*) are also shown. *F*, comparison of membrane surface expression of eGFP–ROMK1 in the presence or absence of wild-type or mutant WNK3. The significance of differences between groups was assessed by a paired *t* test. Data from a representative experiment are shown.

and/or ROMK1, mediators of NaCl and K^+ handling in CD principal cells.

WNK3 does not alter paracellular ion flux in MDCK II cells

Type II Madin-Darby canine kidney cells (MDCK II) are a useful model to study the intrinsic properties and regulation of paracellular ion flux because of their low transcellular ionic conductance and relatively 'leaky' (high conductance) tight junctions (Van Itallie & Anderson, 2004). WNK4 stimulates paracellular Cl^- conductance in tetracycline-responsive MDCK II cells, where it physically associates with, and phosphorylates, claudins – pore-forming tight junction proteins (Yamauchi *et al.* 2004; Kahle *et al.* 2004*b*). Since WNK3, like WNK4, is expressed at tight junctions in the CD, we generated MDCK II cells with tetracycline-responsive WNK3 expression and tested the effects of WNK3 on cell monolayer transepithelial resistance and dilution potential, indices of paracellular ion conductance and selectivity, respectively (Fig. 2*A*–*D*). Similar to WNK4 (Yamauchi *et al.* 2004; Kahle *et al.* 2004*b*), WNK3 localizes to cell–cell borders in MDCK II cells (Fig. 2*B* and *C*). However, in contrast to WNK4 (Kahle *et al.* 2004*b*), induction of WNK3 expression does not alter the paracellular properties of MDCK II monolayers (Fig. 2*D*). Thus, WNK3 is not a modulator of paracellular ion flux in MDCK II cells.

WNK3 does not effect ENaC activity

WNK1 increases the activity of ENaC in *Xenopus laevis* oocytes and mammalian cells (Xu *et al.* 2005; Naray-Fejes-Toth *et al.* 2004); there are no reports of ENaC regulation by WNK4. We directly tested whether WNK3 regulates ENaC activity in a coexpression assay in oocytes, a widely used system for characterizing the function and regulation of this Na^+ channel (Shimkets *et al.* 1997; Valentijn *et al.* 1998; Friedrich *et al.* 2003). In the absence of coexpressed α, β, and γ ENaC subunits, amiloride-inhibitable Na^+ current in oocytes was very low (< 0.1 μA; Fig. 3*A*). Co-expression of the three ENaC subunits without WNK3 gave a significant amiloride-sensitive Na^+ current (Fig. 3*A*); however, this Na^+ current was unaffected by coexpression of WNK3 (Fig. 3*A*). Western blots of homogenates from oocytes co-injected with ENaC and WNK3 revealed robust WNK3 expression (data not shown). We conclude that WNK3 does not affect ENaC activity in oocytes.

WNK3 regulates ROMK1 by modulating surface expression

We tested the effect of WNK3 on the activity of ROMK1 by performing co-expression studies in oocytes. Injection of cRNA encoding ROMK1 tagged with enhanced green fluorescent protein (eGFP–ROMK1) produced a large Ba^{2+}-sensitive K^+ whole-cell current that was markedly inhibited by addition of WNK3 (Fig. 4*A*–*D*; ~5.5-fold reduction at +40 mV, $P < 0.0001$). We observed comparable inhibition with injection of untagged ROMK1 (data not shown), as eGFP–ROMK1 and untagged ROMK1 have identical functional properties (O'Connell *et al.* 2005).

We also tested the effect on ROMK1 activity of a WNK3 mutant harbouring an alanine substitution of a highly conserved aspartate in the catalytic core (WNK3 D294A; Fig. 4*A*). This aspartate is required for catalytic function of a wide variety of kinases, including those of the WNK family, and its mutation renders WNK3 an inactive kinase (Rinehart *et al.* 2005). Kinase-inactive WNK3 inhibits ROMK1 greater than wild-type WNK3 (Fig. 4*A*–*D*; ~2-fold greater inhibition relative to wild-type WNK3 at +40 mV, $P < 0.0001$). These findings indicate that inhibition of ROMK1 by WNK3 does not require WNK3 kinase activity, and that this Asp mutation enhances WNK3's effect on ROMK1.

Kinases alter ROMK1 activity by altering its single channel properties and/or by reducing the number of channels at the cell surface (Hebert *et al.* 2005). To investigate the mechanism for WNK3's action on ROMK1, we first quantified membrane surface expression of eGFP–ROMK1 in oocytes in the presence or absence of WNK3. Wild-type WNK3 caused a marked reduction in expression of eGFP–ROMK1 at the plasma membrane (Fig. 4*E*–*F*; $P < 0.0001$). This reduction was not due to diminished total cellular expression of ROMK1, because Western blotting of whole-oocyte homogenates showed no difference in levels of eGFP–ROMK1 in the presence of WNK3 (data not shown). In concordance with the current data, kinase-inactive WNK3 reduced eGFP–ROMK1 surface expression more than wild-type kinase (Fig. 4*E*–*F*; 2-fold greater inhibition than wild-type WNK3, $P < 0.0001$). Consistent with WNK3 inhibiting ROMK1 primarily through protein trafficking,

Note that both kinase-inactive and PHAII-like mutant WNK3 display a significant increase in inhibition of both ROMK1-mediated K^+ current and eGFP–ROMK1 membrane surface expression relative to wild-type kinase genetic gain-of-function effect. These data demonstrate that WNK3 inhibits ROMK1 activity via a non-catalytic mechanism by altering the expression of the channel at the plasma membrane, and reveal the importance of the conserved WNK acidic domain in a kinase other than WNK4.

WNK3 did not alter ROMK1 single channel properties (conductance or open/closed probability), as determined by single-channel patch clamping experiments in oocytes (Fig. 5). These data show that the effect of wild-type and mutant WNK3 on ROMK1 activity can be fully accounted for by alteration of the expression of this channel at the plasma membrane.

Mutations in WNK4 that cause PHAII cluster within a highly conserved 10 aa acidic domain (Fig. 4*A*); PHAII-causing WNK4 mutants lose their ability to inhibit NCC, but exhibit an *increase* in ROMK1 inhibition compared with wild-type WNK4. We tested the effect on ROMK1 activity and surface expression of a WNK3 mutant harbouring a homologous mutation in this acidic region (Q562E). Similar to the enhanced inhibition of ROMK1 by PHAII-causing mutant WNK4 (Kahle *et al.* 2003; Golbang *et al.* 2005), the PHAII-like WNK3 mutant showed an increase in inhibition of both the K^+ current and surface expression of eGFP–ROMK1 relative to wild-type WNK3 (Fig. 4*C*–*F*; ∼2-fold greater inhibition of K^+ current ($P < 0.0001$) and ∼2-fold greater reduction in surface expression ($P < 0.0001$) compared with wild-type WNK3). This corresponds to an overall ∼85% reduction in K^+ current and ∼90% reduction in surface expression compared with oocytes expressing eGFP–ROMK1 alone (Fig. 4*C*). These data demonstrate for the first time the importance of the conserved acidic domain in members of the WNK family other than WNK4.

Recently, Yang *et al.* (2005) demonstrated that WNK4's inhibitory effect on NCC is mediated by the WNK4 carboxyl terminus (Yang *et al.* 2005). We explored whether WNK3's effect on ROMK1 may similarly depend on its

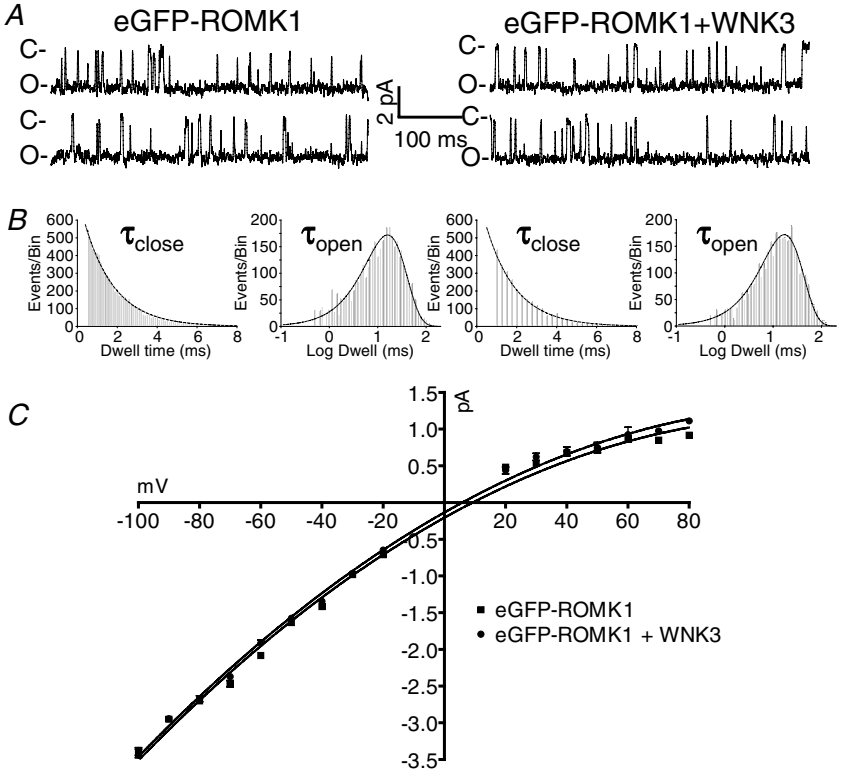


Figure 5. WNK3 does not alter the single channel properties of ROMK1
A, single-channel properties of eGFP–ROMK1 in the presence or absence of WNK3. Closed (C) and open (O) states are indicated. *B*, kinetic properties of eGFP–ROMK1 in the presence or absence of WNK3. Data for histograms were obtained from patches that displayed only one active channel. Approximately 2 min of data were used to determine the mean closed and open dwell times for eGFP–ROMK1 and eGFP–ROMK1 plus WNK3 cell-attached patches at −80 mV. For eGFP-ROMK1, the mean closed time (τ_{closed}) was 1.52 ± 0.05 ms ($n = 3$) and the mean open time (τ_{open}) was 19.3 ± 0.4 ms ($n = 3$); for eGFP–ROMK1 plus WNK3, the mean closed time (τ_{closed}) was 1.47 ± 0.04 ms ($n = 3$) and the mean open time (τ_{open}) was 18.4 ± 0.4 ms ($n = 3$). *C*, conductance properties of eGFP–ROMK1 in the presence or absence of WNK3. Current–voltage (*I–V*) relationships for eGFP–ROMK1 ± WNK3. The inward conductances were measured as 33.1 ± 1.4 pS ($n = 3$) for eGFP–ROMK1 (■), and 34.4 ± 1.2 pS ($n = 5$) for eGFP–ROMK1 plus WNK3 (●) when measured between −100 and −40 mV. These data show that WNK3 does not alter the conductance or open/closed probability of ROMK1, and suggest that ROMK1 regulates ROMK1 primarily through protein trafficking, because WNK3 does not affect the total protein expression of ROMK1 in oocyte homogenates as detected by Western blotting (data not shown).

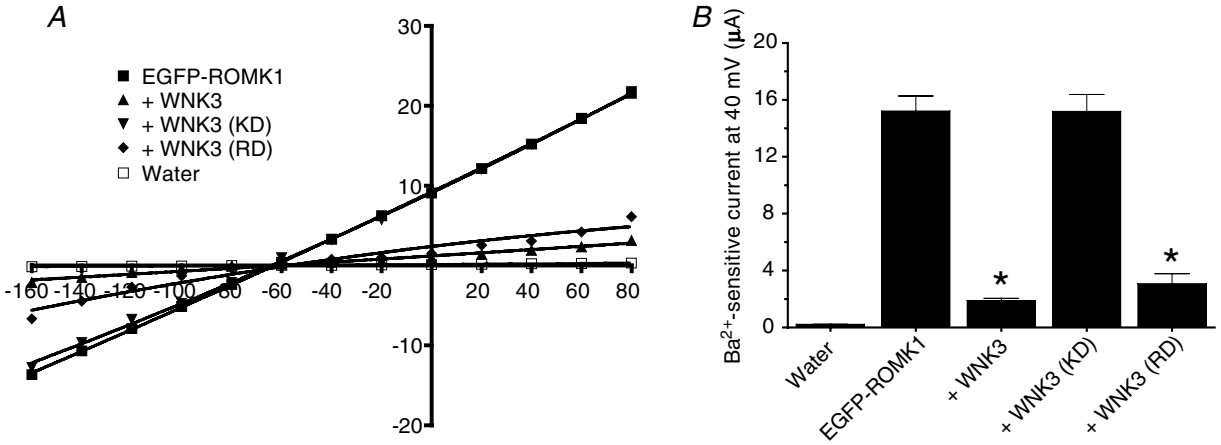
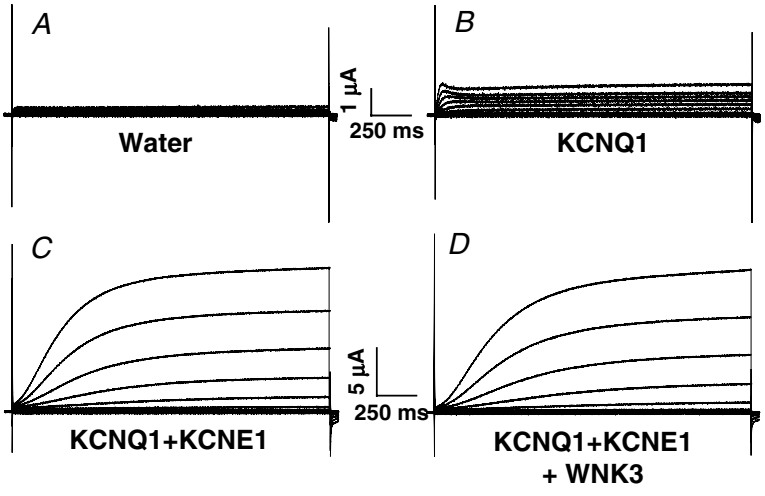


Figure 6. The carboxyl terminus of WNK3 is necessary and sufficient for its regulation of ROMK1
WNK3 mutants that contained the WNK3 kinase domain only (WNK3-KD) or the WNK3 C-terminal regulatory domain only (WNK3-RD) were constructed (see Methods). Ba^{2+}-sensitive whole-cell K^+ currents were recorded from oocytes expressing eGFP–ROMK1 alone, eGFP–ROMK1 plus WNK3, eGFP–ROMK1 plus WNK3-KD, eGFP–ROMK1 plus WNK3-RD, or a water-injected control. *A*, current–voltage (*I–V*) relationships, showing the mean ± S.E.M. of the K^+ currents for each experimental group of oocytes from a representative experiment are indicated. *B*, comparison of K^+ whole-cell currents at +40 mV in oocytes expressing the indicated proteins. Results are expressed as the mean ± S.E.M., and statistical comparisons between groups were made by a paired *t* test. Raw data of a representative experiment are shown. Note that WNK3-RD inhibited ROMK1 K^+ currents similar to wild-type WNK3. In contrast, WNK3-KD lost its ability to inhibit ROMK1 activity. These data demonstrate that WNK3's inhibition of ROMK1 is not dependent on its catalytic activity, and that WNK3's C-terminal regulatory domain is sufficient to confer the effect.



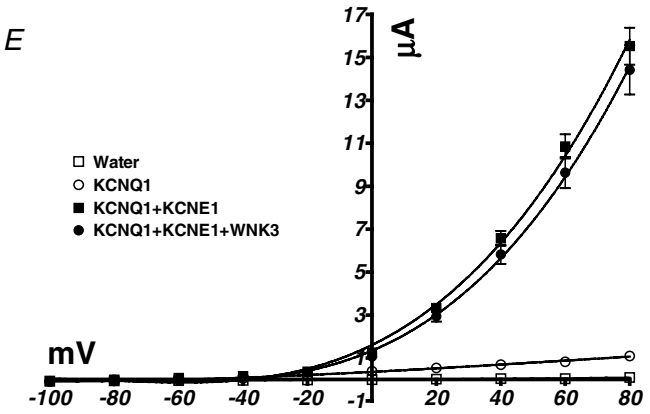


Figure 7. WNK3 does not affect the K^+ channel KCNQ1/KCNE1
Oocytes were injected with cRNAs encoding WNK3, KCNQ1, and/or KCNE1, and currents were elicited with 2-s pulses to potentials of −100 to +80 mV, applied in 20-mV increments from a holding potential of −70 mV. While WNK3 is a potent inhibitor of the kidney-specific K^+ channel ROMK1, it has no effect of the cardiac K^+ channel KCNQ1/KCNE1. These data demonstrate the specificity of WNK3's regulation for the ROMK1 K^+ channel.

C-terminal domain. To investigate this possibility, we constructed a WNK3 mutant that contains the WNK3 N-terminal kinase domain only (aa 1–417; WNK3-KD) or the WNK3 C-terminal regulatory domain only (aa 418–1742; WNK3-RD). WNK3-RD inhibited ROMK1 K^+ currents similar to wild-type WNK3 (Figs 6, $P < 0.0001$). In contrast, WNK3-KD lost its ability to inhibit ROMK1 activity (Fig. 6). These data demonstrate that the WNK3 C-terminal regulatory domain is sufficient to confer WNK3's inhibitory effect on ROMK1.

We tested the specificity of WNK3's effect on ROMK1 by investigating if WNK3 also regulates KCNQ1/KCNE1 activity in oocytes. KCNQ1, the pore-forming subunit of the KvLQT1 K^+ channel mutated in long-QT syndrome, associates with the KCNE1 (minK) subunit to produce a delayed rectifier K^+ current (I_K) responsible for repolarization in ventricular cardiomyocytes (Keating & Sanguinetti, 2001). Similar to previous reports, KCNQ1 had little activity when expressed alone, but produced large K^+ currents when coexpressed with KCNE1 (Fig. 7). In contrast to WNK3's inhibitory effect on ROMK1, WNK3 had no effect on the activity of KCNQ1/KCNE1, as seen by coexpressing these three proteins in oocytes (Fig. 7), despite robust WNK3 expression (data not shown). These data, together with the lack of effect of WNK3 on the ENaC Na^+ channel, demonstrate that WNK3 is not a general regulator of ion channels but distinctively regulates the kidney-specific K^+ channel, ROMK1.

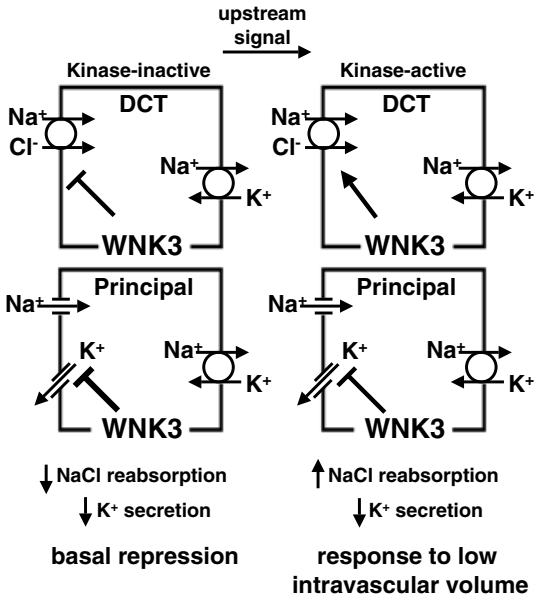


Figure 8. Model for the physiological roles of WNK3
WNK3 is capable of having opposing effects on diverse electrolyte pathways via independent mechanisms – characteristics of a molecular switch and an integrator of systems. In its active state, WNK3 could stimulate NCC-mediated NaCl reabsorption and ROMK1-mediated K^+ secretion; inactive WNK3 could simultaneously both inhibit NCC and increase ROMK1 inhibition. When activated (e.g. due to binding of a ligand), WNK3 might help restore intravascular volume while simultaneously defending serum K^+ in the presence of high circulating aldosterone. Thus, WNK3 could *separately* control two effects of aldosterone, NaCl reabsorption by NCC and K^+ secretion via ROMK1. In its inactive state, WNK3 could mediate basal repression of these NaCl reabsorption and K^+ secretion pathways. WNK3, along with WNK1 and/or WNK4, could be part of the switch mechanism that determines the balance between renal NaCl reabsorption and K^+ secretion. DCT: distal convoluted tubule. Principal: collecting duct principal cell.

Discussion

In summary, WNK3 inhibits the renal ROMK1 K^+ channel by decreasing its expression at the oocyte plasma membrane in a non-catalytic manner. WNK3 harbouring a kinase-inactivating point mutation, or a missense mutation homologous to one in WNK4 that causes disease, produces a gain-of-function effect. WNK3's action is specific: while inhibiting ROMK1, it has no effect on ENaC or paracellular ion flux (different ion transport modes important in the CD), or the cardiac K^+ channel KCNQ1/KCNE1. WNK3's effects are also unique: they are opposite to that of WNK4 at NCC, and are absent at other known WNK targets (ENaC and the paracellular pathway). The magnitude and specificity of WNK3's effects at both NCC and ROMK1, its coexpression with its target proteins in the distal nephron, and the established *in vivo* effect of WNK4 at these same targets provide evidence that WNK3's action is physiologically relevant.

Although WNK3 regulates both ROMK1 and NCC, there are considerable differences in the mechanisms of inhibition. WNK3's activation of NCC is dependent on its catalytic activity (Rinehart *et al.* 2005). Conversely, WNK3's inhibition of ROMK1 does not require WNK3's kinase activity, as a full-length kinase-dead WNK3 mutant and a WNK3 mutant containing only the C-terminal regulatory domain can still inhibit channel activity. These actions are reminiscent of WNK4 on these same targets: WNK4 inhibition of NCC requires its catalytic activity, while WNK4's inhibition of ROMK1 is independent of its kinase activity. Catalytic and non-catalytic functions in a single WNK kinase are not uncommon; WNK1 (Xu *et al.* 2005) and WNK4 (Wilson *et al.* 2003; Kahle *et al.* 2003) both have effects mediated by protein–protein interactions independent of their catalytic activities at downstream targets. WNK3's ability to regulate an electroneutral cotransporter (NCC) *and* a K^+ channel (ROMK1) with different effects (stimulation/inhibition) and mechanisms of regulation (catalytic and non-catalytic) suggest a means to achieve *independent* modulation of ROMK1 and NCC activities.

Since WNK3 alters the expression of ROMK1 at the plasma membrane, but not total ROMK1 protein expression, we hypothesize WNK3 is either enhancing retrieval of channels from the cell surface or inhibiting

the forward trafficking of newly synthesized channels to the membrane. WNK4 inhibits ROMK1 activity by increasing the clathrin-coated pit-mediated endocytosis of channels from the plasma membrane (Kahle *et al.* 2003). WNK3 could be functioning in parallel to or in series with WNK4, facilitating the internalization of K^+ channels in a clathrin-dependent manner. Conversely, WNK3 could affect forward-trafficking of ROMK1, regulating the insertion of channels into the membrane from the endoplasmic reticulum (ER) or subapical vesicle pools that contain 'dormant' channels. In this light, it is interesting that phosphorylation of serine 44 in the cytoplasmic N-terminus of ROMK1 modulates channel surface expression by increasing channel delivery to the plasma membrane consequent to the suppression of a C-terminal ER retention signal (O'Connell *et al.* 2005). This phosphorylation switch of the ROMK1 ER retention signal could provide a pool of mature and properly folded channels for rapid delivery to the plasma membrane (O'Connell *et al.* 2005). WNK3, in a manner independent of its catalytic activity, might facilitate the dephosphorylation of serine 44 or another critical serine/threonine residue to retain ROMK1 in the ER, perhaps via association with phosphatases. Indeed, catalytically inactive WNK3 decreases the phosphorylation of NKCC1 on threonines that are necessary for the plasmalemmal translocation of this cotransporter (Kahle *et al.* 2005). These issues will require future investigation.

The effects of the WNK3 kinase-inactivating point mutation on ROMK1 and NCC are intriguing, and may mimic a normal *in vivo* phenomenon, perhaps achieved by a kinase regulator binding to or dissociating from WNK3 (Fig. 8). In its active state, WNK3 could stimulate NCC-mediated NaCl reabsorption and inhibit ROMK1-mediated K^+ secretion; inactive WNK3 might simultaneously inhibit NCC and increase ROMK1 inhibition. When activated, WNK3 may be important for restoring intravascular volume while simultaneously defending serum K^+ in the presence of high circulating aldosterone – thus controlling two effects of aldosterone (NCC NaCl reabsorption and ROMK1 K^+ secretion) *separately*. In its inactive state, WNK3 may mediate basal repression of these NaCl reabsorption and K^+ secretion pathways. Identifying the proximate factors that alter WNK3 activity *in vivo*, and thereby modulate these different functional states, will be important in future work. The similar effects of the PHAII-causing mutation in WNK4 and the homologous 'PHAII-like' mutation in WNK3 compared to kinase-dead WNK3 on ROMK1 (all mutants show enhanced channel inhibition relative to wild-type kinase) suggest that mutations in the conserved acidic domain in WNKs might alter catalytic activity. Moreover, the 'availability' of the acidic domain may also be regulated by interactions with other proteins, and this interaction could alter the ability of the WNK3 kinase to regulate ROMK1.

WNK3's regulation of ion channels and transporters that are expressed in other nephron segments remains to be fully defined. For example, WNK3 is highly expressed in the proximal convoluted tubule (PCT). KCC3 and KCC4, K^+–Cl^- cotransporters in the *SLC12A* family highly expressed in the PCT, are prime candidates. Furthermore, the functional significance of WNK3 at the tight junction is unclear; while WNK4 facilitates paracellular Cl^- flux in MDCK II cells, WNK3 has no effect on paracellular ion transport in the same model system. However, in these assays, a possible effect of WNK3 on the paracellular flux of Ca^{2+} and Mg^{2+} was not assessed. In this light, it is noteworthy that WNK3 is expressed at the tight junction in the thick ascending limb (Rinehart *et al.* 2005), a nephron segment where these divalent cations are reabsorbed paracellularly.

It is likely that WNK3 is a component of a WNK1/WNK4 signalling network necessary for renal NaCl and K^+ homeostasis. Different members of the WNK family can interact, both physically and catalytically (Lenertz *et al.* 2005). For example, WNK1 has been shown to phosphorylate WNK4 and impair WNK4's regulation of NCC (Yang *et al.* 2003; Yang *et al.* 2005) and WNK1 and WNK3 have been found in the same physical complex in human cells (Vitari *et al.* 2005). The fact that WNK3 affects some downstream targets differently from other WNKs (e.g. WNK3 stimulates NCC, WNK4 inhibits NCC; WNK3 has no effect on ENaC, WNK1 stimulates ENaC), but has similar effects at other targets (both WNK3 and WNK4 inhibit ROMK1), suggests that different WNKs may be recruited individually or in combination to execute a very specific response to a discrete physiological stimulus. Moreover, the multitiered nature of the WNK signalling pathway, which includes the related STE20-family kinases OSR1 and PASK (Vitari *et al.* 2005; Gagnon *et al.* 2006), could provide a means for amplification and transduction of upstream signals into rapid and robust downstream effects.

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