WNK4 kinase is a negative regulator of K\(^{+}\)-Cl\(^{-}\) cotransporters

Tomas Garzón-Muvdi,1 Diana Pacheco-Alvarez,1 Kenneth B. E. Gagnon,2 Norma Vázquez,1 José Ponce-Coria,1 Erika Moreno,1 Eric Delpire,2 and Gerardo Gamba1

1Molecular Physiology Unit, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán and Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Mexico City, Mexico; and 2Department of Anesthesiology, Vanderbilt University Medical Center, Nashville, Tennessee

Submitted 24 August 2006; accepted in final form 16 December 2006

Garzón-Muvdi T, Pacheco-Alvarez D, Gagnon KB, Vázquez N, Ponce-Coria J, Moreno E, Delpire E, Gamba G. WNK4 kinase is a negative regulator of K\(^{+}\)-Cl\(^{-}\) cotransporters. Am J Physiol Renal Physiol 292: F1197–F1207, 2007. First published December 19, 2006; doi:10.1152/ajprenal.00335.2006.—WNK kinases [with no lysine (K) kinase] are emerging as regulators of several membrane transport proteins in which WNKs act as molecular switches that coordinate the activity of several players. Members of the cation-coupled chloride cotransporters family (solute carrier family number 12) are one of the main targets. WNK3 activates the Na\(^{+}\)-driven cotransporters NCC, NKCC1, and NKCC2 and inhibits the K\(^{-}\)-driven cotransporters KCC1 to KCC4. WNK4 inhibits the activity of NCC and NKCC1, while in the presence of the STE20-related proline-alanine-rich kinase SPAK activates NKCC1. Nothing is known, however, regarding the effect of WNK4 on the K\(^{+}\)-Cl\(^{-}\) cotransporters. Using the heterologous expression system of Xenopus laevis oocytes, here we show that WNK4 inhibits the activity of the K\(^{+}\)-Cl\(^{-}\) cotransporters KCC1, KCC3, and KCC4 under cell swelling, a condition in which these cotransporters are maximally active. The effect of WNK4 requires its catalytic activity because it was lost by the substitution of aspartate 318 for alanine (WNK4-D318A) that renders WNK4 catalytically inactive. In contrast, three different WNK4 missense mutations that cause pseudohypoaldosteronism type II do not affect the WNK4-induced inhibition of KCC4. Finally, we observed that catalytically inactive WNK4-D318A is able to bypass the tonicity requirements for KCC2 and KCC3 activation in isotonic conditions. This effect is enhanced by the presence of catalytically inactive SPAK, was prevented by the presence of protein phosphatase inhibitors, and was not present in KCC1 and KCC4. Our results reveal that WNK4 regulates the activity of the K\(^{+}\)-Cl\(^{-}\) cotransporters expressed in the kidney. intraneuronal chloride concentration; transepithelial salt absorption

THE K\(^{+}\)-Cl\(^{-}\) COTRANSPORTERS belong to the cation-coupled chloride cotransporter’s gene family of membrane proteins (SLC12). This family is divided into two branches. One branch is represented by the K\(^{-}\)-driven K\(^{+}\)-Cl\(^{-}\) cotransporters KCC1 to KCC4 and the other one by the Na\(^{+}\)-driven Na\(^{+}\)-Cl\(^{-}\), NCC, and Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporters NKCC1 and NKCC2 (18). Four genes encode isoforms of the K\(^{+}\)-Cl\(^{-}\) cotransporter. These genes are known as SLC12A4, SLC12A5, SLC12A6, and SLC12A7 and encode the K\(^{+}\)-Cl\(^{-}\) cotransporter isoforms KCC1, KCC2, KCC3, and KCC4, respectively. KCC1 exhibits ubiquitous expression and its primary role seems to be cell volume regulation. KCC2 is only present in neurons in which its activity is critical to define intraneuronal chloride concentration. KCC3 and KCC4 are expressed in several tissues, including the central nervous system and the kidney, and may play a role in several physiological processes such as transepithelial salt absorption, renal K\(^{+}\) secretion and reabsorption, myocardial K\(^{+}\) loss during ischemia, and vascular smooth muscle cell relaxation (18, 33, 37).

Several lines of evidence suggest the presence of basolateral K\(^{+}\)-Cl\(^{-}\) cotransporter systems in proximal tubule (PT) (6, 29), thick ascending limb of Henle’s loop (TALH) (3, 22), and collecting duct (CD) (43, 55), as well as in the apical membrane of the distal convoluted tubule (DCT), in which the K\(^{+}\)-Cl\(^{-}\) cotransporter plays a role in K\(^{+}\) secretion (4, 15, 45). Immunolocalization studies in the kidney have shown that KCC3 and KCC4 are present in the basolateral membrane of the proximal tubule and KCC4 is also expressed in the basolateral membrane of the TALH, DCT, and the α-intercalated cells of the CD (7, 35, 46). The distribution of KCC1 protein along the nephron has not been determined. Inactivating mutations of KCC3 are the cause of a rare neurological syndrome known as Anderman’s disease featuring agenesis of the corpus callosum with motor and sensory neuropathy, mental retardation, and psychosis (23). Targeted disruption of KCC3 in mice results in a complex neurological phenotype, without major disturbances in renal function, but with the development of arterial hypertension, probably related to a role of KCC3 in vascular smooth muscle cell relaxation (2, 8, 42). No disease has been linked to KCC4, but targeted disruption of this cotransporter gene in mice produces a syndrome of deafness and renal tubular acidosis (7).

A recently discovered family of serine/threonine kinase proteins named WNK [with no lysine (K)] has been shown to regulate the activity of several membrane proteins (19), in particular members of the SLC12 family and other Cl\(^{-}\) transport pathways (25, 26). Deletions of intron 1 of WNK1 or missense mutations in a conserved acidic region of WNK4 are the cause of a salt-dependent form of arterial hypertension known as pseudohypoaldosteronism type II (PHAII) (48), featuring also hyperkalemia and metabolic acidosis. PHAII-type mutations in WNK4 affect the WNK4-related regulation of the thiazide-sensitive Na\(^{+}\)-Cl\(^{-}\) cotransporter NCC (9, 49, 53), the apical potassium channel ROMK (28), and paracellular claudins (26, 52). In addition to NCC, WNK4 also modulates the function of other Cl\(^{-}\) transport pathways such as the basolateral Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter NKCC1 and the anion exchanger CFEX that mediates several different Cl\(^{-}\) base exchange activities (25). WNK1 modulates the activity of WNK4 (54) and also regulates ROMK (10) and the apical

Address for reprint requests and other correspondence: G. Gamba, Molecular Physiology Unit, Vasco de Quiroga No. 15, Tlalpan 14000, México City, México (e-mail: gamba@biomedicas.unam.mx or gamba@quetzal.imnss.mx).

http://www.ajprenal.org

0363-6127/07 $8.00 Copyright © 2007 the American Physiological Society

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
amiloride-sensitive Na\(^+\) channel in CD (51). Therefore, WNK kinases are emerging as important regulators of Cl\(^-\) transport pathways in both epithelial and nonepithelial cells. In the present study, we thus analyzed the effect of WNK4 on the activity of the renal K\(^+\)-Cl\(^-\) cotransporters KCC1, KCC3, and KCC4.

**METHODS**

*Xenopus laevis* oocyte preparation. Adult female *X. laevis* frogs were purchased from NASCO (Fort Atkinson, WI) and kept at the animal facility under constant control of room temperature (16°C). All animal procedures followed were in accordance with our institutional guidelines. Oocytes were surgically collected from anesthetized animals under 0.17% tricaine and incubated for 1 h under vigorous shaking in Ca\(^2+\)-free medium (10 mM K\(^+\)-gluconate, 1.0 mM Mg\(^2+\), and 5 HEPES/Tris, pH 7.4) with 1 mg/100 ml of gentamicin; this incubation medium was changed every 24 h. On the day of the influx measurement, 2 h before the uptake assay, oocytes were switched to Cl\(^-\)-free ND96 (in mM: 96 Na\(^+\) isethionate, 2 K\(^+\)-gluconate, 6.0 Ca\(^2+\)-gluconate, 1.0 Mg\(^2+\)-gluconate, 5 mM HEPES, 2.5 mM sodium pyruvate, 5 mg/ml gentamicin, pH 7.4).

Assessment of the K\(^+\)-Cl\(^-\) cotransporter function. K\(^+\)-Cl\(^-\) cotransport was assessed by measuring tracer \(^{86}\text{Rb}^+\) uptake (New England Nuclear) in experimental groups of at least 10 oocytes. Since KCC1, KCC3, and KCC4 express minimal activity under isotonic conditions (34, 35), \(^{86}\text{Rb}^+\) uptake was generally assessed in oocytes preswollen by a 30-min incubation period in a hypotonic K\(^+\)/H\(_{11001}\) medium (25) and Cl\(^-\)-free medium [in mM: 50 N-methyl-D-glucamine (NMDG)-gluconate, 4.6 Ca\(^2+\)-gluconate, 1.0 Mg\(^2+\)-gluconate, 5 HEPES/Tris, pH 7.4] with 1 mM ouabain, followed by a 60-min uptake period in a hypotonic Na\(^+\)-free medium (10 mM K\(^+\)-gluconate, 40 mM NMDG-Cl\(^-\)), 1.8 mM CaCl\(_2\), 1 mM MgCl\(_2\), 5 mM HEPES, pH 7.4), supplemented with 1 mM ouabain and 2.0 \(\mu\)Ci of \(^{86}\text{Rb}^+\). To define the amount of tracer \(^{86}\text{Rb}^+\) uptake due to the K\(^+\)-Cl\(^-\) cotransporter activity, uptake in all experimental groups was assessed in parallel in the presence or absence of extracellular chloride. When uptake in isotonic conditions was measured, the isotonic condition was generated by supplementing the incubation and uptake solutions with 3.5 g/100 ml sucrose to reach isosmolar conditions for oocytes (~210 mosmol/kgH\(_2\)O). Ouabain was added to prevent \(^{86}\text{Rb}^+\) uptake via the Na\(^+\)/K\(^+\)-ATPase. The absence of extracellular Na\(^+\) and the hyponoticity of the uptake medium prevented \(^{86}\text{Rb}^+\) uptake via the endogenous Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter that is present in oocytes (17).

All uptakes were performed at 32°C temperature. At the end of the uptake period, oocytes were washed five times in ice-cold uptake solution without isolate to remove extracellular fluid tracer. Oocytes were dissolved in 10% sodium dodecyl sulfate and tracer activity was determined for each oocyte by \(\beta\)-scintillation counting.

**cDNA constructs and mutations.** The full-length rabbit KCC1, human KCC2, human KCC3, and mouse KCC4 cDNAs are subcloned into the high expression vector pGEMHE (34, 35, 44). The full-length mouse SPAK and WNK4 kinases are inserted into the amphibian oocyte expression vector pBF and were previously described (16). Site-directed mutagenesis was performed on WNK4 cDNA to generate kinase dead WNK4 (WNK4-D318A), kinase dead SPAK (SPAK-K104R), or to introduce PHAII-type mutations (E559K, D561A, and Q562E) into the WNK4 sequence. Complementary sense and antisense oligonucleotides containing the appropriate mutations were custom made (Sigma). Mutations were performed using the Quick-Change kit following the manufacturer’s recommendations (Stratagene, La Jolla, CA). In addition, by means of double-step PCR, the sequence of the epitope tag HA was introduced in frame into the wild-type WNK4 and SPAK cDNAs. Then, using the appropriate restriction enzymes the tagged sequence was pasted into the catalytically inactive WNK4-D318A and SPAK-K104R cDNAs. To prepare cRNA, KCC1 and KCC4 cDNAs were linearized at their 3′-end with NheI, and KCC3 cDNA with NotI and transcribed in vitro using a T7 RNA polymerase mMESSAGE kit (Ambion). To prepare cRNA from the wild-type or mutant kinases WNK4 and SPAK cDNAs were linearized with MluI and transcribed in vitro using a SP6 RNA polymerase mMESSAGE kit (Ambion). Transcription product integrity was confirmed on agarose gels and concentration was determined by absorbance reading at 260 nm (DU 640, Beckman, Fullerton, CA). cRNA was stored in aliquots at −80°C until used.

**Nested RT-PCR amplification of SPAK/OSR1 from X. laevis oocytes.** External and internal primers for nested RT-PCR amplification of SPAK/OSR1 from *X. laevis* oocyte mRNA were custom made (Sigma) based on the *X. laevis* SPAK/OSR1 homolog serine theronine kinase 39 sequence deposited in the GenBank database (accession number BC077748). External primer sequences were sense 5′-TCATCAACAGGGACGACTA-3′ and antisense 5′-ATGACCTCTG-GTGCACATC-3′ and amplify a fragment of 563 bp. Internal primer sequences were sense 5′-CATCAAGAATACAACTGG-3′ and antisense 5′-GTCAGATCCATGTCACCCC-3′ and amplify a fragment of 349 bp. Total RNA from *X. laevis* oocytes was isolated using the Tripure system (Roche) following the manufacturer’s recommendations. Reverse transcription (RT) was carried out using 2.5 μg of total RNA at 37°C for 60 min in a total volume of 20 μl using 200 units of the Moloney murine leukemia virus reverse transcriptase (Invitrogen). The 563-bp fragment obtained with external primers was gel purified and used as template for the internal PCR. Products were resolved in 5% acrylamide gels.

**Western blot analysis of wild-type and mutants HA-WNK4 and HA-SPAK**. Total proteins were extracted from 10 to 15 oocytes per group by passing oocytes several times through a 0.4-mm needle syringe in lysis buffer using 4 μl per oocyte (200 mM sucrose; 0.5 mM EDTA, 5 mM Tris-HCl, pH 7.0; inhibitor protease cocktail complete). The homogenates were centrifuged at 14,000 rpm for 10 min at 4°C, and the supernatant was collected. Protein concentrations were assessed in duplicate using a Bio-Rad DC Protein assay (Bio-Rad, Hercules, CA). For Western blot analysis, 20 μg of proteins were diluted in 10 μl loading buffer and subsequently denatured by boiling for 5 min. Proteins were resolved by SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Bio- tech; 2 h at 400 mA). Prestained Rainbow markers (Amersham) were used as molecular mass standards. Nonspecific binding sites were blocked overnight at 4°C in 500 mM NaCl, 20 mM Tris-buffered saline containing 0.4% nonfat dry milk. Thereafter, membranes were incubated with 1:2,000 dilutions of a specific monoclonal anti-HA peroxidase-conjugated antibody (Sigma) diluted in blocking buffer (TTBS, 0.05% Tween 20) for 1.5 h at room temperature. Membranes were subsequently washed three times in TTBS for 10 min and immunoreactive species were detected using the ECL Plus Western blotting detection system (Amersham).

**Statistical analysis.** Statistical significance is defined as two-tailed \(P < 0.05\) and the results are presented as means ± SE. The significance of the differences between groups was tested by nonpaired \(t\)-test when two groups were compared or by one-way ANOVA with multiple comparisons using the Bonferroni correction. Although all experiments were performed at least twice, most of the observations are based on more than two experiments.
RESULTS

WNK4 reduces the activity of K⁺-Cl⁻ cotransporters in swollen oocytes. We previously showed that microinjection of *X. laevis* oocytes with KCC1, KCC3, or KCC4 cRNAs (34, 35) resulted in significant K⁺-Cl⁻ cotransport activity, compared with control oocytes that were injected with water. Activity of these K⁺-Cl⁻ cotransporters is evident, however, only when uptakes were performed under hypotonic conditions, supporting what has been demonstrated in several cell types, that the K⁺-Cl⁻ cotransporters expressed in oocytes are also activated by cell swelling (1). Figure 1 shows the combined results of several experiments in which *X. laevis* oocytes were injected with water, K⁺-Cl⁻ cotransporter cRNA alone, or together with WNK4 cRNA. Tracer ⁸⁶Rb⁺ uptake assays were performed 4 days later under hypotonic conditions in the absence or presence of extracellular chloride. Microinjection with KCC cRNA resulted in a significant increase in ⁸⁶Rb⁺ uptake over the water-injected oocytes. The values observed were KCC1 8,840 ± 434 pmol·oocyte⁻¹·h⁻¹, KCC3 24,893 ± 626 pmol·oocyte⁻¹·h⁻¹, and KCC4 18,146 ± 601 pmol·oocyte⁻¹·h⁻¹. In all cases, the level of uptake was dramatically reduced in the absence of extracellular chloride, indicating that observed uptake was due to the activity of the exogenous K⁺-Cl⁻ cotransporter. As shown in Fig. 1, coinjection of *X. laevis* oocytes with the wild-type WNK4 kinase cRNA resulted in a significant reduction of the ⁸⁶Rb⁺ uptake induced by K⁺-Cl⁻ cotransporters. KCC1 activity was reduced by 60% (KCC1+WNK4 4,274 ± 519 pmol·oocyte⁻¹·h⁻¹, *P* < 0.01 vs. KCC1 alone), KCC3 activity by 40% (KCC3+WNK4 15,592 ± 2,129 pmol·oocyte⁻¹·h⁻¹, *P* < 0.01 vs. KCC3 alone), and KCC4 activity by 55% (KCC4+WNK4 7,464 ± 552 pmol·oocyte⁻¹·h⁻¹, *P* < 0.01 vs. KCC4 alone). Thus coexpression of the K⁺-Cl⁻ cotransporters KCC1, KCC3, and KCC4 with WNK4 resulted in a significant reduction in their activity.

SPAK does not affect the activity of nonneuronal K⁺-Cl⁻ cotransporters in swollen oocytes. We showed previously that the STE20 kinase SPAK modulates the activity of other members of the cation-coupled chloride cotransporter, such as the Na⁺-K⁺-2Cl⁻ cotransporter NKCC1 (16) and interact at the protein-protein level with KCC3 (40). Moreover, KCC3 possesses what appears to be a real SPAK binding motif (RVXF) at the NH₂-terminal domain. Thus we assessed the effect of SPAK on the activity of the K⁺-Cl⁻ cotransporter in microinjected oocytes exposed to hypotonicity. As shown in Fig. 2, however, no effect of SPAK was observed. Tracer ⁸⁶Rb⁺ uptake in KCC1, KCC3, and KCC4 when injected alone was 9,248 ± 502, 24,435 ± 615, and 18,840 ± 580 pmol·oocyte⁻¹·h⁻¹, respectively, while in the presence of SPAK, the ⁸⁶Rb⁺ uptake in KCC1+SPAK, KCC3+SPAK, or KCC4+SPAK cRNA-injected oocytes was 8,319 ± 684, 25,029 ± 543, or 16,493 ± 742, respectively. The values in the presence of SPAK were not statistically different to those observed in its absence.

Presence of SPAK does not change the WNK4 inhibitory effect on K⁺-Cl⁻ cotransporters activity in swollen oocytes. We previously showed that WNK4 effect on NKCC1 is changed by the presence of SPAK (16). We thus analyzed whether SPAK could also change the type of effect that WNK4 has on the K⁺-Cl⁻ cotransporters. Experiments were designed to analyze the effect of the WNK4+SPAK combination on K⁺-Cl⁻ cotransporter activity. *X. laevis* oocytes were injected with KCC1, KCC3, or KCC4 cRNA alone, or in combination with WNK4 cRNA or WNK4 + SPAK cRNA. The combined
results of five experiments for KCC1, four experiments for KCC3, and six experiments for KCC4 are shown in Fig. 3. As shown above, coinjection of KCC cRNA with WNK4 cRNA alone resulted in significant reduction of the K⁺-Cl⁻ cotrans-

Fig. 3. Effect of wild-type SPAK on WNK4-induced inhibition of KCC activity induced by cell swelling under hypotonic conditions. X. laevis oocytes were injected with water or 0.2 μg/μl each of KCC1 (A), KCC3 (B), or KCC4 (C) cRNA alone or together with WNK4 cRNA or WNK4 cRNA and SPAK cRNA, as stated. $^{86}$Rb⁺ uptake was assessed 4 days later in hypotonic conditions (110 mosmol/kgH₂O) in the presence (open bars) or absence (filled bars) of Cl⁻ in the uptake medium. The pooled data from at least 5 different experiments are shown, with means ± SE of at least 50 oocytes for each group. No difference was observed between groups of oocytes injected with KCCs alone or together with SPAK.

Fig. 2. Effect of wild-type SPAK on KCC cotransport activity induced by cell swelling under hypotonic conditions. X. laevis oocytes were injected with water or 0.2 μg/μl each of KCC1 (A), KCC3 (B), or KCC4 (C) cRNA alone or together with SPAK cRNA. $^{86}$Rb⁺ uptake was assessed 4 days later in hypotonic conditions (110 mosmol/kgH₂O) in the presence (open bars) or absence (filled bars) of Cl⁻ in the uptake medium. The pooled data from at least 5 different experiments are shown, with means ± SE of at least 50 oocytes for each group. No difference was observed between groups of oocytes injected with KCCs alone or together with SPAK.
porter activity. Tracer $^{86}$Rb$^+$ uptake in KCC1, KCC3, and KCC4 in the presence of WNK4 was 4,341 ± 596, 16,129 ± 867, and 7,464 ± 552 pmol·oocyte$^{-1}$·h$^{-1}$, respectively. These values were significantly different from those observed in the absence of WNK4. When SPAK cRNA was added to the microinjected cocktail, the uptake observed in KCC1 was further reduced to 3,490 ± 347 pmol·oocyte$^{-1}$·h$^{-1}$. However, the difference did not reach significance. For KCC3 and KCC4 cRNA-injected oocytes, addition of SPAK cRNA did not increase the WNK4-induced inhibition of the K$^+$/Cl$^-$ cotransporter activity. Uptake observed in the presence of WNK4+SPAK for KCC3 was 14,471 ± 981 pmol·oocyte$^{-1}$·h$^{-1}$ and for KCC4 was 7,942 ± 552 pmol·oocyte$^{-1}$·h$^{-1}$. These values were not different from the uptake observed in the presence of WNK4 alone. Thus, in contrast to observations of Gagnon et al. (16) that SPAK does not change the WNK4-induced inhibition of K$^+$/Cl$^-$ cotransporter activity. Uptake observed in the presence of WNK4+SPAK for KCC4 in the presence of WNK4 was 4,341 ± 596 pmol·oocyte$^{-1}$·h$^{-1}$, and for KCC4 was 7,942 ± 552 pmol·oocyte$^{-1}$·h$^{-1}$.

Inhibition of KCCs by WNK4 is dependent on WNK4 catalytic activity. Previous studies with WNK1, WNK3, and WNK4 showed that catalytic activity of these kinases is required for regulation of some, but not for all of the WNKs target proteins. Catalytically inactive WNKs can be obtained by a single point mutation in which an aspartic acid of the kinase domain is substituted by alanine (50). The mutations D368A, D294A, and D318A have been used for WNK1 (36, 38), D368A, D294A, and D318A for WNK3 (41), and WNK4 (9, 47, 49), respectively. For instance, the ability of WNK4 to reduce the NCC activity is completely prevented by eliminating the catalytic activity of WNK4. In contrast, the inhibitory effect of WNK4 on KCC1 was significantly reduced and on KCC3 and KCC4 was completely prevented by eliminating the catalytic activity of WNK4.

Protein expression of wild-type and mutant WNK4 and SPAK in oocytes. Western blot analysis was performed to find out whether the protein expression of the wild-type and mutant forms of WNK4 and SPAK were not affected by introduction of the mutations used to generate the catalytically inactive forms or by coexpression of two kinases together. To perform these experiments, the DNA sequence encoding the HA-tag epitope was introduced in frame into the wild-type and mutant kinases. Then, oocytes were injected with KCC1, KCC3, or
KCC4 cRNA alone or together with wild-type or mutant WNK4 and/or SPAK cRNA. Three to four days later, one-half of the oocytes were used to assess functional expression by measuring $^{86}\text{Rb}^+$ uptake in the absence or presence of extracellular chloride. The other half were used to extract proteins for Western blot analysis using an anti-HA monoclonal antibody. Functional experiments exhibited similar results as those presented in Figs. 1 to 4. That is, when oocytes were incubated in hypotonic conditions, the KCC activity was inhibited by wild-type HA-WNK4, but not by wild-type HA-SPAK or the catalytically inactive HA-WNK4-D318A. The level of inhibition observed by the coinjection of wild-type HA-WNK4 together with the catalytically inactive HA-SPAK-K104R was similar to that observed by HA-WNK4 alone. Thus the presence of the HA epitope did not affect the behavior of the wild-type and mutant kinases. Figure 6 shows a representative Western blot obtained from one of these experiments from KCC4 cRNA-injected oocytes. Results were similar when proteins were extracted from experiments in which KCC1 or KCC3 was the $\text{K}^+/-\text{Cl}^-$ cotransporter cRNA injected (data not shown). As shown in Fig. 6, no protein bands were detected by anti-HA antibodies in proteins extracted from X. laevis oocytes injected with water or with KCC4 cRNA alone. A band corresponding to WNK4 molecular mass (~135 kDa) was detected in oocytes injected with HA-WNK4 cRNA. The presence and intensity of WNK4 bands were similar in oocytes injected with KCC4 plus wild-type HA-WNK4 cRNA compared with those injected with KCC4 plus HA-WNK4-D318A, HA-WNK4 and HA-SPAK, or HA-WNK4 and HA-SPAK-K104R. Thus expression of the mutant WNK4-D318A was similar to wild-type WNK4, and the presence of wild-type or mutant SPAK had no effect on WNK4 expression. In addition, a band corresponding to SPAK molecular mass (~58 kDa) was observed in oocytes injected with wild-type or mutant SPAK. The intensity of the band was similar in the absence or presence of WNK4.

**PHAII-type mutations do not affect the inhibition of KCCs by WNK4.** Missense mutations of an acidic domain of WNK4 are the cause of PHAII syndrome that features arterial hyper-
tension and metabolic acidosis among other clinical manifestations. It has been proposed that these mutations alter the way in which WNK4 regulates the activity of several membrane transport proteins. In some cases, as occurs with the inhibitory effect of WNK4 on NCC (9, 49), the mutations prevent this effect. In other cases, like the WNK4-induced inhibition of ROMK (28) or the WNK4-induced phosphorylation of claudins (26, 52), the PHAI1 mutations increased the effect on its targets. In other words, PHAJ1-type mutations can behave as “loss-of-function” or “gain-of-function” mutations (19). Because KCC4 is expressed in α-intercalated cells of the cortical collecting duct and its targeted deletion resulted in metabolic acidosis (7), we wanted to know whether the PHAI1-type mutations affected the WNK4-induced inhibition of KCC4. The results, however, as shown in Fig. 7 revealed that wild-type WNK4 reduced the activity of KCC4 to a similar degree to WNK4 harboring any of the PHAI1 type mutations E559K, D561A, or Q562E.

Catalytically inactive WNK4 activates KCC2 and KCC3 in isotonic conditions. We showed before that WNK3 in its catalytically active and inactive form is able to bypass the toxicity requirement for regulation of the cation-chloride cotransporters. For example, wild-type WNK3 activates NKCC1 cotransporter, even when oocytes are incubated in hypotonic conditions in which NKCC1 is inhibited. In contrast, catalytically inactive WNK3-D294A inhibits NKCC1, even when oocytes are incubated in hypertonic solutions in which NKCC1 is activated. Similarly, wild-type WNK3 prevents the cell swelling-induced activation of all four K⁺-Cl⁻ cotransporters, while WNK3-D294A is able to activate the four K⁺-Cl⁻ cotransporters in isotonic conditions in which they are normally inactive (11, 27, 41). Therefore, we analyzed the effect of wild-type WNK4 and the catalytically inactive WNK4-D318A on K⁺-Cl⁻ cotransporters in isotonic conditions. We first observed that in isotonic conditions, WNK4 has no effect on the K⁺-Cl⁻ cotransporter’s activity. Then, we performed experiments in which oocytes were injected with the K⁺-Cl⁻ cotransporter cRNA alone or together with WNK4-D318A cRNA, the catalytically inactive form of SPAK (SPAK-K104R) cRNA or both inactive kinases simultaneously (WNK4-D318A + SPAK-K104R). Then, 86Rb⁺ uptake was assessed by incubating the oocytes in isotonic conditions. As shown in Fig. 8, A and B, coinjection of KCC1 or KCC4 with the catalytically inactive forms of WNK4 and/or SPAK had no effect on these cotransporters activity. In contrast, coinjection of KCC2 or KCC3 with WNK4-D318A resulted in a significant increase in the Cl⁻-dependent 86Rb⁺ uptake. 86Rb⁺ uptake in oocytes injected with KCC2 or KCC3 alone was 1,003 ± 74 and 715 ± 133 pmol·oocyte⁻¹·h⁻¹, respectively, while in oocytes injected with KCC2 or KCC3 and WNK4-D318A cRNA 86Rb⁺ uptake was 5,161 ± 723 and 2,446 ± 434 pmol·oocyte⁻¹·h⁻¹, respectively. The increase was significant (P < 0.01) compared with its respective control. In addition, although 86Rb⁺ uptake was not affected by SPAK-K104R, neither in KCC2 nor in KCC3, coinjection of these cotransporters cRNA with WNK4-D318A cRNA and SPAK-K104R cRNA resulted in further increase in activity since 86Rb⁺ uptake in KCC2 oocytes increased to 13,184 ± 1,751 pmol·oocyte⁻¹·h⁻¹ (P < 0.01 vs. KCC2 + WNK4-D4A group) and in KCC3 oocytes to 4,483 ± 603 pmol·oocyte⁻¹·h⁻¹ (P < 0.01 vs. KCC3 + WNK4-D4A group). Thus our data suggest that catalytically inactive WNK4-D318A is able to induce activation of KCC2 and KCC3 in isotonic conditions, but not of KCC1 and KCC4.

The activation of KCC2 and KCC3 observed by WNK4-D318A is similar to what we previously observed with the catalytically inactive form of WNK3 (WNK3-D294A) (11) and it is also similar to what would be expected with activation of the protein phosphatases, suggesting that WNK4-D318A is also able to activate some of the endogenous protein phosphatases in the oocytes. To test this possibility, we assessed the effect of the PP1 inhibitor calcyquin A and/or the PP2B inhibitor cyclosporine A on the WNK4-D318A-induced activation of KCC2 or KCC3 in isotonic conditions. As shown in Fig. 9A, the activity of KCC2 was increased by WNK4-D318A and the increment was partially inhibited by calycycin A or cyclosporine A, and completely prevented by the combination of both inhibitors. Interestingly, the activation of KCC2 achieved by the combination of both inactive kinases WNK4-D318A and SPAK-K104R was not inhibited by calycycin A or cyclosporine A alone but was significantly decreased by the combination of both inhibitors. As Fig. 9B shows, the activation of KCC3 either by WNK4-D318A alone or both inactive kinases together was completely prevented by calycycin A.

**DISCUSSION**

In the present study, we analyzed the effect of the serine/threonine kinase WNK4 on the functional expression of the K⁺-Cl⁻ cotransporter isoforms KCC1, KCC3, and KCC4. We observed that cell swelling-induced activation of K⁺-Cl⁻ cotransporters is partially (~50%) inhibited by WNK4. The catalytic activity of WNK4 is required since the catalytically inactive WNK4-D318A, in which the aspartate 318 was substituted by alanine, lost the inhibitory effect of WNK4. The presence and/or activity of the STE20 kinase SPAK did not
affect the inhibitory effect of WNK4 on KCCs. In addition, PHA-II type mutations in WNK4 did not affect the WNK4 ability to reduce K\(^+\)-Cl\(^-\) cotransporter activity. Finally, when activity of the cotransporters was assessed in isotonic conditions, we observed that catalytically inactive WNK4 is able to bypass the tonicity requirements for activation of KCC3 (and KCC2), but not for KCC1 and KCC4.

WNK kinases are emerging as powerful regulators of several transport pathways in many tissues. Among these transport mechanisms are the cation-coupled chloride cotransporters. The hypothesis that WNK kinases are regulators of salt transport mechanisms is supported by evidence indicating that WNK kinase activity is regulated by hypertonicity and hypotonicity (31). In the kidney, WNK4 is most highly expressed in the distal nephron. In this region, the modulation of NCC and ROMK activity by WNK4 has been proposed to be critical for balancing the renal reabsorption of salt and secretion of potassium. WNK4 inhibits NCC and ROMK by different mechanisms. NCC inhibition requires the catalytic activity of WNK4 and is lost by PHAI-II-type mutations (9, 21, 49), whereas ROMK inhibition is independent of WNK4 catalytic activity and is enhanced by PHAI-II-type mutations (28). Thus it has been proposed that distinct states of WNK4 activity and regulation, presumably by aldosterone, may allow variations in WNK4 activity toward salt reabsorption and K\(^+\) secretion mechanisms, ending the kidney with the ability for K\(^+\) secretion when K\(^+\) in plasma is increased, without having to activate salt reabsorption mechanisms, or increasing salt reabsorption, when required, without having to increase K\(^+\) secretion (28). Outside the kidney, WNK4 is expressed in several polarized epithelial cells and modulates the activity of NKCC1 (16, 25).

Another member of WNK kinases with remarkable effects on SLC12 cotransporters is WNK3. This kinase possesses the ability to bypass the tonicity requirements for activation or inhibition of the cotransporters. Wild-type WNK3 activates NCC, NKCC1, and NKCC2, and inhibits all four KCCs, even during cell swelling. In contrast, the catalytically inactive WNK3-D294A inhibits NCC, NKCC1, and NKCC2, and remarkably activates KCCs, even when oocytes are incubated in isotonic conditions (11, 27, 41). Interestingly, WNK4 without catalytic activity (WNK4-D318A) loses its inhibitory effect on NCC, while WNK3 without catalytic activity not only loses its ability to activate NCC but actually turns into a powerful inhibitor of this cotransporter.

WNK1 does not seem to directly regulate the activity of SLC12 members. However, WNK1 has been proposed to be a major regulator of other WNK kinases. The WNK4-induced inhibition of NCC activity is prevented by WNK1 (53) and biochemical analysis has shown that WNK2 and WNK4 kinases can be phosphorylated and regulated by WNK1 (31). In addition, WNK kinases not only interact with each other. Recent observations indicate that WNK kinases also interact with STE20-like kinases such as SPAK (or PASK) and OSR1, which have been shown to regulate NKCC1 activity (13, 39). Gagnon et al. (16) observed that in the presence of SPAK, the effect of WNK4 on NKCC1 turned stimulatory when oocytes were incubated in isotonic conditions, but has no further effect when oocytes were exposed to hypertonicity, suggesting that WNK4+SPAK together are able to bypass the tonicity requirement for NKCC1 activation. Simultaneously, Vitari et al. (47) and Moriguchi et al. (36) using combinations of WNK1 or WNK4 with SPAK observed that NCC, NKCC1, or NKCC2 become phosphorylated at their NH\(_2\)-terminal domain only.

---

Fig. 8. Effect of catalytically inactive WNK4-D318A and SPAK-K104R on KCCs activity under isotonic conditions. X. laevis oocytes were injected with water or 0.2 μg/μl each of KCC1 (A), KCC4 (B), KCC2 (C), or KCC3 (D) cRNA alone or together with WNK4-D318A cRNA, SPAK-K104R cRNA, or both, as stated. \(^{86}\)Rb\(^+\) uptake was assessed 4 days later in isotonic conditions (220 mosmol/kgH\(_2\)O) in the presence (open bars) or absence (filled bars) of Cl\(^-\) in the uptake medium. The pooled data from 3 experiments are shown.
when both WNK and SPAK kinases were expressed together, suggesting that WNK kinases lie upstream of SPAK (20). Recently, it was shown that WNK1, but not WNK4, is able to regulate OSR1 kinase in HeLa cells (5).

In the present study, we show that WNK4 also modulates the activity of the K⁺-Cl⁻ cotransporter isoforms that are expressed outside the central nervous system. We previously observed that KCC2 is partially inhibited by WNK4 (16). The activity of KCC1, KCC3, and KCC4 is reduced by 50% when these cotransporters where coexpressed with WNK4. Thus the K⁺-Cl⁻ cotransporters are another Cl⁻ transport pathway to be regulated by WNK4. Kahle et al. (25) showed that WNK4 inhibits the activity of NKCC1, whereas Gagnon et al. (16) showed that in the presence of SPAK, WNK4 increases the NKCC1 activity. Supporting these observations, Vitari et al. (47) and Moriguchi et al. (36) presented evidence that WNK1 and WNK4 are able to phosphorylate SPAK, which in turn interacts and phosphorylates the NH2-terminal domain of NKCC1. In the present study, we observed that WNK4 is a negative regulator of the K⁺-Cl⁻ cotransporters and that this type of effect is not changed by the presence of SPAK. This conclusion is based on the following observations. Although X. laevis oocytes exhibit endogenous expression of SPAK/OSR1 (Fig. 4), the activity of KCCs was not affected by coinjection of KCc4 cRNA with SPAK cRNA (Fig. 2), the WNK4 effect on KCCs was not changed by coinjection of the KCCs with WNK4 and SPAK cRNA (Fig. 3), and dominant negative SPAK-K104R did not affect the inhibitory effect of WNK4 (Fig. 4). Thus WNK4 alone inhibits NKCC1 (25) and in the presence of SPAK activates NKCC1 (16, 36, 47). In contrast, WNK4 alone or coexpressed with SPAK inhibits all K⁺-Cl⁻ cotransporters. Therefore, WNK4 (in the presence of SPAK) activates Cl⁻ influx and inhibits Cl⁻ efflux through members of the SLC12 family. This is a similar situation to that observed previously for WNK3 that activates Cl⁻ influx pathways (NCC, NKCC1, and NKCC2) and inhibits Cl⁻ efflux pathways (KCC1 to KCC4).

Three isoforms of the K⁺-Cl⁻ cotransporters are expressed in the kidney. Immunolocalization of KCC1 along the nephron has not been addressed. At the mRNA level, KCC1 transcripts have been shown to be expressed along the nephron (12, 32). KCC3 and KCC4 are present in the PT basolateral membrane (7, 35, 46) in which their role has been proposed to be the regulation of cell volume, since large amounts of solute and water are transported by PT cells (24, 37). For instance, stimulation of the Na⁺-glucose cotransporter in the apical membrane of PT increases the load of salt and water into cells, presumably increasing cell volume, and activates a K⁺ efflux mechanism that is barium insensitive and inhibitable by 1 mM furosemide, strongly suggesting that the activated pathway is a K⁺-Cl⁻ cotransporter (6). Null mice in which KCC3 or KCC4 was disrupted, however, did not show major disturbances in renal function attributable to PT cells. It is possible that the presence of one cotransporter is enough to compensate for the absence of the other, suggesting that double knockout mice will be required to clarify the role of K⁺-Cl⁻ cotransporters in PT cells. In TALH, the presence of a K⁺-Cl⁻ cotransporter pathway has been clearly demonstrated to be present in the basolateral membrane (3, 22) and immunolocalization studies indicate that it is the KCC4 isoform. In DCT KCC4 is expressed in the basolateral membrane. However, physiological studies have also suggested the presence of an apical K⁺-Cl⁻ cotransporter (4, 14, 45). Because neither KCC3 nor KCC4 has been observed in DCT apical membrane, it is highly likely that KCC1 is the isoform responsible for such observations. Finally, KCC4 has a clear role in CD acid secretion. This isoform has not been addressed. At the mRNA level, KCC1 transcripts have been shown to be expressed along the nephron (12, 32). KCC3 and KCC4 are present in the PT basolateral membrane (7, 35, 46) in which their role has been proposed to be the regulation of cell volume, since large amounts of solute and water are transported by PT cells (24, 37). For instance, stimulation of the Na⁺-glucose cotransporter in the apical membrane of PT increases the load of salt and water into cells, presumably increasing cell volume, and activates a K⁺ efflux mechanism that is barium insensitive and inhibitable by 1 mM furosemide, strongly suggesting that the activated pathway is a K⁺-Cl⁻ cotransporter (6). Null mice in which KCC3 or KCC4 was disrupted, however, did not show major disturbances in renal function attributable to PT cells. It is possible that the presence of one cotransporter is enough to compensate for the absence of the other, suggesting that double knockout mice will be required to clarify the role of K⁺-Cl⁻ cotransporters in PT cells. In TALH, the presence of a K⁺-Cl⁻ cotransporter pathway has been clearly demonstrated to be present in the basolateral membrane (3, 22) and immunolocalization studies indicate that it is the KCC4 isoform. In DCT KCC4 is expressed in the basolateral membrane. However, physiological studies have also suggested the presence of an apical K⁺-Cl⁻ cotransporter (4, 14, 45). Because neither KCC3 nor KCC4 has been observed in DCT apical membrane, it is highly likely that KCC1 is the isoform responsible for such observations. Finally, KCC4 has a clear role in CD acid secretion. This isoform has
Because WNK4 is most highly expressed in DCT and CD, it is expected that regulation of KCCs by WNK4 will affect the function of these cotransporters in the distal nephron, adding another pathway for the WNK4-induced inhibition of K⁺ secretion. Interestingly, WNK4 inhibition of the potassium channel ROMK does not require the kinase catalytic activity (28). In contrast, WNK4-induced inhibition of K⁺-Cl⁻ cotransporters requires the WNK4 catalytic activity. This increases the diversity of regulatory possibilities by WNK4, because it is possible that WNK4 is inhibiting two different K⁺ secretory mechanism in DCT by different mechanisms. The requirement of catalytic activity of WNK4 to inhibit K⁺-Cl⁻ cotransporters suggests that WNK4 induces phosphorylation of KCCs because it is known that KCC remains inactive when these proteins are phosphorylated (1). In addition, WNK4 could also be involved in regulating urine acidification. The expression of WNK4 along the CD has been demonstrated by three studies (30, 38, 48). None of them, however, specifically shows that WNK4 is expressed at the α-intercalated cells. If this were the case, then the inhibition of KCC4 by WNK4 could decrease the H⁺ secretion by α-intercalated cells of the CD following the proposed mechanism by Boettger et al. (7). We explored the effect of PHAII-type mutations on KCC4 inhibition by WNK4 as a potential mechanism to explain part of the metabolic acidosis seen in these patients. However, our data do not support this possibility. In contrast to what has been observed for NCC (9, 49), ROMK (28), and claudins (26, 52), PHAII-type mutations in WNK4 do not affect its inhibitory properties on KCC4. Because outside the kidney WNK4 is expressed in several Cl⁻-transporting polarized epithelia (25), the regulation of KCCs in these places could play a role in transepithelial K⁺ transport.

We previously observed that eliminating the catalytic activity of WNK3 by the D294A substitution (41) switch this kinase affects members of the SLC12 family. Wild-type WNK3 activates NCC, NKCC1, and NKCC2, while the catalytically inactive WNK3-D294A completely inhibits the activity of these Na⁺-driven cotransporters (27, 41). A similar situation occurs with KCCs. Wild-type WNK3 completely inhibits the activity of all four KCC cotransporters, even when oocytes were exposed to hypotonicity in which KCCs are maximally active, whereas WNK3-D294A activates the KCC cotransporters, even when oocytes are incubated in isotonic medium during the uptake where it is known that KCCs are inactive (11). KCC activation by WNK3-D294A apparently is due to WNK3-D294A-induced activation of protein phosphatases 1 and 2B (11). Thus WNK3 has the ability to bypass the toxicity requirements for regulation of the SLC12 family members. In the present study, we explored whether a similar situation occurs by eliminating the catalytic activity of WNK4. Interestingly, we observed that KCC2 and KCC3 but not KC1 or KCC4 were activated by WNK4-D318A. When catalytically inactive SPAK-K104R cRNA was added to the coexpression cocktail, further activation of KC2 and KCC3 was observed. These effects of the catalytically inactive kinases were partially or completely prevented by the protein phosphatase inhibitors calyculin A or cyclosporine A (Fig. 9), suggesting that, as we previously observed with the catalytically inactive form of WNK3 (11), WNK4-D318A-induced increase in ⁸⁶Rb⁺ uptake by KCC2 and KCC3 is associated with activation of the protein phosphatases. Because KC1 and KCC4 were not activated in the same way, it is possible that unique sequences or motifs within KCC2 and KCC3 endow these isoforms with the ability to be activated by WNK4-D318A. Further studies will be required to clarify these possibilities.

In summary, in the present study we show that K⁺-Cl⁻ cotransporters are inhibited by WNK4, adding another Cl⁻-transport mechanism that is regulated by this kinase. WNK4 inhibits KCCs by mechanisms in which WNK4 catalytic activity is required. PHAII-type mutations do not change the effect of WNK4 on KCC cotransporters. Finally, the catalytically inactive WNK4 is able to activate KCC2 and KCC3 in isotonic conditions.

ACKNOWLEDGMENTS

We thank all members of the Molecular Physiology Unit for suggestions and assistance.

GRANTS

This work was supported in part by National Institutes of Health Grants DK-36803 and DK-64635 to G. Gamba and NS-36758 to E. Delpire.

REFERENCES

WNK4 REGULATION OF K⁺–Cl⁻ COTRANSPORTERS


