Volume sensitivity of cation-Cl\(^{-}\) cotransporters is modulated by the interaction of two kinases: Ste20-related proline-alanine-rich kinase and WNK4

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Submitted 28 January 2005; accepted in final form 26 May 2005

Volume sensitivity of cation-Cl\(^{-}\) cotransporters is modulated by the interaction of two kinases: Ste20-related proline-alanine-rich kinase and WNK4. Am J Physiol Cell Physiol 290: C134–C142, 2006. First published June 1, 2005; doi:10.1152/ajpcell.00037.2005.—In the present study, we have demonstrated functional interaction between Ste20-related proline-alanine-rich kinase (SPAK), WNK4 [with no lysine (K)], and the widely expressed Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter type 1 (NKCC1). NKCC1 function, which we measured in Xenopus laevis oocytes under both isosmotic (basal) and hyperosmotic (stimulated) conditions, was unaffected when SPAK and WNK4 were expressed alone. In contrast, expression of both kinases with NKCC1 resulted in a significant increase in cotransporter activity and an insensitivity to external osmolarity or cell volume. NKCC1 activation is dependent on the catalytic activity of SPAK and likely also of WNK4, because mutations in their catalytic domains result in an absence of cotransporter stimulation. The results of our yeast two-hybrid experiments suggest that WNK4 does not interact directly with NKCC1 but does interact with SPAK. Functional experiments demonstrated that the binding of SPAK to WNK4 was also required because a SPAK-interaction-deficient WNK4 mutant (Phe997Ala) did not increase NKCC1 activity. We also have shown that the transport function of K\(^{+}\)-Cl\(^{-}\) cotransporter type 2 (KCC2), a neuron-specific KCl cotransporter, was diminished by the expression of both kinases under both isosmotic and hyposmotic conditions. Our data are consistent with WNK4 interacting with SPAK, which in turn phosphorylates and activates NKCC1 and phosphorylates and deactivates KCC2.

bumetanide; Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter; K\(^{+}\)-Cl\(^{-}\) cotransporter; Xenopus oocytes

THE ACTIVITY OF THE THREE prototypical cation-Cl\(^{-}\) cotransporters, the Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\), K\(^{+}\)-Cl\(^{-}\), and Na\(^{+}\)-Cl\(^{-}\) cotransporters, is regulated by phosphorylation and/or dephosphorylation (for review, see Refs. 6, 7, 14, 15, 27). Although regulated in opposite directions, the Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter (NKCC) and the K\(^{+}\)-Cl\(^{-}\) cotransporter (KCC) seem to share common regulatory pathways. Indeed, factors that activate one transporter usually inhibit the other and vice versa. For example, NKCC is activated by cell shrinkage and inhibited by cell swelling, whereas KCC is activated by cell swelling and inhibited by cell shrinkage. Treatment with the protein phosphatase inhibitors calyculin A or okadaic acid inhibits KCC (8, 30) but activates Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransport (16, 24), indicating the importance of phosphorylation in the regulation of cation-Cl\(^{-}\) cotransporters.

The possibility of direct phosphorylation of NKCC type 1 (NKCC1) by PKA and PKC can be eliminated rapidly, because these kinases mediate both cotransporter stimulation and inhibition, depending on cell type (discussed in detail in Ref. 27). Several protein kinases, such as myosin light-chain kinase (13, 22), JNK (12), and Rho kinase (4), have been proposed as candidates for the activation of NKCC1 upon hyperosmotic treatment. Other kinases that are activated upon hyperosmotic stress include p38 MAPK (42), MKK3/p38 (32), and focal adhesion kinase (18). Whether any of these kinases directly phosphorylate and activate NKCC1 in vivo remains to be determined.

We recently identified a Ste20-related kinase (SPAK; or PASK or STK39) that colocalizes with NKCC1 on the salivary gland basolateral membrane and on the choroid plexus apical membrane (26). We also demonstrated that this kinase interacts with the cation-Cl\(^{-}\) cotransporters KCC3, NKCC1, and NKCC2. Interestingly, two binding motifs were identified within the NH\(_2\)-terminal tail of NKCC1, with the second site overlapping a putative protein phosphorylation site (2). Furthermore, the two SPAK-binding motifs are located upstream of three threonine residues involved in NKCC1 activation (3). However, heterologous expression of wild-type SPAK in Xenopus laevis oocytes did not affect NKCC1 activity (25, 26).

Using a yeast two-hybrid screen, we identified WNK4 [with no lysine (K)] as a putative binding partner of SPAK (25). WNK4 belongs to a family of four novel mammalian serine-threonine kinases (WNK1–WNK4) lacking a conserved lysine (K) residue in the catalytic domain (34). Mutations in WNK4 and WNK1 have been linked to pseudohypoaldosteronism type II, an autosomal dominant form of hypertension (36). Subsequent work has demonstrated that wild-type WNK4 prevents or slows down the trafficking of the Na\(^{+}\)-reabsorbing, thiazide-sensitive NCC. In addition, mutations in WNK4 were shown to disinhibit NCC trafficking, resulting in increased cell surface expression of the cotransporter and an increase in Na\(^{+}\) reabsorption (37, 40, 41). In a recent publication, Kahle et al. (11) reported that NKCC1 trafficking also was prevented by WNK4 expression. Because SPAK interacts with both NKCC1 and WNK4, we sought to examine the role of the stress kinase as a putative intermediate between WNK4 and the cotransporter. Herein we present evidence that coexpression of WNK4 and SPAK in NKCC1-injected Xenopus laevis oocytes enhanced the activity of the cotransporter under isotonic conditions and completely desensitized the cotransporter to external osmolarity. Significantly, we also show that WNK4 and SPAK produce the opposite effect on KCC2 function, indicating that the combination of the two kinases results in the phosphorylation of the cotransporters. Our data are therefore consistent with WNK4 interacting with the stress kinase, which in turn phosphorylates the cotransporters.

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EXPERIMENTAL PROCEDURES

Cloning of the mouse WNK4. Sense and antisense oligonucleotide primers were designed to amplify, by performing PCR, the open-reading frame of mouse WNK4. The two primers contained additional restriction sites for directional subcloning into several vectors. High-fidelity, long-range PCR was performed using cDNA reverse transcribed from mouse brain, Expand Long Template PCR buffer, and DNA polymerase mix (Roche Applied Science, Indianapolis, IN). After separation of the PCR reaction using 1% agarose gel electrophoresis, we gel extracted (Qiagen, Valencia, CA) the 3,852-bp band and ligated it into the TA cloning vector pGEM-Teasy (Invitrogen, Carlsbad, CA). Three independent clones were fully sequenced using the BigDye Terminator 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA), and a full-length WNK4 clone free of mutations was created from two of these clones. The full-length WNK4 fragment was inserted into the ambiphasic oocyte expression vector pBF using XhoI and Xhol and into the yeast two-hybrid vector pACTII using ApaI and Xhol.

Mutagenesis of SPAK and WNK4. Complementary sense and antisense oligonucleotides containing the codon AAG (Arg) instead of AAG (Lys) (33) were used to mutate a 491-bp fragment from mouse SPAK cDNA subcloned into pBluescript (QuickChange; Stratagene, La Jolla, CA). The parental DNA was digested with DpnI to cleave methylated GATC sequences. After DpnI treatment of the PCR reaction, a 1-μl aliquot was transformed into E. coli. Several clones were isolated to verify proper sequence and mutation. The EcoRI-EcoRV fragment was then reinserted into the original pSPAK clone in pBF. Comparison of the three-dimensional structure of WNK1 and PKA and comparison of all WNK sequences identified a conserved lysine residue within β-strand 2 of the catalytic domain (38, 39). Because this key lysine residue anchors and orients ATP, its loss resulted in less variability of the data.

Construction of EGFP-NKCC1 cDNA. The multiple cloning site of pBSK+ (Bluescript) was modified by inserting an oligonucleotide adapter between EcoRI and XhoI that contained additional restriction sites (NcoI, BglII, and NarI). The open-reading frame of enhanced green fluorescent protein (EGFP) was transferred from the Invitrogen vector pEGFP-c2 into the modified pBSK vector NcoI + BglII. Subsequently, the NH2-terminus of mouse NKCC1 between the restriction sites NarI and XhoI was added to the EGFP-pBSK clone. Finally, using EcoRI and XhoI, we reinserted the EGFP-NKCC1 fragment into the original NKCC1 clone in pBF.

RNA synthesis. All cDNA clones in pBF were linearized with Msel and transcribed into cRNA using Ambion’s mMessage MaChine SP6 transcription system (Ambion, Austin, TX). RNA quality was verified by performing gel electrophoresis (1% agarose and 0.693% formaldehyde) and then quantitated by measuring absorbance at 260 nm.

Isolation of Xenopus laevis oocytes. Stages V and VI Xenopus laevis oocytes were isolated from 12 different frogs as previously described (25, 31) and were maintained at 16°C in modified L15 medium (Leibovitz’s L15 solution diluted with water to a final osmolarity of 195–200 mosM, supplemented with 10 mM HEPES and 44 μg of gentamicin sulfate). The next day we injected 50 nl of water containing 15 ng of NKCC1 (or KCC2) cRNA and 10 ng kinase cRNA into the oocytes. Control oocytes were injected with 50 nl of water. 86Rb uptake measurements were performed 3 days postinjection. All experiments involving animals were approved by the Vanderbilt University Institutional Animal Care and Use Committee.

Assessment of mouse NKCC1 expression in oocyte plasma membranes. The surface expression of NKCC1 in the oocyte plasma membrane was measured on the basis of fluorescence using an EGFP-NKCC1 construct (see above). Individual oocytes were monitored for EGFP fluorescence using the Zeiss confocal laser-scanning microscope LSM510 (PlanApochromat; ×5 magnification, 0.16 numerical aperture lens). The excitation wavelength was set at 488 nm, and emission signals were collected using a 505-nm band-pass filter. Gain and offset were adjusted manually to contain the EGFP fluorescence signal within the 0–215 intensity range of the eight-bit gray density scale. We captured a Z stack of six optical slices near the middle of the oocyte and chose a single optical section with the largest diameter, indicative of the equatorial center of the oocyte. These settings were used to assess fluorescence of EGFP-NKCC1-injected oocytes expressing SPAK cRNA, WNK4 cRNA, or both kinase cRNA.

Western blot analysis. Xenopus laevis oocytes injected with HA-tagged WNK4, HA-tagged WNK4 (K183M), or HA-tagged WNK4 (F997A) were lysed in a buffer containing 150 mM NaCl, 10 mM Tris-Cl, pH 7.4, 1 mM EDTA, 1% Triton X-100, and protease inhibitors (20 μl/oocyte). After 10-min incubation on ice in lysis buffer, the oocytes were triturated with a pipette, incubated for an additional 15 min on ice, and then spun at 15,000 g for 15 min. An aliquot of the supernatant was saved for a Bradford protein assay (Bio-Rad Laboratories, Hercules, CA). Proteins (70 μg) were resolved by performing 7.5% SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes. Membranes were blocked for 2 h at room temperature with 5% nonfat dry milk in TBST (150 mM NaCl, 10 mM Tris-Cl, and 0.5% Tween 20 [polyoxyethylene-sorbitan monolaurate]) and then incubated overnight at 4°C with horseradish peroxidase (HRP)-conjugated monoclonal anti-HA antibody (1:1,000 dilution; Roche Applied Science) in TBST-5% nonfat dry milk. Membranes were washed extensively in TBST, and protein bands were visualized using enhanced chemiluminescence (ECL Plus; Amersham Biosciences, Piscataway, NJ).

K+ uptake in Xenopus laevis oocytes. Groups of 20 oocytes in a 35-mm dish were washed once with 3 ml of isosmotic saline (in mM: 96 NaCl, 4 KCl, 2 CaCl2, 1 MgCl2, and 5 HEPES buffered to pH 7.4) and preincubated for 15 min in 1 ml of the same isosmotic saline + 1 mM ouabain. The solution was then aspirated and replaced with either 1 ml of isosmotic or hyperosmotic solution containing 5 μCi 86Rb. An additional 60 mM sucrose was added to make the isosmotic solution hyperosmotic. Oocytes injected with KCC2 were washed and preincubated in isosmotic saline (in mM: 48 N-methyl-D-glucamine Cl- + 4 KCl, 2 CaCl2, 1 MgCl2, 90 sucrose, and 5 HEPES, pH 7.4). Hypotonic solution was identical to the isosmotic solution, except that sucrose was omitted. Two 5-μl aliquots of flux solution were sampled at the beginning of each 86Rb uptake period and used as standards. Preliminary experiments have shown that the 86Rb flux is linear during a 3-h period. Therefore, after 1 h of uptake, the radioactive solution was aspirated, and the oocytes were washed four times with 3 ml of ice-cold isosmotic or hypertonic solution. A single oocyte was transferred into glass vials, lysed for 1 h with 200 μl of 0.25 N NaOH, and neutralized with 100 μl of glacial acetic acid. Next, we measured 86Rb tracer activity using β-scintillation counting. NKCC1/KCC2 flux was expressed as nanomolar K+•oocyte−1•h−1.
Statistical analyses. Differences between groups were tested using one-way ANOVA followed by multiple comparisons using the Student-Newman-Keuls, Bonferroni, and Tukey’s posttests. Recognizing that an absence of significance between specific groups might be the result of a type II error, we conducted one-way ANOVA on a logarithmic transformation of the data. The results of these additional analyses are represented by dashed lines in the figures. P > 0.05 was considered to be nonsignificant, whereas P < 0.001 was considered to be very significant. The results are presented as means ± SE (n = 20 oocytes).

Yeast two-hybrid. Portions of PCR-amplified SPAK and NKCC1 were inserted in the yeast vector pGBDUC2 and transformed into competent PJ69-4A yeast. Yeast cells containing SPAK or NKCC1 were then transformed using full-length WNK4 inserted into pACTII. Yeast cells containing NKCC1 were also transformed with SPAK inserted into pACTII. The transformed yeast was plated onto double-dropout plates (−uracil, −leucine) to measure transformation efficiency and onto triple-dropout plates (−uracil, −leucine, −histidine) to determine protein-protein interaction. Yeast survival was assessed after 2–6 days at 30°C.

RESULTS

Three days after injection of mouse NKCC1 cRNA into Xenopus laevis oocytes, the level of K⁺ uptake measured under isosmotic conditions (~195 mosM) was two to four times greater than the level of K⁺ uptake in water-injected oocytes (Fig. 1A). In NKCC1-injected oocytes, the K⁺ uptake was significantly increased when the bath osmolarity was raised to 265 mosM, whereas in water-injected oocytes, the uptake was minimally affected by hypertonicity. Bumetanide, a potent inhibitor of NKCC1, abolished both heterologous and native NKCC1 cotransporter activity, because the level of K⁺ uptake in the presence of 20 μM bumetanide was smaller than the baseline level of uptake in water-injected oocytes. Figure 1B shows that 3 days postinjection, hyperosmotically stimulated K⁺ uptake reached ~80% of maximal uptake. Furthermore, the levels of ⁸⁶Rb⁺ uptake increased progressively with increasing amounts of injected NKCC1, saturating >10 ng of cRNA (Fig. 1C).

Coexpression of SPAK and WNK4 in water-injected oocytes resulted in a small but significant increase in K⁺ uptake, likely due to activation of the native cotransporter (Fig. 2). Expression of each kinase alone in water-injected oocytes had no effect on K⁺ uptake (data not shown). No significant difference was observed in cotransporter function under either isosmotic or hyperosmotic conditions between NKCC1-injected oocytes alone and NKCC1-injected oocytes expressing either SPAK or WNK4 (Fig. 2). When SPAK and WNK4 were coexpressed in NKCC1-injected oocytes, a significant increase in K⁺ uptake was observed under isosmotic conditions. This increase was observed in 20 separate experiments performed with 12 different frogs. The hyperosmotically stimulated activity observed in NKCC1-injected oocytes was reduced by a small but significant amount upon coexpression of both wild-type NKCC1 and NKCC1 with a point mutation in its activation loop (Fig. 2).

Fig. 1. Na⁺⁻K⁺⁻2Cl⁻ cotransporter type 1 (NKCC1) function in Xenopus laevis oocytes. A: under isosmotic conditions, K⁺ uptake measured with ⁸⁶Rb⁺ as a tracer is significantly greater in NKCC1-injected oocytes compared with water-injected oocytes. The marked increase in NKCC1 activity associated with hypertonicity was completely inhibited by 20 μM bumetanide (bumet.). Note the existence of a small native NKCC1 component that was not significantly activated in hyperosmotic conditions. B: hypertonicity-induced K⁺ uptake increased progressively after NKCC1 injection and reached a plateau after 4 days. C: K⁺ uptake was dependent on the amount of NKCC1 injected, with a rapid increase associated with concentrations ranging from 1 to 5 ng of cRNA and saturation at concentrations >10 ng of cRNA. Bars in A and data points in B and C represent means ± SE; n = 20 oocytes.

Fig. 2. Coinjection of Ste20-related proline-alanine-rich kinase (SPAK) and WNK4 [with no lysine (K)] enhanced NKCC1 activity. NKCC1 (15 ng) or water was injected into Xenopus laevis oocytes together with different combinations of SPAK (10 ng) and WNK4 (10 ng). K⁺ uptake was measured under isosmotic (195 mosM) and hyperosmotic conditions (265 mosM); Incubation with 20 μM bumetanide (BUM) confirmed that SPAK and WNK4 were affecting NKCC1. Bars represent means ± SE; n = 20 oocytes. Each experimental condition was repeated twice with similar results. Absolute K⁺ uptake values varied from experiment to experiment.

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type SPAK and wild-type WNK4. The addition of 20 μM bumetanide reduced K⁺ uptake below the level of water-injected oocytes in both isosmotic and hyperosmotic conditions, indicating that the increase in K⁺ uptake was solely a result of NKCC1 activity.

To determine whether expression of the kinases affected the cell surface expression of the cotransporter, we examined the level of EGFP-NKCC1 signal at the membrane using confocal microscopy. This method was previously used to assess cell surface expression of the cotransporter, we examined the result of NKCC1 activity.

Membrane fluorescence was observed 3 days postinjection using confocal laser microscopy. Far left column, 5 individual EGFP-NKCC1-injected oocytes; middle left column, EGFP-NKCC1-injected oocytes expressing SPAK; middle right column, EGFP-NKCC1-injected oocytes expressing both SPAK and WNK4. All images were captured using identical confocal microscopic settings (see EXPERIMENTAL PROCEDURES). The experiment was performed twice with identical results. B: EGFP-NKCC1 (15 ng) was injected into Xenopus laevis oocytes together with different combinations of SPAK (10 ng) and WNK4 (10 ng). K⁺ uptake was measured in isosmotic (195 mosM) and hyperosmotic conditions (265 mosM). Bars represent means ± SE; n = 20 oocytes. Experiment was repeated once with identical results.

Two different types of solutions were used to examine the effect of SPAK and WNK4 on NKCC1 volume sensitivity. The isosmotic solution containing 96 mM NaCl was made hyperosmotic with the addition of sucrose. As shown in Fig. 4A, NKCC1 activity is sensitive to small increases in osmolarity. However, in the presence of SPAK and WNK4, NKCC1 is activated under isosmotic conditions and remains activated with increases in osmolarity. In native tissues and in heterologous expression systems, NKCC1 activity is strongly inhibited by hypotonicity. To determine whether the SPAK and WNK4 stimulation of NKCC1 activity could be shut off by hypotonicity, we modified our solutions to contain only 48 mM NaCl such that in the absence of sucrose, the solution had an osmolarity of −110 mosM. Sucrose at concentrations of 90 and 150 mM were added to create isosmotic (195 mosM) and hyperosmotic (265 mosM) solutions, respectively. Thus K⁺ uptake was measured at three different osmolarities without changing the external Na⁺, K⁺, and Cl⁻ concentrations. As anticipated, hypotonicity abolished cotransporter activity in oocytes injected with NKCC1 alone. However, NKCC1-injected oocytes coexpressing wild-type SPAK and wild-type WNK4 exhibited stimulated cotransporter activity even under hypotonic conditions (Fig. 4B).

Fig. 3. Cell surface expression and function of enhanced green fluorescent protein (EGFP)-NKCC1 in oocytes. A: oocytes were injected with 15 ng of EGFP-NKCC1 cRNA and 10 ng of kinase cRNA. Membrane fluorescence was observed 3 days postinjection using confocal laser microscopy. Far left column, 5 individual EGFP- NKCC1-injected oocytes; middle left column, EGFP-NKCC1-injected oocytes expressing SPAK; middle right column, EGFP-NKCC1-injected oocytes expressing both SPAK and WNK4. All images were captured using identical confocal microscopic settings (see EXPERIMENTAL PROCEDURES). The experiment was performed twice with identical results. B: EGFP-NKCC1 (15 ng) was injected into Xenopus laevis oocytes together with different combinations of SPAK (10 ng) and WNK4 (10 ng). K⁺ uptake was measured in isosmotic (195 mosM) and hyperosmotic conditions (265 mosM). Bars represent means ± SE; n = 20 oocytes. Experiment was repeated once with identical results.

Fig. 4. Loss of NKCC1 volume sensitivity. A: K⁺ uptake was measured in solutions containing 96 mM NaCl and various sucrose concentrations to reach osmolarities ranging from 195 to 265 mosM. B: K⁺ uptake determined in solutions containing 48 mM NaCl and various sucrose concentrations. Absence or addition of sucrose resulted in hyposmotic (~110 mosM) or hyperosmotic solutions (~265 mosM). Closed circles in A and B represent uptake in oocytes into which NKCC1 was injected, whereas closed triangles in A represent oocytes into which water was injected. Open squares in A and B represent uptake in oocytes into which NKCC1 was injected that coexpressed SPAK and WNK4. Note that NKCC1 activity was markedly inhibited in hyposmotic conditions and that the presence of SPAK and WNK4 rendered NKCC1 activity insensitive to changes in osmolarity. Data points represent means ± SE; n = 20 oocytes. Each experimental condition was performed a minimum of 3 times. Data are different from those presented in Figs. 2 and 5.
To determine whether the catalytic activities of SPAK and WNK4 were both required for NKCC1 activation, we mutated the key lysine residue involved in the interaction with the α- and β-phosphates of ATP in both kinases (see Mutagenesis of SPAK and WNK4). Because WNK4 catalytic activity in vitro has not yet been demonstrated (35), the K183M mutant is referred to as nonfunctional rather than catalytically inactive. Expression of catalytically inactive SPAK or WNK4 (K183M) in NKCC1-injected oocytes resulted in a small but significant change in K⁺ uptake under isosmotic conditions. Coexpression of catalytically inactive SPAK with wild-type WNK4 or coexpression of wild-type SPAK with WNK4 (K183M) eliminated the two- to fourfold stimulation of NKCC1 activity observed in the presence of both wild-type SPAK and wild-type WNK4 under isosmotic conditions (Fig. 5). A putative SPAK-binding motif (RFxV), previously identified in cation-Cl⁻ cotransporters (25), was found within WNK4. Previous yeast two-hybrid studies had shown that mutating the phenylalanine residue within this motif into an alanine prevented SPAK interaction (26). Therefore, we mutated phenylalanine-997 of WNK4 into an alanine and demonstrated that under isosmotic conditions, coexpression of WNK4 (F997A) with wild-type SPAK in NKCC1-injected oocytes resulted not in cotransporter stimulation but in a small but significant inhibition (Fig. 5). To eliminate the possibility that WNK4 expression at the protein level was affected mutating the kinase (K183M or F997A), we used Western blot analysis to assess protein expression of HA-tagged wild-type WNK4, HA-tagged WNK4 (K183M), and HA-tagged WNK4 (F997A). No significant difference in the amount of protein was observed between NKCC1-injected oocytes expressing wild-type WNK4 or either WNK4 mutant (see Fig. 5, inset).

To further examine the interaction of SPAK with NKCC1, we coexpressed wild-type SPAK and wild-type WNK4 in oocytes into which two mutant forms of the cotransporter were injected. As anticipated, the NKCC1 mutant that was missing a segment containing both SPAK-binding motifs (26) was not activated in the presence of SPAK and WNK4. However, coexpression of wild-type SPAK and wild-type WNK4 in oocytes into which the second NKCC1 mutant was injected, which contained alterations in each of the two SPAK-binding motifs, exhibited activity similar to that of NKCC1-injected oocytes that coexpressed SPAK and WNK4. This finding was unexpected, because yeast two-hybrid analyses indicated that the binding motifs were necessary for SPAK-NKCC1 interaction (Fig. 5).

The two kinases had different effects on hypertonically stimulated NKCC1 activity. Expression of catalytically inactive SPAK, but not of WNK4 (K183M), resulted in significant inhibition of hyperosmotic cotransporter activity (Fig. 5). Likewise, coexpression of catalytically inactive SPAK with wild-type WNK4, but not coexpression of WNK4 (K183M) with wild-type SPAK, significantly inhibited cotransporter activity. In addition, prevention of the protein-protein interaction between SPAK and WNK4 by coexpressing WNK4 (F997A) with wild-type SPAK in NKCC1-injected oocytes did not alter the hyperosmotic stimulation of the cotransporter. In accordance with Figs. 2 and 4B, coexpression of wild-type SPAK and wild-type WNK4 in NKCC1-injected oocytes reduced hyperosmotic cotransporter activity to the same level observed under isosmotic conditions (Fig. 5).

We had previously shown that SPAK interacts with both the COOH-terminal regulatory domain of WNK4 and the NH₂-terminal region of NKCC1 (26). To establish a possible direct interaction of WNK4 with NKCC1, we performed yeast two-hybrid analysis between WNK4 and the NH₂ and COOH termini of NKCC1. Our data show no direct interaction between WNK4 and either cytosolic tail of the cotransporter (Fig. 6, A and B). To eliminate the possibility that either GAL4-WNK4 or GAL4-NKCC1 fusion proteins were not expressed properly, we tested each with SPAK. Figure 6, C and D, demonstrates an absence of interaction between SPAK and the COOH terminus of NKCC1 but a positive interaction of SPAK with the NH₂ terminus of the cotransporter. We subcloned the WNK4 (F997A) mutant into the yeast vector pACTII and, as demonstrated in Fig. 6E, observed a positive interaction between SPAK and full-length WNK4, but no protein-protein interaction between SPAK and the WNK4 (F997A) mutant (Fig. 6F).

We tested the effect of coexpression of SPAK and WNK4 on KCC2 activity in Xenopus laevis oocytes, because the activation and/or inhibition of the NKCCs and the KCCs seemed to share common regulatory pathways. To eliminate any ⁸⁶Rb (K⁺) uptake through endogenous NKCC1, K⁺ uptake was measured in a Na⁺-free solution with N-methyl-D-glucamine used as the replacement cation. In isosmotic conditions, no significant difference in K⁺ uptake was observed between oocytes injected with KCC2 alone and KCC2-injected oocytes expressing either wild-type SPAK or wild-type WNK4 (Fig. 7). When we coexpressed both kinases in KCC2-injected oocytes, we observed a small but significant inhibition of KCC2 activity. Coexpression of WNK4 (K183M) with wild-type SPAK in KCC2-injected oocytes did not alter K⁺ uptake.
under both isosmotic and hyposmotic conditions (Fig. 7). In contrast, when catalytically inactivated SPAK was expressed with wild-type WNK4 in KCC2-injected oocytes, K⁺ uptake levels were markedly stimulated. In hyposmotic conditions, KCC2-mediated K⁺ uptake was not altered by the expression of wild-type SPAK alone but was reduced by 30% in the presence of wild-type WNK4. Coexpression of both kinases reduced KCC2 activity to levels observed under isosmotic conditions. When WNK4 (K183M) was coexpressed with wild-type SPAK alone but was reduced by 30% in the presence of wild-type WNK4. Coexpression of both kinases reduced KCC2 activity to levels observed under isosmotic conditions. When WNK4 (K183M) was coexpressed with active SPAK, the hyposmotic activation of KCC2 was unaffected; however, when inactivated SPAK was coexpressed with wild-type WNK4, we observed an 40% reduction in the K⁺ uptake observed between NKCC1-injected oocytes alone vs. NKCC1-injected oocytes that expressed wild-type WNK4 under either isosmotic or hyposmotic conditions. These data suggest that the trafficking of the cotransporter to the plasma membrane of Xenopus laevis oocytes was unaffected by the published WNK4 (AY-187027) or the mouse brain isoform that we cloned, which contained an alternative 3' end sequence (data not shown). At this stage, we cannot explain why the same cotransporter (NKCC1, although human vs. mouse), the same kinase (WNK4), and the same expression system (Xenopus laevis oocytes) would yield such diametrically opposite results. In fact, we have demonstrated herein that coexpression of WNK4 and SPAK significantly activated NKCC1 under isosmotic conditions.

The effect of SPAK and WNK4 on NKCC1 function could be a result of 1) increased trafficking of NKCC1 or 2) activation of existing cotransporters at the cell surface. Expression of a functional EGFP-tagged NKCC1 argues against changes in

DISCUSSION

The activity of NKCC1 closely follows the phosphorylation state of the cotransporter (19). Previous studies have identified two kinases involved in the regulation of cation-CI⁻ cotransport activity (25, 26). The first kinase, SPAK, a mammalian serine-threonine kinase related to yeast Ste20 kinases, was identified using a yeast two-hybrid screen (26). Although heterologous expression of wild-type SPAK in Xenopus laevis oocytes failed to affect NKCC1 function (25) (Fig. 2), expression of catalytically inactive SPAK resulted in a decrease of stimulated NKCC1 activity in both human embryonic kidney HEK-293 cells (5) and oocytes (Fig. 5). The second kinase, WNK4, a mammalian serine-threonine kinase lacking a conserved lysine (K) residue in the catalytic domain (34) was identified through mutations leading to increased Na⁺ reabsorption in kidney (37, 40). Of interest is the demonstration, through yeast two-hybrid analysis, that SPAK and WNK4 are binding partners (25).

Cell surface expression of the thiazide-sensitive NCC has been shown to be diminished upon heterologous expression of WNK4, an observation purported to be the basis for increased Na⁺ reabsorption in distal convoluted tubules and increased blood pressure in patients with mutations in the WNK4 gene (37, 40). In a recent report, Kahle et al. (11) demonstrated that WNK4 also interfered with the trafficking of NKCC1 to the cell surface of Xenopus laevis oocytes. However, our yeast two-hybrid analysis indicated no protein-protein interaction between wild-type WNK4 and NKCC1 (Fig. 6). In addition, our experiments demonstrated no significant difference in the K⁺ uptake observed between NKCC1-injected oocytes alone vs. NKCC1-injected oocytes that expressed wild-type WNK4 under either isosmotic or hyposmotic conditions.

The effect of SPAK and WNK4 on NKCC1 function could be a result of 1) increased trafficking of NKCC1 or 2) activation of existing cotransporters at the cell surface. Expression of a functional EGFP-tagged NKCC1 argues against changes in
cell surface expression of the cotransporter by either of the kinases (Fig. 3, A and B). Furthermore, if coexpression of SPAK and WNK4 in NKCC1-injected oocytes resulted in increased cotransporter insertion in the plasma membrane, then under hyperosmotic conditions, a larger K⁺ uptake would be measured compared with oocytes injected with NKCC1 alone. In fact, we observed the opposite: a small but significant decrease in cotransporter activity (shown in Figs. 2, 4B, and 5). Therefore, it is likely that quiescent cotransporters expressed at the cell surface are activated by the combination of the two interacting kinases. To determine whether the catalytic activity of each kinase is required for cotransporter stimulation, we mutated the catalytic domain of each kinase. For SPAK, we used a mutation (K104R) known to render the kinase inactive (33). Our results clearly demonstrate that SPAK kinase activity is required, because the K104R mutant interfered with the stimulation of the cotransporter under both isotonic and hypertonic conditions. Previous studies have shown that substituting a methionine for a lysine residue at position 233 renders WNK1 catalytically inactive (38). Because the catalytic activity of WNK4 has not yet been demonstrated successfully in vitro (35, 41), whether the same substitution (K183M) in WNK4 affects its catalytic activity is unknown. That no cotransporter stimulation was observed with WNK4 (K183M) suggests that the K183M mutation in the catalytic domain presumably affects the kinase activity of WNK4. However, the lack of any effect of our K183M mutation could also be the result of abnormal protein expression. Our Western blot analysis data (see Fig. 5, inset) argue against this possibility, because equivalent protein expression levels were observed in Xenopus laevis oocytes injected with either wild-type WNK4 or WNK4 (K183M). In addition to the necessity for SPAK and WNK4 to be functional, our results with regard to the WNK4 (F997A) mutant indicate that the protein-protein interaction between the two kinases appears to be necessary for cotransporter stimulation under isomotic conditions as well as for the cotransporter inhibition under hyperosmotic conditions. Taken together, our results clearly demonstrate that the stress kinase serves as a putative intermediate between WNK4 and NKCC1 (Fig. 5).

We previously suggested that SPAK serves as a scaffold for other proteins, including WNK4 (25). Our findings allow us to propose a model, shown in Fig. 8B, in which WNK4 affects NKCC1 activity through its interaction with SPAK. This model is supported by a recent finding that SPAK serves as a link between PKC-δ and activator protein 1 (AP-1) activation in the T-cell receptor response. Li et al. (17) demonstrated that the phosphorylation of SPAK required a physical interaction with PKC-δ and that this complex was necessary for SPAK activation of AP-1. Whether AP-1 activation requires the binding of the PKC-δ-SPAK complex to the transcription factor was not addressed in their article. In our system, whether NKCC1 phosphorylation and/or activation requires the binding of the WNK4-SPAK complex (Fig. 7B) has not yet been resolved. The divergent data obtained with the NKCC1 SPAK-deficient and deletion mutants provide interesting clues. The NH₂-terminal region missing in the deletion mutant comprises not only the two SPAK binding motifs but also a stretch of alanine residues that could link the cotransporter to the cytoskeleton. Because yeast two-hybrid and coimmunoprecipitation experiments demonstrated a direct interaction between SPAK and NKCC1, it is possible that there are two separate pools of SPAK: one anchored to the cotransporter that does not participate in its phosphorylation but that might be involved in cell signaling (25) and another associated with WNK4 in the vicinity of the cotransporter, which participates in its activation (Fig. 8B). That SPAK interacts with both NKCC1 and WNK4 through identical binding domains (RFxV) is consistent with the hypothesis regarding the existence of two pools of SPAK. Indeed, it is difficult to conceive that one SPAK molecule could bind simultaneously to both NKCC1 and WNK4 using the same interaction site.

Fig. 8. Models representing WNK4 and SPAK kinases. A: schematic showing WNK4 (1,222 amino acids) and SPAK (556 amino acids). Alignment reveals only 23% homology between the SPAK and WNK4 kinase domains. The position of the domain in each kinase involved in protein-protein interaction (P) is also indicated. The SPAK-binding motif starting with RFQV is located in the COOH terminus of WNK4. Phenylalanine (F997) was mutated into alanine to prevent interaction. The extreme COOH terminus of WNK4. Phenylalanine (F997) was mutated into alanine to prevent interaction. The extreme COOH terminus of WNK4. Phenylalanine (F997) was mutated into alanine to prevent interaction. The extreme COOH terminus of WNK4. Phenylalanine (F997) was mutated into alanine to prevent interaction.

B: representation of a macromolecular complex comprising NKCC1, SPAK, WNK4, and the cytoskeleton. Solid arrows represent putative phosphorylation pathways. Dashed arrow represents cotransport of Na⁺, K⁺, and Cl⁻.
Hyperosmolarity stimulates NKCC1 in a wide variety of native tissues (for review, see Ref. 27). Although native amphibian NKCC1 appeared to be volume insensitive in the present study (Fig. 2), heterologous expression of mouse NKCC1 was stimulated three- to fourfold by hypertonicity (Figs. 1A, 2, 4B, and 5). This indicates that Xenopus laevis oocytes express native kinases that can phosphorylate and activate the cotransporter. Osmotic activation of NKCC1 was not affected by overexpression of either wild-type SPAK or wild-type WNK4 alone (Fig. 2). Moreover, expression of WNK4 (K183M), either alone or in the presence of wild-type SPAK, did not affect the hypertonic stimulation of the cotransporter, suggesting that WNK4 is not part of the hypertonic pathway. In contrast, overexpression of catalytically inactive SPAK in NKCC1-injected oocytes markedly reduced the hyperosmotic stimulation of the cotransporter (Fig. 5), demonstrating that SPAK was directly involved in the hypertonic stimulation of the cotransporter. In addition, the reduction of the hypertonicity-stimulated NKCC1 activity by overexpressing both wild-type kinases also indicates that the SPAK-WNK4 complex interfered with the osmotic sensitivity of the cotransporter. That we observed only a partial reduction indicates that hypertonic regulation of NKCC1 must utilize other components. For instance, if hypertonicity-stimulated NKCC1 activity were the result of protein phosphatase inhibition and the SPAK-WNK4 complex were to release this inhibition, then coexpression of SPAK and WNK4 in NKCC1-injected oocytes would yield identical cotransporter activity, regardless of osmolarity. In fact, we observed identical NKCC1 activity in the presence of both wild-type kinases under both isosmotic and hyperosmotic conditions. Moreover, if SPAK were acting as a scaffold for WNK4, bringing it into proximity to the phosphatase, then regardless of its catalytic activity, the hypertonic inhibition of the protein phosphatase would be released. Indeed, even in the presence of catalytically inactive SPAK, overexpression of wild-type WNK4 resulted in decreased activity of NKCC1. Furthermore, when inactive SPAK was expressed together with wild-type WNK4, the inhibitory effect was amplified as would be anticipated if inactive SPAK were also to compete with a native kinase phosphorylating NKCC1. Alternatively, the hypertonic stimulation of NKCC1 could involve an as yet unidentified kinase, or SPAK itself could be volume sensitive and activate in a WNK4-independent manner upon cytoskeletal rearrangement under osmotic stresses. The role of actin filaments in the regulation of NKCC1 activity has long been recognized (9, 20). In a recent report, Di Ciano-Oliveira et al. (4) showed that small GTPases of the Rho family mediate K⁺ flux. Previous work has focused on identifying a regulator of ion transport and human disease.

GRANTS

This work was supported by a Grant-In-Aid from the National American Heart Association and by National Institute of Neurological Disorders and Stroke Grant NS-36758.

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