Regulation of NKCC2 by a chloride-sensing mechanism involving the WNK3 and SPAK kinases

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The Na^+:K^+:2Cl^- cotransporter (NKCC2) is the target of loop diuretics and is mutated in Bartter's syndrome, a heterogeneous autosomal recessive disease that impairs salt reabsorption in the kidney's thick ascending limb (TAL). Despite the importance of this cation/chloride cotransporter (CCC), the mechanisms that underlie its regulation are largely unknown. Here, we show that intracellular chloride depletion in *Xenopus laevis* oocytes, achieved by either coexpression of the K-Cl cotransporter KCC2 or low-chloride hypotonic stress, activates NKCC2 by promoting the phosphorylation of three highly conserved threonines (96, 101, and 111) in the amino terminus. Elimination of these residues renders NKCC2 unresponsive to reductions of $[Cl^-]_i$. The chloride-sensitive activation of NKCC2 requires the interaction of two serine-threonine kinases, WNK3 (related to WNK1 and WNK4, genes mutated in a Mendelian form of hypertension) and SPAK (a Ste20-type kinase known to interact with and phosphorylate other CCCs). WNK3 is positioned upstream of SPAK and appears to be the chloride-sensitive kinase. Elimination of WNK3's unique SPAK-binding motif prevents its activation of NKCC2, as does the mutation of threonines 96, 101, and 111. A catalytically inactive WNK3 mutant also completely prevents NKCC2 activation by intracellular chloride depletion. Together these data reveal a chloride-sensing mechanism that regulates NKCC2 and provide insight into how increases in the level of intracellular chloride in TAL cells, as seen in certain pathological states, could drastically impair renal salt reabsorption.

ion transport | loop of Henle | protein serine-threonine kinases | hypertension | diuretics

The renal-specific Na^+:K^+:2Cl^- cotransporter (NKCC2) is the major salt transport pathway of the apical membrane of the mammalian thick ascending limb (TAL) of Henle's loop. The activity of NKCC2 is critical for salt reabsorption, countercurrent multiplication, acid-base regulation, and divalent mineral cation metabolism (1). NKCC2 is the main pharmacological target of loop diuretic drugs used worldwide for the treatment of edematous states. The fundamental role of NKCC2 in human physiology and blood pressure regulation has been established by the finding that inactivating mutations in *SLC12A1*, the gene encoding NKCC2, causes Bartter syndrome type I (2), an autosomal recessive syndrome featuring severe volume depletion, hypokalemia, metabolic alkalosis, and hypercalciuria.

NKCC2 is an electroneutral cation-coupled chloride cotransporter (CCC) in the *SLC12* gene family that contains seven members encompassing two different branches. The sodium-driven branch (Na-[K]-Cl) comprises the thiazide-sensitive Na^+:Cl^- cotransporter NCC and two different isoforms of the Na^+:K^+:2Cl^- cotransporter—the ubiquitous NKCC1 and the kidney-specific NKCC2. The potassium-driven branch (KCCs) comprises four different K^+-Cl^- cotransporters, KCC1–KCC4. Studies have shown that CCCs are regulated by intracellular chloride concentration $[Cl^-]_i$ by means of phosphorylation and dephosphorylation events. Phosphorylation activates Na-[K]-CCs and inhibits KCCs, whereas dephosphorylation has the opposite effect (3–5). Extensive work performed with NKCC1 (6–11) has established that cotransporter activation by low intracellular chloride is associated with phosphorylation of specific threonines in its amino terminus. This phosphorylation appears to be due to the activation of a kinase, rather than the inhibition of a protein phosphatase; the existence of a kinase whose activity is modulated by $[Cl^-]_i$ has been proposed to account for these phenomena (12).

In recent years, serine/threonine kinases from two different gene families have been shown to fit the profile of "Cl^--sensing kinases." One is the WNK family, from which two members (WNK1 and WNK4) are mutated in pseudohypoaldosteronism type II, a Mendelian form of human hypertension. WNK3 increases the activity of the sodium-driven cotransporters Na-[K]-CCs, promoting Cl^- influx, but decreases the activity of the potassium-driven cotransporters KCC1–KCC4 to prevent Cl^- efflux. Through these reciprocal actions on cellular Cl^- influx and efflux pathways, WNK3 regulates the level of $[Cl^-]_i$ (13–15). The Ste20-type kinases SPAK/OSR1 become phosphorylated in response to decreases in $[Cl^-]_i$ and also regulate the activity of NKCC1 (12, 16–19). A link between these two different kinase families has been established by the finding that WNK1 and WNK4 interact with and phosphorylate SPAK/OSR1, which enables SPAK/OSR1 to physically associate with, phosphorylate, and activate NKCC1 (20–22).

Here, we show NKCC2 is regulated by $[Cl^-]_i$ and that phosphorylation of two conserved threonines in its amino terminal domain is involved. Activation of NKCC2 by intracellular chloride depletion requires an interaction between WNK3 and SPAK; WNK3 is positioned upstream of SPAK and appears to be the chloride-sensitive kinase. These observations define a regulatory pathway for NKCC2 and provide insight into how increases in $[Cl^-]_i$ in Bartter syndrome type III, which results from inactivating mutations in the TAL's basolateral Cl^- efflux channel CLC-KB, could lead to the inhibition of NKCC2 activity.

Results

NKCC2 Activity and Phosphorylation Are Increased by Intracellular Chloride-Depletion Maneuvers. We evaluated whether NKCC2 is regulated by $[Cl^-]_i$ by employing two experimental approaches

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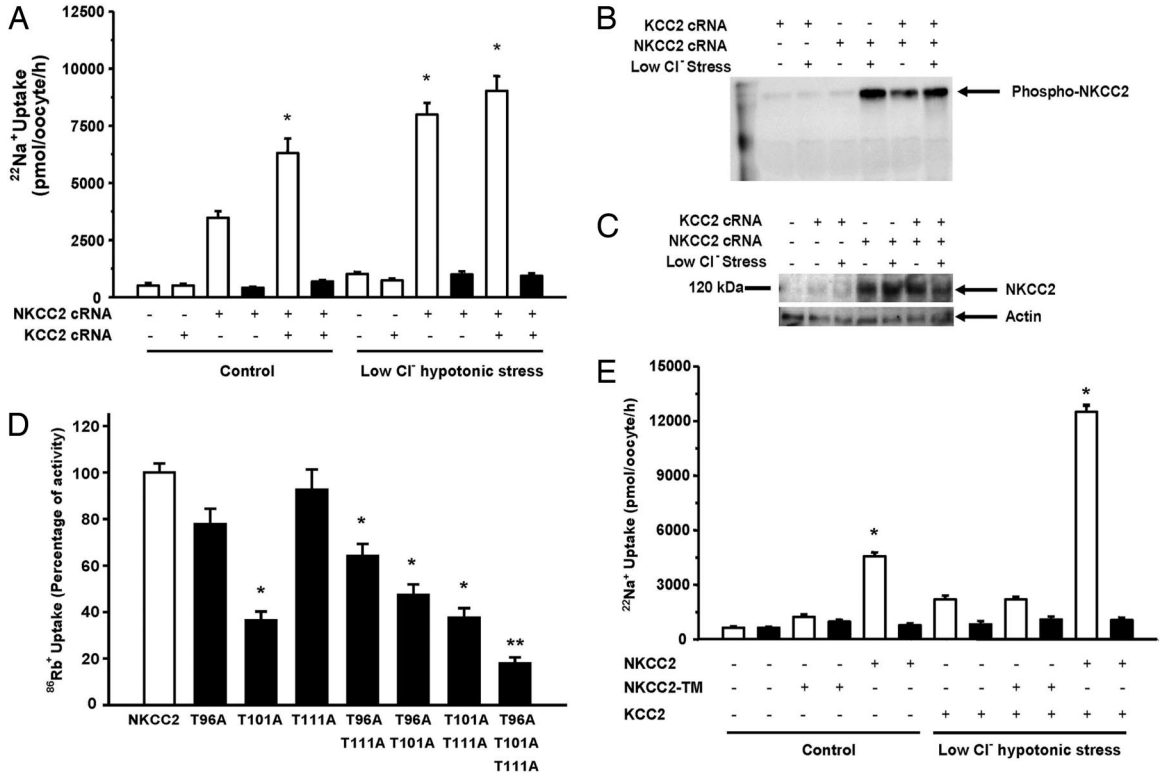

Fig. 1. Regulation of NKCC2 by intracellular chloride-depletion maneuvers. *Xenopus* oocytes were injected with water or 10 ng per oocyte of wild-type or mutant NKCC2 cRNA alone or together with 10 ng per oocyte of KCC2 cRNA, as stated. Four days later, influx experiments or Western blot analyses were performed in control conditions or under low-chloride stress. (*A*) $^{22}Na^+$ uptake was assessed in absence (open bars) or presence (filled bars) of 100 μM bumetanide. (*B*) A representative Western blot analysis performed by using the R5 phosphoantibody (26). (*C*) A representative Western blot analysis performed with polyclonal anti-NKCC2 and anti-actin antibody (41). (*D*) Uptake of $^{86}Rb^+$ was assessed in oocytes injected with water, wild-type, or mutant NKCC2. Mean value of wild-type NKCC2-injected oocytes was taken as 100%, and the value observed in mutants was normalized. *, significantly different from the uptake observed in wild-type NKCC2; **, significantly different to uptake observed in T101A. (*E*) Uptake of $^{22}Na^+$ in oocytes injected with water, wild-type NKCC2 cRNA, or triple-mutant NKCC2 exposed to control conditions and both chloride-depletion maneuvers as stated. Uptake of $^{22}Na^+$ was performed in the absence (open bars) or presence (filled bars) of 100 μM bumetanide. This is a representative experiment, and each bar represents the mean ± SEM of 10 oocytes. *, significantly different from the uptake observed in the corresponding control.

that have been used successfully to induce a sustained depletion of $[Cl^-]_i$ (7, 8, 21, 23, 24). The first strategy used coexpressing KCC2, a transporter that promotes chloride efflux in isotonic conditions (3). The second strategy used exposing oocytes to "low-Cl^- hypotonic stress," which induces the opening of Cl^- channels promoting Cl^- efflux (25). Oocytes expressing NKCC2 alone exhibited an increase in bumetanide-sensitive $^{22}Na^+$ uptake versus water-injected oocytes (Fig. 1*A*). Coexpression with KCC2 or incubation of oocytes in low-chloride hypotonic stress augmented bumetanide-sensitive $^{22}Na^+$ uptake (Fig. 1*A*). Exposing oocytes to both intracellular chloride-depletion maneuvers did not result in further activation of NKCC2.

Intracellular chloride depletion could augment NKCC2 function by increasing the phosphorylation of residues necessary for its activation, as has been shown previously to occur with shark NKCC1 (7) and NCC (24). In this regard, the amino terminal domain threonines that become phosphorylated in NKCC1 (T184 and T189) and NCC (T53 and T58) in response to chloride depletion are conserved in NKCC2 (T96 and T101); a third phospho-acceptor residue implicated in regulation of NKCC1 (T202) and NCC (S71) also is conserved in NKCC2 (T111) (24). R5 antibody, which recognizes phospho-threonines 184 and 189 in NKCC1, also is able to recognize phospho-threonines 96 and 101 in NKCC2 (26), and the phosphorylation of these threonines in NKCC2 has been shown to have a regulatory role *in vivo* (27). By using R5 antibody, phospho-NKCC2 was detected in homogenates extracted from oocytes expressing NKCC2 and exposed to low chloride stress and in homogenates from oocytes expressing both NKCC2 and KCC2, but not in those injected with water or expressing KCC2 and exposed to low chloride stress or oocytes expressing NKCC2 alone and exposed to control conditions during uptake (Fig. 1*B*). The amount of NKCC2 in oocytes was not affected by the intracellular chloride-depletion strategies (Fig. 1*C*). Densitometric analysis revealed that the ratio between phospho-NKCC2 (Fig. 1*B*) over total NKCC2 signals (Fig. 1*C*) of 0.14 in control condition increased to 1.06 after low-Cl^- stress, 1.03 after coinjection of KCC2, and 1.41 when both maneuvers were applied together. Thus, intracellular chloride depletion is associated with increased activity and phosphorylation of NKCC2 at the amino terminal threonines T96 and T101.

Elimination of the Amino Terminal Threonines T96, 101, and 111 Prevents NKCC2 Activation by Intracellular Chloride Depletion. Site-directed mutagenesis was used to substitute threonines 96, 101, and/or 111 of NKCC2 with alanine to produce single, double, or triple NKCC2 mutants. As shown in Fig. 1*D*, no significant effect was observed by the elimination of T96 or T111 on NKCC2 activity. However, NKCC2 activity was reduced 60% by mutation of T101. NKCC2 activity in the double mutant T96,111A was reduced by 40%, and double mutants in which T101 was involved had activity similar to the single mutant T101A. A triple mutant NKCC2 exhibited a further reduction of activity to nearly 85%. Thus, activity in the triple mutant was significantly lower

than in T101A. Thus, the behavior of the NKCC2 mutants differs from that of NKCC1 (7) or NCC (24), in which basal transporter activity depends on the presence of the second threonine. We exposed oocytes expressing wild-type NKCC2 or triple-mutant NKCC2 to both chloride-depletion maneuvers together (coexpression of KCC2 plus low-chloride hypotonic stress). As shown in Fig. 1*E*, a significant increase in NKCC2 activity was observed when both maneuvers were applied to oocytes expressing wild-type NKCC2. In contrast, the activity of triple mutant NKCC2 was not increased by chloride-depletion maneuvers. Thus, threonines 96, 101, and 111 not only become phosphorylated when NKCC2 is activated by intracellular chloride depletion, but also are required for its activation.

Regulation of NKCC2 Activity by WNK3 and SPAK Kinases. We next investigated whether the proposed "Cl^--sensing kinases" WNK3 and/or SPAK were involved in the mechanism by which intracellular chloride depletion increases NKCC2 activity because these kinases have been shown to regulate other CCCs. Similar to our previous findings (15), the coexpression of NKCC2 with WNK3 resulted in a significant increase in NKCC2 activity by >2-fold [supporting information (SI) Fig. S1]. No effect was observed when SPAK was coexpressed with NKCC2, but the coexpression of SPAK with WNK3 and NKCC2 resulted in a greater increase in bumetanide-sensitive $^{86}Rb^+$ uptake than was seen in oocytes expressing WNK3 and NKCC2 (Fig. S1). Because oocytes express WNK and SPAK orthologs that might be important for the functional regulation of heterologous-expressed NKCC2, we analyzed the effect of reducing the endogenous activity of these kinases by coexpressing NKCC2 together with catalytically inactive forms of WNK3 (D294A, termed WNK3-DA) (15) or SPAK (K104R, termed SPAK-KR) that serve as dominant-negative-type mutants or as inhibitors (16). Basal activity of NKCC2 was reduced 65 ± 6.5% ($P < 0.05$) by coinjecting WNK3-DA cRNA and 47 ± 2.6% ($P < 0.05$) with SPAK-KR coexpression. A similar type of inhibition has been observed for NKCC1 when coexpressed with inactive SPAK-KR (12, 19). Thus, although wild-type SPAK had no effect on NKCC2 (Fig. S1), inactive SPAK-KR significantly reduces basal NKCC2 activity. These data show that NKCC2 is inhibited by the catalytically inactive forms of WNK3 and SPAK and suggest that both kinases are required to maintain NKCC2 basal activity.

WNK3 Lies Upstream of SPAK for NKCC2 Activation. WNK1 and WNK4 lie upstream of SPAK in the regulation of NKCC1 (20, 21). Because both WNK3-DA and SPAK-KR inhibit NKCC2, we tested whether WNK3 and SPAK function in the same pathway in the regulation of NKCC2 by injecting WNK3-DA cRNA or SPAK-KR cRNA alone or both inactive kinases together. To increase our ability for detecting additive effects, lesser amounts of each kinase cRNA were injected. Data were normalized to the $^{86}Rb^+$ uptakes in oocytes injected with only NKCC2 (Fig. 2, filled bars). As shown in Fig. 2*A*, coexpression of WNK3-DA or SPAK-KR resulted in a significant 25% inhibition of NKCC2. When both inactive kinases were coexpressed, no further inhibition was observed. These data suggest that WNK3 and SPAK operate in series, and not parallel pathways, to regulate NKCC2.

We next coexpressed inactive WNK3-DA or SPAK-KR in combination with the wild-type SPAK or WNK3, respectively, to determine which kinase is able to rescue NKCC2 from the inhibitory effect of the other's inactive form. Wild-type WNK3 activated NKCC2 in the presence of SPAK-KR (Fig. 2*B*). In contrast, in the presence of WNK3-DA, the coexpression of wild-type SPAK did not activate NKCC2 (Fig. 2*C*). These observations suggest that WNK3 is positioned upstream of SPAK.

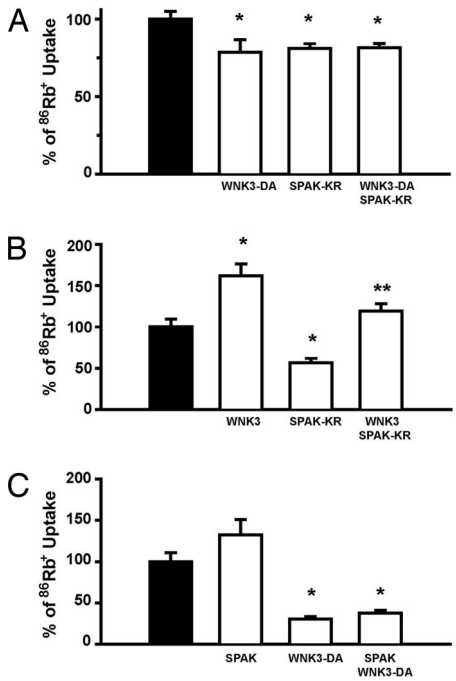

Fig. 2. Effect of the catalytically inactive kinases WNK3-DA and SPAK-KR on the functional expression of NKCC2. For all figures, oocytes were injected with water or 10 ng per oocyte of NKCC2 cRNA alone or together with the active or inactive kinases as stated. Three days later, $^{86}Rb^+$ uptake was assessed. Values observed in oocytes injected with NKCC2 alone were taken as 100% for normalization purpose. (*A*) Oocytes were injected with NKCC2 cRNA alone (filled bar) or together with 2.5 ng per oocyte of WNK3-DA cRNA or SPAK-KR cRNA alone or both inactive kinases together (open bars) as stated. (*B*) Oocytes were injected with NKCC2 cRNA alone (filled bar) or together with 5 ng/oocyte of wild-type WNK3 cRNA with or without 5 ng/oocyte of SPAK-KR cRNA (open bars). (*C*) Oocytes were injected with NKCC2 cRNA alone (filled bar) or together with 5 ng per oocyte of WNK3-DA cRNA with or without 5 ng per oocyte of wild-type SPAK cRNA (open bars). *, significantly different from the uptake observed in the NKCC2 cRNA group; **, significantly different from the uptake observed in the NKCC2 cRNA plus SPAK-KR cRNA group.

A SPAK-Binding Motif in WNK3 Is Necessary for WNK3's Activation of NKCC2. According to a recent genome-wide analysis (28), the sequence GRFQVITI in the carboxyl-terminal regulatory domain of WNK3 represents a unique SPAK-binding site that is conserved in mouse, rat, and human WNK3. Thus, the effect of wild-type or WNK3-F1337A on NKCC2 activity and WNK3-SPAK interaction was analyzed. Wild-type WNK3 was able to increase activity, but this effect was completely absent with WNK3-F1337A (Fig. 3*A*). Consistent with this observation, HA-SPAK was coprecipitated with wild-type c-Myc-WNK3, but not with c-Myc-WNK3-F1337A (Fig. 3*B*), suggesting that to activate NKCC2, WNK3 and SPAK form a protein complex. The lack of WNK3-F1337A effect on NKCC2 could be alternatively explained if the mutation F1337A reduces WNK3 kinase expression and/or activity, but this scenario is unlikely for three reasons. First, expression of c-Myc-WNK3 or c-Myc-WNK3-F1337A was similar (Fig. 3*C*). Second, WNK3 without catalytic activity (Fig. 2*A*) inhibits NKCC2, whereas WNK3-F1337A mutant does not (Fig. 3*A*). Third, the effect of WNK3-F1337A on KCC4 demonstrates that this construct contains catalytic activity. Both wild-type WNK3 and WNK3-F1337A reduced the activity of KCC4 when oocytes were incubated in hypotonic conditions. Because catalytic activity of WNK3 is required for KCC4 inhibition (13), this observation demonstrates that WNK3-F1337A is an active kinase. Interestingly, this observation also shows that the mechanisms by which WNK3 activates

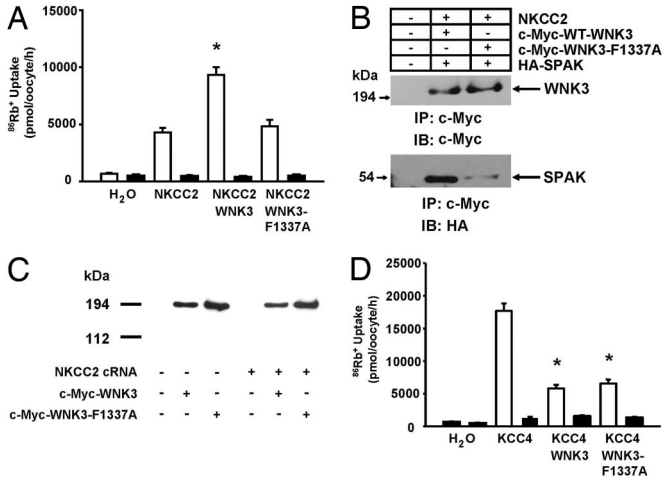

Fig. 3. Effect of elimination of the unique SPAK binding site of WNK3 on the kinase expression and its effects on NKCC2 and KCC4. (*A*) Uptake of $^{86}Rb^+$ in oocytes injected with water, NKCC2 cRNA alone or together with wild-type c-Myc-WNK3 cRNA, or c-Myc-WNK3-F1337A cRNA. Uptake was performed in isotonic conditions in the absence (open bars) or presence (filled bars) of 100 μM bumetanide. *, significantly different from the uptake observed in NKCC2 cRNA group. (*B*) Oocytes were injected with water, NKCC2 cRNA, c-Myc-WNK3 cRNA, c-Myc-WNK3-F1337A cRNA, and/or HA-SPAK cRNA as stated. Three days later, c-Myc-WNK3 or c-Myc-WNK3-F1337A were immunoprecipitated from corresponding protein homogenates, resolved in SDS/PAGE, and transferred to PDFV membranes. Western blot analyses were performed by using anti-c-Myc or anti-HA monoclonal antibodies as stated. (*C*) Representative Western blot analysis of proteins extracted from oocytes 3 days after injection with water or NKCC2 cRNA alone or together with wild-type c-Myc-WNK3 cRNA or c-Myc-WNK3-F1337A cRNA as stated. Similar results were observed in the absence of NKCC2 cRNA injections. (*D*) Uptake of $^{86}Rb^+$ in oocytes injected with water, KCC4 cRNA alone or together with wild-type c-Myc-WNK3 cRNA, or c-Myc-WNK3-F1337A cRNA in hypotonic conditions in the presence (open bars) or absence (filled bars) of extracellular chloride. *, significantly different from the uptake observed in the KCC4 cRNA group.

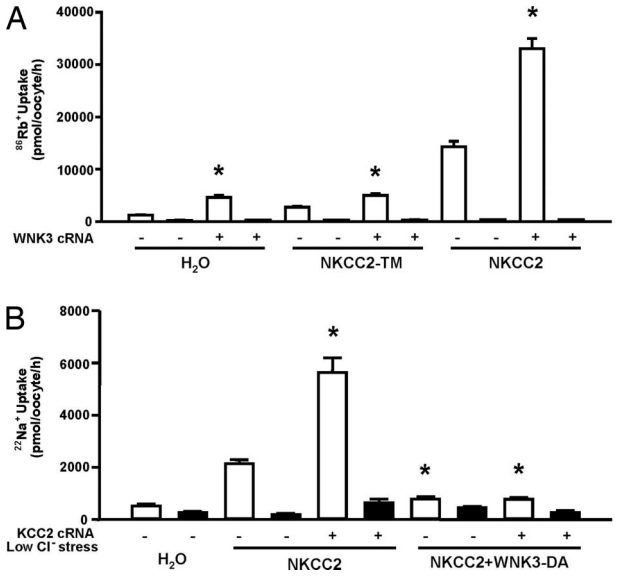

Fig. 4. Effect of elimination of the threonines 96, 101, and 111 in NKCC2 (NKCC2-TM) or coinjection of wild-type NKCC2 and WNK3-DA on the response to wild-type WNK3 or chloride depletion maneuvers. (*A*) Oocytes injected with water, wild-type NKCC2, and triple-mutant NKCC2 were coinjected with WNK3 cRNA as stated. Four days later, $^{86}Rb^+$ uptake was assessed in control isotonic conditions. Values observed in water-injected oocytes were subtracted to all groups. *, significantly different from the uptake observed in the corresponding group in the absence of WNK3. (*B*) *Xenopus laevis* oocytes were injected with water, with NKCC2 cRNA alone, or together with KCC2 cRNA and/or WNK3-DA as stated. Uptakes of $^{22}Na^+$ were performed in control isotonic conditions or under low-chloride hypotonic stress in the absence (open bars) or presence (filled bars) of 100 μM bumetanide. *, significantly different from the uptake observed in the NKCC2 control group.

NKCC2 or inhibits KCCs activity are different: Interaction between WNK3 and SPAK is required for NKCC2 activation, but is not necessary for KCC4 inhibition. In this regard, another difference is that WNK3-DA inhibitory effect on NKCC2 is a dominant-negative type of effect, whereas activation of KCCs is not (Fig. S2). Moreover, we have previously shown that activation of KCCs by WNK3-DA is prevented by protein phosphatase inhibitors (13), although this is not the case for NKCC2 inhibitory effect on WNK3-DA or SPAK-KR (Fig. S3).

Amino Terminal Threonines 96, 101, and 111 Are Required for WNK3-Induced Activation of NKCC2. We have previously shown that increased activity of NKCC2 by WNK3 is associated with phosphorylation of threonines 96 and 101, as detected by the R5 phosphoantibody (15). Thus, we analyzed the effect of WNK3 on NKCC2 harboring the triple mutation (T96,101,111A) that eliminates the response to intracellular chloride depletion (Fig. 1*D*). As shown in Fig. 4*A*, WNK3 increased the activity of wild-type NKCC2, but had no effect on the NKCC2 triple mutant in which all three threonines were eliminated. These data suggest that the activation of NKCC2 by WNK3 is due to phosphorylation and requires the presence of threonines 96, 101, and 111. Because intracellular chloride depletion also activates NKCC2 by phosphorylating these residues, we reasoned that WNK3 could be the kinase translating the decrease of $[Cl^-]_i$, into NKCC2 activation, and, in this case, catalytically inactive WNK3-DA should prevent the activation of NKCC2 by intracellular chloride-depletion maneuvers. We therefore tested the effect of both chloride-depletion maneuvers together (coexpression of KCC2 plus low-chloride stress) on NKCC2 activity coexpressed with WNK3-DA. In the presence of WNK3-DA, the activity of NKCC2 was significantly reduced, as shown previously. Intracellular chloride depletion, however, failed to increase the $^{86}Rb^+$ uptake mediated by NKCC2 under these conditions (Fig. 4*B*). These data show that NKCC2's activation by intracellular chloride depletion is prevented by WNK3-DA.

Discussion

In the present study, we demonstrate that rat NKCC2 expressed in oocytes is activated by intracellular chloride depletion and is associated with phosphorylation of the cotransporter at threonines 96 and 101. Elimination of these two residues, together with T111, renders NKCC2 less active and unresponsive to reduction of $[Cl^-]_i$. The regulation of NKCC2 by intracellular chloride depletion requires interaction between WNK3 and SPAK, in a pathway in which WNK3 lies upstream of SPAK. This is demonstrated by the fact that eliminating the unique SPAK-binding site in WNK3 (F1337A) prevents the activation of NKCC2 by this kinase. Mutation of threonines 96, 101, and 111 also prevented the response of NKCC2 to WNK3, and overexpression of the catalytically inactive WNK3-DA completely prevented the activation of NKCC2 by intracellular chloride depletion. These data together suggest that changes in $[Cl^-]_i$ are sensed by WNK3, which in turn interacts with and activates SPAK, promoting the phosphorylation of NKCC2 at threonines 96, 101, and 111, which results in increased activity of the cotransporter.

The regulation of WNK kinases by $[Cl^-]_i$ has been previously demonstrated. When Xu *et al.* (29) first cloned the cDNA encoding WNK1, they observed that, from a variety of potential stimuli to regulate WNK1, the only positive one was NaCl

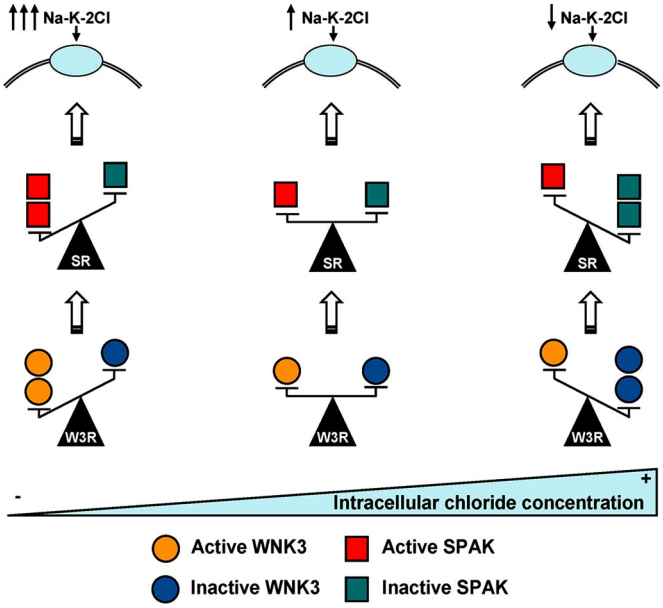

Fig. 5. Proposed model for intracellular chloride, WNK3, and SPAK interaction in the control of NKCC2 activity.

concentration. Later, Lenertz *et al.* (30) showed that WNK1 is activated by osmotic challenges in a variety of cell lines. Moriguchi *et al.* (21) observed in HEK293 cells that the activity of WNK1 and of SPAK/OSR1 kinases is increased by lowering $[Cl^-]_i$ and proposed that WNK1 behaves as an activator of SPAK/OSR1 in response to a decrease in the $[Cl^-]_i$. It remains to be determined how changes in $[Cl^-]_i$ affect the kinase activity of WNK3, if WNK3 directly phosphorylates SPAK, and how a WNK3–SPAK interaction phosphorylates NKCC2.

Fig. 5 depicts our proposed model for the WNK3–SPAK pathway in the regulation of NKCC2. We propose that variations in $[Cl^-]_i$ are associated with changes in the ratio of active versus inactive WNK3 kinase (W3R). Injecting oocytes with wild-type WNK3 cRNA, by increasing W3R, mimics a situation in which $[Cl^-]_i$ is reduced. Increased W3R in turn increases the active versus inactive SPAK kinase ratio (SR) that promotes NKCC2 phosphorylation, thereby increasing the activity of the cotransporter. In contrast, injecting oocytes with WNK3-DA cRNA, by decreasing the W3R, mimics increases in $[Cl^-]_i$, which in turn decreases SR and thus prevents phosphorylation and activation of NKCC2. Injecting oocytes with wild-type SPAK cRNA alone cannot mimic the activation of NKCC2 seen with the reduction of $[Cl^-]_i$ because, although the number of SPAK copies increases, they are not activated by the low amount of WNK3. In contrast, injecting oocytes with inactive SPAK-KR cRNA simulates an increase in $[Cl^-]_i$ because the overexpression of this inactive kinase reduces SR. This model also helps to explain why wild-type SPAK is unable to reverse NKCC2 inhibition by WNK3-DA, but wild-type WNK3 can overcome the inhibition of NKCC2 by SPAK-KR. The latter occurs because, even in the presence of SPAK-KR, overexpression of wild-type WNK3 will increase the SR via activation of endogenous SPAK. By disrupting the SPAK-binding motif of WNK3 (WNK3–F1337A), WNK3 is no longer able to interact with SPAK and thus cannot increase the SR. Thus, NKCC2 activation does not occur. Finally, because the catalytically inactive WNK3-DA exerts a dominant negative-type effect on endogenous WNK3, intracellular chloride depletion no longer activates NKCC2 in the presence of WNK3-DA.

Bartter's syndrome is a heterogeneous disease in which inactivating mutations in NKCC2, the potassium channel ROMK, and the basolateral chloride channel ClC-KB have been shown to cause Bartter's syndrome type I, II, and III, respectively (31). The mechanisms of disease in Bartter's syndrome type I and II are easily understood; inactivation of NKCC2 directly impairs salt flux through transporter, whereas mutations in ROMK disrupt the potassium-recycling mechanism necessary for continued NKCC2 function. Both types are clinically evident since birth or even before as polyhydramnios. In contrast, the mechanisms by which mutations in ClC-KB cause disease are unclear. Patients with the type III variant show a less severe phenotype that is not clinically evident until late in the first year of life, often exhibiting combined features of Bartter's and Gitelman's syndrome (another form of Mendelian hypotension due to mutations in NCC). In the case of Bartter's type III, CLC-KB-inactivating mutations, by preventing chloride efflux from cells, might indirectly impair the activity of NKCC2 in the TAL and in some cases also of NCC in the DCT, but the clinical consequences takes time to develop until the nephron reaches its maximum development in the early postnatal life (32). Consistent with this hypothesis are the data from this and a previous study (24) that NKCC2 and NCC are regulated by intracellular chloride. Thus, we believe that our observations help to define a regulatory pathway of NKCC2 that provides insight into how increases in $[Cl^-]_i$ in Bartter's syndrome type III could lead to the inhibition of NKCC2 (and NCC) activity.

In conclusion, our data demonstrate that NKCC2 is activated by intracellular chloride depletion by a mechanism that involves WNK3 and SPAK kinases. WNK3 appears to be the Cl^--sensing kinase that in turn physically interacts with SPAK by means of a unique SPAK-binding site. The interaction results in the activation of SPAK and then in the phosphorylation of NKCC2 at the amino terminal domain threonines 96, 101, and probably 111, which, in turn, results in an increase in NKCC2 activity.

Methods

Clones and Mutagenesis. The cDNAs used in this study were previously described (15, 33–38). Site-directed mutations (QuikChange; Stratagene) were performed to substitute threonines 96, 101, and/or 111 of NKCC2 and WNK3 phenylalanine 1337 for alanine. c-Myc-epitope-tag was introduced into WNK3 after the ATG starting codon by using double-step PCR. A construct containing a fragment of WNK3 from residues 410–482 was done by PCR, and a flag epitope tag was engineered in frame with the rest of coding region. DNA sequencing was used to confirm all mutations. All primers were custom made (Sigma).

Assessment of the Na^+:K^+:2Cl^- and K^+-Cl^- Cotransporters' Function. rNKCC2 activity was assessed by functional expression in *Xenopus laevis* oocytes as described (15, 33, 39). Oocytes were injected with water or rNKCC2 cRNA (10 ng/oocyte) and exposed to two different conditions that promote a decrease in the $[Cl^-]_i$: coinjection with the K^+:Cl^- cotransporter KCC2 cRNA (10 ng/oocyte), and low-Cl^- hypotonic stress (24). For influx details and all solutions contents, see *SI Methods*.

Western Blot Analysis and NKCC2 Phosphoantibody Studies. Immunoblots were performed by using a rabbit polyclonal anti-NKCC2 antibody following our previously published protocol (41). Expression of proteins harboring the FLAG (for WNK3–410-482 fragment), HA (for SPAK or SPAK-KR), and c-Myc epitopes (for WNK3, WNK3-DA or WNK3-F1337A) were detected by using the appropriate peroxidase-conjugated monoclonal antibodies (Sigma). R5 antibody was used to detect the phosphorylation status of T96 and T101 in NKCC2 as described previously (26).

Data Analysis. All results presented are based on a minimum of three different experiments with at least 10 oocytes per group in each experiment. Statistical significance is defined as two-tailed, with $P < 0.05$, and the results are presented as mean ± SEM. The significance of the differences between groups was tested by one-way ANOVA with multiple comparisons using Bonferroni's correction.

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