Physiological role of SLC12 family members in the kidney

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Bazúa-Valenti S, Castañeda-Bueno M, Gamba G. Physiological role of SLC12 family members in the kidney. Am J Physiol Renal Physiol 311: F131–F144, 2016. First published April 20, 2016; doi:10.1152/ajprenal.00071.2016.—The solute carrier family 12, as numbered according to Human Genome Organisation (HUGO) nomenclature, encodes the electroneutral cation-coupled chloride cotransporters that are expressed in many cells and tissues; they play key roles in important physiological events, such as cell volume regulation, modulation of the intracellular chloride concentration, and transepithelial ion transport. Most of these family members are expressed in specific regions of the nephron. The Na-K-2Cl cotransporter NKCC2, which is located in the thick ascending limb, and the Na-Cl cotransporter, which is located in the distal convoluted tubule, play important roles in salt reabsorption and serve as the receptors for loop and thiazide diuretics, respectively (Thiazide diuretics are among the most commonly prescribed drugs in the world.). The activity of these transporters correlates with blood pressure levels; thus, their regulation has been a subject of intense research for more than a decade. The K-Cl cotransporters KCC1, KCC3, and KCC4 are expressed in several nephron segments, and their role in renal physiology is less understood but nevertheless important. Evidence suggests that they are involved in modulating proximal tubule glucose reabsorption, thick ascending limb salt reabsorption and collecting duct proton secretion. In this work, we present an overview of the physiological roles of these transporters in the kidney, with particular emphasis on the knowledge gained in the past few years.

with-no-lysine kinase 4; serine-proline-alanine-rich kinase; hypertension; salt transport; diuretics

THE SOLUTE CARRIER FAMILY 12 (SLC12) encodes the electroneutral cation-coupled chloride cotransporters. These plasma membrane transporters translocate cations (Na\(^+\) and/or K\(^+\)) coupled to Cl\(^-\) ions; the total cation:anion stoichiometry of 1:1 makes the process electroneutral. Early evidence for electroneutral cotransporter systems emerged from work by Renfro in the winter flounder urinary bladder (108) and by Geck et al. (44) in Ehrlich ascites cells. Later in the 1980s, evidence for the renal physiological relevance of the SLC12 cotransporter family, in addition to its localization along the nephron, became evident from the work of Greger and Schlatter (reviewed in Ref. 50), Hebert and Andreoli (reviewed in Ref. 54) and Ellison et al. (30). Research on the molecular physiology of these cotransporters has increased, particularly because two members serve as receptors for loop and thiazide diuretics, two of the most common drugs prescribed worldwide. By the end of the 1990s, all members of the family were identified at the molecular level (17, 41, 42, 45, 84, 97, 98, 150), providing the tools for detailed analysis of the renal expression of these cotransporters (Fig. 1) to elucidate this family’s role in Mendelian salt-losing nephropathies and to produce transgenic mouse models (25, 52, 142). Finally, in recent years, key regulatory phosphorylation sites were discovered in different SLC12 family members (24, 46, 80, 95, 106, 109, 113), allowing assessment of cotransporter activity using in vitro and in vivo systems. In recent years, the complex regulatory network of kinases and ubiquitin ligases modulating the activity of this cotransporter family was also discovered (83, 102, 144, 147). Thus, a remarkable amount of data on the physiology and pathophysiology of SLC12 cotransporters has accumulated over the years. In this work, we provide an overview of the recent advances in the field.

SLC12 Family

The SLC12 family is composed of nine genes encoding seven functionally identified members and two orphan transporters (4, 38, 55). The family is divided into two branches based on the degree of identity: one branch encodes the Na\(^+\)-dependent Na-K-2Cl cotransporters (NKCC1 and NKCC2) and the Na-Cl cotransporter (NCC). The other branch encodes the Na\(^+\)-independent K-Cl cotransporters known as KCC1 to KCC4. All these proteins are expressed in the kidney, with the exception of KCC2, which is neuron-specific (98). Two additional members, CCC8 and CCC9, exhibit a low degree of identity with other members, and their physiological functions are not yet understood (4, 53).

An important characteristic of the SLC12 family is that the activity of all members is regulated by the phosphorylation/
dephosphorylation of key serine/threonine residues located at
the amino- and carboxy-terminal domains (4, 38). Interest-
ingly, the phosphorylated residues differ between the two
branches. A cluster of serine/threonine residues in the amino-
terminal domain is conserved in NKCC1, NKCC2 and NCC. In
contrast, the four KCC cotransporters mainly have phosphor-
ylation sites in the carboxy-terminal domain. Additionally,
phosphorylation has the opposite effect on the Na⁺/H⁺-de-
pendent and -independent branches. Phosphorylation activates
the NKCCs and NCC but inhibits the KCCs. Conversely, dephos-
phorylation inhibits NKCCs and activates KCCs. Therefore, in
the regulation of SLC12 transporter activity, kinase-induced
phosphorylation appears to be coupled to protein phosphatases
that induce dephosphorylation; thus, the activity at any given
time is the result of the balance between kinases and phosphat-
ases (71).

The major kinases that modulate SLC12 transporters have
been uncovered in the last decade, whereas the major phos-
phatases remain elusive. The kinases that directly phosphory-
late the cotransporters are STE-20 serine-proline-alanine-rich
kinase (SPAK) and oxidative stress response kinase 1 (OSR1)
(35). These kinases bind to target protein domains containing
the RFxV/I motif, usually located near the phosphorylation site
(27). The SPAK and OSR1 activity toward SLC12 members is,
in turn, modulated by a family of with-no-lysine kinases
(WNKs) composed of four members, WNK1 to WNK4 (73).
WNKs interact with and phosphorylate SPAK and OSR1 at a
key residue of the T-loop (107), thus promoting the interaction
and phosphorylation of the SLC12 cotransporters. Elimination
of the SPAK/OSR1 key residue at the T-loop completely
prevents the effect of WNKs on SLC12 cotransporters (107).
WNKs have also been subject of intense research because
mutations in WNK1 and WNK4 cause the salt-sensitive hy-
perkalemic hypertensive disease in humans known as familiar
hyperkalemic hypertension, pseudohypoaldosteronism type II
(PHAIIP) or Gordon syndrome (147).

K-Cl Cotransporters

KCC3 and KCC4 are expressed in many tissues, including
the kidney (Table 1). The KCC3 and KCC4 renal expression
pattern has been studied at the protein level. Two independent
studies showed that KCC3 is exclusively expressed in the
basolateral membrane of the proximal tubule (S1–S3) of mouse
and rat kidneys (11, 81). KCC4 is also present in the basolat-
eral membrane of the proximal tubule, but it is also expressed
in the basolateral membrane of the thick ascending limb of the
loop of Henle (TAL) and in α-intercalated cells in the collect-
ing duct (CD) (10, 79). One study suggested the presence of
KCC4 in the basolateral membrane of the rabbit distal convo-
luted tubule (DCT) (141), but no confirmation has been pub-
lished.

KCC2 is exclusively expressed in neurons, and mutations
on this transporter are the cause of a recessive inherited
disease known as early-infantile epileptic encephalopathy
(OMIM 616645) (130). KCC1 is ubiquitous but the KCC1

![Fig. 1. Schematic illustration of solute carrier
family 12 (SLC12) cotransporters along the
nephron. DCT, distal convoluted tubule; KCC4 and KCC3, K-Cl cotransporter 4 and 3;
NKCC2, Na-K-2Cl cotransporter; PT, proximal
tubule; TAL, thick ascending limb of Henle; NCC, Na-Cl cotransporter.](image-url)
knockout (KO) mice express no phenotype (120). In the kidney, KCC1 has been studied only at the mRNA level and has been found to be expressed in the proximal tubules, DCT, CD, and papillary ducts of Bellini (45, 66). Thus, the role of KCC1 in renal physiology is largely unknown. In this regard, early microperfusion studies presented evidence of the existence of electroneutral K\(^+\)/H\(^+\)-Cl\(^-\)/H\(^+\) cotransport activity in several nephron segments, and the molecular identity of this KCC remains unknown. Wingo (148) reported the existence of a Cl\(^-\)-dependent K\(^+\) secretory mechanism in the rabbit cortical CD that is strongly stimulated when the luminal chloride concentration decreases. Amorim et al. (2) showed that a portion of the Cl\(^-\)-dependent K\(^+\) secretion observed in the rat DCT was sensitive to furosemide, and Ellison et al. (29) and Velazquez et al. (140) presented evidence for a K\(^+\)/Cl\(^-\) transport mechanism in the DCT, CNT, and inner medullary CD. Because immunohistochemical data have not revealed the expression of KCC3 and KCC4 in these locations, the nature of the KCC remains unknown, making KCC1 a possible candidate.

**Table 1. SLC12 cotransporters in the kidney**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Nephron Location</th>
<th>Functional Physiology in the Kidney</th>
<th>KO Phenotype</th>
<th>Genetic Diseases</th>
<th>Affected Gene/Protein</th>
<th>Effect on Cotransporter Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLC12A1</td>
<td>NKCC2</td>
<td>TAL</td>
<td>NaCl reabsorption in the TAL</td>
<td>Bartter-like: Hypotension, metabolic alkalosis, hypercalciuria, high PRA</td>
<td>Bartter Syndrome</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Regulation of calcium excretion; urine concentration</td>
<td></td>
<td></td>
<td></td>
<td>down</td>
</tr>
<tr>
<td>SLC12A2</td>
<td>NKCC1</td>
<td>Basolateral membrane of CD cells; mesangial and afferent arteriole</td>
<td>Provide ions to be secreted</td>
<td>Deafness and inner ear function defects, abnormal saliva secretion, hypotension</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>SLC12A3</td>
<td>NCC</td>
<td>DCT</td>
<td>NaCl reabsorption in the DCT</td>
<td>Gitelman-like: Hypotension, hypokalemia, metabolic alkalosis, hypercalciuria, hypomagnesemia, high plasma aldosterone, high PRA</td>
<td>Gitelman Syndrome</td>
<td>600968</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Regulation of renal potassium and calcium excretion</td>
<td></td>
<td></td>
<td></td>
<td>down</td>
</tr>
<tr>
<td>SLC12A4</td>
<td>KCC1</td>
<td>PT, DCT, CCD, MCD (mRNA)</td>
<td>Possible role in KCI epithelial transport</td>
<td>No clinical phenotype</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>SLC12A5</td>
<td>KCC2</td>
<td>ND</td>
<td></td>
<td>Neonatal death due to motor neuron deficit that impairs respiration</td>
<td>Idiopathic generalized epilepsy OMIM 616645</td>
<td>Loss of function mutations in KCC2</td>
<td>down</td>
</tr>
<tr>
<td>SLC12A6</td>
<td>KCC3</td>
<td>PT</td>
<td>Basolateral K(^+) recycling that supports Na(^+)/Glucose transport in the PT</td>
<td>Andermann-like: Peripheral neuropathy, locomotor deficit, sensorimotor gating deficit, white matter degeneration, adult deafness, hypertension</td>
<td>Andermann Syndrome OMIM 604878</td>
<td>Loss of function mutations in KCC3</td>
<td>down</td>
</tr>
<tr>
<td>SLC12A7</td>
<td>KCC4</td>
<td>PT, TAL, and CD (α-IC cells)</td>
<td>Participates in the pathway for acid excretion in α-IC cells of CD NaCl reabsorption in the TAL</td>
<td>Postnatal hearing loss, renal tubular acidosis</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

LF, loss of function; GF, gain of function; PRA, plasma renin activity. OMIM, Online Mendelian Inheritance in Man; ND, no data; (↑), increase in cotransporter function/activity; (↓), decrease in cotransporter function/activity.
The first evidence of Na\(^{+}\)-independent K-Cl cotransport activity in the basolateral membrane of proximal tubules was presented by Sasaki et al. (123) using isolated perfused neprhon segments. Simultaneously, Avison et al. (7) showed that in vivo incubation of proximal tubules in a high-glucose solution causes an increase in intracellular Na\(^{+}\) content and a decrease in K\(^{+}\) content, which was proposed to be caused by a Ba\(^{2+}\)-resistant but furosemide-sensitive K\(^{+}\) efflux, suggesting a KCC mechanism. It was hypothesized that glucose stimulates Na\(^{+}\) reabsorption in the proximal tubule and that a cell volume increase, which is likely associated with glucose reabsorption, stimulates a KCC transport mechanism that promotes the basolateral efflux of K\(^{+}\) ions through a furosemide-sensitive K\(^{+}\)-Cl\(^{-}\) cotransport system (Fig. 2A). Supporting this proposal, Melo et al. (79) showed upregulation of KCC3 mRNA and protein in the kidney cortex of hyperglycemic rats, and Boettger et al. (11) showed that the regulatory volume decrease (RVD) is impaired in the proximal tubule of KCC3\(^{-/-}\) mice. Interestingly, although KCC4 is also expressed in the basolateral membrane of proximal tubules, the RVD of the proximal tubules in KCC4\(^{-/-}\) mice was not affected (10). Additionally, Melo et al. (79) did not observe changes in the KCC4 expression in the renal cortex of hyperglycemic rats. Thus, KCC3 seems to play a more important role than KCC4 in proximal tubule glucose reabsorption.

KCC4\(^{-/-}\) mice develop renal tubular acidosis (10). It was proposed that acidosis results from impaired urinary acid excretion due to the altered function of transport pathways in \(\alpha\)-intercalated cells. KCC4 is expressed in the basolateral membrane of this renal cell type, where it colocalizes with the Cl/HCO\(_3\) exchanger. According to the proposed model, KCC4 provides a pathway for the efflux of the Cl\(^{-}\) that enters the cell through the Cl/HCO\(_3\) exchanger (Fig. 2B). The Cl\(^{-}\) efflux allows the sustained activity of the Cl/HCO\(_3\) exchanger, which is necessary to maintain the H\(^{+}\) secretion at the apical side.

Supporting this model, Melo et al. (79) observed that mice that ingested high levels of acid (NH\(_4\)Cl-treated mice) provoked an increase in the KCC4 expression in \(\alpha\)-intercalated cells. Such an increase in KCC4 expression was also observed in the renal medulla of hyperglycemic rats; the measurement of urinary pH indicated metabolic acidosis in the animals. Thus, the KCC4 expression in \(\alpha\)-intercalated cells was upregulated in conditions in which increased urinary acid excretion was required.

Finally, the early microperfusion experiments performed by Greger and Schlatter (50) suggested that the K\(^{+}\) efflux through the basolateral membrane of the TAL, which is necessary to maintain N\(a\)/K-ATPase activity, is mainly electroneutrally coupled to Cl\(^{-}\) (Fig. 2C). The KCC4 expression in the TAL was later reported by Velazquez and Silva (141) and Weinstein (146). The Greger proposal is supported by our observation that the KCC4 expression in the TAL is upregulated in the total kidney lysates of mice given a low-salt diet (79); this finding suggests that increased KCC4 activity in the TAL during salt restriction helps to promote the basolateral K\(^{+}\) efflux in the TAL to sustain the large amount of salt transport that is required.

The key phosphorylation sites modulating KCC activity have been uncovered. There are two major sites in the carboxyl-terminal domain (113); for KCC3, there is one additional site in the amino-terminal domain (80). The phosphorylation of KCC in the kidney has not been studied, but it will certainly be evaluated in future studies because phosphorylation is closely related to cotransporter activity.

**Na-K-2Cl Cotransporters**

The SLC12A2 gene encodes NKCC1, which is a ubiquitous protein and a key player in regulation of cell volume, intracellular chloride concentration, and sodium secretion by many epithelial cells (26, 150). In the renal epithelial cells, NKCC1 expression is located at the basolateral membrane of the cells, particularly in the collecting duct (68). However, no particular role has been ascribed to NKCC1 in tubular physiology. NKCC1 is also present in the mesangial cells and afferent arteriole in which it has been suggested to play a role in the suppression of renin secretion, because NKCC1 knockout mice exhibit increased renin release (20, 59). NKCC1 knockout mice present a mild hypotension, and this phenotype has been suggested to be due to loss of NKCC1 effects on both vascular and renal cells (32, 62, 91).

The SLC12A1 gene encodes NKCC2, which is expressed in the apical membrane of the epithelial cells lining the TAL, where it facilitates the salt reabsorption that accounts for \(\sim 10–20\%\) of the total filtered load. NKCC2 is inhibited by loop diuretics (such as furosemide and bumetanide), which are extensively used to treat edema formation in salt-retaining conditions (37, 41, 60). Salt reabsorption by NKCC2 is facilitated by a complex mechanism involving the efflux of Na\(^{+}\) and Cl\(^{-}\) in the basolateral membrane via Na/K-ATPase, the Cl\(^{-}\) channel ClC-Kb, and the K-Cl cotransporter KCC4 coupled with the K\(^{+}\) recycling into the lumen through the apical outer medullary K\(^{+}\) channel (ROMK); this mechanism generates a lumen-positive transepithelial voltage, promoting the paracellular reabsorption of Na\(^{+}\), Ca\(^{2+}\), and Mg\(^{2+}\) (Fig. 3). Inactivating mutations in NKCC2, ROMK, or ClC-Kb result in Bartter syndrome types I, II, or III, respectively, due to a reduction in the TAL salt reabsorption (36), demonstrating the critical role that the integration of these proteins has in the proper function of this nephron segment.

The SLC12A1 gene encoding NKCC2 gives rise to three splice isoforms that result from the alternative transcription of exon 4, which encodes the primary structure of the last half of the second transmembrane domain (TM2) and the first connecting segment between TM2 and TM3 (97). These isoforms are known as B, A, and F and are spatially distributed along the TAL. NKCC2B is only present in the cortical portion of the TAL, and its appearance in the outer medullary TAL (22, 58, 97, 152). Functional analysis demonstrated that the affinity of the isoforms for Cl\(^{-}\), a transport-limiting factor, is B > A > F (48, 70, 72, 104), thus providing an explanation for the higher capacity/lower affinity for transport in the medullary TAL compared with the low capacity/high affinity of the cortical TAL (14, 114). Interestingly, the splicing machinery that produces these isoforms can be modulated by the dietary salt content. A low-salt diet promotes upregulation of the high-affinity variant NKCC2B in the cortical TAL, and its appearance in the outer medullary TAL (124). The molecular mechanisms by which dietary salt variation alters the splicesome to preferentially generate one variant over another are completely unknown. Interestingly, NKCC2B\(^{-/-}\) mice exhibit compensatory mechanisms that en-
Fig. 2. Physiological models for K-Cl cotransporters (KCCs) activity along the nephron. A: role for KCC3 is expressed in the basolateral membrane of proximal tubule cells (PT cell). B: role for KCC4 in the α-intercalated cells of the collecting duct (CD). C: role for KCC4 in the thick ascending limb of Henle (TAL). CA, carbonic anhydrase; ClC-Kb, Cl− channel-Kb; NKCC2, Na-K-2Cl cotransporter; SGLT2, sodium/glucose cotransporter 2.
hance NKCC2A expression (90). Apart from the splice isoforms, in the mouse, the SLC12A1 gene gives rise to variants with a shorter carboxy-terminal domain that have NKCC2-inhibition properties. During cell swelling, the variants also act as a bumetanide-sensitive, but K/H11001-independent Na-Cl cotransporter (76, 103, 105), providing a molecular explanation for the existence of a bumetanide-sensitive Na-Cl cotransporter in the mouse TAL (1, 31, 131).

The NKCC2 functional activity is regulated by various hormones and intracellular events that trigger interconnecting pathways, resulting in the phosphorylation and dephosphorylation of serine and threonine residues in the amino-terminal domain of NKCC2. NKCC2 regulatory pathways can be divided into two main types. One pathway modulates the membrane trafficking of NKCC2-containing vesicles and is associated with intracellular second messengers, such as cAMP, cGMP, and nitric oxide. This pathway is linked to hormones that act through G protein-coupled receptor, such as AVP, ANG II, glucagon, parathyroid hormone, adrenergic stimulation and serum calcium through the activation of the calcium-sensing receptor, CaSR (3, 21, 40, 43). Through the activation of their receptors, activating hormones trigger the production of cAMP by inducing the adenylyl cyclase (AC) cascade, thus activating PKA, which is known to mediate the phosphorylation of NKCC2 at residue Ser-126 in response to AVP (51). The cAMP/PKA pathway has been correlated with the exocytosis of NKCC2-containing vesicles through vesicle-associated membrane protein 2 (15, 16, 92) (Fig. 3A). Of interest, many groups have demonstrated in vitro and in vivo that Ser-126 phosphorylation is AC-6 dependent, and a recent study reported that AC-6-deficient mice exhibit a mild Bartter-like phenotype (111). cGMP and nitric oxide induce activation of phosphodiesterase 2, which inhibits cAMP production, thus decreasing NKCC2 exocytosis (93, 94). In addition to cAMP/
in the UMOD gene promoter have been linked with increased NKCC2.

The second pathway is typically induced by a reduction in the intracellular chloride concentration \([\text{Cl}^-]\), in which NKCC2 is activated by the WNK-SPAK/OSR1 pathway (106, 110) (Fig. 3B). Evidence suggests that NKCC2 phosphorylation at the serine/threonine residues in the amino-terminal domain (Thr-95, Thr-100, and Thr-105 of human NKCC2), which are conserved in NCC and NKCC1, activate the cotransporter (46, 47, 106); however, it is unclear whether phosphorylation of these residues is associated with activation of the cotransporter that is already in the plasma membrane and/or upregulation of the NKCC2 in the apical membrane. In this regard, data from NCC studies strongly suggest that SPAK/OSR1 phosphorylates the cotransporter that is already in the plasma membrane, and the phosphorylated transporter becomes resistant to ubiquitylation and clathrin-related endocytosis, ultimately increasing the NCC expression in the plasma membrane (99, 118). Given the similarities between NCC and NKCC2, it is possible that a similar mechanism applies for NKCC2.

The data obtained from the total SPAK knockout, T245A-SPA-Knockin mice and kidney-specific OSR1 knockout mice revealed that NKCC2 is phosphorylated by OSR1, in contrast to NCC, in which SPAK is the primary kinase that phosphorylates the amino terminal domain residues. The total absence of SPAK (SPA-Knockout) is associated with the opposite effect for NKCC2 vs. NCC, that is, increased and decreased phosphorylation, respectively (151). In contrast, in the SPAK knockin mice in which the kinase is expressed, but cannot be activated due to the absence of the autophosphorylating threonine, both NKCC2 and NCC are reduced (107). In addition, elimination of OSR1 expression in the kidney is associated with marked reduction of NKCC2 expression, leading to a Bartter-like phenotype (67). The finding seems to be related to the presence of shorter, nonactive variants of SPAK that exert a dominant-negative effect on NKCC2 (74, 96). These isoforms are not present in the total SPAK knockout, allowing OSR1 to freely act on NKCC2; in contrast, these isoforms are present in SPAK-knockin mice and inhibit the effect of OSR1 on NKCC2.

In this second regulatory pathway of NKCC2, the main upstream modulators of SPAK are the family of WNK kinases; these include WNK1 (the long isoform L-WNK1, and the kidney-specific isoform KS-SPA-K), WNK3 and possibly WNK4. WNK3 has been shown to enhance NKCC2 phosphorylation and activation in a kinase-dependent manner in vitro (106, 112). However, WNK3 KO mice do not exhibit any renal phenotype (77, 88). In this regard, total knockout animals often develop unexpected compensatory mechanisms. Thus, a conditional WNK3 KO mouse would be desirable to elucidate the physiological role of WNK3 in the kidney. Interestingly, despite the intense research on the WNK4 effect on NCC (see below), there is no information about the WNK4 effect on NKCC2.

Finally, uromodulin regulation of NKCC2 could have implications in the development of hypertension. Polymorphisms in the UMOL gene promoter have been linked with increased risk of chronic kidney disease and hypertension and are associated with higher uromodulin excretion in the urine (89, 136). Interestingly, increasing uromodulin expression in mice is associated with salt-sensitive hypertension that could be due to activation of the SPAK kinase and increased amino-terminal phosphorylation of NKCC2 (85). In addition, uromodulin overexpression is also associated with age-related tubular dilation and casts formation.

**Na-Cl Cotransporter**

The thiazide-sensitive Na-Cl cotransporter, NCC, is exclusively expressed in the apical membrane of the DCT (39). Between 5 and 10% of the salt filtered in the glomerulus is reabsorbed in the DCT, which is divided into two segments: DCT1, in which only NCC is present in the apical membrane, and DCT2, in which the apical sodium channel ENaC is expressed in addition to NCC. Thus, Na+ reabsorption in DCT1 is due solely to NCC, and it is, thus, not coupled with K+ secretion; in contrast, Na+ reabsorption in DCT2 is partially due to ENaC activity, and, thus, it is where the K+ secretion of the distal nephron starts.

There are three Mendelian diseases caused by changes in NCC activity. Gitelman syndrome is due to inactivating mutations in NCC and features hypotension, hypokalemia, metabolic alkalosis, and hypocapnia. SeSAME syndrome is caused by inactivating mutations in the basolateral membrane K+ channel KCNJ10 and causes a Gitelman-like picture, but is accompanied by a complex neurological phenotype (9, 125). Disruption of the KCNJ10 channel decreases the basolateral chloride conductance of the DCT, which, in turn, decreases WNK-SPAK activity and, thus, reduces NCC activity (see below) (154). The third disease is PHAII, in which increased NCC function is thought to be a key factor in explaining the development of hypertension, hyperkalemia, metabolic acidosis, and, in some cases, hypercalciuria. Thus, PHAII is the mirror phenotype of Gitelman and SeSAME syndromes.

Four different types of PHAII have been reported and are caused by mutations in the genes encoding the kinases WNK1 or WNK4 or the ubiquitin ligase complex-forming proteins Kelch-like 3 (KLHL3) or Cullin 3 (CUL3), (13, 69, 147). These four proteins are elements of signaling pathways implicated in the regulation of NCC activity. WNK1 and WNK4 modulate NCC activity (8, 23), and KLHL3 and CUL3 modulate the WNK half-life through a ubiquitylation mechanism (87, 124, 145, 149).

Deletions in intron 1 of the WNK1 gene lead to the overexpression of the long WNK1 isoform (L-WNK1) specifically in the DCT (143). In the case of WNK4, PHAII mutations occur within the coding region of the gene and specifically affect the so-called acidic domain, which is rich in negatively charged amino acids and constitutes the binding site for KLHL3 (Fig. 4). PHAII mutations in the acidic domain of WNK4 prevent binding to KLHL3, decreasing the degradation rate of the kinase and consequently upregulating WNK4 (127, 145, 149). PHAII mutations in KLHL3 and CUL3 lead to increased WNK1 and WNK4 (and perhaps WNK3) expression in the kidney. Mutations in KLHL3 disrupt the formation of the complex, precluding WNK ubiquitylation. In the case of CUL3, the PHAII mutations result in the skipping of exon 9 (13). Interestingly, McCormick et al. (75) showed in cultured cells that a CUL3 protein lacking the amino acids encoded in exon 9 retains the ability to form a functional CRL complex;
however, this complex is, in fact, more active and promotes degradation of KLHL3, impeding WNK ubiquitylation. Thus, in all cases, PHAII appears to be the result of increased WNK expression. However, it is still unclear how this change leads to sustained activation of NCC.

The effect of WNK kinases on NCC has been debated over the years, but recent work has provided partial clarification. In a recent review, we attempted to summarize and reconcile the old and new published information about the role of WNK1 and WNK4 in NCC regulation and the effect of PHAII mutations on these regulatory pathways (8). In brief, it was thought for years that WNK1 had no effect on NCC, but now it is currently accepted that L-WNK1 activity promotes NCC activation via a SPAK/OSR1-dependent mechanism (23, 109, 144). As for WNK4, evidence that has accumulated over the years from in vitro and in vivo models indicated that WNK4 could be either an inhibitor or an activator of NCC. Now, we understand that WNK4 is a chloride-sensitive kinase whose effect on SPAK-NCC is modulated by [Cl\(^-\)] (7). The chloride-binding site was uncovered by Piala et al. (101), who demonstrated that two key leucine residues in the kinase domain of L-WNK1 bind chloride ions, precluding the autophosphorylation and, thus, activation of the kinase. We showed that at high [Cl\(^-\)], WNK4 cannot be autophosphorylated and inhibits NCC due to its dominant-negative effects on WNK1 and WNK3 (23). In contrast, at low [Cl\(^-\)], WNK4 can be autophosphorylated and, thus, activates NCC via a SPAK-dependent mechanism (7). Thus, the inhibition and activation of NCC induced by WNK4 is likely due to the [Cl\(^-\)], of the model used for expression (Fig. 4A). When leucine 322 is removed from...
WNK4, WNK4 becomes a constitutively active kinase that is no longer inhibited by chloride. The sensitivity to chloride is likely different among WNK kinases. Results from Bazúa-Valenti et al. (7a, 8) and Terker et al. (134) suggest that the sensitivity profile is probably WNK4 > WNK1 > WNK3.

The chloride modulation of NCC activity via WNK4 has been observed to be a key mechanism by which extracellular K⁺ remarkably modulates NCC activity (Fig. 4B). Several studies demonstrate that NCC expression/phosphorylation is modulated by dietary potassium intake (18, 34, 137). A high K⁺ diet reduces NCC, increasing the amount of tubular fluid in the distal nephron, resulting in increased K⁺ secretion; in contrast, a low-K⁺ diet increases NCC activity, thus reducing flow to the distal nephron to prevent K⁺ secretion. Additionally, high K⁺ intake is also associated with similar changes in NCC activity (86, 129). Terker et al. (135) first showed that lowering extracellular K⁺ promotes the hyperpolarization of DCT cells because K⁺ ions apparently leave the cell through KCNJ10; this movement promotes the efflux of Cl⁻ ions, presumably through CLC-KB/A, thus decreasing [Cl⁻]; and activating NCC via a WNK4-SPAK-dependent mechanism. Later, Terker et al. (134) also showed that even minor changes in serum K⁺ within the physiological range induce the opposite change in NCC expression/phosphorylation. The higher the serum K⁺ is, the lower the NCC activity.

The K⁺ modulation of NCC activity has gained much attention (28, 100) because it raised questions about the previously accepted role of aldosterone in stimulating NCC expression/activity (6, 61). It is known that NCC can be regulated by ubiquitylation (63, 118), which can occur via the HECT-E3 ligase Nedd4-2 (5) or through modulation of WNK1 ubiquitylation (119). However, aldosterone administration increases urinary K⁺ loss, which, in turn, will reduce serum K⁺ levels and, thus, stimulate NCC activity. A recent study in mice in which the mineralocorticoid receptor was eliminated exclusively along the entire nephron strongly suggested that the aldosterone modulation of NCC is an indirect, rather than, direct effect of the changes in serum K⁺ (133). Furthermore, 11β-hydroxysteroid dehydrogenase type 2 is not present in DCT1, and it is not clear whether it is expressed in DCT2 cells (reviewed in Ref. 57). This enzyme defines the aldosterone-sensitive distal nephron because it inactivates glucocorticoids, thereby preventing binding to mineralocorticoid receptors (MR), allowing regulation of MR function by aldosterone.

As mentioned above, it is not clear how PHAIIs develops, because increased levels and activity of WNK kinases do not appear to easily explain the sustained activation of NCC. Data show that WNK4 and probably L-WNK1 are activated by a low-salt diet (via ANG II pathway) and by a decreased extracellular K⁺ concentration. PHAIIs patients exhibit the phenotype that would be expected to inhibit the activity of WNK kinases, that is, salt-sensitive hypertension and hyperkalemia. However, WNK kinases are not inhibited and remain active. Thus, it not yet clear why, although these kinases are upregulated, they are not inhibited by the PHAIi conditions. This question will be the subject of upcoming research.

NCC activity is modulated by a diverse set of signals as part of the physiological response necessary for the regulation of certain physiological parameters, which include blood pressure, serum potassium, and calcium excretion. These signals include hormones such as ANG II, aldosterone, AVP, insulin, norepinephrine, estradiol, progesterone, prolactin, and parathyroid hormone (recently reviewed in Ref. 115). In brief, all these messengers, except parathyroid hormone, stimulate the increase in NCC amino-terminal phosphorylation, that is, NCC activation. In most cases, this phosphorylation has involved SPAK/OSR1 activation (19, 64, 117, 122, 128, 132, 138, 139). The relevant signaling pathways are beginning to be described. Evidence suggests that WNK4 plays an essential role in the activation of NCC by ANG II (19, 121). In addition, Shibata et al. (126) have shown that the ANG II-stimulated, PKC-mediated phosphorylation of KLHL3 in the Kelch propeller domain (site S433) prevents substrate recognition and, thus, decreases WNK4 degradation. This result would be expected to have a positive effect on the NCC activity in the DCT. S433 is also a target for phosphorylation by PKA (153); therefore, AVP-mediated PKA activation may promote KLHL3 phosphorylation and, thus, regulate WNK4 ubiquitylation and degradation.

Activation of NCC is implicated in the development of hypertension associated with calcineurin inhibitors. The administration of these drugs is associated with an increased hypertension rate and two groups have observed that tacrolimus (56) and cyclosporine (78) in rodents induce phosphorylation and activation of NCC, thus increasing salt reabsorption and hypertension. Inhibition of the phosphatase calcineurin and, thus, prevention of NCC dephosphorylation is the likely mechanism because elimination of the FKBP12-binding protein along the nephron precluded the hypertensive effect of this compound; this protein is required for the tacrolimus inhibition of calcineurin (65). Besides NCC, NKCC2 was also shown in a recent study to be regulated by calcineurin, but it was regulated by a different isofrom. NKCC2 is inhibited by calcineurin β that is expressed in the TAL (12), whereas NCC is inhibited by the α isofrom expressed in the DCT (56). Interestingly, in the clinical setting, patients receiving tacrolimus who develop hypertension after kidney transplantation exhibit higher expression/phosphorylation of NCC in urinary exosomes than those with normal blood pressure (116).

**Summary and Overview**

A great amount of information has been generated over the last years about the molecular physiology of SLC12 cotransporters in the kidney. Although we have a much better understanding of the mechanisms underlying the regulation of these cotransporters, much more progress is needed to fully understand this subject. For instance, little is known about the molecular nature of the protein phosphatases involved in their regulation. The kinases activating the cotransporters have been discovered, but the mechanism by which mutations in these proteins lead to PHAI is still unclear. Little is known about the genomic mechanisms modulating the transcription of SLC12 genes, and little is known also about the regulatory mechanisms of KCCs in the kidney. Hence, we have many questions to answer in the upcoming years.

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rineural deafness, ataxia, mental retardation, and electrolyte imbalance (SeSAME syndrome) caused by mutations in KCNJ10.


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