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Intracellular Ion Control of WNK Signaling

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Abstract

The with no lysine (K) (WNK) kinases are an evolutionarily ancient group of kinases with atypical placement of the catalytic lysine and diverse physiological roles. Recent studies have shown that WNKs are directly regulated by chloride, potassium, and osmotic pressure. Here, we review the discovery of WNKs as chloride-sensitive kinases and discuss physiological contexts in which chloride regulation of WNKs has been demonstrated. These include the kidney, pancreatic duct, neurons, and inflammatory cells. We discuss the interdependent relationship of osmotic pressure and intracellular chloride in cell volume regulation. We review the recent demonstration of potassium regulation of WNKs and speculate on possible physiological roles. Finally, structural and mechanistic aspects of intracellular ion and osmotic pressure regulation of WNKs are discussed.

INTRODUCTION

The with no lysine (K) (WNK) kinases were first discovered in 2000 in a screen for new members of the mitogen–activated protein/extracellular signal–regulated protein kinase family (1). An important insight was that the invariant catalytic lysine found in subdomain II of other protein kinases, which binds ATP, was instead replaced by a cysteine, and this substitution was conserved in homologs from other species. The WNK catalytic lysine is instead found in subdomain I (1). As discussed further below (see section titled Molecular Mechanisms for Ion Regulation of WNK Kinases), this unusual arrangement allows chloride to directly regulate WNKs (2). Another observation from this study was that WNK1 kinase activity could be stimulated by high concentrations of extracellular sodium chloride, a second clue into the importance of ions and osmolarity in WNK regulation (1).

There are four mammalian WNKs, and in 2001, mutations in *WNK1* and *WNK4* were associated with a syndrome of high blood pressure and high blood potassium in humans (3). Since then, understanding of the physiological and pathophysiological roles of WNKs has continued to expand. Recent reviews discuss WNK genetics and biochemistry, and roles of mammalian WNKs in epithelial ion transport, hypertension, chronic kidney disease, the vasculature, immune cells and inflammation, cell volume regulation, development, cancer, autophagy, metabolism, and the nervous system, and provide information about WNKs in *Caenorhabditis elegans*, *Drosophila melanogaster*, zebrafish, and plants (4–11). Here, we focus on recent advances in ion regulation of WNK kinases, including the roles of chloride, potassium, and osmotic effects.

CHLORIDE REGULATION OF WNK KINASES

Intracellular chloride has been implicated in a broad range of cellular processes (12, 13), but the molecular mechanisms underlying chloride signaling remain largely unknown. The emerging understanding of chloride regulation of WNKs has thus served as a paradigm for understanding intracellular ion signaling.

In Search of a Chloride-Sensitive Kinase

Early clues to the existence of a chloride-sensitive kinase came from studies of sodium-potassium-2-chloride cotransporters (NKCCs). NKCCs are members of the SLC12 family of cation-chloride cotransporters and allow the electroneutral, coupled movement of one sodium, one potassium, and two chloride ions across plasma membranes (14). In most cells, intracellular sodium concentrations are low due to the activity of the sodium-potassium ATPase (Na/K-ATPase), which uses the energy of ATP to pump three sodium ions out of the cell and two potassium ions in (15). Therefore, sodium, potassium and chloride ions typically move through NKCCs from the extracellular space to the intracellular space along the sodium gradient (14).

The shark rectal gland is an example of a chloride-secreting epithelium. Its role is to secrete hypertonic saline, allowing sharks to rid themselves of the excess salt imbibed with seawater (16). The CFTR (cystic fibrosis transmembrane conductance regulator) chloride channel is on the apical membrane, secretes chloride, and is stimulated by hormones that signal through cAMP, such as vasoactive intestinal peptide (**Figure 1**). On the basolateral membrane are the Na/K-ATPase, NKCC, and potassium channels (17). Lytle & Forbush (18) demonstrated that forskolin, which stimulates cAMP signaling and, therefore, chloride exit through CFTR, results in NKCC serine and threonine phosphorylation. Hypotonic bathing medium also stimulated transporter phosphorylation (18). In hypotonic conditions, cells swell and then undergo regulatory volume decrease, a process in which potassium and chloride are extruded, followed by water (19). This

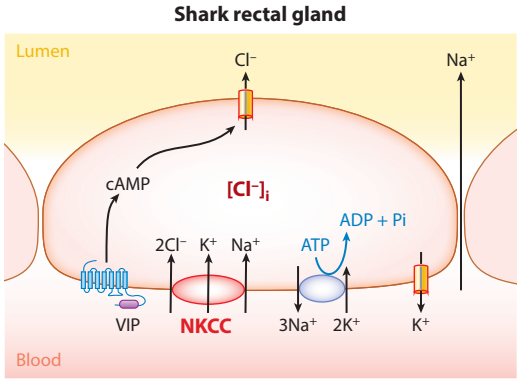

Figure 1

Cl^- coupling in the shark rectal gland. cAMP pathway stimulation of apical Cl^- exit through CFTR results in a drop in $[Cl^-]_i$, which stimulates the phosphorylation and activity of the basolateral NKCC. This replenishes $[Cl^-]_i$ and couples apical Cl^- exit with basolateral Cl^- entry. Na^+ is secreted through the paracellular pathway to achieve secretion of a hypertonic sodium chloride-rich fluid. The Na/K-ATPase lowers intracellular sodium to provide the driving force for ion transport through the NKCC. K^+ is recycled through basolateral K^+ channels. Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; CFTR, cystic fibrosis transmembrane conductance regulator; Cl^-, chloride; $[Cl^-]_i$, intracellular chloride; K^+, potassium; Na^+, sodium; Na/K-ATPase, sodium/potassium-ATPase; NKCC, sodium-potassium-2-chloride cotransporter; Pi, phosphate; VIP, vasoactive intestinal peptide.

restores cell volume and also results in a decrease in intracellular chloride (20). Thus, maneuvers that resulted in decreased intracellular chloride in the rectal gland epithelial cells, such as stimulation of apical chloride efflux or hypotonicity, increased NKCC phosphorylation, implying the existence of a chloride-sensitive kinase. Other work demonstrated that NKCC phosphorylation correlated with transporter activation and also that reduced intracellular chloride activates NKCC activity in many cell types (reviewed in 21). For example, working with internally dialyzed squid giant axons, which allowed precise manipulation of intracellular ion concentrations, John Russell and colleagues (22) demonstrated that low intracellular chloride stimulated NKCC transport activity in a modulatory or regulatory manner.

These findings supported the chloride-coupling hypothesis (18) (**Figure 1**). The stimulation of apical chloride exit lowers intracellular chloride, which activates a chloride-sensitive kinase that is normally inhibited at higher chloride concentrations. NKCC phosphorylation increases basolateral chloride entry, matching apical and basolateral chloride fluxes. This maintains epithelial cell volume during transepithelial ion transport. But what is the identity of the chloride-sensitive kinase?

SPAK and OSR1 Kinases Phosphorylate and Activate NKCCs and NCC

The potassium chloride cotransporters (KCCs) are SLC12 family members related to the NKCCs. Delpire and colleagues (23) used the amino terminus of KCC3a to perform a yeast two-hybrid screen, which identified SPAK (Ste20-related proline-alanine-rich kinase) as a binding partner. OSR1 (oxidative stress response 1) is a second Ste20 kinase paralogous to SPAK and also bound KCC3a. SPAK and OSR1 also interacted with NKCC1 and NKCC2 (23). [NKCC1 is found in nonepithelial cells and, typically, on the basolateral membrane of secretory epithelia, whereas the absorptive NKCC2 is found on the apical membrane of the thick ascending limb

of the kidney (14)]. Soon after, Dowd & Forbush (24) provided evidence that SPAK phosphorylates and activates NKCC1. Additional studies demonstrated that SPAK and OSR1 activate NKCC1, NKCC2, and another related SLC12 transporter, NCC (sodium chloride cotransporter), by phosphorylating the transporters on conserved N-terminal serines and threonines (25–29). SPAK/OSR1 and NCC/NKCC phosphorylation were increased in conditions that lower intracellular chloride (e.g., hypotonic and/or low chloride bathing medium) (24, 25, 28). These results raised the possibility that SPAK and OSR1 could be the chloride-sensitive kinases, with increased autophosphorylation and activation in low chloride, or, alternatively, that an upstream chloride-inhibited kinase may be phosphorylating SPAK and OSR1 to activate them. Autophosphorylation of OSR1 is inhibited in vitro in the presence of increasing chloride concentrations (30), an observation that merits further study. However, attention soon shifted to the kinases just upstream of SPAK and OSR1, the WNK kinases.

WNKs Phosphorylate and Activate SPAK and OSR1

WNK kinases phosphorylate SPAK and OSR1 on a T-loop threonine and an S-motif serine, with the T-loop threonine phosphorylation required for SPAK/OSR1 activation (26, 30–32) (**Figure 2*a***). The activity of WNKs immunoprecipitated from cells was increased when the cells were bathed in hypotonic low chloride medium (25, 32). Furthermore, studies in cultured cells and *Xenopus* oocytes linked low intracellular chloride to WNK autophosphorylation, WNK-dependent OSR1 phosphorylation, and NKCC2 phosphorylation and activation (25, 28, 33, 34). These results suggested that WNKs could be the long-sought chloride-sensitive kinases.

Chloride Inhibits WNKs Through Direct Binding to the Kinase Domain

Definitive proof for chloride regulation of WNKs came from a study demonstrating that chloride stabilizes the WNK1 kinase domain and inhibits its autophosphorylation through direct binding to the active site (2) (**Figure 2*b*,*c***). The mechanisms are discussed in further detail below (see section titled Molecular Mechanisms for Ion Regulation of WNK Kinases). Based on the understanding of the chloride-binding site, this study also generated an important tool for physiologists: WNK mutants with decreased sensitivity to chloride inhibition (2). Introducing these mutations into WNK4 increased NCC transport activity in *Xenopus* oocytes in which NCC and WNK4 were coexpressed, even in the presence of high intracellular chloride, and the mutations had a similar effect on NCC activity as maneuvers that decreased intracellular chloride (35).

Chloride Regulation of WNKs: Physiological Contexts

The physiological significance of chloride regulation of WNKs is an emerging area that continues to be explored. Here, we describe advances in this understanding in the kidney, pancreatic duct, cell volume regulation, nervous system, and inflammation.

Chloride-sensitive WNK signaling in the distal convoluted tubule of the kidney. Sodium chloride is reabsorbed through NCC in the distal convoluted tubule (DCT) of the mammalian nephron, accounting for 5–10% of renal salt reabsorption (36). Inhibition of NCC with thiazide diuretics is a first-line treatment for hypertension (37), and mutations that increase WNK1 or WNK4 activity in the DCT increase NCC activity and cause hypertension in humans (3, 38–40). Thus, NCC activity is an important determinant of blood pressure.

Potassium is a powerful regulator of NCC activity, with high extracellular potassium decreasing NCC phosphorylation and activity, and low extracellular potassium increasing NCC

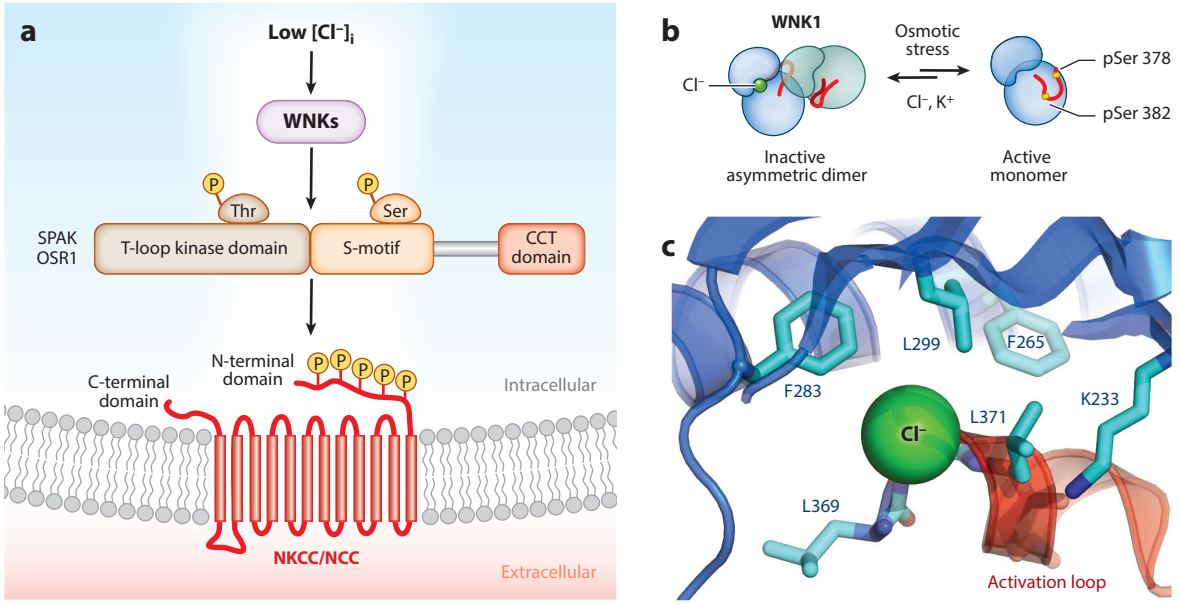

Figure 2

The WNK-SPAK/OSR1-N(K)CC pathway. (*a*) Low $[Cl^-]_i$ activates WNKs, which then phosphorylate the paralogous Ste20 kinases, SPAK and OSR1, on a T-loop threonine (Thr 233 in SPAK, Thr 185 in OSR1) to activate the kinases. WNKs also phosphorylate the SPAK/OSR1 S-motif serines (Ser 373 in SPAK and Ser 325 in OSR1). Activated SPAK and OSR1 go on to phosphorylate NKCC1, NKCC2, and NCC on conserved N-terminal serines and threonines. Transporter phosphorylation (circled P) increases ion transport activity. The SPAK/OSR1 CCT domain interacts with K/RFxV/I motifs on WNKs and N(K)CCs. (*b*) Two WNK kinase domains, each consisting of a smaller N-terminal domain and a larger C-terminal domain, form an inactive, asymmetric dimer that is stabilized by Cl^- ion (*green*) binding to one subunit and possibly by K^+ ion binding as well. The activation loop (*red*) containing the WNK1 Ser 382 and Ser 378 autophosphorylation sites (*yellow*) is buried in the dimer interface in one subunit and occluded from solvent in the second subunit. Osmotic stress or lowering of $[Cl^-]_i$ or K^+ concentrations exposes the activation loop for autophosphorylation. (*c*) Cl^- binding site in Subunit A of unphosphorylated dimeric WNK1. Cl^- contacts backbone nitrogen atoms in a helical turn in the activation loop (*red*). The binding site is otherwise hydrophobic, and the leucine residues Leu 369, Leu 371, and Leu 299 are shown. Two phenylalanines are nearby, Phe 265 and Phe 283. The WNK-specific catalytic lysine, Lys 233, is also nearby. Abbreviations: CCT, conserved C-terminal; Cl^-, chloride; $[Cl^-]_i$, intracellular chloride; F/Phe, phenylalanine; I, isoleucine; K^+, potassium; K/Lys, lysine; L/Leu, leucine; NCC, sodium chloride cotransporter; NKCC, sodium-potassium-2-chloride cotransporter; OSR1, oxidative stress response 1; pSer, phosphorylated serine; R, arginine; Ser, serine; SPAK, Ste20-related proline-alanine-rich kinase; Thr, threonine; V, valine; WNK, with no lysine (K) kinase.

phosphorylation and activity. This influences sodium delivery to the downstream aldosterone-sensitive distal nephron, where potassium is secreted in exchange for sodium reabsorption (41). Thus, increased NCC activity in the face of low potassium decreases sodium delivery to the aldosterone-sensitive distal nephron and reduces potassium secretion in that segment. As discussed above (see subsection titled SPAK and OSR1 Kinases Phosphorylate and Activate NKCCs and NCC), the WNK-SPAK/OSR1 pathway is a major regulator of NCC phosphorylation and activity. An early study showed an inverse relationship between extracellular potassium and OSR1 phosphorylation in COS7 cells, suggesting that changes in extracellular potassium could influence WNK-SPAK/OSR1 signaling (33). Indeed, subsequent in vivo studies in mice demonstrated that the stimulatory effects of low potassium on NCC are dependent on WNK-SPAK/OSR1 signaling (42–45).

How does extracellular potassium influence WNK-SPAK/OSR1 signaling and NCC phosphorylation in the DCT? Terker et al. (43) proposed that low extracellular potassium results

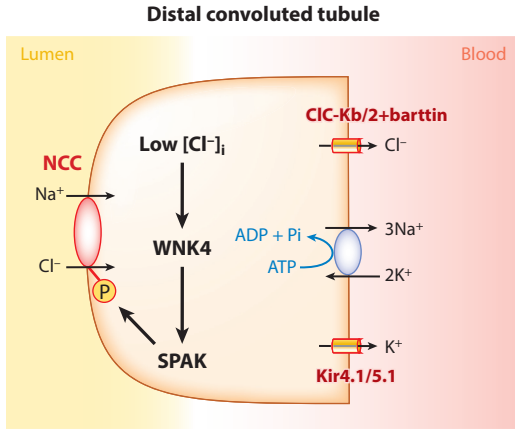

Figure 3

K^+ effects on WNK signaling in the distal convoluted tubule. Low extracellular K^+ results in K^+ efflux through the basolateral Kir4.1/5.1 K^+ channel and hyperpolarization of the membrane potential. This drives Cl^- efflux through the ClC-Kb Cl^- channel and lowers $[Cl^-]_i$. WNK4 (the dominant WNK in the distal convoluted tubule) is activated by low Cl^- and phosphorylates and activates SPAK and OSR1, which phosphorylate (circled P) and activate the NCC. Na^+ delivery to the downstream aldosterone-sensitive distal nephron is decreased, limiting Na^+-dependent K^+ secretion. Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; Cl^-, chloride; $[Cl^-]_i$, intracellular chloride; ClC, chloride channel; K^+, potassium; Na^+, sodium; NCC, sodium chloride cotransporter; OSR1, oxidative stress response 1; Pi, phosphate; SPAK, Ste20-related proline-alanine-rich kinase; WNK, with no lysine (K) kinase. Figure adapted with permission from Reference 147.

in potassium efflux through the basolateral Kir4.1/5.1 potassium channels, hyperpolarization of the membrane potential, and chloride efflux through the basolateral ClC-Kb chloride channel (**Figure 3**). Lowering of intracellular chloride stimulates WNK4, which is the dominant WNK paralog in the DCT, and is also most sensitive to chloride in vitro and in cellular studies (35, 43, 46–48). This model was supported by an elegant series of experiments in HEK (human embryonic kidney) cells (43) and a second independent study in HEK cells (49). In addition, SPAK phosphorylation was decreased in mice in which *Kcnj10*, the gene encoding Kir4.1, was knocked out in the nephron, despite hypokalemia and upregulation of WNK1 and WNK4 (50). A hypomorphic mutation in *Bsnd*, which encodes the barttin accessory subunit required for ClC-Kb trafficking, also abolished the increased SPAK phosphorylation observed with low potassium diet in wild-type mice (51). These studies, as well as other studies of mice with genetic disruptions in *Kcnj10* or *Kcnj16* (encoding Kir5.1), also showed that NCC phosphorylation was no longer sensitive to extracellular potassium and that potassium homeostasis was disrupted (50–55).

Additional support for the role of intracellular chloride in regulating transepithelial ion transport via modulation of WNK signaling came from a study of the *Drosophila* Malpighian (renal) tubule. The Malpighian tubule transports potassium through the main segment principal cell (56). There is a single *Drosophila* WNK ortholog and a single SPAK/OSR1 ortholog, Fray. WNK and Fray regulate transepithelial potassium flux through the regulation of a basolateral NKCC, and hypotonic bathing medium stimulates potassium flux in a WNK-, Fray-, and NKCC-dependent manner (57, 58) (**Figure 4**). Using the transgenic chloride sensor, ClopHensor, Sun et al. (59) demonstrated that intracellular chloride falls in the Malpighian tubule epithelial cells upon exposure to hypotonic medium, and tubule WNK activity increases over 30 to 60 min. Thus, this study linked changes in intracellular chloride with WNK activation and transepithelial ion fluxes. Like

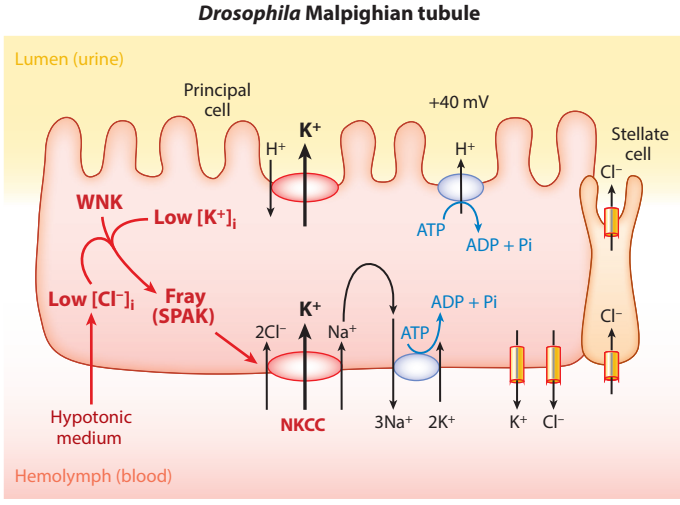

Figure 4

Ion regulation of WNK in the *Drosophila* Malpighian tubule. The *Drosophila* Malpighian (renal) tubule secretes an isosmotic, potassium chloride–rich fluid. The apical vacuolar H^+-ATPase of the principal cell generates a lumen-positive potential difference that drives apical exchange of protons and K^+ as well as transepithelial Cl^- flux through the stellate cells. NKCC is required for normal transepithelial K^+ flux and is regulated by *Drosophila* WNK and Fray, the fly SPAK/OSR1 ortholog. Na^+ entering through the NKCC is recycled by the basolateral Na/K-ATPase. Basolateral K^+ and Cl^- conductances have also been demonstrated. In hypotonic conditions, $[Cl^-]_i$ concentrations fall, WNK is activated, and WNK-, Fray- and NKCC-dependent K^+ flux and fluid secretion are increased. K^+ also inhibits *Drosophila* and mammalian WNKs. Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; Cl^-, chloride; $[Cl^-]_i$, intracellular chloride; H^+, proton; H^+-ATPase, proton ATPase; K^+, potassium; Na^+, sodium; Na/K-ATPase, sodium/potassium-ATPase; NKCC, sodium-potassium-2-chloride cotransporter; OSR1, oxidative stress response 1; Pi, phosphate; SPAK, Ste20-related proline-alanine-rich kinase; WNK, with no lysine (K) kinase. Figure adapted with permission from Reference 59.

the DCT, the Malpighian tubule has basolateral potassium and chloride conductances (60), and WNK activity was also inversely correlated with extracellular potassium in isotonic conditions (59), as predicted by the DCT model.

Su et al. (61) used another transgenic chloride sensor, Cl-Sensor, to measure intracellular chloride in microdissected DCT under different conditions. As in the Malpighian tubule, intracellular chloride decreased in a hypotonic low chloride bath. As predicted, pharmacologic blockade of potassium or chloride channels increased intracellular chloride. Interestingly, acute changes in extracellular potassium resulted in only transient changes in intracellular chloride (~2 min). A possible explanation for this is that when intracellular chloride decreases due to basolateral efflux, WNK4-SPAK-NCC activation restores intracellular chloride. In the Malpighian tubule, intracellular chloride decreased within 10 min of exposure to hypotonic medium, but WNK activation was seen only at 30 min (59). Further studies are required to understand the kinetics of intracellular chloride changes and WNK4 activation in the DCT. One barrier is the difficulty of measuring intracellular chloride. New sensors, and new methods for measuring chloride using existing sensors, may help overcome this problem (62–64).

The chloride-insensitive WNK mutants described by Piala et al. (2) have been exploited to demonstrate the physiological significance of WNK chloride sensing in renal epithelial cells. In HEK cells, expression of chloride-insensitive WNK1 or WNK4 abolished the stimulatory

effect of low potassium medium on SPAK and NCC phosphorylation (43, 65). In the *Drosophila* Malpighian tubule, coexpressing chloride-insensitive *Drosophila* WNK with the scaffold protein Mo25 increased transepithelial ion flux (59). In mice, knocking in mutations that render WNK4 chloride insensitive increased WNK activity (as judged by SPAK/OSR1 phosphorylation) and NCC phosphorylation and activity, with no further increase in SPAK/OSR1 or NCC phosphorylation when the mice were fed a low potassium diet (66). Together, these data provide compelling evidence for the role of intracellular chloride in WNK regulation of N(K)CCs in renal epithelia.

Whether high potassium inhibition of NCC phosphorylation is due to decreased WNK-SPAK/OSR1 signaling, or chloride inhibition of WNK, has been controversial. Some studies showed that a dietary potassium load in rodents, or bathing kidney slices ex vivo in high extracellular potassium, decreased NCC phosphorylation without decreasing SPAK phosphorylation (67–69). Others, however, have observed both decreased SPAK and NCC phosphorylation in ex vivo kidney slices with increasing extracellular potassium concentrations and in vivo in mice undergoing potassium gavage (65, 70). Notably, the in vivo study showed decreased SPAK phosphorylation by immunofluorescence only in the DCT, and not in other segments, nor in Western blots of whole kidney lysates (containing SPAK from multiple nephron segments) (65). This could explain the apparent discrepancy with earlier studies. This study also showed that NCC dephosphorylation in response to potassium gavage was abolished by SPAK/OSR1 double knockout in the nephron, further suggesting a role for turning WNK-SPAK/OSR1 signaling off in the face of high potassium (65). Potassium gavage also failed to suppress NCC phosphorylation in the chloride-insensitive WNK4 knockin mice (66). In contrast, downregulation of total and phosphorylated NCC after administration of a high potassium diet over four days was intact in the chloride-insensitive WNK4 knockin mice, suggesting that chloride plays a role in inhibiting the WNK4-SPAK/OSR1-NCC pathway in response to acute, but not chronic, potassium administration (66). In this regard, additional mechanisms for NCC inactivation also appear to be important for the response to high potassium, including phosphatases and the degradation of WNKs and NCC (67, 69–75).

DCT expression of a constitutively active SPAK mutant, mimicking WNK-phosphorylated SPAK, resulted in DCT hypertrophy and atrophy of the downstream aldosterone-sensitive distal nephron (76). Similarly, feeding mice a low potassium diet for three days increased proliferation in the early DCT, whereas *Kcnj10* knockout resulted in decreased length of the DCT (77). Thus, the role of WNK-SPAK/OSR1 signaling in the DCT may have different effects over shorter and longer timescales. Whether nephron remodeling occurs in the chloride-insensitive WNK4 knockin mice is also an open question.

KS-WNK1 is a kidney-specific isoform of WNK1 lacking the N terminus and most of the kinase domain (78, 79). Knockout of KS-WNK1 amplifies the effects of both low and high potassium on NCC phosphorylation (80), and mutations that increase KS-WNK1 levels increase SPAK, OSR1, and NCC phosphorylation (40). Similarly, KS-WNK1 coexpression with NCC in *Xenopus* oocytes increases SPAK and NCC phosphorylation and NCC transport activity. Intriguingly, this correlates with an increase in WNK4 autophosphorylation, even in high chloride conditions (81). This suggests that KS-WNK1 decreases the inhibitory effect of chloride on WNK4. Although the mechanism is unclear, this effect of KS-WNK1 on WNK4 requires an intact HQ (His Gln) motif required for WNK–WNK interactions (82), suggesting that interactions between WNKs may affect their chloride sensitivity. Studies on WNK3 also hint at this possibility. Human WNK3 expressed in *Xenopus* oocytes, in which native *Xenopus* WNKs are expressed, appears to have little sensitivity to chloride (35, 83). For example, chloride-insensitive WNK3 mutants stimulate NCC activity to a similar degree as wild-type WNK3 (83). In contrast, when the single *Drosophila* WNK is knocked down in the Malpighian tubule and replaced with WNK3, WNK3 activity is

85% greater in tubules expressing chloride-insensitive WNK3 compared to wild-type WNK3, despite a relatively low intracellular chloride concentration of 26 mM (84). Chloride sensitivity of different WNK isoforms expressed in *Xenopus* oocytes also appears to be influenced by their C-terminal domains (83), which are an important locus of WNK–WNK interactions (82). Further studies should help clarify the mechanisms underlying these observations.

Chloride regulation of WNK in the pancreatic duct. The distal pancreatic duct secretes fluid with bicarbonate concentrations of up to 140 mM (85). Like the shark rectal gland, the pancreatic duct expresses an apical CFTR chloride channel. Park et al. (86) demonstrated that CFTR activation decreases intracellular chloride from 20 mM to 5 mM and increases WNK1-SPAK/OSR1 signaling in pancreatic duct cells. CFTR also conducts bicarbonate ions. Remarkably, in low intracellular chloride conditions, WNK1 signaling increased CFTR bicarbonate permeability relative to chloride, which could enable the very high bicarbonate concentrations found in the distal duct (86).

Although WNK1's effects on CFTR were proposed to occur via activation of SPAK and OSR1, this mechanism was revised in a follow-up study from the same group. This study found that WNK1 alone was sufficient to increase CFTR bicarbonate permeability, independent of SPAK and OSR1 (87). Furthermore, although structure-function analysis indicated that the kinase domain was required for WNK1's effect, kinase activity was not, as kinase-dead WNK1 mutants could also increase CFTR bicarbonate permeability when intracellular chloride was low. This suggested that the effects of chloride are independent of effects on WNK1 kinase activity. Rather, chloride modulated the binding of WNK1 to CFTR, with increased binding at lower chloride concentrations. Modeling studies suggested that WNK1 interacts with CFTR via residues found in the WNK dimer interface (88) (**Figure 2*b***). This raises the possibility that low intracellular chloride facilitates WNK1/CFTR interactions by stabilizing the WNK1 monomer and making WNK1 residues available for binding with CFTR that are buried in the inactive WNK1 dimer at higher intracellular chloride concentrations. Further studies are required to investigate this idea.

The cell volume conundrum. In addition to demonstrating increased NKCC phosphorylation in the shark rectal gland in hypotonic conditions, Lytle & Forbush (18) also demonstrated that NKCC phosphorylation was increased when the shark rectal gland was bathed in hypertonic medium. This is consistent with the understanding of the role of NKCC in allowing ion influx after an osmotic challenge that shrinks cells; water follows ions to restore cell volume (19). In duck red blood cells, the NKCC phosphopeptide maps were similar after cell shrinkage or lowering of intracellular chloride, suggesting that the same kinase may be both cell volume and chloride sensitive (89). Indeed, hypertonicity activates WNKs, particularly WNK1 and WNK3, and WNKs are required for cell volume regulation in response to hypertonic stress (1, 83, 90–94). However, this poses a problem, as intracellular chloride increases during shrinkage, which should inhibit WNK. Multiple solutions have been proposed to this problem. First, work in both the internally dialyzed squid giant axon and duck red blood cells demonstrated that NKCC phosphorylation and activity were regulated by shrinkage and chloride in an interdependent fashion, such that hypertonic stress decreased chloride inhibition of NKCC (89, 95). A possible mechanistic explanation for this is illustrated in **Figure 2*b*** and discussed further below (see section titled Molecular Mechanisms for Ion Regulation of WNK Kinases), based on findings of direct osmotic effects on the WNK kinase domain (88). Cells also recruit additional mechanisms, such as the activation of chloride channels, to restrain the rise in intracellular chloride and its inhibitory effects on WNK during shrinkage (96).

Chloride regulation of WNK signaling in the nervous system. The neurotransmitters GABA (γ-aminobutyric acid) and glycine signal through neuronal ligand-gated chloride channels to elicit hyperpolarization (inhibition) if intracellular chloride is low or depolarization (activation) if intracellular chloride is high. NKCCs and KCCs are major determinants of intracellular chloride, which is high during development in most neurons, and low in adulthood (97). In addition to activating NKCCs, WNK-SPAK/OSR1 signaling inhibits KCCs (5). WNKs have been implicated as upstream regulators of neuronal chloride concentrations, for example, by inhibiting KCCs during development to maintain high intracellular chloride concentrations (98). This raises the question of how WNK activity is maintained in immature neurons with high intracellular chloride and vice versa for mature neurons. Inoue et al. (99) shed light on this problem in immature neurons, demonstrating that taurine bypasses high chloride inhibition of WNK by stimulating WNK1 activity and inhibitory KCC phosphorylation, thereby maintaining high intracellular chloride (**Figure 5*a***). Interestingly, taurine is an osmolyte, and WNK1 Ser 382 phosphorylation was increased by taurine, as also seen in the case of cellular or in vitro osmotic stress conditions (1, 88). However, extracellular taurine concentrations in this study were only 3 mM, making an osmotic effect less likely (99). Additional studies are required to determine the mechanism for taurine activation of WNKs and how taurine interacts with chloride WNK inhibitory mechanisms.

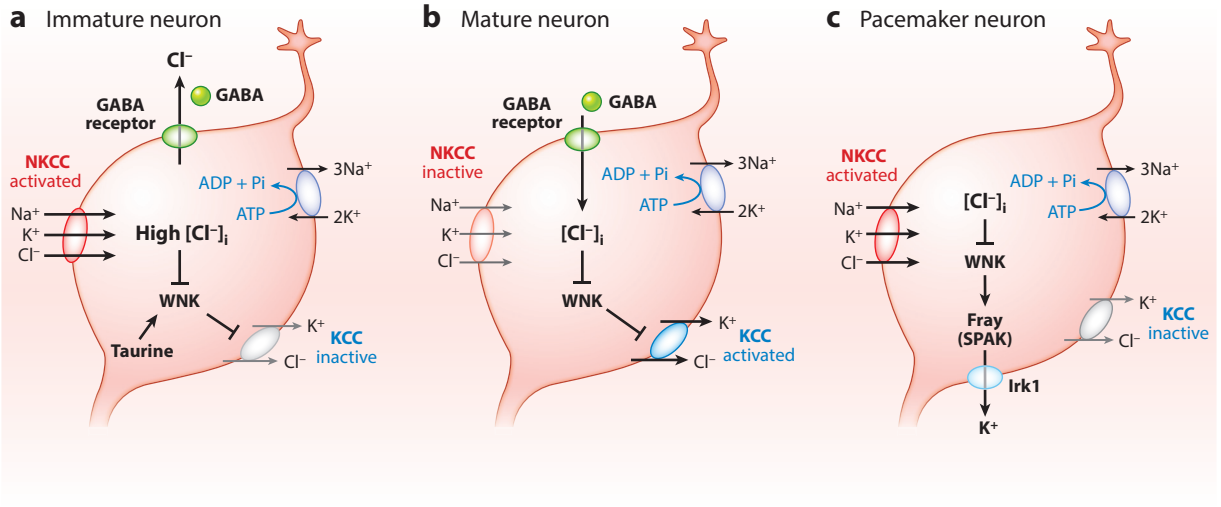

Figure 5

Cl^- regulation of neuronal WNK. (*a*) $[Cl^-]_i$ is typically high in immature neurons and low in mature neurons. As a result, Cl^- movement through $GABA_A$ receptor Cl^- channels will be outward (depolarizing) in immature neurons and inward (hyperpolarizing) in mature neurons. NKCC and KCC are major determinants of $[Cl^-]_i$. Phosphorylation of KCC by the WNK-SPAK/OSR1 pathway inhibits its activity. In immature neurons, taurine activation of WNK bypasses WNK Cl^- inhibition to enable inhibitory KCC phosphorylation. (*b*) In mature neurons, low NKCC activity and high KCC activity maintain low $[Cl^-]_i$ concentrations. Chloride influx through $GABA_A$ receptors may help tune $[Cl^-]_i$. Influx of Cl^- through the $GABA_A$ receptor will inhibit WNK, which in turn will relieve WNK-SPAK/OSR1 inhibition of KCC to stimulate Cl^- efflux through KCC and restore $[Cl^-]_i$ to low concentrations. (*c*) In *Drosophila* sLN_v circadian pacemaker neurons, an NKCC-dependent increase in $[Cl^-]_i$ over the course of the morning inhibits the WNK-Fray pathway and activation of Irk1. Disruption of this pathway lengthens the circadian period. Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; Cl^-, chloride; $[Cl^-]_i$, intracellular chloride; GABA, γ-aminobutyric acid; Irk1, inwardly rectifying potassium channel 1; K^+, potassium; KCC, potassium chloride cotransporter; Na^+, sodium; NKCC, sodium-potassium-2-chloride cotransporter; OSR1, oxidative stress response 1; Pi, phosphate; sLN_v, small ventral lateral neuron; SPAK, Ste20-related proline-alanine-rich kinase; WNK, with no lysine (K) kinase.

WNK1 has also been implicated in the tuning of intracellular chloride in response to GABAergic activity. Heubl et al. (100) demonstrated that pharmacologic antagonism of the $GABA_A$ receptor chloride channel, which is predicted to decrease intracellular chloride, increased WNK1 Ser 382 and SPAK/OSR1 phosphorylation. KCC phosphorylation and lateral membrane diffusion were increased, and KCC clustering and surface expression were decreased, in a WNK-SPAK/OSR1-dependent manner. This resulted in increased intracellular chloride. Conversely, increased chloride influx through the $GABA_A$ receptor is predicted to inhibit WNK-SPAK/OSR1 phosphorylation of KCC, increase KCC activity, and relower intracellular chloride after GABA-ergic signaling, matching KCC activity to $GABA_A$ chloride channel activity to maintain intracellular chloride homeostasis (**Figure 5*b***).

In 1997, Wagner et al. (101) proposed that oscillating intracellular chloride concentrations in central pacemaker neurons of the mammalian suprachiasmatic nucleus (SCN) altered GABA-ergic tone in the day versus night, with excitatory signaling during the day and inhibitory signaling at night. Other investigators also observed oscillating chloride concentrations, as inferred from the GABA reversal potential, but the effects of GABA during the day versus night have varied across studies (102–107). A recent study (108) used the Cl-Sensor transgenic sensor, expressed in a specific subpopulation of SCN neurons, to measure intracellular chloride across the day-night cycle in mice. This study demonstrated increased intracellular chloride during the day, and decreased intracellular chloride at night, consistent with the prior study of Wagner et al. (101). However, GABA application resulted in chloride influx in both day and night, suggesting that the difference in intracellular chloride was not, in fact, affecting GABA-ergic neurotransmission in the SCN (108). This raised the question of whether intracellular chloride could have some other role in pacemaker neurons, such as a signaling role.

The molecular mechanisms underlying circadian rhythms in *Drosophila* have been extensively studied and are highly conserved in mammals (109, 110). The small ventral lateral (sLN_v) neurons are pacemaker neurons in the *Drosophila* central brain (111). A recent study demonstrated circadian oscillation in sLN_v neuron intracellular chloride, with an NKCC-dependent increase in chloride between two and six hours after lights on (112). Flies with NKCC mutations had altered circadian behavior in both light/dark conditions and in constant darkness; the latter reflects dysfunction of the free-running clock. While a normal circadian period is ~24 h, NKCC mutants had a circadian period of ~26 h. This long-period phenotype was suppressed (normalized) by heterozygous loss of KCC, or KCC knockdown in the sLN_v neurons, suggesting it was due to the abnormally low intracellular chloride in NKCC mutants. Low intracellular chloride was hypothesized to activate the WNK-Fray pathway. Indeed, knockdown of either WNK or Fray in the sLN_v neurons also normalized the period of NKCC mutants. Conversely, expression of chloride-insensitive WNK (either *Drosophila* WNK or human WNK3), or constitutively active Fray, resulted in a long-period phenotype, as seen in the NKCC mutants. Importantly, wild-type WNK expression had no phenotype, highlighting the importance of chloride regulation of WNK in this process. Additional studies showed that the long-period phenotype was due to Fray activation of an inwardly rectifying potassium channel (112) (**Figure 5*c***). Like SCN pacemakers, the sLN_v neurons undergo circadian oscillation in the resting membrane potential and neuronal activity, which could be affected by changes in potassium channel activity (113, 114). Thus, these results suggest that intracellular chloride in the sLN_v neurons regulates the circadian period through the WNK-Fray regulation of an inwardly rectifying potassium channel (Irk1). Interestingly, in other *Drosophila* circadian neurons, WNK and Fray appear to operate upstream of NKCC and KCC transporters to regulate intracellular chloride and the response to GABA, highlighting the distinct actions of this signaling pathway in the nervous system (115).

Chloride regulation of WNKs in inflammation. Two hundred billion cells are turned over through apoptosis every day and removed by phagocytic cells in the process of efferocytosis. Perry et al. (116) observed that phagocytic cells that engulf apoptotic corpses have lower intracellular chloride, which was associated with upregulation of proinflammatory pathways. They proposed that low chloride activates the WNK1-SPAK/OSR1 pathway to activate NKCC1 and inhibit KCC1. This will increase intracellular chloride and serve as a brake on further phagocytosis and inflammation (**Figure 6*a***). Consistent with this model, genetic loss of WNK1, SPAK, OSR1, or NKCC1 increased phagocytosis and loss of NKCC1 upregulated phagocyte proinflammatory pathways, which was ameliorated by increasing intracellular chloride using the ionophore tributyltin chloride. Furthermore, in a mouse model of lipopolysaccharide-induced acute lung inflammation, pharmacologic inhibition of NKCC1 or WNK increased inflammatory cytokines and molecular signatures of inflammation (116).

The macrophage NLRP3 (NOD-, LRR-, and pyrin domain–containing protein 3) inflammasome is an intracellular sensor of extracellular microbes, danger signals, and environmental irritants, and its activation leads to the release of proinflammatory cytokines [e.g., interleukin

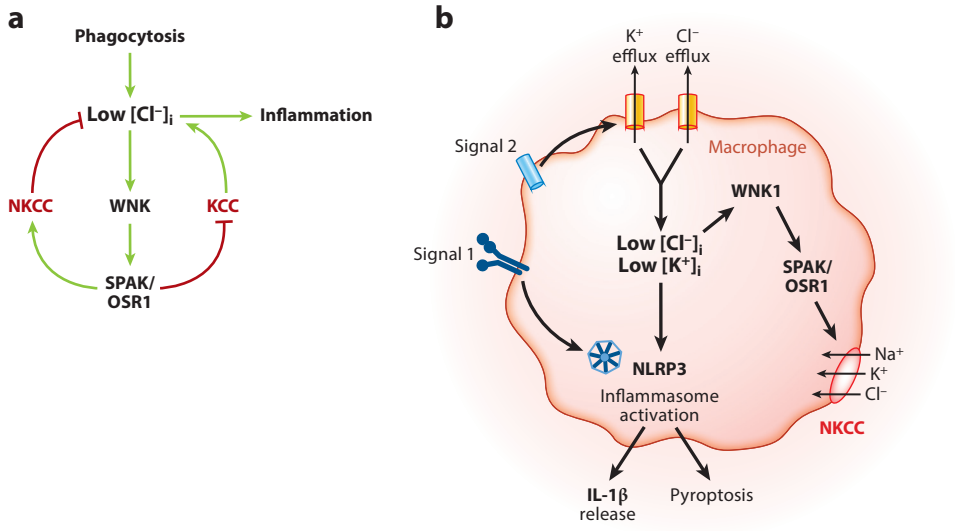

Figure 6

Cl^- regulation of WNK in inflammation. (*a*) In phagocytes that have internalized apoptotic corpses (efferocytosis), an efflux of Cl^- leads to low $[Cl^-]_i$ concentrations, which is proinflammatory. Low Cl^- activation of the WNK-SPAK/OSR1 pathway stimulates NKCC1 activity, and inhibits KCC1, to increase $[Cl^-]_i$ and blunt the inflammatory effects of low Cl^- in these cells. Thus, loss of WNK, SPAK/OSR1, or NKCC1 is proinflammatory. (*b*) The NLRP3 inflammasome in macrophages is activated in the presence of a priming signal 1 (e.g., lipopolysaccharide, TNF, or IL-1β) and an activating signal 2 (e.g., microbes, pore-forming toxins, ATP, or particulates or crystals). Signal 2 stimulates the efflux of K^+ and Cl^-, leading to low $[Cl^-]_i$ concentrations, which are required for NLRP3 activation and the release of inflammatory cytokines (IL-1β) and pyroptosis (inflammatory cell death). WNK1 is activated by low $[Cl^-]_i$ and K^+, and restores concentrations of these ions through SPAK/OSR1 and NKCC1 activation (and perhaps KCC inhibition) to curb excess inflammation. Abbreviations: ATP, adenosine triphosphate; Cl^-, chloride; $[Cl^-]_i$, intracellular chloride; IL-1β, interleukin 1β; K^+, potassium; $[K^+]_i$, intracellular potassium; KCC, potassium chloride cotransporter; Na^+, sodium; NKCC, sodium-potassium-2-chloride cotransporter; NLRP3, NOD-, LRR-, and pyrin domain–containing protein 3; OSR1, oxidative stress response 1; SPAK, Ste20-related proline-alanine-rich kinase; TNF, tumor necrosis factor; WNK, with no lysine (K) kinase.

(IL)-1β] and pyroptosis, a form of inflammatory cell death (117). Efflux of potassium and chloride, with consequent lowering of intracellular potassium and chloride concentrations, has been causally implicated in NLRP3 activation in studies going back to the 1990s (reviewed in 117). Mayes-Hopfinger et al. (118) examined the role of WNK signaling in this process. Pharmacologic inhibition of WNK or SPAK/OSR1, or knockout of WNK1 (but not WNK4) in macrophages, increased NLRP3 inflammasome activation, cytokine release, and pyroptosis in response to several NLRP3 inflammasome activators. This suggests that, as in the process of efferocytosis, WNK-SPAK/OSR1 signaling serves as a brake on macrophage proinflammatory signaling. Like WNK1 knockout, bathing macrophages in chloride-free medium increased NLRP3 activation, IL-1β release, and pyroptosis, and there was no further effect of chloride-free medium on WNK1 knockout cells, suggesting that WNK1 signaling is stimulated by ionic changes. Measured intracellular potassium also decreased in the chloride-free medium, which could also contribute to WNK1 activation, given the recent demonstration of WNK inhibition by potassium (84; discussed in the next section). Interestingly, decreases in both intracellular potassium and chloride in response to several NLRP3 inflammasome activators were exaggerated in WNK1 knockout cells, suggesting that the role of WNK1 is to restore intracellular ion homeostasis, and that failure to do so results in the observed increases in NLRP3 activation and inflammation. Consistent with this idea, bathing macrophages in chloride-free medium resulted in exaggerated decreases in both intracellular chloride and potassium in WNK1 knockout cells. This was accompanied by IL-1β release (up to ~450 pg/mL) in response to chloride-free medium in WNK1 knockout cells but not in wild-type cells. Similarly, decreases in intracellular chloride and potassium were exaggerated in WNK1 knockout macrophages bathed in potassium-free medium, with even more striking increases in IL-1β release (up to ~2,500 pg/mL) in WNK1 knockout macrophages as compared to wild-type cells. Conversely, increasing extracellular chloride or potassium decreased NLRP3 activation, IL-1β release, and pyroptosis (118). Hypotonic medium, and the consequent fall in intracellular potassium and chloride during regulatory volume decrease, was also shown to activate NLRP3 in an earlier study (119). Mayes-Hopfinger et al. (118) showed that NLRP3 activation, IL-1β release, and pyroptosis were also exaggerated in WNK1 knockout macrophages bathed in hypotonic medium. Neutrophil infiltration and IL-1β release were also increased in peritoneal exudates from mice with macrophage WNK1 knockout after injection of monosodium urate (a stimulator of the NLRP3 inflammasome). Thus, the studies by Perry et al. (116) and Mayes-Hopfinger et al. (118) both implicate loss of WNK1 signaling with excess inflammation due to derangements in intracellular ion homeostasis (**Figure 6**).

POTASSIUM REGULATION OF WNK KINASES

The chloride-coupling hypothesis, i.e., that intracellular chloride couples apical and basolateral chloride transport via kinase signaling, informed the hypothesis of a chloride-sensitive kinase, as discussed above (see subsection titled In Search of a Chloride-Sensitive Kinase). The Malpighian tubule principal cell is a potassium-secreting epithelial cell (**Figure 4**). Therefore, Pleinis et al. (84) hypothesized that in addition to sensing chloride and osmolarity, WNKs may also sense potassium. Low potassium bath was previously shown to stimulate endogenous *Drosophila* WNK activity in the Malpighian tubule, whereas high potassium bath was inhibitory (59). However, changes in extracellular potassium can change intracellular chloride, as discussed (**Figures 3** and **4**; see subsection titled Chloride-Sensitive WNK Signaling in the Distal Convoluted Tubule of the Kidney). Therefore, to determine whether there are effects of extracellular potassium independent of changes in intracellular chloride, Pleinis et al. designed a series of bathing media in which extracellular potassium was varied, but measured intracellular chloride was kept constant

(by manipulation of extracellular chloride). High extracellular potassium decreased the activity of Malpighian tubule *Drosophila* WNK at both higher (~26 mM) and lower (~13 mM) intracellular chloride concentrations, suggesting independent effects of the two ions on WNK activity. High extracellular potassium also inhibited mammalian WNK3 or WNK4 when *Drosophila* WNK was knocked down and replaced with the mammalian WNKs. High potassium inhibited both wild-type and chloride-insensitive WNK3 or WNK4 mutants, also suggesting that the effect of potassium is independent of chloride. Ouabain, an inhibitor of the Na/K-ATPase that is expected to decrease intracellular potassium, stimulated *Drosophila* WNK activity (84).

Because changes in extracellular potassium can influence other ions or the membrane potential, additional studies examined whether there are direct effects of potassium on WNK kinases, analogous to the effects of chloride. Like chloride (2), increasing potassium concentrations stabilized the WNK1, WNK3 and *Drosophila* WNK kinase domains. Potassium also inhibited autophosphorylation of *Drosophila* WNK and WNK3 (as well as WNK1 and WNK4; A. Rodan, unpublished observations) (84), again similar to the effects of chloride (2). Experiments with full-length WNK3 and WNK4 across a broad range of potassium concentrations indicated that the kinases were most sensitive to changes in potassium within the physiological range (100–150 mM), with an IC_{50} of 122 mM for potassium inhibition of WNK4. Interestingly, the inhibition curves were slightly different for WNK3 and WNK4, and Malpighian tubule-expressed wild-type or chloride-insensitive WNK4 was activated by low potassium bath, whereas WNK3 was not (84). This hints at possible differences between WNK3 and WNK4 potassium sensitivity, akin to differences observed for chloride and osmotic sensitivity (35, 48, 83), although the mechanisms for these differences have yet to be elucidated. Thus, this study demonstrated that WNK is a potassium-sensitive kinase. Possible mechanisms for potassium regulation of WNKs are under study, with hypotheses shown in **Figure 2*b*** and discussed below (see section titled Molecular Mechanisms for Ion Regulation of WNK Kinases).

Chloride concentrations vary between cell types (12) and, as discussed in previous sections, can also vary within a cell type across time. In contrast, intracellular potassium concentrations are typically more constant due to the activity of the Na/K-ATPase and the role of potassium in determining the resting membrane potential in many cell types. However, there are a number of examples of changes in intracellular potassium in (patho)physiological conditions.

The decrease in intracellular potassium that is required for NLRP3 inflammasome activation and the failure of macrophages to maintain intracellular chloride and potassium homeostasis after activation of the inflammasome in the absence of WNK1 are discussed above (see subsection titled Chloride Regulation of WNKs in Inflammation). Similarly, WNK1 knockdown resulted in lower chloride and potassium concentrations in primary glioma cells (94). Thus, WNK chloride and potassium sensing may be important for maintaining intracellular homeostasis of these ions.

Increasing intracellular potassium stimulates DNA synthesis, with a particularly sharp rise in the range of 90 to 130 mM (120). Intracellular potassium depletion inhibits protein translation and arrests receptor-mediated endocytosis and bulk fluid pinocytosis (120, 121). The mechanisms for these effects are unknown. Pathogenic G1 or G2 variants in *APOL1*, which increase the risk of kidney disease, also cause potassium efflux and lowering of intracellular potassium concentration (122). McFarlin et al. (123) recently demonstrated that muscle potassium concentration decreases from 115 mM to 87 mM in mice fed a potassium-deficient diet for 10 days. Interestingly, SPAK, OSR1, and NKCC1 phosphorylation was increased in muscle in these conditions, suggesting increased WNK activity (123).

Like the shark rectal gland, salivary and sweat glands are chloride-secreting epithelia with a basolateral NKCC and express WNK1 and WNK4 (14, 85, 124–126). Potassium concentrations decrease in salivary and sweat gland epithelial cells upon cholinergic stimulation (127–130),

presumably due to activation of basolateral potassium channels (85). The changes in intracellular potassium were substantial: from 92 mM to 32 mM in the rhesus sweat gland and from 116 mM to 81 mM in the mouse submandibular salivary gland (127, 128), which could increase WNK4 activity by 15–30% (84). Interestingly, two of the studies demonstrating a decrease in intracellular potassium in the salivary gland showed either no change in intracellular chloride or even an increase (129, 130). In salivary gland acinar cells, cholinergic stimulation causes an immediate (within seconds) drop in intracellular chloride concentration, presumably due to apical chloride efflux, and a partial recovery within minutes, likely due to stimulation of WNK signaling and activation of NKCC1 activity (131). Thus, rapid restoration of intracellular chloride and a more sustained depression of intracellular potassium could potentially increase and prolong WNK activation, although further study is required to investigate these ideas.

In the kidney, decreased intracellular potassium was observed in the proximal and distal tubule of rats chronically fed a low potassium diet (132–134). Conversely, intracellular potassium increased in the distal nephron of rats fed a high potassium diet (133, 135). Intracellular chloride in the DCT has been measured (135) or modeled (43, 136) to increase by 2–4 mM in response to physiologically relevant changes in extracellular potassium. Intracellular potassium changes have been measured between 8 and 14 mM in rats fed low or high potassium diets (132–135). A 4-mM increase in intracellular chloride, from 13 mM to 17 mM, is predicted to inhibit WNK4 kinase activity by ~3%, based on in vitro studies (48). Similarly, an 8-mM change in intracellular potassium is predicted to inhibit WNK4 kinase activity by ~4% (84), indicating that changes in intracellular potassium could have similar effects as those proposed for chloride. The effects of chloride and potassium may also be additive (84) and amplified by downstream signaling events. Another possibility is that there are temporal differences in changes to intracellular chloride and potassium, as discussed above for the salivary gland. For example, if changes in extracellular potassium result in transient changes in intracellular chloride (61), due to WNK4-SPAK-NCC activation and apical chloride influx, but changes in intracellular potassium are more prolonged, this could provide a mechanism for ongoing WNK activation. Further studies are required to test this idea, including careful measurements of intracellular chloride and potassium over time.

The findings of constitutively increased NCC phosphorylation in the chloride-insensitive WNK4 mice, and a lack of further increase in NCC phosphorylation in response to a low potassium diet, provide compelling evidence for a role for intracellular chloride-regulated WNK signaling in the DCT response to low potassium (66). A role for potassium regulation of WNK in the DCT response to low potassium is not excluded, however, as the mutations may activate WNK4 to a greater degree than occurs with physiological decreases in intracellular chloride. Some studies have also questioned the role of chloride in the effects of potassium on NCC phosphorylation (67, 136), and the downregulation of total and phosphorylated NCC after four days of high potassium diet was intact in the chloride-insensitive WNK4 knockin mice, indicating that this effect is independent of WNK4 chloride sensing (66). Whether potassium regulation of WNKs plays a role in the DCT requires further study.

High potassium diet also decreases sodium absorption in the thick ascending limb of the kidney (137). The bulk of sodium reabsorption in this segment occurs through NKCC2, which is regulated by WNK4 (138). However, there was no change in total or phosphorylated NKCC2 levels, nor NKCC2 in vivo activity, in the chloride-insensitive WNK4 knockin mice, suggesting different roles for intracellular chloride in regulating WNK signaling in the DCT versus the thick ascending limb (66). Additional studies are required to determine the role of potassium regulation of WNK in the thick ascending limb.

Recently developed potassium sensors (139, 140) will help define the degree to which intracellular potassium may be changing in different cell types and under different (patho)physiological

conditions. Understanding the mechanisms underlying potassium regulation of WNK and the development of potassium-insensitive WNK mutants, analogous to the chloride-insensitive WNK mutants, will also be important for probing the physiological role of potassium regulation of WNKs.

MOLECULAR MECHANISMS FOR ION REGULATION OF WNK KINASES

In a search for crystallization conditions for the unphosphorylated kinase domain of WNK1 (uWNK1), graduate student Thomas Moon discovered that sodium chloride stabilized uWNK1 in thermal shift assays (2). Anomalous scattering X-ray diffraction was conducted using bromide ion, a good scatterer anticipated to bind similarly to chloride. The anomalous difference map revealed a single peak in the active site of Subunit A of dimeric uWNK1 (**Figure 2*c***). The site is a mixture of hydrogen bonding to backbone amide groups and hydrophobic interactions. The backbone amides come from the N terminus of an inhibitory helix in the activation loop (**Figure 2*c***). This helix is a feature in several inactive protein kinases (141). One of the hydrophobic amino acids, Leu 371, emanates from this helix. Another, Leu 369, is close by, and is in the sequence DLG (Asp Leu Gly), homologous to the DFG (Asp Phe Gly) motif present in the majority of protein kinases. Mutation of the hydrophobic amino acids in general led to overall enhanced activity of uWNK1 and reduced chloride inhibition of autophosphorylation and activity (2).

The special catalytic lysine in WNKs (1) may relate to the chloride binding site, as this lysine, Lys 233, is permissive to the formation of the chloride binding inhibitory helix (**Figure 2*c***), where the normal catalytic lysine placement is not (2).

The chloride ion binds to an inactive structure that is distinct from active kinase structures [142, 143; Protein Data Bank (PDB) file 6CN9]. The structure is an asymmetric dimer (144) (**Figure 2*b***). The interactions that form the dimer are present as lattice contacts in other WNK1 crystal structures (e.g., PDB file 5DRB) (145). In contrast, phosphorylated WNK structures are generally monomeric (PDB files 5O26, 5O21, and 5W7T). A concentration-dependent molecular weight has been demonstrated for WNK3 (88), forming dimers and higher-order aggregates above 25 μM uWNK3 in 150 mM sodium chloride. Our model for the inhibition of WNKs by chloride is that it stabilizes an inactive dimeric structure that is not competent to autophosphorylate (**Figure 2*b***). Chloride also inhibits phosphorylated forms of WNKs (2), but it is so far unclear whether the mechanism and chloride binding mode for this inhibition are the same as they are for uWNKs.

The inactive uWNK1 dimer is also an osmotic pressure sensor. The structure possesses unique buried waters bound to residues conserved in WNK kinases (88). This suggests that waters serve as allosteric ligands promoting the inactive structure of WNKs when water is plentiful. We propose that this uWNK conformer is in equilibrium with a dryer autophosphorylation-competent conformer promoted when there is a demand on solvent (**Figure 2*b***). Additional studies are required to determine whether the shift toward the autophosphorylation-competent monomer may allow WNK activation during cell shrinkage in response to hypertonic stress, despite an increase in intracellular chloride.

The activation of WNKs may be multistaged, as there are two phosphorylation sites in the activation loop (Ser 382 and Ser 378 in WNK1), and crystallography of monophosphorylated forms of WNK1 (146) and WNK3 (PDB file 5O21) do not adopt protein kinase-canonical active configurations.

How potassium binds WNKs is unknown. It is clear from in vitro studies that it binds and regulates the kinase domain directly (84). Given the difference in charge between chloride and

potassium it is unlikely that they will bind to the same site, and potassium inhibits WNKs with mutations in the chloride site (84). It will be interesting to discover whether the mechanism follows the same rules as for chloride, namely, binding to an inhibitory dimer and preventing autophosphorylation.

CONCLUSION

How cells sense and respond to changes in intracellular ion concentrations and osmolarity has long been a mystery. WNK kinases are emerging as sensors of the intracellular ionic and osmotic milieu and provide a blueprint to understanding this process at a molecular and physiological level. Ongoing study of these fascinating kinases is sure to provide additional insights, as well as serve as a template for the study of other ion-sensing proteins.

DISCLOSURE STATEMENT

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