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REVIEW

Electroneutral absorption of NaCl by the aldosterone-sensitive distal nephron: implication for normal electrolytes homeostasis and blood pressure regulation

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Abstract Sodium absorption by the distal part of the nephron, i.e., the distal convoluted tubule, the connecting tubule, and the collecting duct, plays a major role in the control of homeostasis by the kidney. In this part of the nephron, sodium transport can either be electroneutral or electrogenic. The study of electrogenic Na^+ absorption, which is mediated by the epithelial sodium channel (ENaC), has been the focus of considerable interest because of its implication in sodium, potassium, and acid–base homeostasis. However, recent studies have highlighted the crucial role played by electroneutral NaCl absorption in the regulation of the body content of sodium chloride, which in turn controls extracellular fluid volume and blood pressure. Here, we review the identification and characterization of the NaCl cotransporter (NCC), the molecule accounting for the main part of electroneutral NaCl absorption in the distal nephron, and its regulators. We also discuss recent work describing the identification of a novel "NCC-like" transport system mediated by pendrin and the sodium-driven chloride/bicarbonate exchanger (NDCBE) in the β-intercalated cells of the collecting system.

Keywords NCC · Distal nephron · Pendrin · Chloride channels · WNK

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Introduction

The kidney plays a critical role in almost all physiological processes, including blood pressure, cell volume and pH regulation, muscle contractility, and neuron excitability, as it keeps constant the concentration, or the body content, of the different ions and water. To ensure homeostasis, a very large amount of plasma and solute is filtered. The different renal epithelial cell types, which can achieve selective reabsorption or secretion of water and ions, then modify the composition of this ultrafiltrate. As a consequence, the daily excretion of water and ions into urine exactly matches the daily intake brought about by the diet. Since the daily intake of each substance can vary considerably from one individual to another, and from time to time, the amount of the different solutes or water absorbed or secreted by epithelial cells is tightly controlled. Schematically, three different zones of the nephron can be functionally distinguished. The proximal tubule (PT) achieves a massive reabsorption of water and solutes. The loop of Henle accounts for the creation and maintenance of a cortico-papillary gradient of solutes, and hence of osmoles, required for the concentration of final urine. The terminal part of the nephron, which includes the distal convoluted tubule (DCT), the connecting tubule (CNT), and the collecting duct (CD), is

responsible for the fine-tuning of all electrolytes and water balances.

Among the different substances transported across the renal epithelium, the sodium ion is of particular importance. Indeed, sodium chloride is the main source of osmoles in the extracellular fluid, including the plasma, and therefore it is one of the critical determinants of blood pressure. The importance of the renal regulation of sodium balance for blood pressure regulation has been initially proposed by Arthur Guyton who observed that volume regulation, and the relationship between blood pressure and renal sodium handling, are abnormal in human individuals affected by hypertension [1]. In fact, a central component of the feedback system for long-term control of arterial pressure is the pressure-natriuresis mechanism, whereby an increase in renal perfusion pressure leads to a decrease in sodium reabsorption and increase in sodium excretion. Guyton's theory was based upon a complex mathematical model of blood pressure regulation. However, according to his pioneering hypothesis, most of the genes mutated in patients with Mendelian syndromes of altered blood pressure have indeed all turned out to be involved in the control of renal NaCl absorption [2]. The finding that all known inherited and acquired forms of hypertension ultimately operate via the same common pathway has led to the proposal that common forms of hypertension should feature increased renal sodium reabsorption as well [2].

The sodium ion is not only important because it sets blood pressure. The main bioenergizer of animal cell membranes is generally the Na^+/K^+ P-ATPase, which converts the energy derived from metabolism into steep sodium and potassium gradients across the cell membrane [3, 4]. The inwardly directed sodium gradient is then used to energize the uptake of many other solutes into the cells via sodium-dependent secondary active cotransporters or exchangers. For example, along the renal tubule, sodium absorption drives the absorption of glucose, amino acids, phosphates, bicarbonate, and chloride. When the process that accounts for sodium absorption across the renal epithelium is electrogenic, sodium transport can also be the primary determinant of the transepithelial voltage difference (V_{te}) that develops across several specific parts of the nephron. In the thick ascending limb (TAL) of Henle's loop, the V_{te} drives the passive absorption of various cations, particularly Ca^{2+} and Mg^{2+} [5]. In the CD, V_{te} drives the secretion of both K^+ and H^+ [6]. Therefore, any change in the rate of sodium absorption in the different nephron segments not only affects sodium balance but can also lead to other electrolyte imbalances.

This interaction of sodium ions with other ions is particularly important in the distal nephron, which comprises the distal convoluted tubule (DCT), the connecting tubule (CNT), and the collecting duct (CD). There, the sodium ion can be absorbed along with chloride via electroneutral processes, or can be absorbed by the electrogenic epithelial sodium channel (ENaC), a mechanism which in turn drives potassium and proton secretion. Many physiological or pathophysiological conditions indicate that the respective proportion of electrogenic versus electroneutral sodium absorption is central for the coordinated (or independent) control of blood pressure, blood K^+ concentration, and acid–base status. For instance, genetic or pharmacologic inactivation of the electroneutral transport in the DCT favors electrogenic sodium absorption by the CD and leads to the development of hypovolemia along with hypokalemia and metabolic alkalosis [7, 8], while its excessive activation provokes hyperkalemia and metabolic acidosis [8–10].

In summary, sodium transport in the distal nephron plays a central role in the control of fundamental physiological processes. Many excellent reviews have described in detail the properties, regulation, and roles of the electrogenic epithelial sodium channel ENaC [6, 11–14]. The purpose of the present review is to summarize the knowledge obtained recently about the different mechanisms of electroneutral NaCl transport processes identified to date in the distal nephron.

Electroneutral NaCl transport by the kidney: from thiazide diuretics to NCC

The discovery of the different ion transporters accounting for the renal absorption of Na^+ is relatively recent. It was the conclusion of intense research efforts aimed at understanding the mechanisms of action of diuretics, and to identify the molecular basis of rare Mendelian diseases characterized by a phenotype mimicking the use of these drugs. Chlorothiazide is one of the oldest diuretics identified [15]. It was originally designed empirically, without the knowledge of renal ion transporters, by modifying acetazolamide, a carbonic anhydrase blocker with weak natriuretic properties [16]. Interestingly, the authors of the seminal article describing the effects of chlorothiazide administration to dogs [15] observed that this drug markedly differs from carbonic anhydrase blockers in that the excretion of sodium promoted by the drug is accompanied by chloruresis rather than by bicarbonaturia. Several years later, in vitro studies reached the conclusion that three modes of transepithelial chloride transport exist: (1) the first is the passive diffusion of the chloride anion through the paracellular pathway driven by transepithelial differences in concentration and electrical potential, like in the toad bladder [17], (2) the second involves electroneutral Cl^-/HCO_3^- exchange [18], and finally (3) J.L. Renfro [19] discovered that in the urinary bladder of the teleost

Pseudopleuronectes americanus (i.e., the winter flounder) active Cl^- transport can also be directly coupled to Na^+. Importantly, a subsequent study performed by J.B. Stokes demonstrated for the first time that the NaCl cotransport system of the winter flounder's urinary bladder is inhibitable by thiazide compounds [20–23]. However, the identification of the molecule targeted by thiazides in mammalian kidneys remained a matter of controversy for a long time because thiazides retain some of the properties of acetazolamide in that they are able to inhibit carbonic anhydrase and thereby a parallel counter-transport system for Na^+/H^+ and Cl^-/HCO_3^- exchange [24, 25]. In order to gain insight into the molecular nature of the NaCl absorptive pathway targeted by thiazides, several groups tried to use a tritiated derivative of metolazone, a thiazide-like compound. Beaumont et al. [26, 27] showed that [^{3}H]-metolazone binds a "high affinity receptor" located at the apical membrane of distal convoluted tubule cells. The binding of [^{3}H]-metolazone could be displaced by several different thiazide derivatives [26], or inhibited by Cl^- [28]. In addition, the density of this receptor was regulated under physiological conditions known to modulate NaCl transport, like changes in dietary sodium or chronic diuretic administration [29, 30]. Ellison et al. [31] were able to solubilize and purify this [^{3}H]-metolazone receptor from rabbit kidney cortex, and used this material to generate a monoclonal antibody that turned out to recognize specifically a unique 125-kDa protein. This protein, again, localized to the apical membrane of cells in the distal convoluted tubule [32]. However, the identification of the molecule accounting for thiazide-sensitive NaCl cotransport did not come from these elegant biochemical and physiological studies. Indeed, the major breakthrough came, again, from studies performed in fish. Indeed, Gamba et al. [33], using a functional expression cloning strategy, finally identified the molecule accounting for the NaCl cotransport activity of the urinary bladder of the winter flounder described originally by J.L. Renfo [19, 33]. The gene encoding the $Na^+/K^+/2Cl^-$ cotransporter NKCC1 of the rectal gland of the shark, *Squalus acanthus*, was subsequently cloned [34], and the superfamily of cation-chloride cotransporters SLC12 was defined. Both genes turned out to share remarkably high sequence homologies, thus enabling the identification of other members of this superfamily through the search of conserved sequences in the genomic databases (for review see Ref. [35]). Gamba et al. [36] isolated the cDNAs encoding the rat isoform of NaCl and $Na^+/K^+/2Cl^-$ cotransporters very shortly after the first cloning in fish. Finally, another branch of the SLC12 gene family, including four distinct KCl cotransporters, was subsequently identified [37–40].

The importance of the NaCl cotransporter (encoded by the *SLC12A3* gene) of the DCT in renal sodium homeostasis is highlighted by the fact that NCC is a target of aldosterone, the main hormone controlling renal Na^+ transport [41, 42]. Moreover, inactivating mutations of *SLC12A3* in humans [43] cause Gitelman syndrome, an inherited recessive disease characterized by low blood pressure; even heterozygous inactivating mutations confer a low blood pressure or a protection against arterial hypertension [44]. In contrast, excessive activity of NCC is central to the phenotype of patients with familial hyperkalemic hypertension (FHHt) [9], also known as Gordon's syndrome or Pseudohypoaldosteronism type II, a rare inherited disease characterized by hypertension that is highly sensitive to thiazide compounds. However, NCC activation in this disease is not caused by activating mutations in the *SLC12A3* gene but by mutations in genes involved in regulatory pathways controlling NCC [9, 45–48]. The link between the molecular and the physiological regulators of NCC has since been intensively studied.

Regulation of NCC activity: from hormones to cellular pathways

In the adult kidney, NCC expression is exclusively restricted to the distal convoluted tubule (DCT) of the kidney [49–51]. NCC mRNA and protein are particularly abundant in the early DCT and decrease gradually along the late DCT in mouse, rat, and human. The rabbit DCT does not show a gradual decrease in NCC expression but a rather abrupt transition with the CNT cells [49]. DCT cells are mitochondria-rich cells with long basolateral infoldings. It is also associated with the highest Na^+–K^+-ATPase activity of any nephron segment [52], probably reflecting the high rate of transport activity of this segment.

Hormones controlling renal salt balance, particularly from the renin–angiotensin–aldosterone system, are known to regulate NCC and tune the intracellular NCC regulatory mechanisms to modify NaCl transport and balance. Other hormones such as vasopressin [53–56] or PTH [57] are known to regulate NCC. Finally, three different groups recently reported that NCC is regulated by insulin, making a potential bridge between hyperinsulinism and salt-sensitive hypertension [58–60].

NCC regulation by the renin–angiotensin–aldosterone system

From the discovery of the remarkable effects of thiazides on blood pressure, it has been obvious that NCC plays a critical role in renal sodium handling. Therefore, it was intuitively proposed that NCC might be regulated by hormones of the renin–angiotensin–aldosterone system (RAAS).

While the effects of the RAAS on ENaC are quite clear, its importance in NCC regulation is still not completely elucidated. The mineralocorticoid hormone aldosterone binds to the cytosolic mineralocorticoid receptor, translocates to the nucleus, and activates the transcription of its target genes. During NaCl restriction, the secretion of aldosterone increases, thereby activating both the transcription and protein abundance of the three subunits composing ENaC [61]. This in turn stimulates Na^+ retention by the distal nephron. However, glucocorticoids (e.g., cortisol) have the same affinity for the mineralocorticoid receptor than aldosterone and are present in the blood at much higher concentrations than aldosterone. Thus, it is predicted that without any protective mechanism the mineralocorticoid receptor should mostly be activated by glucocorticoid, which would prevent any action of aldosterone. The 11β-hydroxysteroid dehydrogenase type 2 (11BHSD2) is an intracellular enzyme that degrades glucocorticoids but not aldosterone. Thus, 11BHSD2 prevents the mineralocorticoid receptor from being activated by glucocorticoids. All aldosterone-sensitive cells are thought to express 11BHSD2. However, 11BHSD2 is absent from the early DCT (the nephron segment characterized by high NCC expression) and is only detectable in the late DCT (a nephron segment characterized by low NCC expression) [62]. Therefore, it was initially proposed that aldosterone is not active in the DCT, and hence could not regulate NCC. However, several groups reported that aldosterone stimulates thiazide-sensitive Na^+ reabsorption in the DCT [42, 63], an effect correlated with an increase in NCC abundance [41]. How the DCT cells are protected against illegitimate activation of the mineralo-corticoid receptor by glucocorticoid remains elusive. Further, during chronic exposure to primary aldosteronism, the kidney has the ability to decrease its sensitivity to aldosterone and thereby minimize Na^+ retention by a phenomenon called "aldosterone escape". Wang et al. [64] showed that NCC abundance is strongly repressed in rats during primary hyperaldosteronism while ENaC is continuously stimulated by a chronic administration of aldosterone. The authors proposed that the downregulation of NCC in this setting account for the escape. The mechanisms blocking NCC responsiveness to aldosterone are still unknown. Nevertheless, the absence of 11BHSD2 in DCT cells and the observation that NCC can be inhibited while aldosterone's secretion is increased both suggest that the effects of aldosterone on NCC might not be direct but rather require some additional factors.

The second factor from the RAAS that affects NCC is angiotensin II (AngII), a vasoactive peptide produced by cleavage of angiotensin I by the angiotensin-converting enzyme (ACE). Angiotensin I itself is produced by renin from angiotensinogen. The production of AngII is stimulated during volume depletion to keep blood pressure constant by favoring renal NaCl retention and vascular vasoconstriction. Consequently, inhibition of AngII generation by ACE inhibitors and angiotensin II receptors antagonists are commonly used as anti-hypertensive drugs. In the kidney, angiotensin II stimulates most of Na^+ transporters, among which NCC [65–69]. NCC is indeed targeted to the DCT apical membrane upon angiotensin II infusion [65]. Until recently, it was unclear whether most, if not all, effects of AngII require aldosterone. In fact, aldosterone's secretion by the adrenals is stimulated by AngII. Thus, the increase in NCC expression observed during AngII treatment could be mediated by aldosterone rather than being a direct effect of AngII. However, a study has recently shown that locally produced AngII rather than circulating AngII plays a crucial role in the regulation of NCC [70]. The hypertensive response and NCC upregulation are indeed blunted in a mouse model devoid of renal ACE. The proposed model is that circulating AngII activates ACE in the proximal tubule, thus increasing the intra-renal production of AngII. This locally synthesized AngII then stimulates Na^+ transporters along the entire distal nephron and particularly enhances NCC phosphorylation and abundance [70]. According to this paradigm, the effects of AngII cannot be mediated by aldosterone. Moreover, another study conducted in rats by Van der Lubbe et al. [71] showing that NCC expression is still increased by a chronic infusion of AngII in adrenalectomized rats further supports the possibility that AngII directly affects NCC expression or activity.

NCC regulation by sodium and potassium intake

NCC is not exclusively regulated by the RAAS. Recent studies have established that chronic and acute K^+ loading decrease NCC activity [72, 73]. An increase in K^+ intake stimulates K^+ secretion by the distal nephron and a reduced NaCl reabsorption in the DCT through NCC is proposed as one of the mechanisms. Indeed, a decrease in NCC-mediated NaCl absorption increases Na^+ and Cl^- delivery to the CNT and the CD. This is then expected to stimulate electrogenic Na^+ reabsorption via ENaC and thus promote potassium secretion by principal cells. The effects of acute K^+ loading on NCC are independent of the accompanying anion since $KHCO_3$ and KCl loading produce the same decrease in NCC [72]. The effects of K^+ loading are also independent of plasma aldosterone levels, as mice that do not generate aldosterone (aldosterone synthase-deficient mice) are able to decrease NCC phosphorylation levels during acute K^+ loading. The latter observation supports the existence of an unidentified kaliuretic factor, regulating NCC [74, 75].

The downregulation of NCC by K^+ loading seems in contradiction with the stimulation of NCC by aldosterone, as an increase in K^+ intake is the major stimulus for

aldosterone secretion. NCC is therefore inversely regulated in two situations of elevated aldosterone, i.e., increased during NaCl restriction or and decreased during K^+ loading. The difference between the two situations is the level of circulating, and therefore intra-renal, AngII. While AngII level is high during NaCl restriction, it is low during K^+ load. AngII level is also reduced when Na^+ intake increases and this is associated with a decrease in NCC expression [76]. NCC could therefore be regulated by AngII rather than by aldosterone. One study, however, supports the direct regulation of NCC by aldosterone. Using adrenalectomized rats submitted to a chronic aldosterone infusion, van der Lubbe and collaborators [71] showed that NCC activation by aldosterone is not inhibited in vivo by losartan, an angiotensin II receptor inhibitor. These contradictory results illustrate the complexity of NCC regulation, and more generally of the coordinated regulation of Na^+, K^+, and Cl^- balance by the distal nephron. Many more studies will be required before a clear physiological model could be established.

The molecular mechanisms by which NCC expression and activity are regulated have started to be unraveled over the last few years. Two main mechanisms have been identified: phosphorylation/dephosphorylation and degradation of the co-transporter.

NCC regulation by phosphorylation

As mentioned above, NCC belongs to the SCL12 family of electroneutral cation-coupled chloride cotransporters, which contains two branches, i.e., the sodium-driven cotransporters (NCC, NKCC1, and NKCC2) and the potassium-driven cotransporters (KCC1-4). Many in vitro studies had shown that NKCC1 activity is modulated by phosphorylation (for review, see [77]) and five threonine residues (Thr175, Thr179, Thr184, Thr189, and Thr202 of shark NKCC1), located in the amino-terminal intra-cellular domain of the protein, were then identified as being subjected to phosphorylation and modulating NKCC1 activity [78, 79]. The phosphorylation of only one of these residues, Thr189, is absolutely required for the cotransporter activity. The phosphorylation of the other residues is modulatory; phosphorylation of Thr184 and Thr202, for example, increases the sensitivity of NKCC1 to changes in intracellular chloride concentration [78]. These five residues are conserved in NCC and NKCC2. These residues are Thr46, Thr50, Thr55, Thr60, and Ser73 in human NCC [80] and, for the sake of simplicity, we will rename them Thr1, Thr2, Thr3, Thr4, and Ser1 in the remaining review, as cDNAs from different species were used in the cited articles. An additional phosphorylation site, without any homology to NKCCs, was identified (Ser91 in human NCC, renamed Ser2 here) by Richardson and collaborators in cells submitted to intracellular chloride depletion [80].

The mutation of Thr4 of rat NCC, corresponding to Thr189 in shark NKCC1, to alanine abolishes sodium transport in *Xenopus laevis* oocytes [81], thus demonstrating that phosphorylation of this residue is essential for NCC activity. This was later confirmed in transfected HEK293 cells [80]. Pacheco-Alvarez further tested the functional importance of Thr3 and Ser1 (corresponding to Thr184 and Thr202 of shark NKCC1), and the results differ from what was obtained for NKCC1. While the mutation of Thr3 inhibits NCC activity only moderately (25 % decrease), like NKCC1, the mutation of Ser1 strongly reduces NCC activity (75 % decrease), when it had almost no effect on NKCC1 basal activity. These differences might result from species and/or conformational and/or amino-acid-sequence differences between NKCC1 and NCC. Importantly, the mutation of Thr4 markedly reduced the phosphorylation of Thr1, Thr3 and, to a lesser extent, Ser2 in HEK293 cells [80]. The abrogation of NCC activity by this mutation could therefore result from a loss of phosphorylation of these three residues in combination with the loss of Thr4 phosphorylation. This study prompted the development of antibodies recognizing the phosphorylated residues of the cotransporter and the use of NCC phosphorylation level as an index of NCC activity in vivo.

Like any transporter or channel, NCC activity can be regulated by modifying its transport capacity or its insertion at the plasma membrane. By performing immuno-electron microscopy on rat kidneys, the group of A. McDonough indeed showed that phosphorylated NCC is found only in the apical membrane while total NCC is found both in the apical membrane and in intracellular vesicles [82]. Whether phosphorylation of the five aforementioned residues affects one or the other or both is still a matter of debate, as reviewed in [83]. The group of G. Gamba showed in *Xenopus laevis* oocytes than the mutation of the phosphorylated residues to alanine does not affect the cell surface expression of the cotransporter [81]. In addition, a NCC cDNA bearing mutations in all three residues fails to be activated by intracellular chloride depletion, which strongly activates wild-type NCC [81]. These results thus suggest that phosphorylation regulates NCC activity and/or sensitivity to intracellular chloride concentration but not its insertion at the apical membrane. However, Richardson and collaborators showed that the transfection of a human cDNA bearing a mutation of the Thr4 residue into alanine in HEK293 cells prevents the insertion of NCC at the plasma membrane [84]. This study therefore suggests that phosphorylation of the N-terminal residues stimulates NCC activity only by increasing its insertion into the plasma membrane [82].

The kinases that phosphorylate NCC were once more identified by homology with NKCC1. In 2002, the group of E. Delpire identified the SPAK (Ste20-related proline-alanine-rich kinase) and OSR1 (oxidative stress response 1) kinases through a yeast two-hybrid screen using KCC3 as a bait [85]. It was then shown that both kinases can also bind NKCC1, NKCC2, and NCC. SPAK and OSR1 phosphorylate NCC-activating residue (Thr4) [80] as well as Thr1, Thr3, and Ser2. The kinase(s) responsible for NCC phosphorylation on Thr2 and Ser1 remain(s) to be identified.

SPAK and OSR1 are both expressed in the DCT but also expressed in the Thick Ascending Limb of Henle's loop, consistent with a role in the regulation of NCC and NKCC2 [56]. The importance of SPAK for NCC phosphorylation in vivo was demonstrated by the characterization of several mouse models, in which SPAK is either knocked-out [86, 87] or bears a missense mutation that prevents its activation (see below; [88]). In all cases, NCC phosphorylation is dramatically reduced (by 60–85 %), which results in the development of a Gitelman-like syndrome in mutant mice, with decreased blood pressure, hypokalemia, and hypocalciuria. Importantly, these studies show that OSR1 cannot compensate for the lack of SPAK and thus probably plays only a very minor role in NCC regulation in vivo. This is supported by the fact that NCC expression and phosphorylation are increased rather than decreased in $OSR^{+/-}$ mice, which display a 50 % reduction in OSR1 expression [89].

However, SPAK is, as its substrates, activated by phosphorylation and yeast two-hybrid screens identified WNK1 and WNK4 as responsible for SPAK phosphorylation [90, 91]. WNK1 and WNK4 belong to the WNK (With No lysine (K)) subfamily of serine-threonine kinases and became the focus of numerous studies related to NCC regulation when mutations in the *WNK1* and *WNK4* genes were found in patients affected by familial hyperkalemic hypertension (FHHt) [47]. This rare Mendelian disorder is characterized by moderate hypertension, hyperkalemia, and hyperchloremic metabolic acidosis. One of the trademarks of the disease is the sensitivity of patients to a very low dose of thiazides. FHHt was therefore believed to be the consequence of NCC activation. Consistent with this hypothesis, WNK1 and WNK4 are both expressed in the DCT [92, 93]. In addition, this hypothesis was confirmed by the characterization of two FHHt mouse models, expressing a mutated WNK4 cDNA, which display increased NCC expression and phosphorylation [9, 10].

The mechanisms by which WNK1 and WNK4 could regulate NCC phosphorylation have been quite extensively studied, mainly in vitro, but many results remain controversial, even though they were obtained in similar models. As mentioned above, WNK1 and WNK4 both bind and phosphorylate SPAK. Phosphopeptide mapping studies demonstrated that WNK1 phosphorylates SPAK at a residue located within the T-loop of the catalytic domain (Thr233 in human SPAK) and a serine residue located within a C-terminal non-catalytic region (Ser373 in SPAK) [91]. Further studies showed that phosphorylation of the T-loop residue is sufficient to activate SPAK, as its mutation into alanine impairs NCC phosphorylation both in vitro and in vivo [80, 88]. The role of the second phosphorylated residue remains unclear [91]. These studies strongly suggest the existence of a WNK1-SPAK-NCC phosphorylation cascade in the DCT, in which NCC is activated by phosphorylation by SPAK, itself activated by phosphorylation by WNK1. We confirmed this hypothesis in vivo, in a mouse model harboring an activation of WNK1 [94]. We observed an increased phosphorylation of SPAK near the apical membrane of DCT cells in the mutant mice, while it was more diffuse in the cytoplasm of control DCTs. This observation suggests that phosphorylation by WNK1 may be required for bringing the SPAK kinase closer to its substrate NCC, in the subapical compartment, thus allowing the phosphorylation and membrane insertion of the co-transporter.

The regulation of NCC phosphorylation by WNK4 appears more complex. Studies performed in *Xenopus laevis* oocytes showed that WNK4 inhibits NCC activity [48, 95] by reducing its membrane insertion through enhanced lysosomal degradation (see below). These in vitro studies were first confirmed by in vivo studies. A mouse transgenic mouse model overexpressing WNK4 indeed exhibits decreased NCC expression [9]. These results are, however, in contradiction with studies showing that WNK4 can phosphorylate SPAK in vitro, even if to a lesser extent than WNK1 [91, 96]. The situation became even more complex with the characterization of a *WNK4* knock-out model and a new transgenic model of WNK4 overexpression. $WNK4^{-/-}$ mice indeed display a dramatic reduction in NCC phosphorylation and expression and thus a Gitelman-like syndrome [97], similar to what is observed in SPAK mutant mice [86–88]. The group of S. Uchida very recently generated a new transgenic model of WNK4 overexpression: surprisingly, this model displays the exact opposite phenotype of the previous one, i.e., increased NCC expression and phosphorylation [98]. Taken together, these two in vivo studies suggest that WNK4 is an activator of NCC, rather than an inhibitor. Unfortunately, no clear explanation has been found yet for these contradictory results. One hypothesis is that WNK4 could exhibit positive or negative effects on NCC activity depending on the physiological situation and that the net effect could depend upon the expression level of WNK4 relative to WNK1, as they have been shown to interact through their carboxy-terminal domain and phosphorylate each other in vitro [99]. A recent study by Na and collaborators [100] supports the "dual effect" of WNK4 towards NCC. The authors characterized the

sensitivity of WNK4 kinase activity to intracellular calcium concentration. The initial hypothesis was that WNK4 missense mutations identified in FHHt patients could modify this sensitivity. Most of the mutations are indeed located in an acidic motif, rich in negatively charged amino-acid residues, and result in an alteration of the negative charge [47]. The negatively charged acidic domain could act as a calcium-sensor and its mutations could modify its sensitivity to Ca^{2+} ions. Na and collaborators first showed that OSR1 phosphorylation by WNK4 is stimulated when Ca^{2+} concentration increases. This change in kinase activity is not observed when a WNK4 FHHt-mutant is used [100]. These data suggest that WNK4 could be switched from an inhibitory or at least from a "weak activator" mode to an activator mode when intracellular calcium concentration increases. WNK1 kinase activity could be similarly stimulated as the acidic domain is extremely conserved between members of the WNK1 family. These observations are supported by in vivo studies. SPAK phosphorylation is indeed increased in mouse models expressing a WNK4 mutant, which display all the clinical signs of FHHt [10]. Furthermore, the inactivation of SPAK in these mice corrects their blood pressure and biological phenotype [101].

An increase in intracellular calcium concentration is known to be induced by angiotensin II (angII), which could therefore change WNK4 kinase activity. This is particularly interesting in the context of the results obtained by San-Cristobal and collaborators, who showed that NCC inhibition by WNK4 is abrogated by angiotensin II in a SPAK-dependent manner in *Xenopus* oocytes [69]. Accordingly, SPAK phosphorylation is stimulated by angII in vitro and in vivo [69, 102]. In addition, the stimulation of NCC phosphorylation by angII is abrogated by WNK4 inactivation in mice [97]. The characterization of the activation status of WNK1 and WNK4 during angII treatment is hampered by the lack of antibodies recognizing the phosphorylated activated form of the kinases in the mouse kidney. Similarly, the implication of WNK1 in angII-mediated stimulation of NCC is hampered by the lack of a pertinent mouse model, as *WNK1* knock-out leads to embryonic death, caused by cardiovascular development defects [103]. The study of Na and collaborators thus provides a mechanism by which angII could activate NCC through the WNK-SPAK cascade [100].

Several years ago, it was shown that aldosterone also activates NCC [41]. This again could be mediated by the WNK-SPAK cascade. The team of E.J. Hoorn indeed showed that SPAK phosphorylation and abundance are increased in adrenalectomized rats receiving chronic aldosterone infusion and losartan treatment, thus permitting the characterization of the effects of aldosterone alone on NCC regulation [71]. They also showed that WNK4 abundance is increased by this treatment.

In conclusion, we have gained a lot of information regarding the regulation of NCC abundance and/or activity by phosphorylation over the past decade. However, crucial questions remain, especially regarding the physiological situations in which the different kinases are stimulated and by which hormone(s). In particular, the observation that aldosterone could activate NCC is puzzling. Aldosterone is indeed secreted in response to sodium depletion or potassium load, two situations which require opposite regulations of NCC. While NCC needs to be activated during sodium depletion, it has to be downregulated during potassium load (see above). How aldosterone leads to opposite changes in NCC phosphorylation during these physiological challenges remains to be understood.

NCC regulation by degradation

Studies in *Xenopus* oocytes showed that WNK4 inhibits NCC activity by reducing its surface expression [48]. This could be achieved by stimulating the endocytosis or by attenuating the surface delivery rate of the cotransporter. Two groups first showed that clathrin-dependent endocytosis is not involved in WNK4-mediated inhibition of NCC [104, 105], thus favoring the second hypothesis. This was confirmed by direct measurements of NCC forward trafficking, which revealed that WNK4 inhibits the anterograde movement of cotransporters traveling to the plasma membrane from the trans-Golgi network [106]. This is achieved through an increased interaction of NCC with the lysosomal-targeting receptor sortilin [107] and the AP-3 adaptor complex, which facilitates cargo transport to lysosomes [106].

A second set of studies, however, showed that WNK4 could also stimulate NCC endocytosis. Like many other transporters, NCC surface expression is reduced by the phorbol ester TPA. Ko and collaborators [108] showed that TPA does not exert this effect through the classical PKC pathway but via activation of the Ras-guanyl-releasing protein RasGRP1, resulting in downstream activation of ERK1/2. Phosphorylated ERK1/2 then stimulates the ubiquitination and dynamin-dependent endocytosis of NCC. Interestingly, the team of H. Cai showed that WNK4 also stimulates ERK1/2 phosphorylation [109]. The in vivo relevance of this pathway was assessed in the rats fed a low- or high-NaCl diet. A low-NaCl diet decreases while a high-NaCl diet increases ERK1/2 phosphorylation [110]. Taken together, these studies suggest that WNK4 could stimulate NCC ubiquitination and endocytosis via an ERK1/2-dependent pathway.

The regulation of NCC surface expression by ubiquitination is reminiscent of that of the epithelial sodium (Na)

channel ENaC. ERK1/2 phosphorylation indeed facilitates the interaction of the β- and γ-subunits of the channel with the ubiquitin ligase Nedd4-2, thereby promoting the ubiquitination, endocytosis, and proteosomal degradation of the channel [111]. It was recently demonstrated that NCC is also ubiquitinated by Nedd4-2 [112]. As for ENaC, sgk1 prevents NCC ubiquitination by phosphorylating and thus inhibiting Nedd4-2. The importance of NCC regulation through Nedd4-2-dependent degradation was confirmed in vivo in mice bearing a nephron-specific inactivation of the ubiquitin ligase, which display increased NCC expression [113]. This pathway once more links NCC to aldosterone. It is indeed well known that sgk-1 expression and phosphorylation are induced by aldosterone, thus leading to increased ENaC surface expression and activity. The inhibition of Nedd-4-2 dependent ubiquitination of NCC could therefore contribute to the activation of the cotransporter by aldosterone.

Identification of a NCC-like transport system in the renal β-intercalated cells

Overview of the mechanisms of chloride absorption by the connecting tubule and the cortical collecting duct

In contrast to the DCT, which is composed of a single cell type, the downstream segments (i.e., the CNT and CCD) are characterized by a cellular heterogeneity. They harbor a mixture of three main cell types: the principal/CNT cells (PCs), the α- and β-intercalated cells (ICs). Until recent studies, Na^+ and Cl^- transport in the CNT and CCD were thought to be achieved and regulated independently. Na^+ reabsorption was thought to be exclusively achieved through ENaC working in tandem with the basolateral sodium pump (Na^+/K^+ P-ATPase), both expressed by CNT cells and principal cells of CCD. In this paradigm, Cl^- transport does not occur through PCs but rather through the paracellular route or through ICs [114], where it is closely related to bicarbonate transport [115].

Non α-intercalated cells (i.e., β-ICs and non α- non β-ICs) express an electroneutral Na^+-independent Cl^-/HCO_3^- exchanger at the apical membrane, which has been identified as pendrin (Pds), the product of the *SlC26A4* gene [116]. Cl^- absorption in the mouse CCD is eliminated with genetic ablation of *Slc26a4* [117]. Conversely, Cl^- absorption is increased in CCDs of mice overexpressing Pds in ICs [118]. Thus, in non α-ICs, apical uptake of Cl^- occurs through pendrin, while basolateral efflux is likely mediated by the ClC-K Cl^- channel (ClC-KB in humans, Clc-k2 in rodents) associated to barttin, a regulatory sub-unit [119, 120]. The potassium chloride cotransporter KCC4, located at the basolateral plasma membrane in intercalated cells [121, 122], also appears to facilitate Cl^- exit in α-IC [121]. Whether KCC4 is also expressed in non α-IC and participate to Cl^- absorption in these cells is currently unsettled.

In the paracellular reabsorptive process, Cl^- transport is driven by the transepithelial voltage difference (V_{te}) generated by electrogenic Na^+ absorption through ENaC [123]. The contribution of the amiloride-sensitive (i.e., ENaC-dependent) component of Cl^- absorption is variable between studies (see Table 1). In CCDs isolated from NaCl-restricted mice, even though amiloride eliminated both the V_{te} and K^+ secretion, it had no effect on transepithelial Cl^- absorption [124], indicating that virtually all Cl^- take the transcellular rather than the paracellular route. Cl^- absorption was not observed in the CCD of pendrin-null mice treated with DOCP and supplemented with $NaHCO_3$, indicating that Cl^- absorption in the CDD under these conditions was completely dependent on pendrin [117]. However, in perfused CCDs isolated from deoxycorticosterone pivalate-treated rats, in the presence of vasopressin, amiloride, which completely eliminated the lumen-negative voltage, decreased chloride absorption by ~50 % [125]. We observed similar results in mice treated with deoxycorticosterone pivalate, ~50 % of the transepithelial Cl absorption was insensitive to amiloride (unpublished results). In contrast, in isolated and perfused CCDs from aldosterone-treated mice in the presence of angiotensin II in the bath solution, benzamil, a derivative of amiloride, reduced Cl^- absorption by 66 % and reduced lumen-negative V_{te} by 75 % [126]. Differences in relative contributions of the paracellular Cl^- pathway between studies might result from differences in the physiological state of the tubules due to different in vivo and ex vivo conditions. To this regard, previous studies support the notion that chronic deoxycorticosterone treatment causes a decrease in the Cl^- conductance of the paracellular pathway [127]. Recent studies proposed that claudins, transmembrane proteins of tight junctions, are modulators of the permeability properties of the paracellular pathway. Claudin-4, -7, and -8 are expressed in the collecting duct. Studies of ion permeability and selectivity using overexpression or knock-down of claudin-7 in cell cultures led to controversial results [128, 129]. Claudin-7-deficient mice have renal salt wasting and chronic dehydration, suggesting that claudin-7 is crucial for the barrier function of the tight junction [130]. Based on studies in cell culture, claudin-4 is thought to form a paracellular pore. siRNA knockdown of claudin-4 or claudin-8 in cultured mouse collecting duct cells significantly decreased the paracellular Cl^- permeability without affecting the Na^+ permeability [131]. Claudin-8 was not found to affect Cl^- permeability by itself but rather to be necessary for the recruitment of claudin-4 to tight junctions [131]. Claudin-4-deficient

Table 1 Key observations supporting the existence of a luminal amiloride-resistant, thiazide-sensitive NaCl transport in the CCD and its importance in maintaining Na^+ balance

References	Experimental models	Observations
[140, 141]	Isolated and perfused CCDs from DOCP-treated rats (7–10 days before) in presence of AVP in the bath solution	Bradykinin caused a 40–50 % inhibition of Na^+ and Cl^- absorption without affecting lumen-negative V_{te} or K^+ secretion
[125]	Isolated and perfused CCDs from DOCP-treated rats (7–10 days before) in absence or presence of AVP in the bath solution	HCTZ reduced Na^+ and Cl^- absorption without affecting lumen-negative V_{te} Amiloride eliminated lumen-negative V_{te} but decreased Na^+ and Cl^- absorption only by 50 %
[117]	Isolated and perfused CCDs from DOCP-treated Pendrin-null or control mice drinken $NaHCO_3$ for 5–10 days	Cl^- absorption was detected in the CCD from wild type mice but not in CCDs from pendrin-null mice
[124]	Isolated and perfused CCDs from Na^+-depleted mice (10–15 days)	Amiloride completely eliminated the lumen-negative V_{te} and K^+ secretion and decreased Na^+ absorption by 50 % but had no effect on Cl^- absorption
		Combined HCTZ and amiloride completely eliminated Na^+ and Cl^- absorption
		HCTZ alone decreased Na^+ absorption by 50 %
		Both pendrin and NDCBE activities are blocked by HCTZ (100 μM)
	Isolated and perfused CCDs from NCC-null mice	No effect of amiloride on Na^+ and Cl^- absorption and
		HCTZ completely eliminated Na and Cl absorption
	Isolated and perfused CCDs from Na^+-depleted collecting-duct specific ENaC-null mice	CCDs reabsorbed Na^+ and Cl^-, did not secrete K^+ and did not develop lumen-negative voltage
	Isolated and perfused CCDs from Na^+-depleted NDCBE-null mice	No detectable amiloride-resistant Na^+ and Cl^- absorption
	Pendrin expressing Xenopus laevis oocytes	HCTZ (1 mM) decreased pendrin activity by 50 %
[126]	Isolated and perfused CCDs from aldosterone treated mice (5–7 days) in presence of angiotensin II in the bath solution	Benzamil reduced Cl^- absorption by 66 % and lumen-negative V_{te} by 75 %
[157]	In vivo studies on double knock-out mice for Ncc and pendrin	Double deletion caused severe salt wasting, volume depletion and renal failure
[154]	Isolated and perfused CCDs from mice lacking the B1 subunit of the v-H^+-ATPase fed either a normal or a Na^+-depleted diet	These mice displayed a renal loss of NaCl and developed hypovolemia and lower blood pressure but their CCDs did not reabsorb NaCl
[118]	Isolated and perfused CCDs from mice overexpressing pendrin in IC	Overexpression of pendrin stimulated NaCl absorption but did not lead to K^+ secretion and lumen-negative V_{te}

mice have been recently generated. No conclusion regarding claudin-4 and its paracellular Cl^- channel function in native tissue could be drawn from these mice as they develop lethal hydronephrosis and obstructive uropathy due to urothelial hyperplasia [132]. Acute aldosterone treatment modulates claudin-4 phosphorylation and increased paracellular Cl^- conductance in cultured rat cortical collecting duct cells [133]. Aldosterone also upregulates claudin-8 transcription in the distal colon [134]. If the expression of the gene encoding claudin-8 is regulated similarly in the ASDN as in the distal colon, aldosterone is expected to upregulate claudin-8 and to increase paracellular Cl^- conductance. Taken together, claudin-4 provides a potential molecular mechanism for coupling paracellular Cl^- transport to Na^+ reabsorption in the collecting duct in response to aldosterone stimulation. Abnormal increases in paracellular Cl^- absorption across the tight junction in the collecting duct was first advanced to explain FHHt [135, 136]. The serine threonine kinase WNK4, especially the mutant WNK4 which produces FHHt, can phosphorylate claudin-4 [137] or claudin-7 [138] and promote paracellular Cl^- permeability in cultured cells [137–139]. This is compatible with the original hypothesis that a gain-of-function in chloride shunt conductance could cause the syndrome. However, transgenic mouse models harboring the FHHt mutations reveal no difference in paracellular Cl^- permeability of the collecting duct [10].

Identification of a novel electroneutral thiazide-sensitive NaCl transport system in the intercalated cells

Studies performed in the 1990s found that even though the expression of NCC is restricted to the DCT, approximately 50 % of Na^+ and Cl^- absorption in the rat CCD is blocked by thiazides, a compound that does not target ENaC [125, 140, 141]. Using an approach combining the use of different mouse models bearing a genetic ablation of sodium transporters along the distal nephron with physiological studies, we demonstrated that thiazide-sensitive NaCl absorption in the CCD results from the functional coupling of two bicarbonate transporters: pendrin and the Na^+-driven Cl^-/HCO_3^- exchanger (NDCBE/SLC4A8) [124, 142]. Experiments conducted in isolated and perfused CCDs to access whether thiazides inhibit amiloride-resistant NaCl absorption by blocking NDCBE, and/or pendrin demonstrated that thiazides block NDCBE and pendrin in intact tubules [124]. The latter study was also consistent with the classical view that the apical epithelial Na^+ channel ENaC and the apical K^+ channel ROMK are responsible for Na^+/K^+ exchange in PCs of the CCD. Indeed, in CCDs isolated from Na^+-depleted mice, thiazides completely abolished chloride absorption but did not affect V_{te} and K^+ secretion, while amiloride had the converse effects.

Pendrin/NDCBE-dependent NaCl absorption by intercalated cells is energized by a proton pump

The identification of an electroneutral NaCl absorption by two bicarbonate transporters in ICs raises several issues. Indeed, the luminal bicarbonate concentration in the CNT and CCD is expected to be very low due to avid reabsorption of bicarbonate in the proximal tubule and the loop of Henle. Hence, one can assume that the bicarbonate required for sustaining NDCBE activity comes from active bicarbonate secretion by pendrin. Moreover, chloride accumulation into the cells through pendrin is expected to favor sodium and bicarbonate uptake via NDCBE. Pendrin has been shown to be energized by an outwardly directed bicarbonate gradient, which results from primary active proton extrusion by the H^+ V-ATPase [126, 143]. Moreover, ICs are thought to have very low Na^+/K^+ P-ATPase activity [144]. These considerations raise the question of the dependence of transepithelial NaCl absorption in β-ICs on either the Na^+/K^+ P-ATPase or the H^+ V-ATPase. To address this issue, we tested the effect of ouabain, a blocker of the Na^+/K^+ P-ATPase, or bafilomycin A1, a blocker of the H^+ V-ATPase, on NaCl absorption by ICs. The Na^+ flux in CCDs was only partially inhibited by either amiloride or ouabain. The simultaneous application of both blockers did not lead to significant additive effects, demonstrating that ouabain alone is sufficient to block the amiloride-sensitive component of Na^+ absorption (i.e., ENaC activity) but does not affect amiloride-resistant Na^+ transport (i.e., Pds/NDCBE activity). Conversely, Cl^- transport was not affected by application of amiloride, ouabain, or simultaneous application of both compounds. By contrast, basolateral application of bafilomycin A1 fully inhibited the amiloride-resistant component of Na^+ and Cl^- absorption. These experiments demonstrate that Na^+ absorption by principal cells is primarily energized by the Na^+/K^+ P-ATPase, whereas NaCl transepithelial absorption by β-ICs is energized by the H^+ V-ATPase.

The putative anion exchanger AE4/SLC4A9 is involved in NaCl absorption by intercalated cells

The aforementioned studies also indicate that basolateral NaCl exit from β-ICs is independent of the Na^+/K^+ P-ATPase. In the absence of the Na^+/K^+ P-ATPase, the parallel action of pendrin and NDCBE is predicted to lead to net accumulation of Na^+ and HCO_3^- into the cell. Thus, we tested whether Na^+ transport across the basolateral membrane of β-ICs could be mediated by a bicarbonate-dependent sodium transporter. AE4, encoded by the *SLC4A9* gene, has been reported to be specifically expressed in β-ICs [145]. The localization and transport characteristics of AE4 were to some extent controversial. First described as a

4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS)-insensitive Na^+-independent Cl^-/HCO_3^- exchanger, AE4 shares more similarities with Na^+–HCO_3^- cotransporters than with anion exchangers of the SLC4 superfamily [146, 147]. Subsequently, AE4 was reported to be rather DIDS sensitive [148]. Finally, others suggested that AE4 might mediate Cl^--independent Na^+–HCO_3^- cotransport rather than Cl^-/HCO_3^- exchange [147, 149].

The subcellular localization and function of AE4, and its potential role in Na^+ extrusion across the basolateral membrane of ICs, were assessed using *Slc4a9* disrupted mice. AE4 is exclusively detected at the basolateral membrane of β-ICs [144]. Experiments performed on isolated CCDs from *Slc4a9*$^{+/+}$ and *Slc4a9*$^{-/-}$ mice demonstrated that AE4 mediates basolateral Na^+–HCO_3^- cotransport when expressed in its normal environment [144]. Furthermore, we also confirmed that it mediates sodium extrusion from renal β-ICs as AE4 inactivation, like NDCBE, blocked amiloride-resistant NaCl absorption by these cells [124, 144].

A new paradigm of ion transport by the collecting duct

Based on these studies, we propose a new model for Na^+, Cl^-, and K^+ transport in the CCD. Principal cells mediate Na^+ reabsorption in exchange for K^+ and this process is energized by the outwardly directed gradient of Na^+ generated by the Na^+/K^+ P-ATPase. In intercalated cells, the H^+ pump favors the generation of HCO_3^- by extruding H^+. This generates an outwardly directed HCO_3^- gradient that in turn drives uphill accumulation of Cl^- into the cell. Then, the outwardly directed Cl^- gradient drives the uptake of 1 Na^+ and 2 HCO_3^- ions. The basolateral efflux of Cl^- might occur through a Cl^- channel or a KCl cotransporter while $Na^+(HCO_3^-)_n$ efflux occurs via AE4 (Fig. 1).

Physiological relevance of NaCl absorption by intercalated cells

The role of pendrin in both the maintenance of chloride balance and the regulation of blood pressure is supported by expression and functional in vivo studies. Pendrin expression is primarily and inversely regulated by dietary chloride intake [150] and by factors associated with changes in distal chloride delivery [151]. Furthermore, and like NCC, pendrin expression is stimulated by components of the renin–angiotensin–aldosterone system. Accordingly, targeted inactivation of pendrin induces hypotension [152], which is aggravated when the animals are fed a NaCl-depleted diet [117].

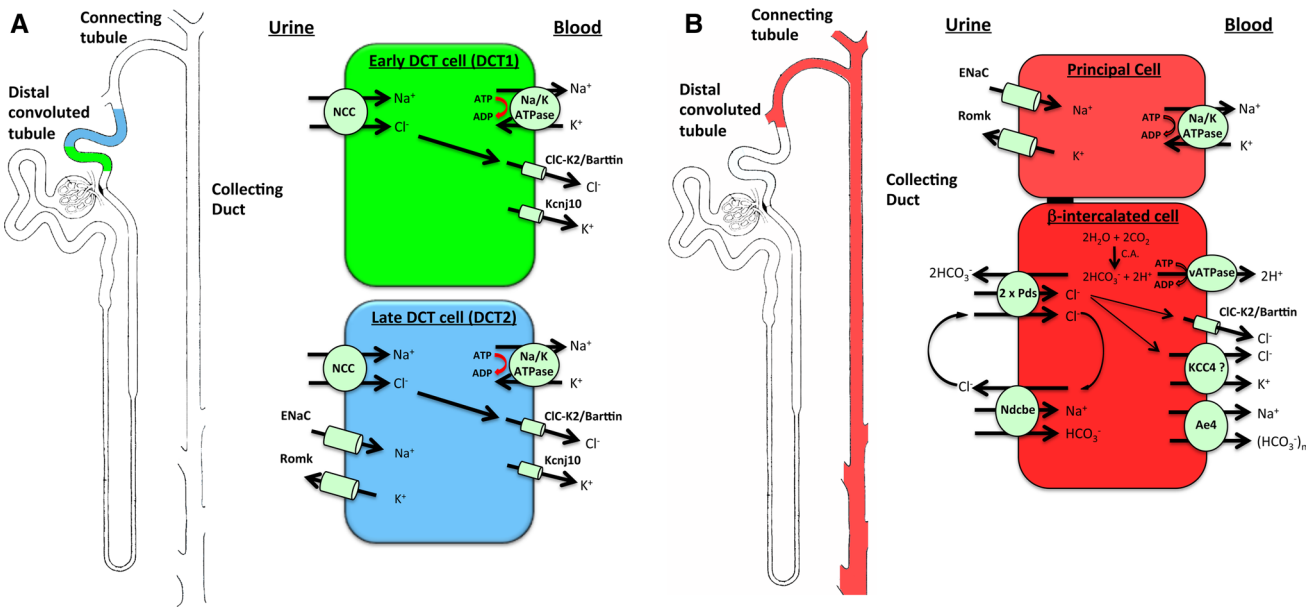

Fig. 1 Electroneutral sodium chloride transport in the distal nephron. Two main electroneutral NaCl transport pathways are found in the distal nephron. **a** In the distal convoluted tubule (DCT), NaCl uptake is mediated by the NaCl cotransporter (NCC) at the apical pole of the DCT cells. Na^+ transport is energized by the basolateral sodium/potassium ATPase (Na/K ATPase). Several channels and transporters are known to participate in NaCl transport. The potassium channel Kir4.1 (Kcnj10) recycles K^+ across the basolateral membrane to support Na/K ATPase activity and the Cl^- channel Clcnk2 and its regulatory subunit Barttin account for the chloride exit across the basolateral membrane. **b** In the β-intercalated cells of the connecting tubule and cortical collecting duct, a second electroneutral NaCl transport pathway has been identified. It involves the apical Cl^-/HCO_3^- exchanger pendrin (PDS) and the Na^+-driven Cl^-/HCO_3^- exchanger (NDCBE). The basolateral Na^+ exit is mediated by the $NaHCO_3$ cotransporter Slc4a9 (AE4). The mechanism of chloride exit is still unknown but could involve the KCL cotransporter KCC4 and also, like in DCT cells, the Cl^- channel Clcnk2 and its regulatory subunit Barttin. In this case, NaCl transport is energized by the basolateral vacuolar proton pump (vATPase) and not by the Na/K ATPase

Pendrin disruption also protects the mice against mineralocorticoid-induced hypertension [153]. Similarly, mice with disruption of the gene encoding the B1 subunit of the proton pump, which also exhibit very low level of pendrin expression, display a renal loss of NaCl causing hypovolemia and lower blood pressure [154]. Conversely, we recently published results showing that mice overexpressing pendrin in ICs develop salt-sensitive hypertension [118]. They exhibit a delayed increase in urinary NaCl and ultimately develop hypertension when exposed to a high-salt diet, indicating that a primary abnormality of renal chloride reabsorption can also lead to NaCl-sensitive hypertension.

The involvement of NDCBE in renal Na^+ handling has not been assessed yet. In a previous study, we showed that NDCBE-deficient mice fed a Na^+-depleted diet could not upregulate the amiloride-resistant, thiazide-sensitive NaCl reabsorption pathway in the cortical collecting duct [124]. As this system would tend to enhance Na^+ retention, one can expect NDCBE dysfunction to be associated with volume depletion.

The key observations published up to now, which led to the conclusion that a luminal amiloride-resistant, thiazide-sensitive NaCl transport in the CCD exists and is accomplished by the parallel action of the Cl^-/HCO_3^- exchanger pendrin and the Na^+-driven $Cl^-/2HCO_3^-$ exchanger (NDCBE/Slc4a8), and is important in maintaining Na^+ balance, are summarized in Table 1.

Crosstalk between β-intercalated and principal cells

During NaCl restriction, pendrin-null mice excrete more Na^+ and Cl^- than wild-type mice and therefore display an apparent vascular volume contraction and lower blood pressure. Higher natriuresis in pendrin-deficient mice after either dietary NaCl restriction or administration of aldosterone was associated with decreased ENaC expression [152]. It was also shown that in mice given furosemide and a high-salt diet, conditions known to increase ENaC function, ENaC-mediated current was lower in CCDs from pendrin-null mice than from wild-type mice [152]. It has therefore been proposed that pendrin could also work in tandem with ENaC to reabsorb NaCl. However, since pendrin and ENaC are expressed in two different types of cell, this should involve modulation of ENaC activity by an extra-cellular signal. Pech et al. [155] tested the hypothesis that this signal is mediated by luminal bicarbonate. They showed that in pendrin-null mice, increasing distal delivery of bicarbonate restores ENaC activity by increasing β- and γ-ENaC protein abundance and, more importantly, γ-ENaC proteolytic cleavage, a process associated with an increase in the channel activity [156]. The authors came to the conclusion that luminal alkalinization due to HCO_3^- secretion by pendrin could enhance ENaC in PCs. This is in line with recent studies by Gueutin et al. [154]. In this study, isolated and perfused CCDs from mice lacking the B1 subunit of the v-H^+-ATPase (*Atp6v1b1*$^{-/-}$ mice), which were shown to develop hypovolemia, did not absorb NaCl and did not develop lumen-negative transepithelial voltage, indicating that ENaC and NDCBE/pendrin activities were both impaired [154]. Of interest, in these mice, pendrin expression was virtually suppressed and ENaC expression was decreased specifically in the cortex as in pendrin-null mice [152]. The authors described a new mechanism that can fully explain these observations; they demonstrated that blockade of the basolateral v-H^+-ATPase in β-ICs leads to ATP release, which in turn triggers PGE_2 release by acting on luminal calcium-coupled purinergic receptors, presumably P2Y2 receptors, resulting in inhibition of ENaC in neighboring PCs. In summary, these studies introduce a new paradigm of crosstalk between PC and IC and provide further evidence that both cell types are important in maintaining Na^+ balance and thus blood pressure.

In conclusion, there are two thiazide-sensitive systems mediating electroneutral NaCl reabsorption in the distal nephron, and not one as originally thought. The first one consists of one co-transporter, NCC, and is present exclusively in the distal convoluted tubule. The second one consists of two exchangers, NDCBE and pendrin, in the β-intercalated cells of the connecting tubule and cortical collecting duct. If the inactivation of one of these systems can be compensated for by the other, as observed in *Ncc*$^{-/-}$ mice [124], the combined deletion of NCC and pendrin causes severe salt wasting, volume depletion, and renal failure [157]. This last observation highlights the crucial part played by the electroneutral Na–Cl reabsorption in the maintenance of NaCl balance and hence blood pressure. Recent studies introduce a new paradigm of crosstalk between PCs and ICs and provide evidence that β-ICs are important in maintaining Na^+ balance and thus normal blood pressure also by controlling ENaC activity in neighboring PCs through release of paracrine factors.

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