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The K-Cl cotransporter-3 (KCC3) in the mammalian kidney

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Abstract

Purpose of review: We recently localized a new KCC3 transporter to the apical membrane of type-B intercalated cells. This gives us an opportunity to revisit the roles of the K-Cl cotransporters-3 in kidney and integrate the new findings to our current knowledge of the biology of the bicarbonate secreting cells.

Recent findings: Here we review the basic properties of the K-Cl cotransporter with a particular attention to the responsiveness of the transporter to cell swelling. We summarize what is already known about KCC3b and discuss new information gained from our localizing of KCC3a in type-B intercalated cells. We integrate the physiology of KCC3a with the main function of the type-B cell, i.e., bicarbonate secretion through the well-characterized apical Cl^-/HCO_3^- exchanger and the basolateral Na-HCO_3 cotransporter.

Summary: Both KCC3b and KCC3a seem to be needed for maintaining cell volume during enhanced inward cotransport of Na-glucose in proximal tubule and Na-HCO_3 in intercalated cells. In addition, apical KCC3a might couple to pendrin function to recycle Cl^-, particularly in conditions of low salt diet and therefore low Cl^- delivery to the distal tubule. This function is critical in alkalemia, and KCC3a function in the pendrin-expressing cells may contribute to the K^+ loss which is observed in alkalemia.

Keywords

K^+ transport; Cell volume; Distal nephron; Type-B intercalated cells; bicarbonate

Introduction

The K-Cl cotransporter mediates the electroneutral, Cl^- coupled, movement of K^+ ions across plasma membranes. The transporter belongs to the SLC12 family of solute carriers, itself a member of a superfamily of amino acid/polyamine/organocation transporters (1). The gene encoding K-Cl cotransport originated in protists and duplicated twice during evolution, resulting into four distinct genes in higher vertebrates: SLC12A4 (KCC1), A5 (KCC2), A6 (KCC3), and A7 (KCC4).

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Conflicts of interest

The authors declare no conflicts of interest.

The K-Cl cotransporter was originally described as a functional transport unit in sheep and human red blood cells (2, 3). The transporter was shown to be activated by N-ethylmaleimide (2), cell swelling (3), and inhibitable by high doses of furosemide (4). Like the Na-K-2Cl cotransporter (5), the K-Cl cotransporter functional unit is a dimer (6). The recent cryo-electron microscopy (cryo-EM) structures of NKCC1 (7**, 8*), KCC1-KCC4 (9**–11) confirms the dimeric nature of the transporters. As cells express multiple K-Cl cotransporters (12–14), it is believed that they likely express heterodimers. Work performed in heterologous expression systems has provided evidence for cotransporter heterodimerization (6, 15).

Swelling activation is a key property of K-Cl cotransporters

K-Cl cotransport activity is activated by dephosphorylation and inhibited by phosphorylation (16, 17). A simple two-state model fits nicely the data of swelling activation of K-Cl cotransport (18) with swelling principally inhibiting the *kinase* that shuts *off* KCC function. The current consensus in the field is that individual transporters at the membrane are either *on* (dephosphorylated) or *off* (phosphorylated), and the overall transport measured in a cell, depends upon the number of transporters expressed at the membrane that are in the *on* state. K-Cl cotransporters possess at their amino terminus a binding site for Ste20p-like kinases (19) and it is the phosphorylation of two key threonine residues, located in the COOH tail of the cotransporter, that inactivates KCC (20). Despite the recent resolution of the structure of the transporters, their activation/de-activation by phosphorylation are still poorly understood.

Expression of KCC3 in kidney

The cloning of human KCC3 revealed the existence of two alternative start sites or alternative promoters (21–23). Mount and Race independently cloned KCC3 from human muscle (21) and placenta (22), respectively. Hiki and colleagues also isolated KCC3 from human umbilical vein endothelial cells (23). As shown in Figure 1, the 109 kb hKCC3 gene starts with exon 1a and finishes with exon 25. Exon 1b is located some 17.6 kb downstream of exon 1a and 43 kb upstream of exon 2. Exon 2 is a small 45 bp alternatively spliced cassette exon encoding a short (15 amino acid long) peptide. Detailed transcript analysis revealed that KCC3a, the isoform containing exon 1a, is highly expressed in muscle and brain, whereas KCC3b, the isoform starting at exon 1b, is predominantly found in the kidney (14). KCC3 is expressed at the basolateral of proximal tubule epithelial cells (24). However, until recently, no antibodies could distinguish between the KCC3a and KCC3b isoforms. After generating and validating a KCC3a-specific antibody, we examined KCC3a localization along the nephron (25**). Except for specific cells labeled in a discontinuous pattern in the cortex, the KCC3a signal was extremely low in kidney sections from wild-type mice. We localized KCC3a in intercalated cells of type-B or type-nonA/nonB using co-staining with pendrin antibody, an intercalated cell marker (26). While the majority of the signal was detected at the apical membrane alongside pendrin, a few cells showed weaker signal at the basolateral membrane (25**). Additional co-labeling experiments revealed that the KCC3a signal was only present in V-ATPase positive cells, distinct from cells expressing the sodium chloride cotransporter (NCC), the epithelial sodium channel (ENaC), and calbindin-28, indicating that KCC3a expression was absent in the distal convoluted

tubule (DCT, identified with antibody reacting with phosphorylated NCC or pNCC), and CNT principal cells (identified with ENaC and calbindin-28). The absence of KCC3a signal in the proximal tubule also indicated to us that the KCC3 signal observed in the proximal tubule must be originating from KCC3b.

Function of KCC3 in the kidney

Creatinine, urea, Na^+, and H^+ serum concentrations in KCC3-KO mice do not differ from controls. However, hourly diuresis and increased water consumption were observed in KCC3 knockout mice, although with no variation in urine osmolality or electrolyte concentration (27). In a separate study, Gerardo Gamba's group showed that while Na^+ or K^+ depletion had no effect on KCC3 abundance, hyperglycemia caused a 2-fold increase in KCC3 expression in the rodent kidney (28). On this observation, they inferred that KCC3 function in the proximal tubule was to either maintain sodium-glucose cotransporter-2 (SGLT2) activity by promoting the basolateral outflow of Na^+ through the Na^+ pump, or to prevent SGLT2 activity from causing the epithelial cells to swell (28). The cell swelling hypothesis is attractive as mathematical models of active epithelial ion transport, e.g., in DCT, indicate the importance of adding a K-Cl cotransporter for the cells to maintain their volume (29).

In two recent papers, we addressed the role of KCC3a in the connecting tubule. In the first manuscript, we demonstrated that KCC3a expression was increased by a variety of manipulations that led to alkalosis. For instance, we observed increased KCC3a expression following 24-h water restriction or salt deficient diet (25**). This observed increase can be accounted for by contraction alkalosis, as for a defined amount of HCO_3^- in the blood, a decrease in plasma volume (contraction) will result in increased HCO_3^- concentration or alkalosis. Furthermore, the condition that resulted in the largest increase in KCC3a expression was feeding the mice with 280 mM bicarbonate in the drinking water (25**). As this treatment resulted in increased pendrin and KCC3a abundance and KCC3 deletion resulted in decreased pendrin abundance, we proposed a functional relationship between pendrin and KCC3a (25**). In conditions of alkalemia, the cortical collecting duct secretes HCO_3^- via pendrin, an apical, electroneutral Cl^-/HCO_3^- exchanger. This function is mediated by type-B intercalated cells. Pendrin secretes HCO_3^- in exchange of Cl^- in the filtrate, thus contributing to acid-base balance (30–34). Many studies have shown pendrin stimulated by metabolic alkalosis (35–40).

K^+ loss is typically observed in alkalemia (41). It is well known that the intracellular storage of K^+ and the capacity of principal cells to secrete K^+, which harbor renal outer medullary potassium channels (ROMK), and large conductance calcium-activated potassium channels known as Maxi-K or BK channels, can be influenced by acid-base balance (42). Increased luminal or systemic levels of HCO_3^-, pH, distal Na^+ and fluid flow all stimulate ROMK, and BK channel, which in turn causes an increase in distal K^+ secretion (42–45). Further studies are needed to dissect the contributions of ROMK, the BK channel, and KCC3a to the K^+ loss observed in alkalemia.

Although, as just discussed, the K^+ loss is often associated with K^+ channel activity like ROMK in principal cells, a recent study demonstrated that pendrin was required for K^+ excretion under a high $KHCO_3$ diet (46). In our recent study, we hypothesized that pendrin and KCC3a are functionally coupled (Figure 2A), thereby accounting for the electroneutral K^+ loss that occurs through KCC3a when Cl^- is recycled to sustain pendrin activity (25**). Note that an alternative model involves cell volume regulation mediated by KCC3. Indeed, bicarbonate transport across the intercalated cell may cause cell swelling (47, 48). Therefore, instead of Cl^- recycling, water may be recycled via KCC3 activity, again to the detriment of K^+ (Figure 2B). This hypothesis would also be consistent with the fact that KCC3 activity is otherwise silent under isosmotic conditions. Once again, this KCC function was mathematically modeled in the rat DCT; the transporter was needed to maintain cell volume during various stages of epithelial transport (29).

Our two models of Figure 2 assumed that the gradients support the efflux of K^+ and Cl^- (and water) into the lumen. According to microperfusion studies in the distal nephron, the luminal concentrations of K^+ and Cl^- in the distal nephron are 2 mM and 30-60 mM, respectively (referenced in (29)). Since the outside product of $[K^+]$ x $[Cl^-]$ is 20–30 times smaller than the inner product, it would require an excess of 40–50 mM luminal K^+ to reverse the gradients. Therefore, transport through apical KCC3 is poised in the direction of secretion or K^+ loss. If KCC3a facilitates the loss of K^+, one would anticipate a decreased expression of KCC3a under low K^+ diet. Against this expectation, we observed increased KCC3a abundance in mice fed with a K^+ deficient diet. While this increase in KCC3a can be explained by the K^+ deficient diet causing alkalosis (49, 50), it is surprising that under low K^+ availability, the kidney would upregulate a transporter that facilitates the loss of K^+. One could postulate that under these conditions the transporters are mostly silent. However, this would be inconsistent with recent data showing that when pendrin knockout mice are placed on a diet low in Na^+, Cl^- and K^+, they excrete a far greater amount of K^+ than wild-type mice (51*). Under normal settings, pendrin-KO mice can maintain a normal acid-base balance (52), but when they are maintained on a NaCl + K^+ deficient diet, they experience severe metabolic alkalosis. The enhanced K^+ excretion seen in pendrin-KO mice fed a diet deficient in both K^+ and NaCl would therefore be consistent with additive or synergistic overexpression of the KCC3a.

Other transporters affecting pendrin function

Several studies have shown that the cystic fibrosis transmembrane conductance regulator (CFTR) interacts with pendrin possibly to stabilize the exchanger in the membrane (53–55). Under baseline conditions, deletion of CFTR has no impact on pendrin abundance (56), in contrast to deletion of KCC3 having a significant impact on pendrin (25**). Evidence suggests, however, that under base loading “stimulated” conditions, CFTR is required for proper pendrin function (56). Moreover, another study reported decreased apical expression and enhanced intracellular localization of pendrin in CFTR-KO mice compared with wild-type mice during bicarbonate loading (57). Thus, there seems to also be a functional link between the exchanger and the Cl^- channel, maybe like the relationship between ROMK and NKCC2 in the thick ascending limb. Note that while CFTR might very well interact to facilitate pendrin function, the Cl^- channel cannot explain the K^+ loss observed in alkalemia.

A similar function had been assigned to the Na^+ driven Cl^-/HCO_3^- exchanger (NDCBE), which makes recycling of Cl^- and HCO_3^- possible. NDCBE is necessary to preserve intravascular volume and Na^+ balance when the body is depleted of salts (58). However, nonA and nonB intercalated cells do not express NDCBE and have higher pendrin abundance and activity (26, 59). As the abundance of KCC3a overlaps with pendrin in the cortex, it suggests expression of KCC3a in nonA/nonB intercalated cells; thus, the cotransporter is more likely to recycle Cl^- than NDCBE. A net result of this operation would be $KHCO_3$ secretion in a state of alkalemia. It appears, however, that pendrin can couple with a variety of transporters and, thus, a particular physiological need may dictate which transporter will functionally pair with pendrin. Further studies are required to identify these different physiological conditions and the corresponding transporters that pendrin couples with.

The entry pathway(s) of K^+ and HCO_3^- into the type-B intercalated cells are still unclear. According to textbooks, proton pumps instead of Na^+/K^+ pumps power renal intercalated cells. However, the Na^+/K^+-ATPase is in fact expressed in intercalated cells (60) and thus, like in many other cells, K^+ enters intercalated cells through the Na^+/K^+ pump. In contrast to type-A intercalated cells which express NKCC1 at the basolateral membrane (61, 62), type-B cells lack the cotransporter. Thus, aside from the pump, it is unclear what other transport pathways participate to the entry of K^+ at the basolateral membrane of type-B intercalated cells. As far as bicarbonate is concerned, it can enter the cell as CO_2 diffusion followed by hydration to HCO_3 by carbonate anhydrases or enter through the Na^+ dependent Cl^-/HCO_3^- exchanger - AE4 (*SLC4A9*) (63). According to recent study, AE4 serves as both a HCO_3^- entry channel into type-B intercalated cells and a HCO_3^- sensor at the basolateral membrane (64**). It is possible that AE4 is as critical for the KCC3a function as it is for the pendrin function.

Regulation of KCC3a in the distal nephron

In our 2022 paper, we showed that deletion of KCC3 decreased the abundance of pendrin in mice (25**). This suggests that pendrin may be dependent on KCC3 function. Whether the reverse situation was true, i.e., deletion of pendrin affects KCC3 expression, had yet to be addressed. Furthermore, the mechanism by which alkalemia stimulates KCC3a expression was unknown. Are aldosterone and/or angiotensin-II involved in the process? Our most recent KCC3a study published in 2023 focused on these issues. In this paper, we showed that KCC3a abundance at the apical membrane of the intercalated cells increased after 24 hours and remained elevated after 7 days of $NaHCO_3$ treatment (65*). Other study, however, has demonstrated that after 7 days of the HCO_3^- load, pendrin abundance returns to baseline, even though pendrin abundance initially increases with HCO_3^- (39). We also investigated whether KCC3a abundance changed when acute acidosis was induced via NH_4Cl treatment. We observed that a 24-hour NH_4Cl treatment had no effect on the abundance of KCC3a or pendrin, contrary to a previous observation that pendrin abundance decreased upon chronic NH_4Cl treatment (66). This difference in pendrin behavior could be explained by differences in sample preparations: membrane protein preparation versus crude protein preparation, which contains both membrane and cytosolic proteins. Another possible explanation is that KCC3a abundance was already at its lowest in the baseline condition.

However, we might observe the effect of NH_4Cl on KCC3a when KCC3a expression is already upregulated.

Our work also demonstrated that KCC3a abundance was increased with $NaHCO_3$, $KHCO_3$ or K-citrate treatments (65*). K-citrate would function as $KHCO_3$ because citrate enters the Krebs cycle, producing CO_2 alongside ATP, leading to the formation of HCO_3^-. These results confirmed that it is the organic anion and not the cation that increases KCC3a abundance. As pendrin expression in intercalated cells is affected by aldosterone and angiotensin-II (67, 68), we addressed the role of these factors in KCC3a activation. We showed that KCl supplementation in the diet, which is known to stimulate aldosterone production, did not increase KCC3a abundance. KCC3a abundance is increased by 23 hours of water restriction, Na^+ deficient diet, or otherwise known as contraction alkalosis (25**); however, angiotensin-II, a key and significant signaling molecule under these circumstances, may also contribute to the increase in KCC3a abundance (69). High salt diet suppresses angiotensin-II release (70), yet we found that HCO_3^- under either high salt diet or normal salt diets increased the abundance of KCC3a and pendrin, compared with high salt diet alone (65*). In addition, systemic suppression of angiotensin-II functions by blocking the angiotensin A1 receptor with losartan had no effect on KCC3a abundance on normal or Na^+ deficient diets, thus ruling out any significant role for angiotensin-II in the increase in KCC3a abundance after 23 hours of water restriction or Na^+ deficient diet (65*). Taken together, our findings indicate that the main signaling molecule is HCO_3^-, not aldosterone or angiotensin-II. Experiments performed in mouse cortical M-1 cells, which express the intercalated cell markers pendrin, V-ATPase, and Gpr116 (71, 72) showed that KCC3a expression in the cells was significantly increased in bicarbonate-containing culture media, compared to HEPES-containing culture media, whereas pH had no effect (65*). Thus, HCO_3^- seems to be the primary signaling molecule responsible for the increased KCC3a abundance.

In contrast to KCC3b, whose mRNA levels are increased in hyperglycemia (28), the increase in KCC3a expression observed in alkalemia does not involve transcription (65*). In fact, despite the significant increase in protein abundance, KCC3a mRNA levels remained unchanged in the kidney of mice fed with bicarbonate. This observation suggests alterations in protein translation or, more likely, changes in protein degradation. This discovery presents a new avenue for further investigation.

The lack of reliable antibodies against phosphorylated KCC3 poses a challenge in studying KCC3 function. The availability of a KCC3 knockout mouse model should in principle help confirming the involvement of KCC3 in K^+ loss in alkalemia. However, due to the severe peripheral neuropathy disorder, we lost two cohorts of KCC3 knockout mice in metabolic cages. As we developed a conditional KCC3 knockout mouse model (73), we should be able to drive KCC3 deletion specifically in intercalated cells. One important confounding factor is ROMK, which constitutes a major pathway for K^+ secretion (74). As mentioned above, whether the contribution of KCC3 to K^+ loss will be significant enough to challenge the contribution of ROMK is an important question to answer. Maybe KCC3 function will be most relevant when ROMK participation in K^+ loss is minimal, e.g., in conditions of low Na^+ delivery to the distal nephron. This is consistent with the elegant study of Charles

Wingo who provided evidence for cortical collecting tubule K^+ and Cl^- secretion in rabbits adapted to a $KHCO_3$-rich and NaCl-deficient diet (75**).

In conclusion, two separate isoforms of KCC3 are expressed in kidney: KCC3a and KCC3b. Early work showed abundant KCC3b transcript, while barely detectable KCC3a mRNA in mouse kidney. Recent work by us identified the site of KCC3a expression: the apical membrane of type-B intercalated cells. Our work showed that KCC3a expression is stimulated by contraction alkalosis and metabolic alkalosis, thereby constituting a pathway for non-conductive K^+ secretion in alkalemia. This discovery opens new avenues of research but also an opportunity to develop drugs that would minimize excessive loss of K^+ in alkalemia.

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- Distinct KCC3 transporters are expressed in the kidney cortex: KCC3b in proximal tubule and KCC3a in distal tubule.
- KCC3a is observed alongside pendrin at the apical membrane of type-B (or nonA/nonB) intercalated cells.
- KCC3a expression is stimulated by contraction alkalosis and metabolic alkalosis.
- KCC3a mediates K^+ secretion (or K^+ loss) in alkalemia.
- Bicarbonate itself seems to be the major factor leading to increased KCC3a protein expression.

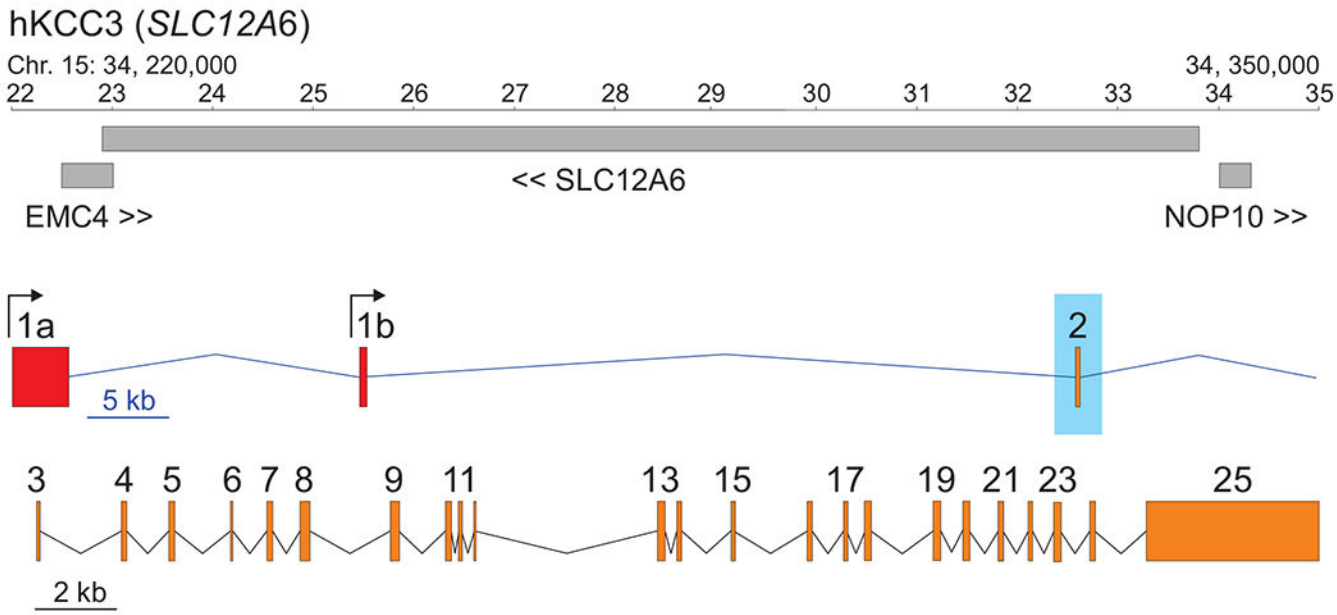

Figure 1. Structure of the *SLC12A6* gene.

The human SLC12A6 gene is located on Chr. 15: 34,229,784-34,338,060 (minus strand) between ER membrane protein complex subunit 4 (EMC4 – 34,225013-34,230156) for which the two genes overlap their 3' end, and ribonucleoprotein, NOP10 (34,339,159-34,343,180). *SLC12A6* is composed of 26-27 exons, depending on whether exon 1a is a single exon (as in mouse) or broken up into 2 exons. Transcription starts at either exon 1a (for KCC3a) or exon 1b (for KCC3b). Exon 2 is a small 45 bp alternatively spliced cassette exon encoding a 15 amino acid peptide. There is evidence that this exon is alternatively spliced in tissues expressing KCC3a or KCC3b.

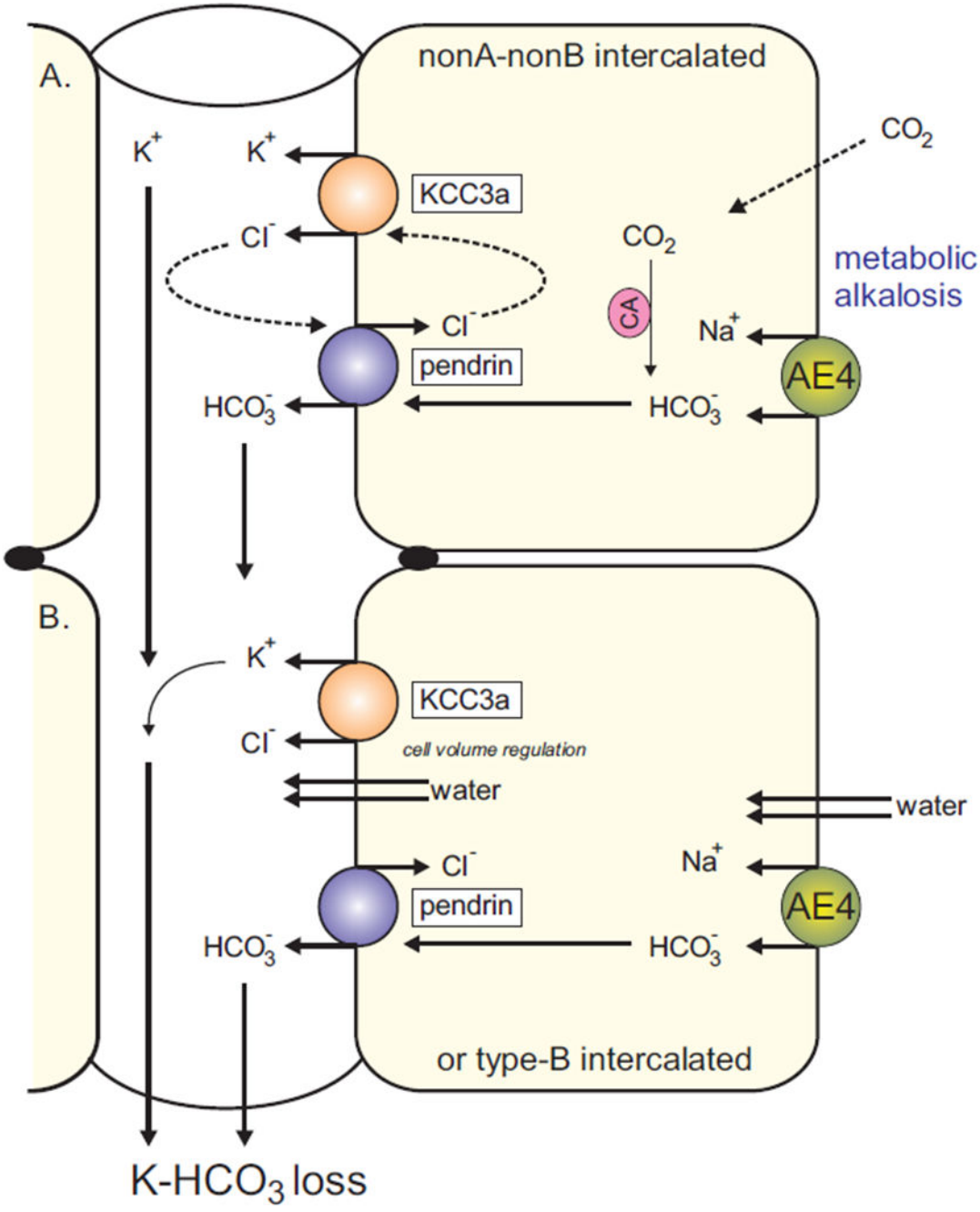

Figure 2. Working model of KCC3a roles in type-B (or nonA/nonB) intercalated cells.

KCC3 is expressed at the apical membrane where it mediates the efflux on K^+ and Cl^-. A: Under conditions of bicarbonate excretion, the Cl^- ion that enters the cell through the $Cl^-/HCO3^-$ exchanger recycles through KCC3. This activity results into the excretion (loss) of K^+. The Na-HCO_3 cotransporter AE4 is a major pathway $HCO3^-$ entry in the cell. B: AE4 activity (import of ions) leads to cell swelling, while pendrin activity (exchange of ions) would be volume neutral. A role for KCC3a would be to maintain cell volume during

bicarbonate transport. Cl^- recycling and volume regulation are not mutually exclusive but complementary.