New perspective of ClC-Kb/2 Cl\(^{-}\) channel physiology in the distal renal tubule

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Submitted 15 December 2015; accepted in final form 14 January 2016

The molecular profile of ClC-Kb/2 channels. ClC-Kb/2 belongs to the CIC family of anion channels/transporters found in the early 1990s upon the cloning of the first member, CIC-0, from the electric organ of Torpedo marmorata (39). CIC proteins function as dimers having two identical anion conducting pathways operating in a discreetional manner (14, 15, 38). Each CIC monomer has 18 helices, but not all of them project across the membrane (14). Upon reconstitution, two identical current steps with independent kinetics can be detected by single channel analysis, which is consistent with a channel having two pores (also often referred as “double-barreled” appearance) (reviewed in Ref. 38). A fast gate leads to an independent opening of each pore upon depolarization, whereas a common slow gate simultaneously controls both pores during hyperpolarization. Crystal structure of the bacterial CIC-ec channel suggests a critical role of glutamate at the position 148 in governing the “fast gate” during channel opening and conferring voltage- and Cl\(^{-}\)-dependent properties (15, 38). It was also proposed that protonation status of this negatively charged residue may convert at least some CICs from a channel into an exchanger (Cl\(^{-}\)/H\(^{+}\) or NO\(_{3}^{-}/\)Cl\(^{-}\)) mode (1, 2, 59, 65). Interestingly, ClC-Kb/2 and its closely structurally related CIC-Ka (CIC-K1 in rodents) have a hydrophobic valine at this position and do not demonstrate noticeable voltage dependence (46, 85). The detailed analysis of ClC-Kb/2 structure has been recently reviewed (4).

The unique characteristic of both CIC-K channels is their ability to interact with barttin (a two-transmembrane domain protein; a product of the BSND gene), which serves as an essential β-subunit critical to form a functional channel (17). Overexpression of either human CIC-Kb or CIC-Ka fails to produce a notable anionic current in the absence of barttin coexpression. In contrast, mouse and rat CIC-K1 (but not CIC-K2) can form a functional channel at the expense of a reduced activity (46, 66). It was demonstrated that barttin binds to the transmembrane domains of CIC-K2 thereby promoting its translocation to the plasma membrane (77). Moreover, CIC-K2 is retained in the Golgi in the absence of barttin (30). Similarly, only a small portion of CIC-K1 can be detected at the plasma membrane when barttin is not coexpressed (66). It has been recently proposed that palmitoylation of the two conserved cysteine residues on positions 54 and 56 of barttin is necessary for proper plasma membrane insertion of CIC-K/barttin complexes (76).

Similar to other CICs, CIC-K channels expressed in oocytes produce anion-selective current with a permeability sequence of Cl\(^{-}\) > Br\(^{-}\) > NO\(_{3}^{-}\) > I\(^{-}\) (17, 46, 84). Extracellular Ca\(^{2+}\) increases CIC-K activity likely via a direct binding to the I-J loop (17, 22, 23, 46, 84). In addition, the channels are extremely sensitive to proton concentration. Basic pH (8.0) increases CIC-K activity, whereas acidic pH (<6.0) practically closes the channels (17, 46, 52, 55, 84, 93). A critical role of a histidine residue (H497) in proton-induced inhibition of CIC-K has been proposed (22). Extremely high alkaline media (pH = 11) can also inhibit CIC-Kb likely via deprotonation of a highly conserved lysine (K165) (24) although it is not clear.
whether this is relevant for CIC-Kb/2 function in the kidney since the channel is still active at pH = 10.

**CIC-Kb/2 expression in the kidney.** CIC-Kb/2 and its highly homologous partner CIC-Ka/1 (~90% identity) were originally cloned from both human and rat kidneys (42, 81). The “K” in CIC-K is due to predominantly kidney-specific expression. However, it is recognized that both channels can also be found in the inner ear serving as an important component of the ion. However, it is recognized that both channels can also be found in the inner ear serving as an important component of the basolateral Cl\^- conductance that participates in K\(^+\) secretion by these epithelia (17, 64). With the use of molecular tools, it was originally proposed that rat CIC-K2 can be detected in all segments of the nephron, including the glomerulus, but these results were not consistent (42, 92). A critical advance in discovery of CIC-K2 expression in the kidney was made by Uchida’s group upon developing CIC-K1^+/− mice (53). Using antibodies recognizing both CIC-K1 and -K2 in the knockout mice, they demonstrated that CIC-K2 is expressed on the basolateral membrane of the thick ascending limb (TAL), distal convoluted tubule (DCT), connecting tubule (CNT), and intercalated cells of the collecting duct (CD) with a proposed role in mediating Cl\^- reabsorption in these sites (45). Consistent with these results, barttin expression was also detected in TAL, DCT, CNT, and intercalated but not principal cells of the CD (17). Despite close structural homology, the expression patterns of CIC-K1 and CIC-K2 almost do not overlap. CIC-K1 is present predominantly on both apical and basolateral plasma membranes of the thin ascending limb contributing to generation and maintenance of the hypertonic medullary interstitium and countercurrent mechanism (53, 82). CIC-K1^+/− mice exhibit classical nephrogenic diabetes insipidus, including polyuria and resistance to exogenous administration of vasopressin (53).

Consistent with the sites of CIC-K2 expression in the renal tubule, an anion-selective channel with 10 pS conductance has been detected on the basolateral membrane of freshly isolated cortical TAL (25, 87), DCT (52), CNT, and cortical CD (55, 93). It recapitulates major biophysical properties of CIC-K channels, namely anion selectivity sequence, pH dependence, and activation by extracellular Ca\(^{2+}\). Unfortunately, there are no reports monitoring single channel CIC-Kb/2 in overexpression systems to perform direct comparison with the basolateral 10-pS Cl\^- channel. On the contrary, multiple reports document CIC-Kb/2-mediated macroscopic whole cell current (17, 22–24, 66, 84). This may potentially indicate that the channel operates in a transporter mode upon overexpression, precluding its electrical resolution at the molecular level. Future studies are necessary to resolve this intriguing possibility. Notably, the recorded 10-pS Cl\^- permeable channel has slow kinetics and does not exhibit a double-barreled appearance (i.e., full and half openings), which is characteristic of the CIC ion channel family (15, 18). Again, the nature of this “breaking-the-pattern” gating is enigmatic. Nevertheless, CIC-K1 produces a single channel with 40 pS conductance upon overexpression in HEK293 cells (46). This apparent difference in single channel properties between CIC-K1 and the natively expressed Cl\^- permeable channel further supports the view that CIC-K2 activity underlies the 10-pS Cl\^- channel in distal tubular segments of the nephron.

**CIC-Kb/2 and Bartter’s syndrome.** Bartter’s syndrome is an autosomal recessive tubulopathy that presents in early childhood and is characterized by urinary salt wasting, hypokalemia, metabolic alkalosis, low blood pressure, resistance to loop diuretics, such as furosemide, and secondary compensatory hyperaldosteronism (44, 68). Bartter’s syndrome patients also commonly exhibit abnormalities in divalent cation balance and nephrocalcinosis due to altered tubular reabsorption of Mg\(^{2+}\) and Ca\(^{2+}\), respectively, while hypomagnesemia is rare (16, 26). Five different types of Bartter’s syndrome have been identified due to mutations in genes encoding specific transport or signaling proteins predominantly expressed in the TAL (44). Missense and nonsense mutations in the Clcnkb gene encoding CIC-Kb underlie Bartter’s syndrome type 3, directly demonstrating a role of this Cl\^- channel in renal salt reabsorption and blood pressure homeostasis (70). The detailed analysis of different disease-causing mutations in CIC-Kb has been recently provided by an excellent review by Andrini and colleagues (4). Similarly, mutations in the BSND gene encoding barttin underlie Bartter’s syndrome type 4 (7). Recall, barttin serves as β-subunit of CIC-Kb essential for channel function and translocation to the plasma membrane (17, 66). No Bartter’s syndrome mutations in the gene encoding highly homologous CIC-Ka channel have been reported in humans. In contrast to Bartter’s syndrome type 1 (due to loss-of-function mutations in the NKCC2 transporter) (71) or type 2 (KCNJ1 or ROMK1 channel) (72), patients with Clcnkb mutations do not develop nephrocalcinosis with variable (from low to high) urinary Ca\(^{2+}\) excretion (36, 70, 96). In general, Bartter’s syndrome type 3 manifestations are reminiscent of Gitelman’s syndrome, caused by loss-of-function mutations in the gene encoding electroneutral NCC cotransporter (Slc12A3), expressed in the DCT (73). This apparent similarity most likely reflects a critical role of CIC-Kb in mediating basolateral Cl\^- exit in the DCT as well. Indeed, insensitivity to thiazide diuretics (targeting NCC) has been described in patients with Bartter’s syndrome type 3 (56).

**Insights into the functional role of CIC-Kb/2 in different nephron segments.** CIC-Kb/2 function in the kidney is an important regulator of electrolyte balance, circulating volume, and, by its extension, blood pressure. As has been outlined in the previous section, loss-of-function mutations in CIC-Kb and barttin cause urinary salt wasting, hypochloremia, and low blood pressure. Systemic administration of CIC-K channel inhibitors increased urinary volume and lowered blood pressure in rats, suggesting that CIC-K blockers can be potentially used as a new class of diuretics (50). On the other hand, gain-of-function polymorphism CIC-Kb\(^{T481S}\) in humans is associated with elevated blood pressure, presumably due to enhanced renal salt retention (37, 69). Similarly, Milan hypertensive rats and Dahl salt-sensitive rats exhibit increased CIC-K2 expression in the DCT and renal medulla, respectively, which can at least partially contribute to salt sensitivity of blood pressure in these models (11, 12).

CIC-Kb/2 is expressed on the basolateral membrane of three different tubular segments: TAL, DCT, and CNT/cortical collecting duct (CCD) (3, 45). However, much less is known about specific functions and the relative contribution of CIC-Kb/2 in each region. In TAL and DCT, this channel is expressed virtually in all cells, where it is thought to take part in transcellular Cl\^- reabsorption working in tandem with the apically localized transporters NKCC2 and NCC, respectively (Fig. 1A). In addition, the existence of the electroneutral basolateral K\(^+\)-Cl\^- pathway, most notably via the KCC4
The cotransporter (Slc12a7), has been reported in TAL, DCT, and CCD (9, 83). However, this coupled K\(^+\) and Cl\(^-\) exit cannot compensate for the loss of function of either ClC-Kb/2 or K\(^+\) Kir4.1/5.1 channels (see below). No clinical syndrome associated with KCC4 mutations has been described so far. It should be noted though that mice deficient of KCC4 develop renal tubular acidosis probably resulting from impaired Cl\(^-\) recycling across the basolateral membrane of acid-secreting α-intercalated cells of the CCD (9).

It is interesting that ClC-Kb2 expression in the distal renal tubule largely aligns with that of the K\(^+\) channel KCNJ10/16 (K\(_{r}\)4.1/5.1). The latter is also found on the basolateral mem-
brane in the TAL, DCT, and CNT/CCD (20, 47, 88, 95, 97). KCNJ16 (K_\text{ir}4.5.1) alone fails to form a functional channel (10). However, it heteromerizes with K_\text{ir}4.1 to form a channel with distinct biophysical properties compared with a K_\text{ir}4.1 homomer (78). It was further demonstrated that K_\text{ir}4.1/5.1 is the predominant K\(^{+}\) channel in the DCT (51, 97) and CNT/CCD (47, 95). Coordinated actions of the Na\(^+-K\(^{+}\)-ATPase and K_\text{ir}4.1/5.1 mediate K\(^{+}\) recycling across the basolateral membrane, thereby modulating sodium reabsorption in the distal nephron (29). Loss-of-function mutations in the gene encoding K_\text{ir}4.1 cause SeSAME/EAST syndrome in humans, leading to electrolyte imbalance reminiscent of Gitelman’s syndrome, including salt wasting, hypocalciuria, hypomagnesemia, and hypokalemic metabolic alkalosis (8, 67). It is also thought that K_\text{ir}4.1/5.1-mediated K\(^{+}\) efflux contributes to setting the resting basolateral membrane potential to establish a favorable electrochemical driving force for transcellular and paracellular transport in DCT and CCD (88, 94, 97). Despite the fact that CIC-Kb/2 activity underlies electrogenic Cl\(^{-}\) movement across the basolateral membrane, a role of this channel in establishing membrane potential has not been reported. Moreover, the pan-specific Cl\(^{-}\) channel inhibitor NPPB had no effect on the basolateral resting membrane potential of intercalated cells in the CCD prominently expressing CIC-Kb/2 (93). Thus, the current view is that CIC-Kb/2 uses a negative basolateral membrane potential generated by coordinated actions of K_\text{ir}4.1/5.1 and Na\(^{-}-K\(^{+}\)-ATPase to drive Cl\(^{-}\) from cytosol to the interstitium.

In the TAL and DCT, CIC-Kb/2 and K_\text{ir}4.1/5.1 are expressed in the same cells (Fig. 1A). Furthermore, recent evidence from Wang’s group documented a functional coupling between these channels (97). It was proposed that K_\text{ir}4.1/5.1 plays a dominant role in determining basolateral membrane voltage creating a favorable driving force for basolateral Cl\(^{-}\) exit. Genetic ablation of K_\text{ir}4.1 not only depolarizes the basolateral membrane but also reduces NPPB-dependent Cl\(^{-}\) current. The nature of the functional coupling between K_\text{ir}4.1/5.1 and CIC-Kb/2 is not completely understood but may involve downregulation of Ste20-like proline-alanine rich kinase (SPAK) (97). SPAK is a well-established critical downstream effector of the with-no-lysine (WNK) signaling (most notably WNK1 and WNK4), a master signaling network governing electrolyte handling in the distal renal tubule by phosphorylating and controlling abundance of a number of transporters and ion channels, including NCC, NKCC2, and ROMK1 (33, 34). Mutations in WNK1 and WNK4 cause familial hyperkalemia hypertension in humans (also known as pseudohypaldosteronism type 2 or Gordon syndrome) (89). It is interesting that intracellular Cl\(^{-}\) through its binding to the catalytic site stabilizes the inactive conformation of WNK1, preventing kinase autophosphorylation and activation (58). Similarly, regulation of NCC by WNK4 also depends on intracellular Cl\(^{-}\) (6). Of note, WNKs have different sensitivity to Cl\(^{-}\), with WNK4 being inhibited at much lower Cl\(^{-}\) concentrations than WNK1 (79). Therefore, CIC-Kb/2-mediated Cl\(^{-}\) exit might be an important determinant in defining the functional status of the WNK network in the distal tubule depending on Cl\(^{-}\) delivery and activity of the apical transporters. Future studies will likely test this plausible hypothesis. In addition, it is also not known whether a reciprocal regulation of CIC-Kb/2 by WNKs or their downstream effectors, such as SPAK, can occur.

The alliance between CIC-Kb/2 and K_\text{ir}4.1/5.1 ends in the CCD (Fig. 1B). This segment contains two fundamentally different cell types. The majority (~70% of total) are principal cells expressing the epithelial sodium channel (ENaC) and ROMK on the apical side and have abundant Na\(^{+}-K\(^{+}\)-ATPase expression on the basolateral membrane (57). These cells control Na\(^{+}\) reabsorption or Na\(^{+}/K\(^{+}\) exchange (57, 74). The remaining 30% are intercalated cells, which are primarily, but not exclusively (20, 32, 48), involved in regulation of acid-base balance and transcellular Cl\(^{-}\) reabsorption (63, 86). They possess a different set of transporting proteins and can be further subdivided into acid-secreting A type, base-secreting B type, and non-A non-B, also called “transitioning” type. The interested reader may refer to a recent comprehensive review for more details about classification and morphological aspects of intercalated cells (63). Fluorescent and electrophysiological studies suggest that principal and intercalated cells are not electrically coupled through gap junctions (54, 90). Indeed, they are able to maintain different resting basolateral membrane voltages that are close to the equilibrium potential for K\(^{+}\) (approximately ~70 mV) for principal and for Cl\(^{-}\) (approximately ~15 mV) for intercalated cells (54, 94). It is interesting that CIC-K2 channel and its regulatory subunit barttin are located on the basolateral membrane of intercalated cells only (45, 55, 80), whereas K_\text{ir}4.1/5.1 expression is restricted to the basolateral membrane of principal cells (47, 94, 95). Using patch-clamp electrophysiology in freshly isolated CCDs, Teulon’s group failed to detect CIC-K2 and K_\text{ir}4.1/5.1 in the same patch (47). However, there is a small overlap between functional expression of both channels in the CNT (55), likely reflecting a transition from nephron segments where CIC-K2 and K_\text{ir}4.1/5.1 are expressed in the same cell (TAL and DCT) to those where the channels are “divorced” to distinct principal and intercalated cells (CCD).

While the exact physiological role of CIC-Kb/2 in the intercalated cells of the CCD is not firmly established, it is viewed that this channel is likely involved in trans-cellular Cl\(^{-}\) reabsorption driven by the electroneutral Cl\(^{-}\)/HCO\(_3\)\(^{-}\) transporter Slc26a4 (pendrin) in base-secreting B type of intercalated cells and the Slc4a11 transporter acting as an electrogenic Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchanger or a Cl\(^{-}\) channel in acid-secreting A type of intercalated cells on the apical side (Fig. 1B) (91). It is interesting that functional expression of CIC-K2 in intercalated cells is comparable with that in TAL and DCT cells (52, 87). Taking into account much lower capacity of CCD to reabsorb Na\(^{+}\) (3 vs. 25% for TAL and 5–10% for DCT), CIC-Kb/2 may also be involved in processes other than equimolar Cl\(^{-}\) transport at this site. Because of its remarkable pH sensitivity, CIC-Kb/2 can participate in regulation of acid-base balance performed by intercalated cells. As has been described above, Bartter’s syndrome type III caused by loss-of-function mutations in CIC-Kb is characterized by metabolic alkalosis (70). Thus, it is possible that channel dysfunction in base-secreting B-type intercalated cells may at least partially be responsible for the altered systemic pH balance. Again, future studies are necessary to explore a possible role of CIC-Kb in urinary excretion of acid.

**Regulation of CIC-Kb/2 by insulin and insulin-like growth factor-1 in CCD.** Despite the unequivocal role of CIC-Kb/2 in controlling water and electrolyte balance in the kidney, little has been done toward uncovering systemic factors regulating
expression and function of the channel. Using native CCDs, our group has recently reported a direct action of insulin and insulin growth factor-1 (IGF-1) on single channel ClC-K2 activity (93). Insulin and IGF-1 have substantial structural similarity, and the same is also true for their respective insulin and IGF-1 receptors (43, 62). Apart from action on metabolism, insulin and IGF-1 are known to regulate urinary excretion of electrolytes affecting tubular transport in multiple segments, including CCD (5, 27, 28). It has been directly demonstrated that insulin and IGF-1 augment ENaC-mediated sodium reabsorption in CCD principal cells (35, 60, 75). In general, both hormones reduce urinary sodium excretion in humans (13, 21). However, they considerably differ with respect to their actions on systemic blood pressure. Thus, in patients with acromegaly, augmented circulating IGF-1 levels result in antinatriuresis and hypertension, which can be corrected with ENaC inhibitor amiloride (40, 41). In contrast, the effects of insulin are primarily determined by plasma glucose and K+ levels and often do not lead to salt retention and elevation in blood pressure (19, 49). Furthermore, IGF-1 reduces renal K+ excretion (21), whereas insulin can promote kaliuresis (19), particularly when plasma K+ levels are exogenously clamped (31, 61).

Electrogenic ENaC-mediated sodium entry across the apical membrane of principal cells creates a favorable electrochemical gradient that can then be used to drive K+ secretion via ROMK as well as paracellular and transcellular Cl− reabsorption (reviewed in Refs. 57 and 74). This is accompanied by the coordinated actions of the basolaterally localized Na⁺-K⁺-ATPase and Kᵢ4.1/5.1 to extrude Na⁺ and maintain high negative basolateral membrane potential (20, 88, 94). Both insulin and IGF-1 augment Na⁺ reabsorption in the CCD by stimulating ENaC and Kᵢ4.1/5.1 and hyperpolarizing the basolateral membrane (94). Interestingly, our group demonstrated that IGF-1 also stimulates CIC-K2 activity in the intercalated cells of freshly isolated murine CCDs in a phosphatidylinositol 3-kinase-dependent manner, whereas insulin blocks the Cl− channel via a mechanism involving stimulation of MAPK (93). This unexpected observation provides an important insight into mechanisms underlying distinct patterns of urinary electrolyte excretion in response to insulin and IGF-1 (Fig. 2). Thus, IGF-1, by stimulating ENaC-mediated sodium transport in principal and CIC-K2 channels in intercalated cells, facilitates cooperative NaCl reabsorption (i.e., volume retention), thus reducing the driving force for K+ secretion by the CCD. In contrast, insulin, by stimulating ENaC but inhibiting CIC-K2,

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**Fig. 2.** Proposed role of ClC-Kb/2 in separating Na⁺ and K⁺ fluxes in CCD upon insulin and insulin growth factor-1 (IGF-1) actions. **A**: insulin stimulates ENaC and Kᵢ4.1/5.1 in principal cells but inhibits CIC-K2 in intercalated cells. This favors coupling of ENaC-mediated Na⁺ reabsorption with K⁺ secretion via apically localized ROMK channels in principal cells. **B**: IGF-1 stimulates both ENaC-mediated sodium transport in principal cells and CIC-K2 channels in intercalated cells, thereby facilitating cooperative sodium and Cl− reabsorption in the CCD. For clarity, only the B type of intercalated cells is shown.
favors coupling of Na⁺ reabsorption with K⁺ secretion (without volume retention) at the apical membrane of principal cells contributing to kaliuresis (Fig. 2). It is also plausible to speculate that modulation of CIC-K2 activity in the CCD might be of clinical importance to dissociate sodium reabsorption from K⁺ secretion at this site, avoiding potentially life threatening hyperkalemia as a result of adverse actions of diuretics targeting ENaC, such as amiloride.

Apart from insulin/IGF-1 effects, other systemic factors may also affect CIC-Kb/2 function in the kidney. Thus, increased dietary salt intake had no influence on CIC-K2 mRNA in the cortex but reduced CIC-K2 mRNA expression in the outer and inner medulla of Dahl salt-resistant rats and Dahl salt-sensitive rats (12). This reduction was much more prominent in salt-resistant compared with salt-sensitive Dahl rats. Thus, increased medullary salt reabsorption may contribute to an inability of these animals to excrete an increased salt load (12). It is unknown if changes in circulating aldosterone levels contribute to the regulation and whether this also translates into changes of CIC-K2 protein expression.

Concluding remarks. It has been long recognized that the CIC-Kb/2 channel is a significant contributor to water and electrolyte handling by the kidney with dysfunction leading to Bartter’s syndrome type 3. Despite this, CIC-Kb/2 is probably one of the least studied channels in the kidney. We have achieved significant progress in characterization of the disease-causing mutations and uncovering sites of CIC-Kb/2 expression on the basolateral membrane of distal tubular segments starting from TAL to CCD. Apart from insulin and IGF-1, little is known about cellular and systemic mechanisms controlling channel function and expression. Furthermore, supportive evidence indicates that the channel may serve different purposes in each tubular segment. It is clear that significant experimental effort needs to be invested to thoroughly investigate CIC-Kb/2 function in the kidney since targeting activity of this might be of clinical relevance for blood pressure management with little systemic effects considering its almost exclusive localization to the nephron.

GRANTS
This research was supported by National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Grant DK-095029 (to O. Policyn) and American Heart Association Grant AHA-15SDG2555010 (to M. Mamenko). V. Bhalla was supported by NIDDK Grant DK-091565.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCE
ROLE OF CIC-Kb2 IN THE DISTAL NEPHRON

Review


