Chloride Transport in the Kidney: Lessons from Human Disease and Knockout Mice

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Knockout mouse models and human inherited diseases have provided important new insights into the physiologic role of chloride transport by CLC Cl^- channels and KCC K-Cl co-transporters. ClC-K/barrtin Cl^- channels are important for renal salt reabsorption and possibly for acid secretion by intercalated cells. The endosomal ClC-5 protein is crucial for proximal tubular endocytosis. Its disruption in mice and patients with Dent's disease leads to hypercalciuria and kidney stones through a pathologic cascade that may be entirely explained by an impairment of endocytosis. KCC4 is important for recycling Cl^- for the basolateral anion exchanger in intercalated cells, as is evident from the renal tubular acidosis resulting from its knockout. Finally, both KCC3 and KCC4 are crucial for proximal tubular cell volume regulation.

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Although transepithelial transport of anions, for obvious reasons, is quantitatively as important as cation transport, it has received less attention. This is probably due in part to the influence of concepts from neurobiology, which stressed the importance of Na^+, K^+, and Ca^{2+} for cellular excitability but also to the overwhelming role of Na^+ homeostasis in the regulation of the extracellular volume and of BP.

Anions not only are passively dragged across epithelia by the transepithelial voltage created by cation transport but also are themselves transported by a plethora of different transport proteins. These include proteins that directly couple their transport to cations, anion exchangers, anion channels, and, as discovered recently, also Cl^-/H^+ exchangers. Despite the completion of several genome projects and the molecular identification of an astounding variety of ion transport proteins, it seems that many transporters and channels have not yet been identified at the molecular level. This is particularly true for the Cl^- channel field, where many currents and channels identified at the functional, biophysical level could not yet be correlated with a specific gene product. For instance, we may still lack the right clones for important channel classes such as swelling-activated or Ca^{2+}-activated Cl^- channels.

Although the progress in molecular biology of ion channels and transporters for some time seemed to displace classical renal physiologic techniques such as those used by Homer W. Smith to a second rank, the generation of genetic mouse models carrying targeted mutations in ion channel and transporter genes have bridged the gap to integrative physiology and will undoubtedly lead to a resurgence of classical methods of renal physiology such as the perfusion of isolated tubules. The pathologies observed in knockout (KO) mice and in human genetic disease have led to invaluable and often unexpected insights into the physiologic functions of specific transport proteins.

Here I review the renal aspects of our work on chloride transport, which focuses on CLC Cl^- channels (and transporters) and on electroneutral KCC K^+/Cl^- co-transporters. The generation and analysis of mouse models have revealed that they play important roles in processes as diverse as salt reabsorption, acid secretion, cell volume regulation, endocytosis, and lysosomal protein degradation, as well as kidney stones.

CLC Gene Family of Cl^- Channels and Transporters

The CLC gene family, which was originally identified by the expression cloning of a chloride channel (ClC-0) that is highly expressed in the electric organ of the marine ray *Torpedo marmorata* (1), is present in all phylae from bacteria to humans. Mammals express nine distinct CLC genes (the gene *CLCNX* encodes the protein ClC-X). With the exception of the skeletal muscle isoform ClC-1 (2), all of these genes are expressed to some degree in kidney. None of these isoforms, however, is kidney specific. Human mutations in genetic disease and the analysis of KO mice have shown that four of these (ClC-Ka, ClC-Kb, ClC-5, and ClC-7) have important impact on kidney function.

Before addressing the renal functions of specific isoforms, it is useful to recall some basic features of these Cl^- transport proteins. CLC channels are dimers, with each of the two subunits having its own pore ("double-barreled channel"). This was concluded from single-channel experiments on native *Torpedo* channels reconstituted into lipid bilayers (3) and convincingly shown by studying WT-mutant ClC-0 concatemers (4,5) and ClC-0/ClC-2 heteromers (6). The dimeric structure of CLC proteins was confirmed impressively by the crystal structure of bacterial CLC proteins (7–9), very strongly suggesting that this architecture applies to all CLC proteins. In addition to ho-

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modimers, CLC proteins can form heterodimers, which may be restricted to members of the same homology branch (6,10,11). However, the physiologic relevance of heteromer formation is currently unclear. In addition, some CLC dimers may associate with accessory β-subunits. The only known cases, so far, concern the two ClC-K isoforms, which associate with barttin (12). The crystal structure of bacterial CLC proteins showed a complicated architecture with 17 intramembrane but not necessarily transmembrane helices (7) and three distinct Cl^- binding sites (8). It also revealed a glutamate side chain that seemed to block the access of extracellular Cl^- to the narrowest part of the pore (7,8). There is very good evidence (8,13) that this glutamate plays a role in Cl^--dependent gating of CLC chloride channels (14), as well as in the coupling of a countertransport of H^+ to Cl^- fluxes in the bacterial ClC-e1 protein (15).

Whereas many CLC proteins, such as the *Torpedo* channel ClC-0 or the mammalian ClC-1, ClC-2, and ClC-Ka/barttin and ClC-Kb/barttin, unambiguously function as Cl^- channels, the bacterial protein ClC-e1, which was used for crystallization studies (7,8), surprisingly mediates an electrogenic $2Cl^-/H^+$-exchange (15). Although mutating the glutamate that is also responsible for chloride-dependent gating in CLC channels abolished the H^+ coupling of anion fluxes, the detailed mechanism of and the structural basis for the exchange activity are not yet clear.

As ClC-6 and ClC-7, which are expressed in late endosomes and lysosomes, could not yet be expressed functionally in the plasma membrane (16), it is unclear whether they function as Cl^- channels or Cl^-/H^+ exchangers. Upon heterologous expression, a small fraction of the endosomal ClC-3, −4, and −5 proteins reach the plasma membrane and yield Cl^- currents (17–19). However, their very steep outward rectification precluded a reliable determination of reversal potentials that could be used to differentiate Cl^- channels from Cl^-/H^+ exchangers (15). Experiments are undoubtedly under way to determine whether some of these endosomal CLC proteins might serve as electrogenic exchangers.

ClC-K/Barttin and Transepithelial Transport

ClC-Ka and ClC-Kb are two highly homologous isoforms (20) (>90% identity at the protein level) that were first found in the kidney (hence the suffix K) (21) but are also found in epithelia of the inner ear (12,22). As discussed in detail below, both ion-conducting ClC-K isoforms need the accessory β-subunit barttin for functional expression (12).

The localization of both ClC-K isoforms on 1p36 (16) in close proximity to each other (23) suggests a recent gene duplication. The high degree of similarity precluded the assignment of species orthologs by sequence alone. Therefore, the rodent CLC isoforms were called ClC-K1 and -K2 (20,21,24,25) as opposed to the human ClC-Ka and -Kb (20). Fortunately, the comparison of expression along the nephron and of functional characteristics suggests that ClC-K1 corresponds to ClC-Ka, and -K2 to Kb (20,23,26). Reverse transcription–PCR of dissected nephron segments (20,21,27), *in situ* hybridization (28), and immunocytochemistry (12,24,29,30) were used to determine the expression of either isoform along the nephron. As the two isoforms are roughly 90% identical, generation of isoform-specific antibodies is difficult. The immunocytochemical investigation of ClC-K2 in a ClC-K1 KO mouse (31) and reporter genes driven by ClC-K promoters in transgenic animals (32) provided important additional insights. ClC-K1 (-Ka) is predominantly expressed in the thin limb of Henle's loop, whereas ClC-K2 (-Kb) is a basolateral channel in the thick ascending limb (TAL), the distal convoluted tubule (DCT), connecting tubule (CNT), and intercalated cells (IC) of the collecting duct. It cannot be excluded that segments that express ClC-K2 also express ClC-K1 (Ka) to some degree. Whereas ClC-K2 (-Kb) is clearly restricted to basolateral membranes of these epithelial cells, ClC-K1 was reported to be in both basolateral and apical membranes by Uchida *et al.* (25) but was detected only in basolateral membranes by Vandewalle *et al.* (30).

The crucial role of ClC-Kb in salt reabsorption became evident when Simon *et al.* (23) showed that mutations in *CLCNKB*, the human gene encoding ClC-Kb, underlie Bartter syndrome type III. Other forms of Bartter syndrome are caused by mutations in the apical, reabsorptive isoform of the Na-K-2Cl cotransporter NKCC2 (Bartter I) (33), in the apical K^+ channel ROMK (Kir1.1) (34), and in the Cl^- channel β-subunit barttin (Bartter IV) (35). Bartter-like symptoms were also seen with certain activating mutations in the extracellular Ca^{2+}-sensing protein, a G protein–coupled receptor (36,37). This last syndrome may be classified as Bartter V.

These observations led to an integrated transport model for NaCl reabsorption in the TAL (Figure 1): Powered by the Na^+ gradient generated by the basolateral (Na,K)-ATPase, the apical NKCC2 accumulates K^+ and Cl^- within the cell. Cl^- leaves the cells through basolateral Cl^- channels formed by ClC-Kb and its β-subunit barttin, whereas K^+ is recycled across the apical membrane through ROMK. The influence of the Ca^{2+}-sensing receptor is less clear and might involve an inhibition of apical NKCC2 or ROMK (36). Mutations in NKCC2 and ROMK generally lead to the severe antenatal Bartter syndrome, whereas the clinical symptoms associated with ClC-Kb mutations are variable and are mostly associated with the less severe "classical" Bartter syndrome. As ClC-Kb is found not only in the TAL but also in more distal nephron segments, including the DCT and CNT, mutations in ClC-Kb are sometimes not associated with high or normal urinary Ca^{2+}, as is typical for Bartter syndrome, but rather with hypocalciuria and hypomagnesemia (38–40), abnormalities typically found in the related Gitelman syndrome. That syndrome is typically caused by mutations in the thiazide-sensitive NaCl co-transporter (41) that is expressed in the DCT. That ClC-Kb mutations are generally not associated with the severe, antenatal form of Bartter syndrome suggests the presence of other basolateral Cl^- exit pathways in the TAL. These might be provided by ClC-Ka/barttin or by the K-Cl co-transporter KCC1 (42). However, this is pure speculation at this point.

The function of ClC-K1 (human ClC-Ka) was elucidated in a KO mouse model (26). Their phenotype resembled diabetes insipidus. The magnitude of the diffusion potential across isolated perfused TAL, a major site of ClC-K1 expression, was drastically changed in the KO (26,43) and had lost its sensitivity

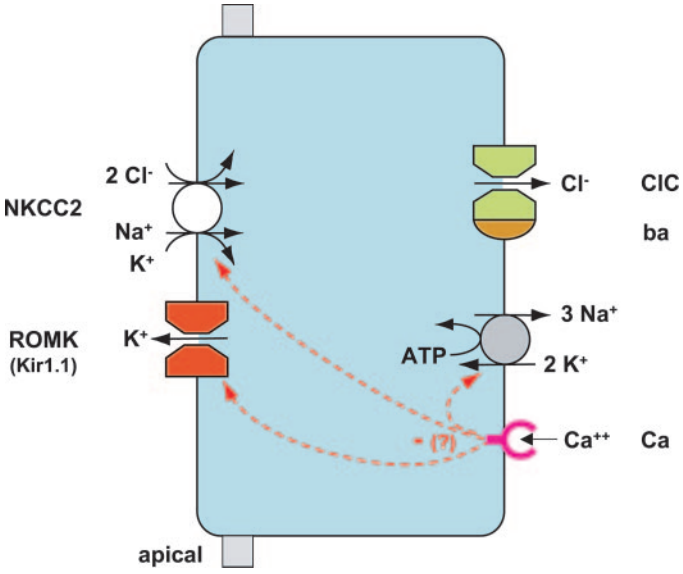

Figure 1. Model for NaCl reabsorption in the thick ascending limb of Henle's loop (TAL). Powered by the Na^+ gradient established by the basolateral (Na,K)-ATPase, the apical NKCC2 transports Na^+, K^+, and Cl^- ions into the cell. K^+ is recycled through apical ROMK (Kir1.1) K^+ channels, and Cl^- crosses the basolateral membrane through Cl^- channels that are heteromers of pore-forming ClC-Kb subunits and auxiliary barttin subunits. Mutations in the genes encoding NKCC2, ROMK, ClC-Kb, and barttin cause Bartter syndrome I to IV. Certain activating mutations in the basolateral Ca^{2+} sensing receptor CaSR, a G protein–coupled receptor, underlie Bartter V. The receptor might inhibit NKCC2, ROMK, or the (Na,K)-ATPase.

to pH and the Cl^- channel inhibitor NPPB (43). The lack of Cl^- permeability in this segment was suggested to lead to the observed impaired accumulation of osmolytes (including NaCl and urea) in the inner medulla (44) and, as a consequence, the diabetes insipidus–like phenotype of ClC-K1 KO mice.

With the enigmatic exception of rodent ClC-K1, which gave small currents when expressed alone in *Xenopus* oocytes (13,21), ClC-K proteins did not yield plasma membrane currents (20). Chimeras between human ClC-Kb and rat ClC-K1 indicated that a segment that included domains D9 to D12 (which includes helices M through R that were revealed in the crystal structure of bacterial CLC [7]) of ClC-K1 was needed for functional expression of the fusion protein (13).

The problem of expressing ClC-K channels was solved after *BSND* was identified by Hildebrandt and coworkers (35) as the gene underlying Bartter syndrome type IV, which combines severe renal salt loss with congenital deafness. This gene encodes barttin, a small protein with two predicted transmembrane domains close to its aminoterminus. Consistent with barttin's being an essential β-subunit of ClC-K channels, immunocytochemistry revealed that it is co-expressed with ClC-K in all renal membranes that express ClC-K1 or ClC-K2, *i.e.*, the thin ascending limb and TAL of Henle's loop, DCT, CNT, and IC of the collecting duct (12). As discussed below, it is also co-expressed with ClC-K in epithelial cells of the inner ear.

Co-expressing barttin with ClC-Ka or ClC-Kb in *Xenopus* oocytes led to significant anion currents with a Cl>Br>I selectivity (12). Although ClC-K1 yielded Cl^- currents by itself, these currents were drastically increased by co-expressing barttin. Barttin enables or drastically increases the surface expression of ClC-K proteins (12) and may also modify their biophysical properties as the sensitivity to external Ca^{2+} (45). Currents from ClC-K1 expressed by itself (13,21), as well as currents of ClC-Ka/barttin and ClC-Kb/barttin (12), are increased by extracellular alkalinization or by raising extracellular Ca^{2+}. Whether these properties are important for their physiologic function, however, is unclear. ClC-K/barttin currents show little evidence of voltage-dependent gating (12). It is interesting that ClC-K proteins lack a highly conserved glutamate (13). The crystal structures of bacterial CLC suggested that its negatively charged side chain might be responsible for the voltage- and chloride-dependent gating of CLC channels (7,8). Indeed, changing the valine at this position to glutamate introduced drastic changes in voltage dependence into ClC-K1 (13).

Attempts have been made to compare native Cl^- channels in the DCT to ClC-K channels (46). However, this comparison did not involve a full check of their biophysical "fingerprint," which includes a Cl>I selectivity and a sensitivity to external Ca^{2+} and pH. Indeed, the reported permeation properties for Cl^- and I^- (46) would argue against such an identity.

The cytoplasmic, carboxyterminal "tail" of barttin displays a peptide sequence (PPYVRL) that resembles so-called "PY" motifs as found in ClC-5 (47) or ENaC (48) or might represent a tyrosine-based signal for endocytosis (YxxL). Compatible with either notion, mutating the tyrosine to alanine increased currents and surface expression in oocytes by a factor of roughly 2 (12). PY motifs can bind to tryptophane-containing WW domains of ubiquitin ligases. Ubiquitination of the respective protein then is a signal for their endocytosis. Although a recent report suggested that Nedd4 might be involved in regulating ClC-K/barttin (49), we did not find such a regulatory relationship using dominant negative constructs of this and several other WW domain–containing ubiquitin ligases (Estévez and Jentsch, unpublished observations). It remains unknown whether a downregulation of ClC-Kb/barttin by ubiquitination or constitutive endocytosis plays a role in regulating Cl^- reabsorption in the kidney.

The functional investigation of several polymorphisms in the human *CLCNKB* gene identified a sequence change (T481S) that led to a dramatic approximately seven-fold increase in ClC-Kb currents (50). Equivalent mutations had similar effects in ClC-Ka but not in ClC-2 or ClC-5. The structural basis for the effect of this mutation, that changes a residue between intramembrane helices O and P, is not clear. If ClC-Kb/barttin Cl^- channels were rate limiting for Cl^- reabsorption, then this polymorphism, which was found in 20% of the used control population, might increase salt retention and hence hypertension. Indeed, a correlation between the presence of this allele and slightly elevated BP was found in some cohorts (51). However, these results should be repeated in other cohorts before drawing firm conclusions (52). A recent Japanese study did not find a correlation between the T481S polymorphism and high

BP (53). The frequency of this polymorphism in that Japanese cohort, however, was low (approximately 3%).

In addition to renal epithelia, ClC-K proteins and barttin are co-expressed in epithelia of the inner ear (12). Expression is limited to basolateral membranes of marginal cells of the stria vascularis and to dark cells of the vestibular organ. Both cell types secrete potassium, thereby creating the high K^+ concentration of the scala media that is crucial for the function of sensory hair cells. The transport model postulates that ClC-K/barttin channels are involved in recycling of Cl^- for the basolateral NaK2Cl co-transporters NKCC1, a role resembling that of the ROMK channel in recycling K^+ for NKCC2 in the TAL (Figure 1). Both ClC-K1 and ClC-K2 are expressed in the cochlea (12). A co-expression of both isoforms in marginal cells, possibly as oligoheteromers, may easily explain that mutations in the common β-subunit barttin cause deafness in Bartter IV (12,35) but that loss-of-function mutations in ClC-Kb in Bartter III cause renal salt loss without deafness (23). This model has been confirmed by identifying a single pedigree with symptoms of Bartter IV that did not carry mutations in barttin but that carried a point mutation in ClC-Ka and a deletion in ClC-Kb (54).

Thus, the deafness observed with a lack of barttin is due to a loss of both ClC-Ka and ClC-Kb. The loss of both channels additionally suggests that the renal phenotype in Bartter IV should be more severe than in Bartter III and may include features of diabetes insipidus as found in ClC-K1 KO mice (26). Indeed, Bartter IV patients present with very severe salt loss and growth retardation and often develop renal failure (55).

Finally, some considerations on a possible role of ClC-Kb/barttin in αIC of the collecting duct. ClC-K/barttin is found in its basolateral membranes (Figure 2, B and C), where it is co-expressed with the anion exchanger AE1 (12). αIC secrete acid into the tubular lumen via an apical H^+-ATPase. Transport of acid equivalents over the basolateral membrane occurs through AE1-mediated extrusion of bicarbonate in exchange for Cl^- (Figure 2C). This requires basolateral Cl^- recycling, which may occur through ClC-K/barttin channels (similar to the Cl^- recycling for the NaK2Cl co-transporter in marginal cells of the stria vascularis). If ClC-K/barttin were rate limiting for recycling, then one would expect a defect in acid secretion, *i.e.*, distal renal tubular acidosis, with loss-of-function mutations in this channel. However, patients with Bartter syndrome present with hypokalemic metabolic alkalosis, which is an in-

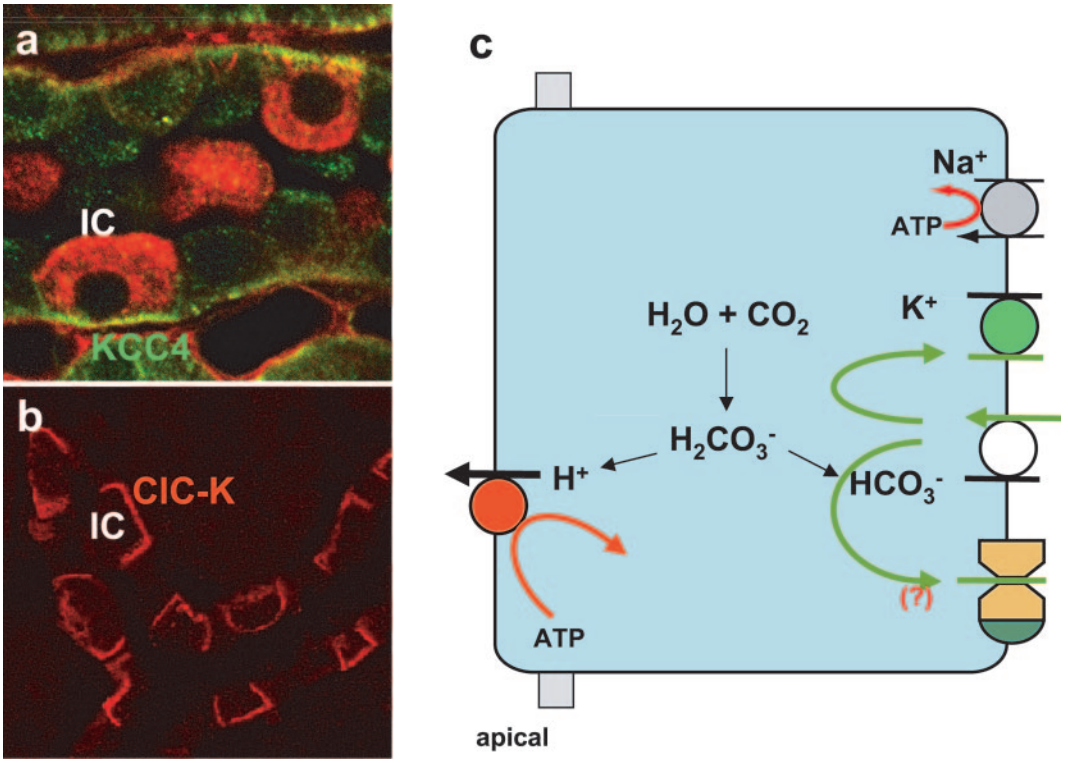

Figure 2. Role of KCC4 and ClC-Kb/barttin in α-intercalated cells (αIC). (A) Immunocytochemistry of a cortical collecting duct. αIC are identified by staining (in red) for the apical proton pump, which is located to a large degree in vesicles. Green staining identifies the K-Cl co-transporters KCC4 in the basolateral membrane. Reprinted from reference 56. (B) αIC also express ClC-K channels (in red) and barttin in their basolateral membranes. Reprinted from reference 12. (C) Model for proton secretion by αIC. Protons are secreted by a V-type H^+-ATPase that is inserted from intracellular vesicles into the apical membrane. Protons are derived from the dissociation of carbonic acid. Bicarbonate ions leave the cell basolaterally through the Cl^-/HCO_3^- exchanger AE1. The exchange for Cl^- requires basolateral Cl^- recycling. There is experimental evidence that KCC4 plays a major role in this recycling process, as its disruption in mice led to renal tubular acidosis (56). The presence of ClC-K/barttin in the same membrane suggests that it may play a similar role.

direct consequence of the severe salt loss in the TAL. Thus, segment-specific gene disruption might be necessary to delineate a role of ClC-Kb/barttin in αIC. However, IC also express KCC4 in their basolateral membranes (Figure 2, A and C) (56). As discussed below, the KO of this transporter indeed leads to renal tubular acidosis.

Some CLC pathologies may be inherited either as autosomal recessive or as dominant traits. For instance, mutations in the skeletal muscle Cl^- channel ClC-1 can cause autosomal recessive and dominant myotonia (57–60), and mutations in ClC-7 underlie recessive malignant infantile osteopetrosis (61) as well as autosomal dominant osteopetrosis (62). In contrast, no dominant form of Bartter syndrome has been described as yet. Because CLC channels and transporters function as dimers, certain mutations may lead to proteins that retain their ability to assemble with their WT counterparts and thereby inhibit their function. In the case of ClC-1, this almost always involves a shift of their voltage-dependent activation to positive voltages, where the channel cannot contribute to the repolarization of muscle action potentials (58). The dominant negative effect results from a changed voltage dependence of the common gate that acts on both pores in parallel (63). The structural basis for the common gate remains poorly understood. However, it also might be influenced by the "gating glutamate" that is missing in ClC-K proteins and the side chain of which blocks the access of anions to the pore in other CLC proteins. It therefore is tempting to speculate that the absence of gating in ClC-K channels severely limits the propensity of random mutations to cause dominant negative effect. Another hypothesis to explain the apparent absence of dominant Bartter might be the severity of the disease that is apparent already in infancy. This severity might have prevented the spread of dominant negative alleles in the population.

ClC-5: An Endosomal CLC that Is Important for Renal Proximal Tubular Endocytosis

Whereas ClC-K/barttin channels are important for transepithelial transport, many other CLC proteins rather function in intracellular vesicles of the endosomal/lysosomal pathway. Among these vesicular CLC, ClC-5 is understood best. The disruption of ClC-5 results in impaired proximal tubular endocytosis (64) and in human Dent's disease (65), an X-linked human inherited kidney stone disorder.

ClC-3, -4, and -5 display approximately 80% sequence identity and form their own branch of the CLC gene family. They are expressed in membranes of the endosomal/lysosomal pathway but can reach the plasma membrane to some degree upon heterologous expression. Whether this occurs *in vivo* is not yet clear. When present at the plasma membrane, they yield strongly outwardly rectifying anion currents that show only minor differences between isoforms (17–19). Currents were detectable only in the positive voltage range. They displayed a $NO_3^- > Cl^- > Br^- > I^-$ conductance sequence and were inhibited by extracellular acidic pH. Their extreme outward rectification precludes reliable determinations of the reversal potential. Therefore, one cannot exclude the possibility that these currents are due to an electrogenic Cl^-/H^+-exchange activity (15) instead of being mediated by a Cl^- channel. The swelling-activated Cl^- currents ascribed to ClC-3 (66) show very different biophysical characteristics and most likely represent endogenous currents of the expression system, as indicated by ClC-3 KO mice (67) and biophysical experiments (18,68).

ClC-5 but not ClC-3 or ClC-4 displays a PY motif between the two CBS domains in its carboxyterminus (47). PY motifs are known to interact with WW domains of ubiquitin ligases. A peptide corresponding to the PY motif of ClC-5 was shown to interact with WW domains of the WWPII ubiquitin ligase *in vitro* (69). Mutating this motif in ClC-5 led to an increase in surface expression and ClC-5 currents, as did the co-expression of a dominant negative mutant of WWP2 that carried an inactivating mutation in the HECT ubiquitin ligase domain (47). Hence, the PY motif results in an increased rate of internalization of ClC-5 from the plasma membrane, an effect that is most likely triggered by ubiquitination (47). Indeed, ClC-5 can be ubiquitinated (70). It is not yet clear whether ubiquitination regulates ClC-5 function *in vivo* and, if so, whether it only influences the endocytosis from the plasma membrane or affects other intracellular transport steps.

ClC-5 is predominantly expressed in the kidney but is also found in other tissues, such as intestinal epithelia (19,71,72). In these tissues, it is present in vesicles of the endosomal pathway. In the kidney, expression is highest in the proximal tubule (PT) and in αIC and βIC of the collecting duct (71,73,74). In the PT, ClC-5 is present in apical endosomes and co-localizes with the H^+-ATPase and proteins at early time points after endocytosis (71) (Figure 3, A and B). It co-localized with endocytosed proteins and with the endosomal marker protein rab5 in cultured cells (71). These data, together with the observation that a constant symptom of Dent's disease is low molecular weight proteinuria (75), suggested a role of ClC-5 in endocytosis (71).

Mice disrupted for ClC-5 indeed confirmed that ClC-5 plays a major role in proximal tubular endocytosis (64,76,77). Like patients with Dent's disease, ClC-5 KO mice display low molecular weight proteinuria. Proximal tubular endocytosis was studied *in vivo* by following the fate of labeled low molecular weight proteins that were injected into the bloodstream of anesthetized mice using confocal microscopy (64). As the gene encoding ClC-5 is located on the X chromosome, female mice that are heterozygous for the disrupted allele display mosaic expression, *i.e.*, some cells express ClC-5, whereas other cells of the same tubule lack it (Figures 3, A and C, and 4A). This expression pattern provides an ideal control. It can also clarify which effects of ClC-5 are cell-autonomous, rather than being due to secondary systemic changes (*e.g.*, in hormones) (64). The analysis of heterozygous females revealed that the disruption of ClC-5 affected both fluid-phase and receptor-mediated endocytosis in a cell-autonomous manner (Figure 3). Megalin, a member of the LDL family of proteins, is the major endocytotic receptor in the PT (78). It was downregulated in a cell-autonomous manner in PT cells lacking ClC-5 (Figure 4, A and B) (64). The total amount of megalin was reduced in KO kidneys as analyzed by Western blots (Figure 4C) (64). Staining for megalin was less broad in cells lacking ClC-5 (Figure 4B). These

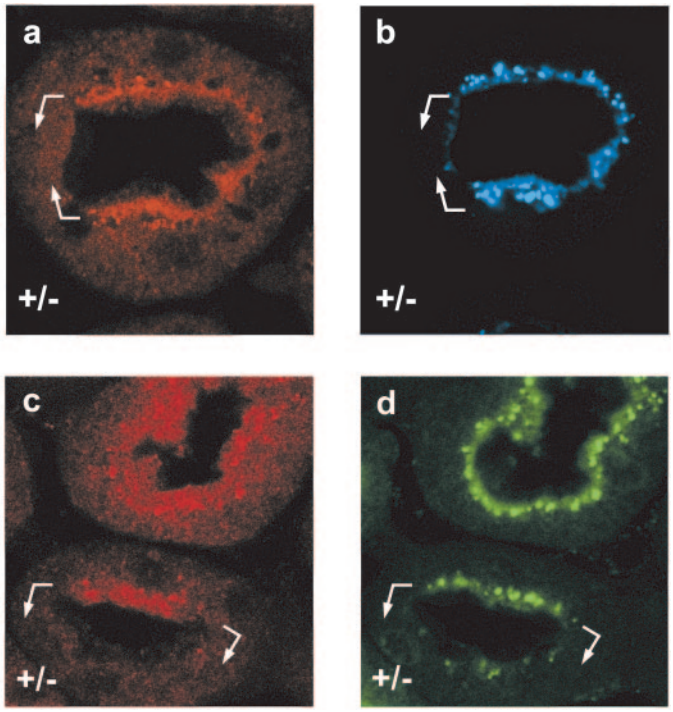

Figure 3. Cell-autonomous effects of disrupting ClC-5 on receptor-mediated endocytosis (A and B) and fluid-phase endocytosis (C and D). Female mice that are heterozygous for the disrupted ClC-5 allele express ClC-5 in some cells of the proximal tubule, whereas others lack ClC-5, as shown in A and C by confocal microscopy (ClC-5 is shown in red). The cells lacking ClC-5 are indicated by the arrows. Endocytosis was investigated by injecting into mice lactoglobulin that was labeled with a fluorescent dye to test for receptor-mediated endocytosis (B) or FITC-dextrane as a fluid-phase marker (D). Kidneys were fixed by perfusion a few minutes after the tracer was injected. These experiments revealed a cell-autonomous defect in both receptor-mediated and fluid-phase endocytosis. Reprinted from reference 64.

results suggested that ClC-5 might play a role in recycling megalin back to the apical brush border membrane (Figure 4D) and may also explain the decreased amount of megalin in the urine of patients with Dent's disease (64,76). The decrease of megalin (and of its co-receptor cubulin) was confirmed later by electron microscopy and cell fractionation (79). The cell-autonomous decrease in megalin (64) suggests that receptor-mediated endocytosis is more severely impaired by a lack of ClC-5 than fluid-phase endocytosis (76).

The disturbances in renal phosphate and calcium handling and eventually the kidney stones in Dent's disease might be secondary symptoms of the impaired renal tubular endocytosis and metabolism of calciotropic hormones (64,76). Parathyroid hormone (PTH) and VitD (bound to its binding protein) are endocytosed in the PT in a megalin-dependent manner (80,81). The defective endocytosis upon disruption of ClC-5 hence is predicted to lead to a loss of PTH into the urine, as is indeed observed in ClC-5 KO mice (64) and in patients with Dent's disease (82). The urinary loss of PTH is unlikely to have any physiologic effect *per se*, as this peptide hormone would undergo lysosomal degradation after proximal tubular endocytosis. However, the decreased endocytosis will lead to increased luminal concentration of PTH in later segments of the PT, which will result in an enhanced stimulation (64) of apical PTH receptors in proximal tubular cells (Figure 5) (83,84). This, in turn, will stimulate the endocytosis of the apical Na-coupled phosphate transporter NaPi-2a (85), which is responsible for the bulk of phosphate reabsorption in the PT. Consistent with this hypothesis, immunocytochemistry revealed that NaPi-2a was found predominantly in intracellular vesicles of ClC-5 KO kidneys, whereas it was expressed prominently in the brush border of WT PT (64). Although difficult to quantify, NaPi-2a localization was not changed in early segments of the PT. This is expected as shortly after glomerular filtration, the luminal concentration of PTH approximates that found in serum. In contrast to the effect of ClC-5 disruption on endocytosis and megalin localization, analysis of heterozygous females indicated that the change in localization of NaPi-2a was not cell-autonomous (64). This finding is consistent with the changes of luminal PTH concentrations. The decrease in apical NaPi-2a can fully explain the hyperphosphaturia of ClC-5 mice (64) and of patients with Dent's disease. One may argue, however, that the lack of ClC-5 might also impair the endocytosis of NaPi-2a. It therefore was essential to perform control experiments that showed that PTH-induced endocytosis of NaPi-2a was slowed but not abolished in ClC-5 KO mice (64).

Another important effect of PTH on proximal tubular cells is the transcriptional stimulation of 25(OH)vitaminD_3 1α-hydroxylase (86,87), the mitochondrial enzyme that converts the inactive precursor 25(OH)-VitD_3 into the active hormone 1,25$(OH)_2$-VitD_3 (Figure 6). As predicted by increased luminal PTH concentrations (64), 25(OH)vitaminD_3 1α-hydroxylase mRNA levels were markedly elevated in ClC-5 KO mice (76). The ensuing increase in enzymatic activity correlated with an increased *ratio* of 1,25$(OH)_2$-VitD_3/25(OH)-VitD_3 in the serum of KO mice (64,76). However, the *absolute* serum concentration of the active hormone 1,25$(OH)_2$-VitD_3 was decreased as a consequence of a severe loss of VitD together with its binding protein into the urine. This complex is normally endocytosed in the PT in a megalin-dependent manner (81).

Thus, there are two opposing effects of the impaired endocytosis in ClC-5 KO mice on serum 1,25$(OH)_2$-VitD_3 levels: The ensuing elevated luminal levels of PTH increase the amount of the enzyme that generates the active hormone 1,25$(OH)_2$-VitD_3, whereas the decrease of apical endocytosis of 25(OH)-VitD_3 severely limits the availability of its precursor. Because 1,25$(OH)_2$-VitD_3 stimulates intestinal Ca^{2+} absorption, an increase in its serum concentration might indirectly lead to hypercalciuria and kidney stones. Indeed, most patients with Dent's disease have moderately elevated levels of this hormone (75,88). In contrast, our ClC-5 KO mice have decreased levels of 1,25$(OH)_2$-VitD_3 and do not display hypercalciuria and kidney stones (64). An independent ClC-5 KO mouse model, however, presents with hypercalciuria (77) and has elevated levels of 1,25$(OH)_2$-VitD_3 (89). Hence, the balance between the stimulation of 1α-hydroxylase and

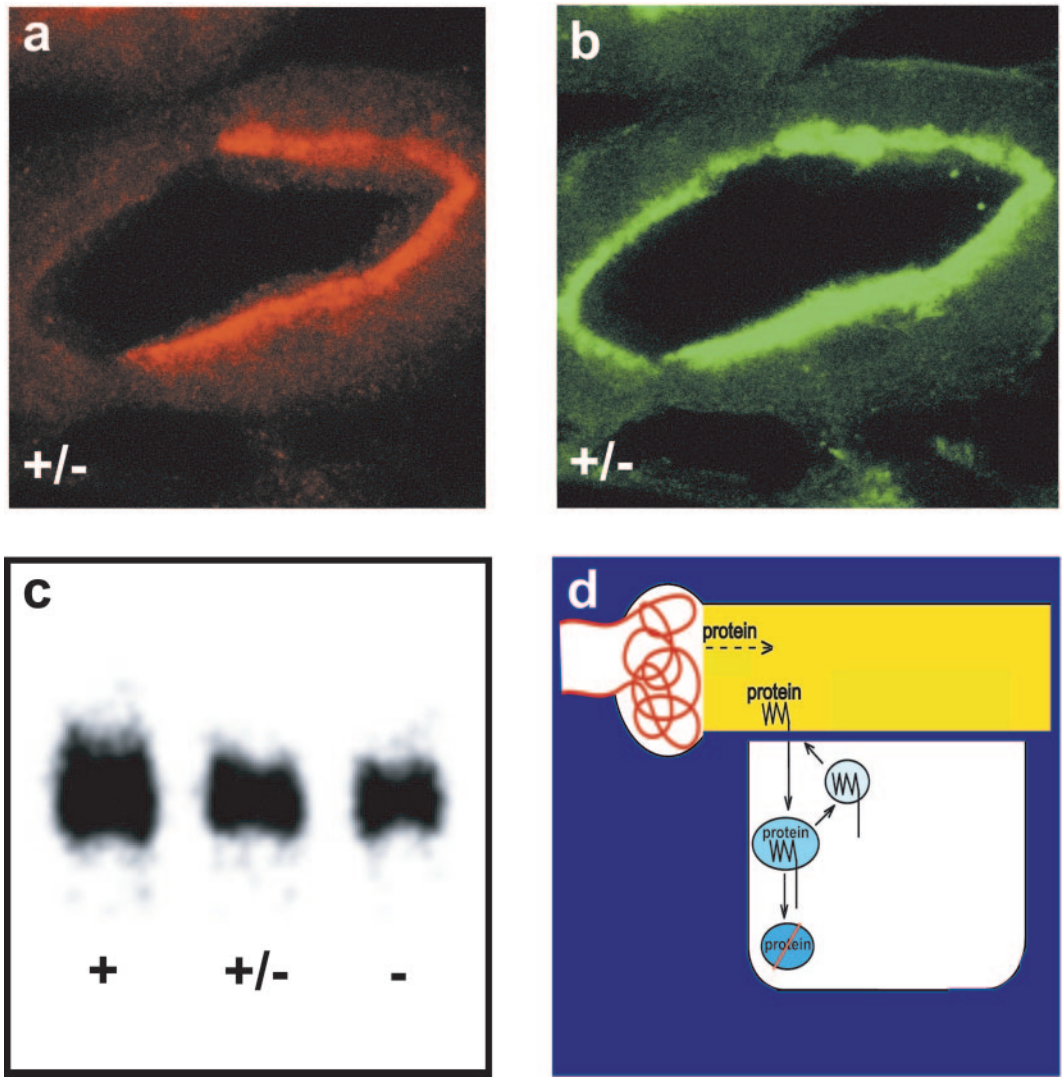

Figure 4. Cell-autonomous effect of ClC-5 disruption on the expression of the endocytotic receptor megalin. (A and B) Confocal immunocytochemistry of a proximal tubule of a female mouse heterozygous for the disrupted ClC-5 allele. (A) Half of the cells of a proximal tubular cross-section lack ClC-5 (shown in red). (B) These cells express less megalin, which seems to be present in a less broad apical area. (C) Western blot analysis of megalin in WT kidneys (+) and kidneys from heterozygous (+/−) and total knockout (KO; −) kidneys reveal a decrease in megalin levels in KO kidneys. Data from reference 64. (D) Model for megalin function. Megalin is a recycling receptor for the apical endocytosis of a broad range of proteins and other substances. The cell-autonomous decrease in megalin suggests a role of ClC-5 in transporting megalin to the apical brush border.

the urinary loss of VitD, which both are due to a primary defect in PT endocytosis, might explain the clinical variability of Dent's disease in a unifying model (Figure 6).

The defect in endocytosis and vesicle trafficking may also impinge on other transporters and receptors in addition to NaPi-2a and megalin (64). It is tempting to speculate that the aminoaciduria and glucosuria observed in patients with Dent's disease (75) might be due in part to altered trafficking of the respective transport proteins. However, no changes in the morphology of the endocytotic apparatus could be detected in ClC-5 KO mice (64,79), and the expression and subcellular distribution of the H^+-ATPase was unchanged in ClC-5 KO mice (64).

How does a lack of ClC-5 impair endocytosis? The lumen of compartments of the endosomal/lysosomal pathway is acidified by electrogenic V-type H^+-ATPases. For its efficient operation, it needs an electric shunt. Otherwise, it would generate a voltage across the vesicular membrane that would inhibit further pumping. It has been known for a long time that the acidification of endosomal and lysosomal compartments depends on anions, suggesting the presence of an anion conductance. To test this hypothesis, we used the pH-dependent fluorescence of acridine orange to investigate the acidification of renal cortical endosomes *in vitro* (64,76). Adding ATP led to an acidification that depended on the presence of Cl^- and was significantly reduced in ClC-5 KO endosomes (76). The remaining acidification in the KO was explained by the impurity of the preparation and by the possibility that other ion-conductive

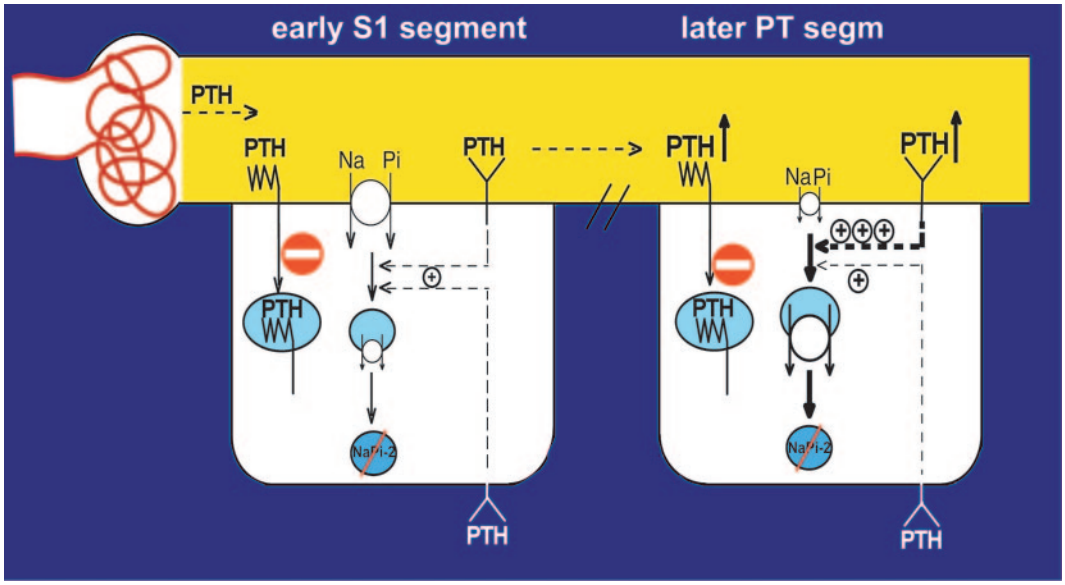

Figure 5. Model for hyperphosphaturia resulting from a disruption of ClC-5 (64,76). Parathyroid hormone (PTH) passes the glomerular filter into the primary urine. In the proximal tubule (PT), it is normally endocytosed after binding to megalin. This process is severely impaired when ClC-5 is missing, resulting in an increase of luminal PTH levels in later segments of the PT. The increased concentration of PTH leads to an enhanced stimulation of apical PTH receptors, which in turn triggers the endocytosis and lysosomal degradation of apical NaPi-2a Na-phosphate co-transporters.

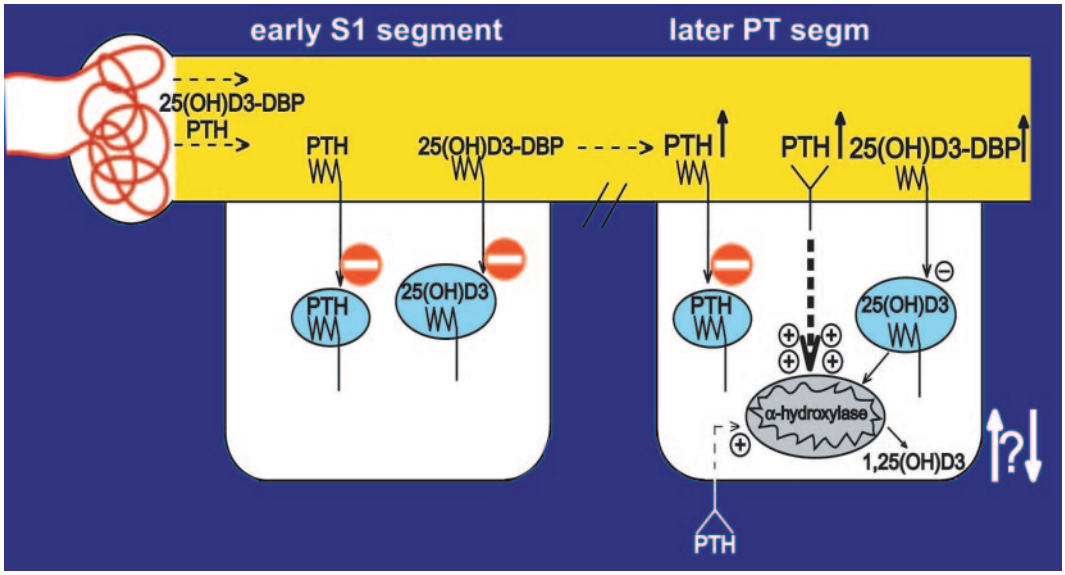

Figure 6. Model for changes in vitamin D levels caused by a lack of ClC-5 (64,76). Both PTH and VitD (bound to its binding protein DBP) are filtered into the primary urine. As already explained in Figure 5, the impairment of endocytosis increases PTH concentration in later PT segments. The augmented stimulation of apical PTH receptors stimulates the transcription of the mitochondrial enzyme 25(OH)vitaminD_3 1α-hydroxylase that creates the active hormone 1,25$(OH)_2$VitD_3 from the inactive precursor 25(OH)VitD_3. As the availability of the precursor is severely reduced as a result of the defect in apical endocytosis, the overall outcome (increase or decrease of 1,25$(OH)_2$VitD_3) is difficult to predict and may explain the difference between KO mouse models (64,76,77,89) and the clinical variability of Dent's disease (75).

pathways might be present in the same vesicles that also express ClC-5 (76).

How might luminal acidification influence endosomal function and trafficking? Several receptor/ligand systems of the endosomal pathway are regulated by luminal pH, as exemplified by the interaction of the transferrin receptor/transferrin interaction. Luminal pH additionally influences endosomal trafficking, as is evident, for example, from the pH-dependent formation of transport vesicles destined for late endosomes (90). The binding to endosomes of some regulatory proteins

that are involved in vesicle transport, such as βCOP (90) and ARF6/ARNO (91), depends on the luminal pH of this compartment.

The role of ClC-5 in acidifying endosomes is similar to that of other vesicular CLC. For instance, ClC-3 is involved in acidifying synaptic vesicles (67) and endosomes (92). ClC-7 is important for the acidification of the resorption lacuna of osteoclasts (61). Surprisingly, although ClC-7 is expressed on lysosomes, no change in steady-state lysosomal pH was detected (93). On the basis of more indirect evidence, ClC-4 has also been implicated in endosomal acidification (11,94). Although ClC-3, -4, and -7 all are expressed in the kidney, none of the respective KO has a defect in renal proximal tubular endocytosis (61,67) (Schaffer and Jentsch, unpublished observations for ClC-4 KO mice). However, the disruption of ClC-7 leads to lysosomal storage disease in the central nervous system and in proximal tubular cells (93), supporting the importance of ClC-7 for the endosomal/lysosomal pathway.

In addition to the H^+-ATPase, the sodium proton exchanger has been suggested to acidify endosomes and to have a role in endocytosis (95,96). As endosomes have a high luminal [Na^+] shortly after pinching off from the plasma membrane, the Na^+ gradient across endosomal membranes would be suited to acidify their lumen. However, the PTH-stimulated endocytosis of NHE3-containing vesicles was significantly slowed in ClC-5 KO PT cells, demonstrating the predominant role of ClC-5 in endocytosis (64).

Renal Roles of KCC Electroneutral Potassium-Chloride Co-Transporters

Electroneutral K-Cl co-transporters belong to the superfamily of electroneutral cation-chloride co-transporters (which also includes, *e.g.*, NKCC1 and NKCC2) and are encoded by four genes (KCC1 through KCC4) (97). Whereas KCC1 is broadly expressed, KCC2 is almost exclusively expressed in neurons. In addition to KCC1, the kidney expresses KCC3 and KCC4. Both latter isoforms are also expressed in several other tissues. This includes the brain for KCC3 but not for KCC4.

K-Cl co-transport has been implicated in transepithelial transport, cell volume regulation, and the lowering of intracellular Cl^- in neurons. To differentiate between the functions of the different isoforms, we disrupted the genes encoding KCC2, -3, and -4 in mice and analyzed their phenotypes (56,98,99). Mice lacking KCC2 died postnatally as a result of a disturbance in GABAergic neuronal inhibition that resulted from an increase in the intraneuronal Cl^- concentration (99). The KO of either KCC3 (98) or KCC4 (56) resulted in deafness, which was tentatively attributed to an impairment of K^+ recycling in the inner ear. The disruption of KCC3 additionally led to severe central and peripheral neurodegeneration (98), which correlated well with the pathology of human Anderman syndrome that is due to loss-of-function mutations in the human gene encoding KCC3 (100).

In addition to those phenotypes, both KCC3 and KCC4 KO mice displayed renal abnormalities. In the kidney, KCC3 was detected exclusively in basolateral membranes of the PT (98). KCC4 was co-expressed with KCC3 in these membranes and was additionally found in IC of the collecting duct (Figure 2A) (56). As discussed above in the section on ClC-K/barttin Cl^- channels, acid-secreting αIC need to recycle Cl^- ions that are taken up through basolateral AE1 Cl^-/HCO_3^- exchangers (Figure 2C) (56). KCC4 may be better suited for this purpose than ClC-Kb/barttin Cl^- channels, because the coupling to K^+ will lead to lower intracellular Cl^-. An inhibition of basolateral HCO_3^- transport by impaired Cl^- recycling should lead to less H^+ secretion by the apical proton pump. KCC4 KO mice indeed displayed renal tubular acidosis, as indicated by an alkaline pH of the urine and a decrease in blood base excess (56). In support of an impairment of basolateral Cl^- exit, electron microprobe analysis of intracellular electrolytes revealed an increase in intracellular Cl^- concentration in IC (56). It will be interesting to test experimentally whether and to which extent recycling through ClC-K/barttin channels takes place in these cells.

No overt renal phenotype was detected in KCC3 KO mice. These mice display hypertension (98), but the apparently exclusive expression of KCC3 in PT makes a renal origin of this phenotype unlikely. Isolated perfused PT were used to assess the role of KCC in cell volume regulation. The KO of KCC4 and, to a lesser extent, of KCC3 partially inhibited the regulatory volume decrease of PT cells (98). PT cells might need efficient mechanism for cell volume regulation as different loads of solutes that are reabsorbed by these cells (*e.g.*, amino acids, glucose) might lead to osmotic changes. The disruption of neither transporter, however, led to significant renal salt or water loss. It remains to be seen whether KCC1, which may be expressed in most nephron segments (42), plays a crucial role in transepithelial transport.

Summary and Outlook

Bridging the gap between genes and integrative physiology by targeted gene disruption in mice, as well as by analyzing human inherited disease, has dramatically furthered our understanding of renal physiology and pathology. Increasingly sophisticated approaches to gene targeting, combined with a revival of "classical" methods of renal physiology and with morphologic techniques, will undoubtedly provide us with exciting new insights into the function of the kidney, a fascinating organ that serves as a paradigm for transport physiology.

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