Pflugers Arch - Eur J Physiol (2003) 445:456–462
DOI 10.1007/s00424-002-0950-6

MOUSE MODELS AND HUMAN DISEASES

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The ClC-5 chloride channel knock-out mouse – an animal model for Dent's disease

Received: 25 April 2002 / Accepted: 10 May 2002 / Published online: 29 November 2002
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Abstract Mutations in the gene *CLCN5* encoding the vesicular chloride channel ClC-5 lead to Dent's disease, an X-linked renal disorder. Dent's disease is characterised by proteinuria, hyperphosphaturia and hypercalciuria, which eventually lead to kidney stones and nephrocalcinosis. As it was unclear how mutations in a chloride channel might cause these symptoms, we and others have generated genetic mouse models to elucidate the underlying pathophysiological mechanisms. We review results obtained from these three mouse models and present new data on endosomal acidification and vitamin D metabolism in ClC-5 knock-out (KO) mice. ClC-5 is expressed in apical endosomes of proximal tubular cells where it colocalizes with endocytosed proteins and the proton ATPase. ClC-5 may provide an electric shunt for the efficient operation of the electrogenic H^+-ATPase. We confirmed this hypothesis by showing that endosomes from *CLCN5* KO mice are acidified at a significantly lower rate than wild-type endosomes. This probably results in the drastic impairment of endocytosis observed in ClC-5 KO mice. Parathyroid hormone (PTH) is filtered into the lumen of the nephron, where it is endocytosed and degraded by proximal tubular cells. The defective endocytosis in ClC-5 KO mice entails an increased luminal concentration of PTH, subsequent stimulation of apical PTH receptors which causes an increased endocytosis of the phosphate transporter NaPi and phosphaturia. We now show that it also results in up-regulation of proximal tubular α-hydroxylase that generates the active form of vitamin D from its precursor. We discuss how the primary defect in endocytosis leads via secondary changes in calciotropic hormones to the tertiary symptoms hyperphosphaturia, hypercalciuria and kidney stones.

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Keywords Proteinuria · Kidney stones · Parathyroid hormone · Nephrocalcinosis

Introduction

Dent's disease and ClC-5

Dent's disease is an inherited renal Fanconi syndrome characterised by low-molecular-weight proteinuria, aminoaciduria, hypercalciuria, hyperphosphaturia, nephrocalcinosis and the development of kidney stones [47]. Dent's disease, as well as the closely related syndromes X-linked recessive nephrolithiasis (XRN) [12, 38], X-linked recessive hypophosphataemic rickets (XLRH) [2] and idiopathic low-molecular-weight proteinuria of Japanese children (JILMWP) [22], are due to mutations in the X-chromosomal *CLCN5* gene [21] that encodes the ClC-5 chloride channel. The common name "Dent's disease" has been proposed for these inherited disorders.

The clinical symptoms of Dent's disease are variable. The most consistent abnormality is the urinary loss of low-molecular-weight proteins, which is even observed in carrier females and children and can occur in the absence of significant hypercalciuria or kidney stones [22]. The proximal tubule is responsible for the efficient reabsorption of proteins and many other solutes that pass the glomerular filter. Proximal tubular cells are endowed with an apical endocytotic machinery that has been histologically and functionally well characterised [6, 27]. As in other cells, endosomes are acidified by a V-type proton ATPase [5]. Consistent with the high capacity of proximal tubular endocytosis, the proton pump is highly expressed in these cells. For its efficient operation [10], a parallel conductance, known to be predominantly carried by chloride, is required to ensure overall electroneutrality of transport [35]. Immunocytochemistry has shown the ClC-

5 chloride channel to co-localise in the proximal tubule with endocytosed protein early after uptake [14] and with the V-type H^+-ATPase [7, 14, 25, 36]. ClC-5 also co-localizes with endosomal markers in transfected cells [14] and subcellular fractions obtained by density centrifugation [7]. Taken together, this suggests that the proteinuria in Dent's disease is caused by defective endocytosis due to a lack in endosomal chloride conductance [14].

In addition to proximal tubular cells, ClC-5 is expressed highly in both types of intercalated cells of the renal collecting duct [7, 14, 25, 36], again residing in intracellular vesicles at the apical pole. In acid-secreting α-intercalated cells ClC-5 co-localizes with the apically expressed H^+-ATPase at the light microscopic level, but its subcellular localisation diverges significantly from the basally expressed proton pump in β-intercalated cells [14, 36]. ClC-5 is also expressed in minor to moderate levels in liver, brain and intestine [34, 41, 44]. Similar to the proximal tubule, in enterocytes and colonic epithelial cells the ClC-5 protein and the V-type proton ATPase co-localise again in vesicles below the apical brush border membrane [44].

Heterologous expression of ClC-5 in *Xenopus* oocytes [11, 41] and mammalian cells [11, 37] elicits strong outwardly-rectifying currents with a conductance sequence of $NO_3^- > Cl^- > Br^- > I^-$ [11]. These currents, which are decreased upon extracellular (intravesicular) acidification, are decreased or abolished with mutations found in Dent's disease [21]. Similar currents are observed with the close homologues ClC-4 [11, 45] and ClC-3 [20]. The extreme outward rectification, which yields detectable currents only at voltages more positive than +20 mV, remains enigmatic.

CLC chloride channels and disease

ClC-5 is a member of the CLC chloride channel family (for a recent review, see [16]). As strongly supported by mutagenesis studies [24, 29] and recently confirmed by crystal structure analysis [8], the basic unit of CLC channels is a dimer of identical proteins, each of which forms a pore on its own. In addition, CLC proteins may associate with accessory β-subunits, as recently exemplified by barttin, a small protein that is necessary for proper function of ClC-K channels [9].

The CLC gene family is evolutionarily conserved, with representatives found in organisms ranging from bacteria and archae to yeasts, plants and animals. There are nine different CLC genes in mammals. Based on homology, they can be grouped into three branches. The first branch (including ClC-1, ClC-2, ClC-Ka and ClC-Kb) encodes channels that function primarily in the plasma membrane. The other two branches, comprising ClC-3, -4 and -5, and ClC-6 and -7, respectively, are now recognised to function primarily in intracellular vesicles [16]. However, at least ClC-7 may be inserted into the plasma membrane (of osteoclasts) under certain circumstances, where it has an important function [18].

The phenotypes of human inherited diseases and of mouse models have shed considerable light on the important and varied physiological functions of CLC channels. In the first (plasma membrane) branch, mutations in the muscle channel ClC-1 cause myotonia [17, 40] and mutations in the kidney channel ClC-Kb Bartter syndrome (associated with severe renal salt loss) [39]. The disruption of ClC-K1 in mice leads to symptoms resembling renal diabetes insipidus [28], while ClC-2 KO mice present with retinal and testicular degeneration [3]. Further, human mutations in the β-subunit barttin lead to Bartter syndrome type IV, a severe salt-losing tubulopathy associated with congenital deafness [1, 9].

The pathophysiology of ClC-5 disruption, the topic of this work, will be described in detail later. The ClC-3 channel has been disrupted in mice. Surprisingly, this leads to a dramatic postnatal degeneration of the hippocampus, which is replaced in adults by a cavity contiguous with the ventricular system. In addition, ClC-3 KO mice had severe retinal degeneration. Similar to ClC-5, ClC-3 is present in endosomal compartments. In addition, it is significantly expressed in synaptic vesicles where it contributes to their acidification [42]

ClC-7 is expressed later in the endosomal pathway [18]. Immunocytochemistry and subcellular fractionation localised it to late endosomes and lysosomes. While being ubiquitously expressed, ClC-7 expression is particularly prominent in osteoclasts. In these cells, ClC-7 can be inserted together with the proton ATPase into the ruffled border that lines the reabsorption lacuna. Similar to the roles of ClC-5 and ClC-3 in endosomes and synaptic vesicles, it is important for the acidification of this "extracellular lysosome". Accordingly, its disruption in mice leads to a severe osteopetrotic phenotype. Guided by this observation, it was found that also some forms of human osteopetrosis are caused by mutations in this channel [18].

To clarify the role of ClC-5 in endocytosis, and to elucidate the enigmatic mechanism by which a disruption of a chloride channel causes hyperphosphaturia, hypercalciuria and kidney stones, we generated and analysed a mouse model in which the *CLCN5* gene was disrupted constitutively by homologous recombination [34]. Another, similar mouse model has been generated by Guggino's group [46], while Yu's group have described a mouse in which the abundance of the ClC-5 protein is decreased by the transgenic over-expression of a ribozyme [26]. We will first review the results gleaned from our ClC-5 KO mouse model, add unpublished data on endosomal acidification and vitamin D metabolism and then compare the three published mouse models of Dent's disease.

The ClC-5 KO mouse

We generated a complete, constitutive ClC-5 KO by deleting genomic sequences that encode an essential part of the protein that is directly involved in pore formation

[34]. Although this strategy might lead to the generation of a severely truncated protein, no signal could be detected in Western blots of *CLCN5*$^{-/-}$ kidneys using a specific amino-terminal antibody, suggesting that the predicted truncated protein or its mRNA might be unstable.

ClC-5 KO mice reproduced the most consistent symptom of Dent's disease, low-molecular-weight proteinuria. Western blot analysis showed a massive increase in the urinary concentration of vitamin D binding protein and retinol binding protein [34]. Whereas injected radioactively labelled β_2-microglobulin accumulated in the cortex of the wild-type (WT) kidney (which contains proximal tubules), it was excreted into the urine of KO mice. Injection of fluorescently labelled lactoglobulin, horseradish peroxidase or fluorescein dextran into the bloodstream of anaesthetised mice and subsequent fixation of kidneys within minutes after injection demonstrated that both receptor-mediated and fluid-phase endocytosis are severely reduced in KO kidneys [34]. We showed that the defect in endocytosis is cell autonomous by exploiting the X-chromosomal localisation of ClC-5: due to random X-chromosomal inactivation in females, ClC-5 is disrupted in every other cell in heterozygous (*CLCN5*$^{+/-}$) females. The severe decrease in endocytosis correlated strictly with the absence of ClC-5 [34].

This defect in endocytosis, the endosomal localisation of ClC-5 and its co-localisation with the H^+-ATPase in apical vesicles of proximal tubular cells suggests strongly that this Cl^- channel is needed for the efficient acidification of endosomes by providing an electrical shunt. Indeed, our preliminary experiments had confirmed this hypothesis [34]. The results of such experiments are now shown in Fig. 1. We isolated endosomal fractions from kidney cortex of WT and KO animals and measured intravesicular acidification using quenching of acridine orange fluorescence. When ATP was added to the medium, both WT and KO endosomes acidified. However, the final level of acidification was significantly reduced in KO endosomes and their rate of acidification was slowed by a factor of about 2 (Fig. 1). The addition of the protonophore carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP) at the end of the experiments confirmed that the change in fluorescence was indeed due to intravesicular acidification. These experiments thus demonstrate the importance of the ClC-5 chloride channel for the acidification of renal cortical endosomes. On the other hand, the acidification of KO endosomes was still dependent on the presence of chloride in the medium (data not shown). This may be explained by the fact that the endosomal preparation most likely does not only contain vesicles that normally express ClC-5. However, it cannot be excluded that additional, different Cl^-channels are present in ClC-5-containing vesicles.

In the proximal tubule, the apical protein megalin, which belongs to the low-density lipoprotein (LDL) superfamily, mediates receptor-mediated endocytosis of proteins and many other substances [19, 32]. Using our KO mice, we have shown that the amount of megalin is decreased about twofold in a cell autonomous manner [34]. This decrease in protein levels of the endocytotic receptor will further exacerbate the defect in endocytosis. We hypothesised that the cell-autonomous decrease in megalin might be due to a decreased fidelity of sorting in the endocytotic pathway, where megalin is normally recycled back to the plasma membrane. These findings may also explain the low amount of megalin shed into the urine of patients with Dent's disease [31].

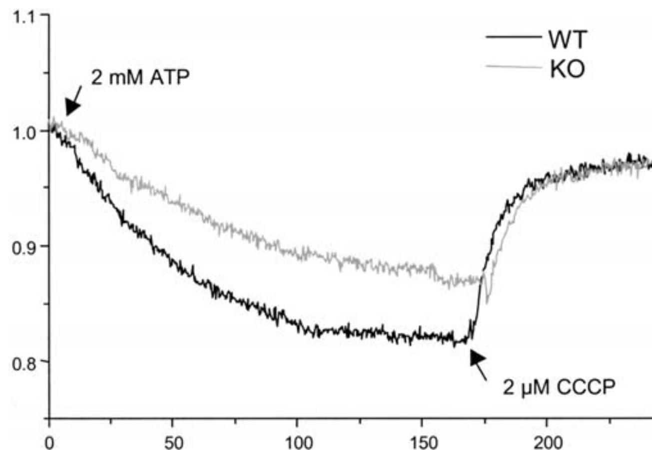

Fig. 1 Acidification of endocytotic vesicles isolated from wild-type (*WT*) and ClC-5 knock-out (*KO*) mouse kidneys. Acidification is quantified by pH-dependent trapping and quenching of the fluorescent dye acridine orange in the lumen of vesicles. Acidification by the proton pump is initiated by adding ATP. At the end of the experiment, the pH gradient was collapsed by addition of the protonophore carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP). Vesicles derived from ClC-5 KO mice display a slower acidification rate and reach lower steady-state levels of acidification. Data represent an average of five assays and were confirmed in three independent experiments

Using our mouse model, we have shown that the other symptoms of Dent's disease, hyperphosphaturia, hypercalciuria and kidney stones, are most likely tertiary consequences of the primary defect in endocytosis [34]. Calciotropic hormones such as vitamin D (bound to its cognate binding protein) or parathyroid hormone (PTH) are filtered into the primary urine. Both hormones are normally endocytosed in a megalin- and ClC-5-dependent pathway (Fig. 2). The urinary PTH concentration of megalin KO mice is increased about fourfold [15]. We therefore reasoned that *CLCN5*$^{-/-}$ mice should also have a reduced rate of PTH endocytosis. This was confirmed by the demonstration of a ~1.7-fold increase in urinary PTH in the presence of a normal blood concentration [34]. Proximal tubular cells have functional PTH receptors both in their basolateral and apical membranes [43]. The rise in tubular PTH concentration, which is predicted to increase from the glomerulus to the S3 segment, should lead to an abnormally high stimulation of apical PTH receptors in later parts of the PT ≙. A well-studied effect of PTH in the proximal tubule is the down-regulation of apical NaPi-2, the main transporter involved in proximal tubular

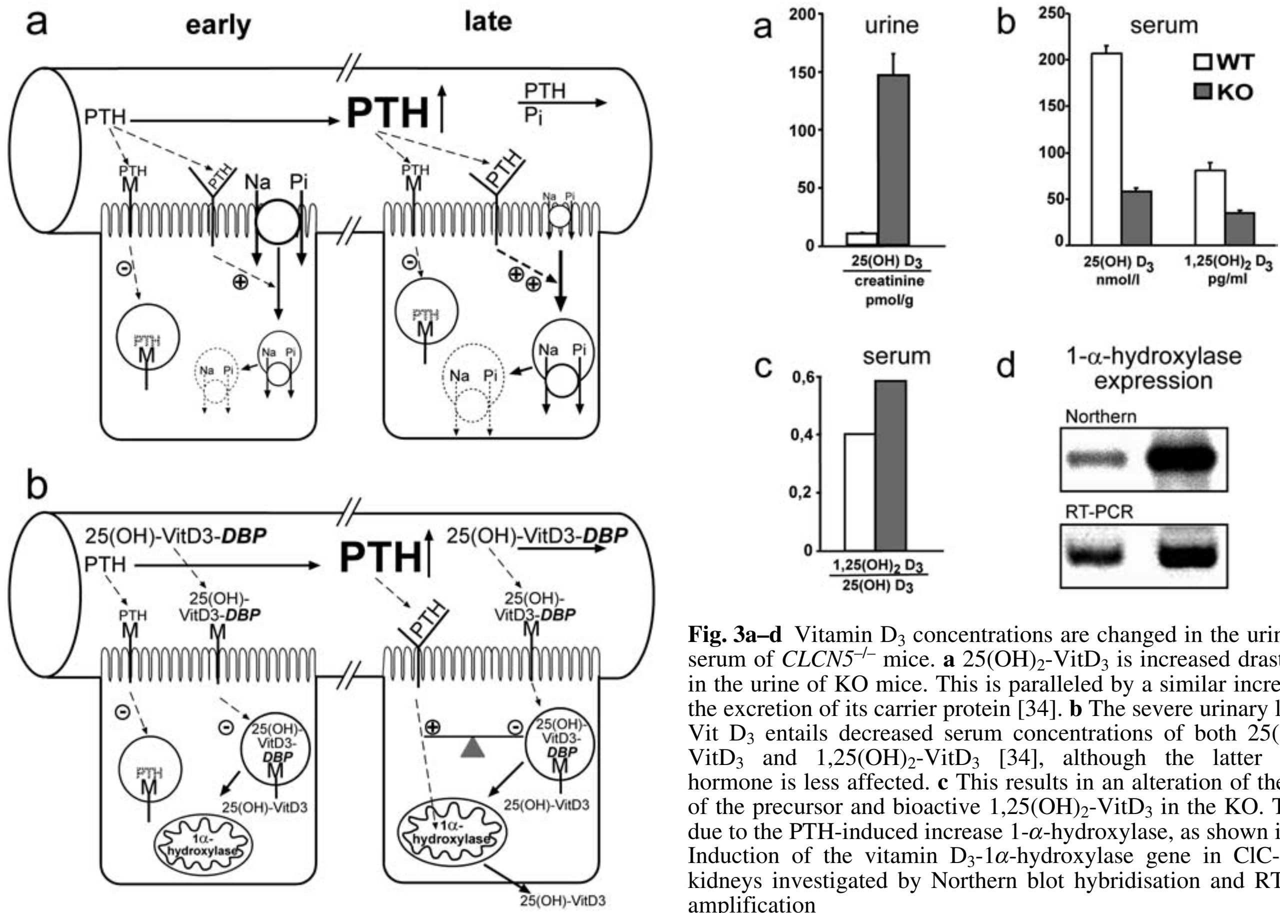

Fig. 2a, b Schematic drawing of the alteration of calciotropic hormone metabolism in proximal tubules of ClC-5 KO kidneys. **a** The defect in ClC-5 dependent endocytosis, which is further exacerbated by the secondary decrease in the apical scavenger receptor megalin, results in a progressive increase of PTH along the lumen of proximal tubules. This leads to an enhanced stimulation of luminal PTH receptors (*Y*) in later PT $\triangleq$ segments, resulting in an increased endocytosis and lysosomal degradation of the apical NaPi-2 cotransporter. This in turn causes the phosphaturia observed in the KO mouse and in patients with Dent's disease. **b** $25(OH)_2$-$VitD_3$, the second key player in calcium homeostasis, is converted in the proximal tubule to its active form $1{,}25(OH)_2$-$VitD_3$ by the mitochondrial 1-α-hydroxylase. The prohormone is transported bound to its carrier protein vitamin D binding protein (*DBP*) and is taken up by an endocytotic process that requires megalin and ClC-5. Elevated PTH in more distal segments of the proximal tubule stimulates the expression of 1-α-hydroxylase. In the ClC-5 KO, the synthesis of $1{,}25(OH)_2$-$VitD_3$ thus depends on the balance of reduced supply and increased enzymatic activity (illustrated by the balance in Fig. 2b). If this results in increased levels of serum $1{,}25(OH)_2$-$VitD_3$, the resulting stimulation of intestinal calcium reabsorption must be balanced by increased renal calcium excretion, eventually resulting in kidney stones

Fig. 3a–d Vitamin D_3 concentrations are changed in the urine and serum of *CLCN5*$^{-/-}$ mice. **a** $25(OH)_2$-$VitD_3$ is increased drastically in the urine of KO mice. This is paralleled by a similar increase in the excretion of its carrier protein [34]. **b** The severe urinary loss of Vit D_3 entails decreased serum concentrations of both $25(OH)_2$-$VitD_3$ and $1{,}25(OH)_2$-$VitD_3$ [34], although the latter active hormone is less affected. **c** This results in an alteration of the ratio of the precursor and bioactive $1{,}25(OH)_2$-$VitD_3$ in the KO. This is due to the PTH-induced increase 1-α-hydroxylase, as shown in **d**. **d** Induction of the vitamin D_3-1α-hydroxylase gene in ClC-5 KO kidneys investigated by Northern blot hybridisation and RT-PCR amplification

phosphate reabsorption. Within minutes, injection of PTH leads to endocytotic retrieval of NaPi-2 from the plasma membrane [23]. The transporter is then targeted to lysosomes for degradation [33] (Fig. 2a). Indeed, whereas NaPi-2 is prominently expressed in the brush border membrane of WT proximal tubular cells, we have shown that it is endocytosed in the majority of proximal tubular segments of KO mice [34]. An exception was found in the early part of the S1 segment, readily explainable by the fact that these cells are superfused with normal blood levels of PTH as the defect in endocytosis has not yet had a chance to change its concentration. The trafficking of NaPi-2 is significantly slowed by the disruption of ClC-5, but still depends on PTH. We thus explain the hyperphosphaturia in terms of a broad defect in proximal tubular endocytosis.

What about hypercalciuria, another prominent symptom of Dent's disease, which is probably of greater significance for the formation of kidney stones and nephrocalcinosis? The proximal tubule is the major site in the body for the enzymatic conversion of 25(OH)-$VitD_3$ to the active metabolite $1{,}25(OH)_2$-$VitD_3$. This reaction is catalysed by the mitochondrial enzyme α-hydroxylase, which is up-regulated by PTH at the transcriptional level [4, 30]. We thus argued that the increase in tubular PTH concentration resulting from the lack of ClC-5-dependent endocytosis should raise the activity of α-hydroxylase in the KO [34]. Indeed, the amount of its mRNA was increased strongly in *CLCN5*$^{-/-}$ kidneys (Fig. 3). Assuming unchanged availability of the precursor, this predicts elevated levels of the active hormone $1{,}25(OH)_2$-$VitD_3$ (Fig. 3). In fact, $1{,}25(OH)_2$-$VitD_3$ concentrations are slightly elevated in patients with Dent's disease [38]. This

is predicted to enhance intestinal absorption of calcium, which would then have to be excreted by the kidney. Thus, the lack of ClC-5 dependent endocytosis may lead, via changed luminal PTH levels, increase in PT $\hat{=}$ 1-α-hydroxylase, elevated 1,25$(OH)_2$-VitD_3 levels and increased intestinal calcium absorption, to hypercalciuria and kidney stones [34].

There is, however, one complication. The main supply of the precursor 1(OH)-VitD_3 to PT $\hat{=}$ cells is via apical, megalin-dependent endocytosis [32]. As a consequence of the impaired endocytosis, we found dramatically increased levels of the VitD binding protein and VitD in the urine of ClC-5 KO mice [34]. Although the 1,25$(OH)_2$-VitD_3:1(OH)-VitD_3 ratio was increased in the KO, reflecting the increase in α-hydroxylase levels (Fig. 3), the absolute serum concentrations of the precursor and of the active form of VitD were decreased due to the urinary loss of VitD (Fig. 3). Accordingly, there was no hypercalciuria in our KO mouse [34]. We predict that a delicate balance between the reduced supply of the precursor and the stimulation of the hydroxylase, both of which are the result of a lack of ClC-5 dependent endocytosis, will determine the presence or absence of hypercalciuria and kidney stones. This may well explain the clinical variability of Dent's disease. As discussed later, this may also explain the difference between our model and the mouse model described by Guggino's group [46].

We also examined the effect on NHE3, a Na^+/H^+ exchanger highly expressed in the PT $\hat{=}$ brush border. Endocytosis of NHE3 is stimulated by PTH. In contrast to NaPi-2, NHE3 is not targeted subsequently to lysosomes, but recycled back to the plasma membrane [48]. Consistent with the increased PTH concentration in the lumen of KO proximal tubules, NHE3 was largely present in intracellular vesicles in *CLCN5*$^{-/-}$ proximal tubular cells. Although this might suggest a more alkaline urine, the urine of KO mice was rather was slightly more acidic for reasons that are not yet clear [34]. The PTH-induced endocytosis of NHE3 was slowed, similar to that of NaPi-2 [34]. This is an interesting point: it has been suggested previously that Na^+/H^+ exchangers participate in acidifying early endosomes and, indeed, amiloride, an inhibitor of this exchanger, reduces endocytosis of proteins [13]. Such a mechanism appears feasible as endocytotic vesicles will have initially the high luminal [Na^+] of the extracellular medium, providing a favourable gradient for intravesicular acidification. The fact that NHE3 endocytosis was significantly slower in the KO suggests, however, that the ClC-5-dependent acidification by the proton pump is the dominant mechanism.

Comparison of the different mouse models for Dent's disease

To date, three different genetically modified mouse models with reduced or abrogated ClC-5 expression have been reported [26, 34, 46]. Luyckx and co-workers [26] created a transgenic mouse line expressing a ribozyme designed to inactivate specifically the mRNA for ClC-5. Surprisingly, they reported moderately reduced level of ClC-5 protein although ClC-5 mRNA levels were unchanged. Under certain feeding conditions mice developed hypercalciuria. It was not reported whether these mice display low-molecular-weight proteinuria, the most consistent symptom of Dent's disease.

Shortly after our mouse model was published [34], Wang and co-workers [46] reported the establishment of another ClC-5 KO mouse strain. Disruption of exon VI of the *CLCN5* gene also led to the complete absence of ClC-5 protein. Urine analysis showed significant polyuria, hypercalciuria, hyperphosphaturia, low-molecular-weight proteinuria and generalised aminoaciduria. These mice did not develop rickets and did not show alterations of serum calcium concentrations as found in many British patients suffering from Dent's disease. Although radiology and magnetic resonance imaging have revealed normal kidneys, a slight positive von-Kossa-staining for nephrocalcinosis was observed at the cortico-medullary junction [46].

These findings differ in part from those in our mouse model [34] in which no hypercalciuria or nephrocalcinosis is observed. The knock-out strategy was similar, and should lead to a total loss of function and no significant dominant negative effect in either case. Moreover, both groups worked on a mixed background of C57BL/6J and 129SvJ mice. The latter was used for the generation of ES cells, but since different ES cell lines were used, there may be slight genetic differences. Given the hypothesis presented above and illustrated in Fig. 2b, namely that the delicate balance between the loss of 25(OH)-VitD_3 and the induction of α-hydroxylase determines whether hypercalciuria and kidney stones occur [34], it would not be surprising that slight differences in genetic background and/or diet come into play. In this respect, it is interesting to note that Piwon et al. [34] used lab chow with a vitamin D3 content of 1 IU/g, while the diet fed to mice by Wang et al. [46] was supplemented with 4.5–3.3 IU/g vitamin D3. Unfortunately hormone values have not been reported for the latter model, making a direct comparison of the hormone status of the different mouse models of Dent's disease currently impossible.

In summary, the generation and analysis of ClC-5 knock-out models has not only explained the role of this intracellular channel in endocytosis, but has also clarified the hitherto enigmatic pathway leading from a defect in a chloride channel to kidney stones. There is very strong evidence that the primary defect in endocytosis leads to a changed endocytosis and metabolism of calciotropic hormones, which in turn results in hyperphosphaturia and kidney stones. As these are tertiary symptoms, and since the defect of endocytosis acts in two opposing directions on the concentration of active vitamin D, the clinical variability of Dent's disease is not surprising. These mouse models now provide means to study the environmental and pharmacological factors that may

prevent or mitigate the severe symptoms of Dent's disease.

Materials and methods

Endosomal acidification assay

Acidification of endosomes was measured using vesicle preparations from between eight and ten mouse kidneys that were finely minced after removal of the papilla and resuspended in 300 mM mannitol, 12 mM TRIS/HCl (pH 7.4). The suspension was homogenised by 20 strokes of a Dounce homogenizer. Debris was removed by low speed centrifugation at 2,500 *g*, for 15 min. Endosomes in the supernatant were recovered by centrifugation at 20,000 *g* for 15 min. After rehomogenisation of the pellet, 32 g of the suspension in mannitol/TRIS/HCl (pH 7.4) was combined with 6.1 g Percoll (Pharmacia) and centrifuged for 30 min at 48,000 *g* in a JA20 rotor (Beckman). A 4-ml aliquot of the highest density fraction was aspirated and diluted to 30 ml with (in mM) 300 mannitol, 100 K-gluconate, 5 $MgSO_4$, 50 HEPES/TRIS (pH 7.0). Endosomes were pelleted for 30 min at 48,000 *g* and repelleted twice at 1,400 *g* in Eppendorf tubes. Protein concentration was determined and aliquots with a protein content of 20 mg/ml were shock frozen and stored in liquid nitrogen (n=10).

To measure the rate of vesicular acidification, 150 µl thawed endosomes was equilibrated in 1 ml (in mM) 300 mannitol, 100 KCl, 5 MgSO4, 50 HEPES/TRIS (pH 7.0) containing 6 µM acridine orange at room temperature. Fluorescence measurements were performed using a SAFAS flx spectrofluorimeter (SAFAS, Monaco) with excitation at 490 nm (slit width 5 nm) and emission at 525 nm (slit width 5 nm). Acidification was initiated by addition of 2 mM ATP and the protonophore CCCP (2 µM) was used to dissipate the proton gradient.

Expression of the 25-OH vitamin D3-1-α-hydroxylase

Northern blot analysis

Poly-(A^+) RNA (2 µg) isolated from ClC-5 KO and WT mouse kidneys was separated on a 1% formaldehyde agarose gel and transferred to nylon membranes (Hybond, APBiotech). A ^{32}P-radiolabelled probe specific for mouse 25-OH vitamin D_3 1-α-hydroxylase (Genbank Accession No.: AB006034) was generated using appropriate primers and RT-PCR. Specifically bound probe was detected using a phosphoimager (Fuji).

RT-PCR analysis

Poly-(A^+) RNA (5 µg) isolated from ClC-5 KO and WT mouse kidneys was reverse transcribed using Superscript II RT (Invitrogen). Amplification of the cDNA with a primer set specific for mouse 1-α-vitamin D3 hydroxylase (forwards: GGCAGAGGC-TCCGAAGTCTTC; reverse: GGTATAAGGTGGTGCAACCAG) was achieved by 28 cycles of 20 s at 94 °C, 30 s at 60 °C and 60 s at 72 °C. The 329-bp amplification products were separated on a 1.5% agarose gel.

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