Effect of Diuretics on Renal Tubular Transport of Calcium and Magnesium

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Word count: 10748
Abstract word count: 250
Running head: Diuretic effects on Ca²⁺ and Mg²⁺ transport

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Abstract
Calcium ($\text{Ca}^{2+}$) and Magnesium ($\text{Mg}^{2+}$) reabsorption along the renal tubule is dependent on distinct trans- and paracellular pathways. Our understanding of the molecular machinery involved is increasing. $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ reclamation in kidney is dependent on a diverse array of proteins, which are important for both forming divalent cation permeable pores and channels, but also for generating the necessary driving forces for $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ transport. Alterations in these molecular constituents lead to profound effects on tubular $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ handling. Diuretics are used to treat a large range of clinical conditions, but most commonly for the management of blood pressure and fluid balance. The pharmacological targets of diuretics generally directly facilitate sodium ($\text{Na}^{+}$) transport, but also indirectly affect renal $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ handling, i.e. by establishing a prerequisite electrochemical gradient. It is therefore not surprising that substantial alterations in divalent cation handling can be observed following diuretic treatment. The effects of diuretics on renal $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ handling are reviewed in the context of the current understanding of basal molecular mechanisms of $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ transport. Acetazolamide, osmotic diuretics, NHE3 inhibitors and antidiabetic SGLT blocking compounds, target the proximal tubule, where paracellular $\text{Ca}^{2+}$ transport predominates. Loop-diuretics and ROMK inhibitors block thick ascending limb transport, a segment with significant paracellular $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ transport. Thiazides target the distal convoluted tubule, however, their effect on divalent cation transport is not limited to that segment. Finally, potassium-sparing diuretics, which inhibit electrogenic $\text{Na}^{+}$ transport at distal sites, can also affect divalent cation transport.
Introduction

Diuretics are used in the management of hypertension, oedema, and a large array of other diseases affected or ameliorated by altering electrolyte transport. In kidney, diuretics often target transport proteins or mechanisms that are critically important for renal reabsorption of sodium, chloride (NaCl) and water. Given that calcium (Ca$^{2+}$) and magnesium (Mg$^{2+}$) transport in the kidney is dependent on electrochemical gradients established by tubular NaCl transport processes, it is not surprising that diuretic treatment can cause significant alterations in renal Ca$^{2+}$ and Mg$^{2+}$ handling. This review aims to highlight the actions of diuretics in the context of our current knowledge of the molecular mechanisms driving tubular Ca$^{2+}$ and Mg$^{2+}$ transport.

Ca$^{2+}$ and Mg$^{2+}$ transport in the proximal tubule

Approximately 55% of Ca$^{2+}$, the fraction in plasma not bound to protein, is freely filtered by the glomerulus into the proximal tubule (PT) (77, 79, 96). A slightly larger fraction of Mg$^{2+}$, approximately 70%, is not bound to plasma proteins and hence freely filterable by the glomerulus (54). The majority, or 60-70% of the filtered Ca$^{2+}$ load, is reabsorbed along the course of the PT (67, 137, 214, 226). The Ca$^{2+}$ concentration in the convoluted portion of the PT accessible to micropuncture remains nearly equivalent or slightly higher (1.0-1.2) to that in the ultrafiltrate (138, 226). This observation supports the generally accepted premise that the majority of PT Ca$^{2+}$ reabsorption occurs via a passive paracellular process, driven by active solute and subsequent water reabsorption (28, 169, 214, 226). Proximal tubular Mg$^{2+}$ reabsorption is also thought to occur via a passive...
paracellular process, driven by solute and water reabsorption, in a fashion similar to Ca\(^{2+}\).

However, only 10-25% of the filtered load of Mg\(^{2+}\) is reabsorbed from the PT, much less than that reported for Ca\(^{2+}\) (138, 139, 193, 194). Consequently, the luminal Mg\(^{2+}\) concentration may almost double along the proximal convoluted tubule (138). This is likely the consequence of greatly reduced relative permeability for Mg\(^{2+}\) \((P_{Mg})\) across this segment amounting to \(1 \cdot 10^{-5}\) cm/s, in comparison to the permeability for Ca\(^{+}\) \((P_{Ca})\), which has been reported to reach \(8 – 27 \cdot 10^{-5}\) cm/s (78, 119, 169).

In the PT, Ca\(^{2+}\) reabsorption closely follows Na\(^{+}\), suggesting that Na\(^{+}\) transport could be a prerequisite for driving Ca\(^{2+}\) reabsorption across the epithelium (214, 226). Na\(^{+}\) is the major physiological solute in the ultrafiltrate. At a molecular level, Na\(^{+}\) reabsorption from the PT occurs via a transcellular process with apical entry predominantly occurring through the Na\(^{+}\)-proton (H\(^{+}\)) exchanger isoform 3, NHE3 (210) (Figure 1). This Na\(^{+}\) uptake is driven by the steep Na\(^{+}\) gradient maintained by the Na\(^{+}\), potassium (K\(^{+}\))-ATPase, which pumps 2 K\(^{+}\) into the cell and 3 Na\(^{+}\) out, thereby maintaining a low cellular Na\(^{+}\) concentration and a steep voltage gradient across the cell membrane. The epithelium of the PT has a high osmotic water permeability due to abundant expression of aquaporin 1 (AQP1) in both the apical and basolateral membrane (171). Thus, Na\(^{+}\) reabsorption via NHE3 facilitates water removal from the luminal fluid.

The proximal tubular epithelium is also very leaky, displaying a transepithelial resistance of approximately \(10 \, \Omega \cdot \text{cm}^2\) (19, 167, 213). Consequently, significant water reabsorption also occurs through the paracellular shunt, although the majority moves transcellular via AQP1 (208, 209) (Figure 1). Importantly, the movement of water through the paracellular shunt facilitates the movement of other ions via convection in a process known as solvent
drag. This process contributes to significant paracellular Na$^+$ reabsorption along the PT (38, 168). Regardless, both Na$^+$ and water are reabsorbed paracellularly from the PT, driven by the transcellular reabsorption of Na$^+$. Ca$^{2+}$ reabsorption follows this passive paracellular mechanism. Whether Ca$^{2+}$ transport occurs due to water removal resulting in a slightly higher electrochemical gradient favouring paracellular Ca$^{2+}$ transport or via the process of solvent drag has yet to be clearly delineated (Figure 1). Hence, volume expansion, which reduces Na$^+$ and water transport from the PT, also reduces Ca$^{2+}$ reabsorption and may lead to significant urinary Ca$^{2+}$ wasting. In line with this, volume contraction reduces urinary Ca$^{2+}$ excretion (228). However, volume status does not appear to affect Mg$^{2+}$ transport in the PT (186). Why Mg$^{2+}$ reabsorption is not as tightly coupled to Na$^+$ reabsorption from the PT is likely due to the nature of the shunt, which is discussed further below.

The presence of an increased fractional excretion of Ca$^{2+}$ in Nhe3 null mice, underlines the importance of Na$^+$ coupled Ca$^{2+}$ reabsorption from the PT (179). Although specific examination of NHE3 coupled Ca$^{2+}$ flux has not been measured by micropuncture or microperfusion, further evidence implicating NHE3 in driving paracellular Ca$^{2+}$ flux is provided by work performed in a proximal tubular cell culture model. When NHE3 was overexpressed, transepithelial Ca$^{2+}$ flux doubled (179). This effect of increased NHE3 mediated transepithelial Ca$^{2+}$ flux was prevented by blocking Na$^+$/H$^+$ exchange, through the removal of extracellular Na$^+$. In line with paracellular Ca$^{2+}$ flux being coupled to NHE3-dependent Na$^+$ transport is ex vivo Ussing chamber studies on intestine (210). Nhe3–deficient mice display reduced Ca$^{2+}$ flux across both the duodenum and cecum, highlighting the dependence of Ca$^{2+}$ absorption on NHE3 activity.
Ultimately, increased renal losses and decreased intestinal absorption of Ca\textsuperscript{2+} led to reduced bone mineral density in \textit{Nhe3} knockout mice (179).

Passive paracellular Ca\textsuperscript{2+} flux requires not only a driving force, but also for the tight junction to be permeable to Ca\textsuperscript{2+}. The molecular identity of the tight junction proteins conferring permeability to cations in the PT has yet to be clarified. The claudin (CLDN) family of tight junction proteins provides the permeability characteristics of an epithelium. Thus, a combination of claudins expressed in the proximal tubule likely confers the leaky permeability characteristics of this nephron segment permitting Ca\textsuperscript{2+} flux. CLDN2, CLDN10a and CLDN17 have been identified in the proximal tubule of adult animals (71, 100, 124, 134). When CLDN2 is expressed in cell culture models, it consistently forms a cation selective pore (11, 81, 250). The renal expression of CLDN2 is restricted to the PT and early thin descending limb. Microperfusion studies on proximal tubules from mice lacking the \textit{Cldn2} gene confirm a role of CLDN2 in making a cation selective paracellular pore across the PT \textit{in vivo} (167). Unfortunately, Ca\textsuperscript{2+} flux across the proximal tubule of \textit{Cldn2} knockout mice has not been measured. However, \textit{Cldn2} knockout mice display hypercalciuria (167). Thus, it is very likely that CLDN2 contributes paracellular Ca\textsuperscript{2+} permeability across the PT. However, one must also consider that \textit{Cldn2} deficient mice display altered tight junction structure and reduced PT reabsorption of Na\textsuperscript{+}, Cl\textsuperscript{−}, and water (167). Thus, it cannot be definitively concluded that \textit{Cldn2} null mice do not display urinary Ca\textsuperscript{2+} loss because of reduced solvent drag or an altered Ca\textsuperscript{2+} gradient across the segment, as mentioned above, altered Na\textsuperscript{+} and water flux across the PT strongly correlates with changes in PT Ca\textsuperscript{2+} transport (6, 18, 69, 229). Further, it is unlikely that claudin-2 forms a Ca\textsuperscript{2+} permeable paracellular pore in isolation,
as the thin descending limb has a much lower apparent Ca\(^{2+}\) permeability than the PT (202).

Electroneutral bicarbonate (HCO\(_3^-\)) reabsorption in the early portion of the PT occurs at the expense of Cl\(^-\), as illustrated in figure 1. This leads to an increased concentration of Cl\(^-\) in the lumen when the filtrate reaches the later portions of the PT. The accumulation of Cl\(^-\) in the lumen allows Cl\(^-\) flux down its chemical gradient, through the paracellular pathway, leaving a net lumen positive, potential difference (16). This lumen positive transepithelial voltage drives Na\(^+\) reabsorption and favours passive Ca\(^{2+}\) reabsorption via the paracellular shunt (13, 16). Although the majority of Ca\(^{2+}\) is reabsorbed via the paracellular pathway across the PT, there is evidence supporting a small, yet significant, transcellular component of Ca\(^{2+}\) transport across both the convoluted (20% of the total absorption), and straight (10%) portions of the PT (28, 203, 238). Finally, the tight junction proteins that confer selectivity for Ca\(^{2+}\) over Mg\(^{2+}\) in the PT are completely unknown and an area requiring further study.

**NHE3 inhibition/Tenapanor**

Unlike the other nephron segments, currently there is not a direct inhibitor of apical sodium entry from the PT that is employed clinically (note carbonic anhydrase inhibitors indirectly prevent Na\(^+\) absorption from this segment, as discussed below). S3226, is a specific NHE3 inhibitor, that has been shown to attenuate ischemia-induced renal failure in an animal model (120). However, this drug has not made it to the clinic and we are unaware of information on its affects on renal divalent cation handling. Given the calciuria seen in Nhe3 deficient mice (180), it is not unreasonable to suggest such
compounds may increase urinary Ca\(^{2+}\) excretion. More recently, a non-absorbable NHE3 inhibitor, tenapanor, has been generated and is undergoing clinical trials. NHE3 is also expressed in the intestine, where it mediates significant Na\(^{+}\) and water reabsorption (210). It is therefore not unexpected that this compound inhibits intestinal Na\(^{+}\) absorption and lowers blood pressure in rats (135). Surprisingly, it also prevents intestinal phosphate absorption and lowers plasma phosphate in patients on dialysis (25, 135). Effects on divalent cation handling have not been reported for these compounds. Although given the previous findings of decreased Ca\(^{2+}\) absorption from duodenum and cecum of NHE3 null mice (180, 200), it likely also attenuates intestinal Ca\(^{2+}\) absorption. It is unclear whether other mechanisms would compensate for this.

### Osmotic diuretics

An osmotic diuretic is a compound that is not reabsorbed along the nephron and in addition exerts an osmotic force, thereby impairing osmotic water reabsorption from the tubule. This results in a diuretic effect. Mannitol is such an osmotic compound and is most commonly employed as an osmotic diuretic. Similar to Ca\(^{2+}\), water is reabsorbed from the PT, down an osmotic gradient created by the reabsorption of Na\(^{+}\). It is therefore not surprising that infusion of D-mannitol interferes with osmotically driven Ca\(^{2+}\) reabsorption as well. Consistent with this, administration of 10-20\% D-mannitol solutions increases the fractional urinary excretion of Ca\(^{2+}\) (67, 246). Moreover, micropuncture data confirms this diuretic reduces proximal Ca\(^{2+}\) reabsorption (246), although infusion of a lower concentration of D-mannitol did not induce calciuria as evaluated by standard renal clearance techniques (27), perhaps due to transcellular proximal tubular Ca\(^{2+}\)
transport in the PT (28), or compensatory pathways in the distal tubule. Mannitol administration also increases urinary Mg\(^{2+}\) excretion (36, 181), however, whether this is via reduced PT paracellular reabsorption, or an effect on the thick ascending limb (TAL), where the majority of Mg\(^{2+}\) is reabsorbed in a paracellular fashion, is not clear.

**Carbonic anhydrase inhibitors and divalent cation transport**

Carbonic anhydrase inhibitors are a group of drugs that act by blocking the carbonic anhydrase enzymes and hence the catalysis of H\(_2\)CO\(_3\) to CO\(_2\) and water and *vice versa*. Without the carbonic anhydrases, the above listed interconversion, would occur at a much slower rate. They are employed for a diverse array of indications including as antiglaucoma agents, diuretics, antiepileptics and in the treatment of acute altitude sickness. Acetazolamide is most commonly used clinically. Acetazolamide increases urinary Ca\(^{2+}\) excretion (15, 157, 230). There are multiple carbonic anhydrase isoforms expressed in multiple tissues including red blood cells, bone as well as multiple isoforms in the kidney epithelium, including the PT and the intercalated cells of the collecting duct (CD)(189). Therefore, ascribing the effect of acetazolamide or other carbonic anhydrase inhibitors on urinary Ca\(^{2+}\) excretion to specific inhibition of carbonic anhydrase in the PT is not straightforward. In particular, these compounds have also been found to inhibit PTH secretion in nephrectomized rats (242). However, acetazolamide exhibits a rather low membrane permeability (204) and the diuretic effect in kidney is thought to be the result of inhibiting H\(^{+}\) recycling across the apical membrane of the PT, where, luminal carbonic anhydrases play a key role in converting filtered HCO\(_3^-\) and secreted H\(^+\) into H\(_2\)O and CO\(_2\). Thus, inhibition of H\(^+\) recycling, needed for Na\(^+\) influx, will impair Na\(^+\)
uptake into the cell, Na\(^+\) reabsorption from the PT, and consequently water reabsorption
from the PT (Figure 1), which ultimately will inhibit PT reabsorption of Ca\(^{2+}\). Consistent
with this, micropuncture studies on dog kidney found reduced Ca\(^{2+}\) reabsorption from the
PT after administration of acetazolamide (18). It is therefore not unreasonable to suggest
that reduced proximal tubular Ca\(^{2+}\) reabsorption is at least in part responsible for calciuria
induced by this drug.

**Na\(^+\)/glucose cotransporter inhibitors**

Na\(^+\)/glucose cotransporter type 2 (SGLT2) inhibitors are a new class of antidiabetic
drugs, which prevent reabsorption of glucose from the PT (Figure 1). The compounds
themselves are not considered diuretic agents, but their ability to increase luminal glucose
concentrations in the PT, can lead to a diuretic response. In normal physiology, filtered
glucose is almost completely reabsorbed from the PT, in a process coupled to Na\(^+\)
reabsorption. Apical uptake occurs through the transporter SGLT2 in the convoluted
portion and to a lessor extent via SGLT1 in the straight PT. Basolateral efflux of glucose
is mediated via type 2 glucose transporters (GLUT2) (Figure 1). As a consequence,
reabsorption of glucose may also contribute to osmotic water removal. As such, one
might predict that deletion or inhibition of proximal tubular Na\(^+\) coupled glucose
transport, i.e. SGLT2, would lead not only to glucosuria but also Ca\(^{2+}\) wasting, with
glucose itself acting as an osmotic diuretic. Consistent with this hypothesis is the
presence of increased urinary Ca\(^{2+}\) excretion in a mutant mouse model devoid of Sglt2
expression (151). This animal also has increased urinary Mg\(^{2+}\) excretion. Furthermore, D-
glucose provokes significant calciuria when infused (27). To date, there is only a
significant body of literature looking at the effects of canagliflozin, a SGLT2 inhibitor, on Ca\(^{2+}\) homeostasis in patients with type 2 diabetes (reviewed in (7, 24)). There has been significant interest in this area as patients treated with this drug have displayed reduced bone mineral density in the hip (although not at other sites). At pharmacological doses canagliflozin does not significantly elevate urinary Ca\(^{2+}\) or Mg\(^{2+}\) excretion. This might be due to incomplete blockage of the transporter, compensation by SGLT1 and/or increased Ca\(^{2+}\) absorption from the distal nephron. Although patients treated with SGLT2 inhibitors display osmotic diuresis, their polyuria is significantly less and the degree of glucosuria significantly lower than that observed in the knockout animal, consistent with incomplete inhibition or better compensation in patients.

**Ca\(^{2+}\) and Mg\(^{2+}\) transport in the thick ascending limb of Henle’s loop**

The TAL plays a critical role in the renal reabsorption of both Ca\(^{2+}\) and Mg\(^{2+}\). Consequently alterations in transport function within the TAL can have profound effects on urinary excretion of divalent cations. Detailed physiological measurements using micropuncture indicate that the loop reclains significant amounts of Ca\(^{2+}\) and Mg\(^{2+}\) (52, 137, 226). Reabsorption of these divalent cations is thought to occur primarily through a common paracellular pore between epithelial cells in the TAL, amounting to 25% of the Ca\(^{2+}\) and more than 60% of Mg\(^{2+}\), filtered by the kidney (52, 137, 226). As such, paracellular transport of Ca\(^{2+}\) and Mg\(^{2+}\) appears largely in the cortical TAL, with only a minor contribution from the medullary portion of the segment (53, 61, 216, 227). The lumen-positive transepithelial voltage gradient is an absolute requirement for driving divalent cation flux across the TAL epithelium (59, 190). This can best be appreciated
when artificially reversing the transepithelial potential to a lumen negative voltage, in
isolated perfused cortical TAL segments. Such alterations results in reversal of fluxes,
with both Ca\(^{2+}\) and Mg\(^{2+}\) being secreted back into the tubular lumen (59). The lumen
positive diffusion potential is generated in part by the asymmetrical secretion of ions
across the TAL, a result of the polarized expression of specific electrolyte transport
proteins in the apical and basolateral membranes of the epithelial cells in this segment.

The Na\(^{+}\), K\(^{+}\)-ATPase is situated in the basolateral membrane of the TAL cell
(Figure 2). The inward entry of Na\(^{+}\), K\(^{+}\), and Cl\(^{-}\) (in the stoichiometry of 1:1:2) across the
apical membrane occurs via the furosemide-sensitive cotransporter, NKCC2 (93, 152,
219). Due to a limited amount of K\(^{+}\) in the tubular fluid, the uptake of monovalent ions
via NKCC2 is highly dependent on efficient recycling of K\(^{+}\) across the apical membrane.
Apical K\(^{+}\) influx is mediated by the renal outer medullary K\(^{+}\) (ROMK) channels (93, 220,
248). The two Cl\(^{-}\) ions, transported inward via the NKCC2 cotransporter, exit the cell via
basolaterally located CLC-Kb channels, a process favoured by the transmembrane
potential difference (92, 218). These channels require the cofactor Barttin, for Cl\(^{-}\)
conductance (72). The luminal recycling of each K\(^{+}\) coupled with the basolateral exit of
Cl\(^{-}\) gives rise to the transepithelial potential difference, forming a lumen positive
transepithelial voltage of +5 to +10 mV across the segment (93, 94). Furthermore, the
transepithelial potential difference may reach values close to +30 mV in the cortical TAL
(91, 117, 154, 201), however, the mechanism underlying this observation remains to be
defined in detail.

The permeability of Na\(^{+}\) (\(P_{Na}\)) has been reported to be as much as 2-10 times
higher than that of Cl\(^{-}\) (\(P_{Cl}\)) in the TAL (34, 161), with comparatively low transepithelial
resistances of 11-34 Ω · cm² (34). Anatomically, the TAL stretches from the inner stripe of outer medulla (TAL_{ISOM}), trough the outer stripe of outer medulla (TAL_{OSOM}), to the cortical bend encompassing the TAL_{CTX}, where it is gradually replaced by the macula densa cells and cells of the distal convoluted tubule (DCT). The low water permeability of the TAL enables it to function as a diluting segment. Thus the NaCl concentration of the luminal fluid gradually falls along the TAL, reaching values of 30-60 mM in the earliest accessible portion of the distal tubule (34). In the medullary TAL, as much as 50% of the Na⁺ is taken up via the paracellular shunt, driven by the electrochemical gradient (106). Given that the TAL is responsible for reclaiming some 20-25% of NaCl from the filtrate, it is not surprising that altered transport within the segment leads to distorted renal handling of NaCl. This is highlighted by mutations in NKCC2, ROMK, CLC-Kb, and Barttin, which underlie various types of Bartter syndrome, a disease characterized by significant NaCl wasting (22, 218-220).

Permeation of Ca²⁺ and Mg²⁺ across the cortical TAL, driven by the lumen positive transepithelial voltage gradient, occurs almost exclusively via the paracellular shunt (53, 61, 216, 227). Overall, the paracellular pathway in the TAL is cation selective (60, 91). These permeation characteristics are determined by specific molecular interactions between claudin tight junction proteins, expressed within the segment. Two main claudins, CLDN16 and CLDN19, primarily participate in forming the paracellular pores, whereby Ca²⁺ and Mg²⁺ permeate the TAL epithelium. Both genes were found to participate in this process via their mutation in the genetic syndrome of Familial Hypomagnesemia with Hypercalciuria and Nephrocalcinosis (FHHNC)(129, 221). Affected family members display severe alterations in Ca²⁺ and Mg²⁺ balance due to
renal wasting of these ions. Consequently, patients suffering from this autosomal recessive disorder have marked hypomagnesemia, hypercalciuria and nephrocalcinosis (129, 160, 187, 221). Further implication of alterations of renal CLDN16 and CLDN19 expression as the main defect (12, 129, 221), is provided by a FHHNC transplant recipient receiving an unaffected kidney, which normalized urinary divalent cation excretion and stabilized serum Mg$^{2+}$ concentrations (187).

Elucidation of ion selectivity conferred by individual claudins has been attempted in various cell model systems. Yet, substantial variability in the effect of CLDN16 and CLDN19 overexpression on permeability characteristics of cell monolayers has been reported. The sources of this variability are discussed in detail elsewhere (9, 101), but likely relate to the cell type and the resulting interactions between the overexpressed claudins under investigation, with endogenously expressed claudins in the cell line employed. Some studies suggest that CLDN16 and CLDN19 are involved in conferring cation permeability of the junctional complex, with only minor effects on divalent cation permeability (118). This data infers that FHHNC mutations increase Cl$^{-}$ backflux, thereby impairing diffusion potentials in the cortical TAL and hence diminishing the lumen-positive voltage gradient, leading to Ca$^{2+}$ and Mg$^{2+}$ wasting. However, these changes should also result in NaCl wasting, which is not observed in FHHNC patients, whom have normal aldosterone levels and Na$^{+}$ balance (23). These observations might be reconciled by very recent studies from Milatz and Himmerkus et al, which suggest that there may be different transport pathways for Ca$^{2+}$ and Mg$^{2+}$ versus Na$^{+}$ along the length of the TAL (161). The authors show that CLDN10, another cation permeable claudin expressed within the TAL is absent from tight junctions where CLDN16 and CLDN19
are localized. Using an elegant series of experiments coupling $P_{Na}$ and $P_{Mg}$ measurements of single microperfused TAL tubules, with subsequent immunohistochemical staining of the perfused tubule, the authors were able to elucidate the $P_{Na}$ and $P_{Mg}$ of the epithelium, and correlate it with the total length of the tight junction that expressed either CLDN10 or CLDN16. Using this approach, permeability measurements on the TAL tubules revealed a high cation selectivity of the paracellular shunt in the TAL-ISOM, which dropped in the TAL-OSOM/TAL-CTX. Furthermore, the ratio of $P_{Mg}$ over $P_{Na}$ increased by more than 3-fold, moving from the TAL-ISOM to the more cortical TAL segments with the highest expression of CLDN16. Concurrently, they found that TAL-ISOM almost exclusively expresses CLDN10, while CLDN16 predominates in the TAL-OSOM/TAL-CTX, ranging from 37% to 97% (161). These new findings support the existence of two paracellular pathways for cation permeation in the TAL, where CLDN10 allows permeation of Na$^+$, while the CLDN16/CLDN19 complex permits permeation of Mg$^{2+}$ and presumably Ca$^{2+}$ as well. This hypothesis is further supported by the observation that genetic ablation of the Cldn10 gene specifically from the distal nephron, leads to an augmented lumen-positive transepithelial voltage gradient, due to an alteration in permeability characteristics within the paracellular shunt (31). Here the $P_{Na}$ over $P_{Cl}$ is markedly decreased, thereby raising the lumen-positive voltage gradient. As the short circuit current was undisturbed, indicating no overall changes in transcellular transport, these findings support a role for CLDN10 in forming a Na$^+$ permeable pore (31). Furthermore, $P_{Ca}$ and $P_{Mg}$ increased substantially over that of $P_{Na}$ in Cldn10 deficient mice, suggesting alterations within the paracellular shunt (31). The findings of altered Ca$^{2+}$ and Mg$^{2+}$ permeability in the Cldn10 knockout mice is in part explained by a redistribution of CLDN16 and CLDN19, to the
now, CLDN10 depleted junctions (161). In combination with an augmented lumen positive voltage in Cldn10 deficient mice, these adaptations could form the basis for enhanced reabsorption of divalent cations trough the CLDN16/19 complex (31, 161). As such, Cldn10 deficient mice show increased renal divalent cation reabsorption, with hypermagnesemia and nephrocalcinosis (31). Furthermore, Cldn10 deficient mice display increased Clcn14 expression. This is a pore-blocking tight junction protein expressed in the TAL and strongly regulated by the Ca^{2+}-Sensing Receptor (CaSR) (31, 62), as discussed below.

CLDN14 was originally identified in a genome-wide association study (236), The investigators found a strong association between kidney stones and hypercalciuria with single nucleotide polymorphisms (SNPs) located within the genomic region of the CLDN14 gene (236). However, the investigators were unable to assign a direct mechanistic role for Cldn14 in regulating either Ca^{2+} balance or stone disease. Furthermore, patients with loss of function mutations in the human CLDN14 gene do not have alterations in Ca^{2+} balance, but suffer from nonsyndromic deafness, as CLDN14 is expressed in the epithelial cells located within the organ of Corti (245). Additional investigations found that Cldn14 was expressed in the TAL (89). Here, regulation of the tight junctional claudin was shown to be highly dependent on alterations in CaSR activity (62, 89). In fact, any elevation in serum Ca^{2+} concentrations or by pharmacological alterations of CaSR sensing using calcimimetics, greatly increased Cldn14 expression (62, 88, 89). Mechanistically, CLDN14 overexpression in various cell lines was found to reduce both the $P_{Na}/P_{Cl}$ ratio as well as the permeability to Ca^{2+}, suggesting that CLDN14 may act to block paracellular permeation of cations (20, 62, 89). In cells, CLDN14 seems
to interact with CLDN16, but not directly with CLDN19 (89). It is therefore likely that
CLDN14 primarily acts by blocking divalent permeation in kidney through the
CLDN16/CLDN19 complex. These findings are in line with the observations that mice
receiving a high Ca\textsuperscript{2+} diet have increased CLDN14 expression, but only in the cortical
region of the kidney, where the CLDN16/CLDN19 complex is expressed (185). Thus, the
increase in CLDN14 induced by hypercalcemia, may block paracellular permeation of
Ca\textsuperscript{2+} and thereby increase urinary excretion of Ca\textsuperscript{2+}, to stabilize serum Ca\textsuperscript{2+} levels (62). A
recent genome-wide association study found a correlation between a SNP in the \textit{CLDN14}
gene and the ratio of Mg\textsuperscript{2+}/Ca\textsuperscript{2+} in urine (44). As such, \textit{Cldn14} deficient mice show a
lower fractional excretion of both Ca\textsuperscript{2+} and Mg\textsuperscript{2+}, when placed on a high Ca\textsuperscript{2+} diet (89).
Furthermore, overexpression of the \textit{Cldn14} gene in the TAL leads to an increase in the
fractional excretion of both Ca\textsuperscript{2+} and Mg\textsuperscript{2+} (88).

**Loop diuretics**

Loop diuretics are a class of sulfamoylbenzoic acid derivatives that include furosemide,
bumetanide, torasemide, piretanide, and benzmetanide, as well as ethacrynic acid that
lacks a sulfonamide substituent. Among loop diuretics, furosemide is by far the most
commonly employed clinically. Furosemide treatment is implemented to correct edema
formation and may be administered in the treatment of chronic heart failure, hepatic
cirrhosis, renal disease, and acute pulmonary edema (74, 82, 85, 212). Furosemide is a
weak organic anion that is predominantly protein bound in plasma. Therefore, only a
minor fraction gets filtered by the kidney and the majority is delivered into the luminal
fluid by proximal tubular anion transporters (183). Furosemide is the diuretic of choice
when targeting the TAL and functions as an effective pharmacological inhibitor of NKCC2-dependent transport, by competing for the Cl⁻ binding site (174) (Figure 2). Understandably, given the important role of the TAL in Na⁺ reabsorption, targeting the NKCC2 transporter leads to a marked natriuretic response (69, 224). As such, patients with antenatal manifestations of Bartter syndrome do not respond appropriately to furosemide when tested (126).

Since administration of furosemide diminishes the lumen-positive transepithelial voltage gradient across the TAL epithelium (35, 55, 90), by inhibiting the primary mechanism generating this potential difference, it is not surprising that Ca²⁺ and Mg²⁺ transport in TAL is significantly inhibited by furosemide administration (59, 190). Loop diuretics such as furosemide and ethacrynic acid promote hypercalciuria and hypermagnesiuria in experimental animals (65, 69, 70, 141). Urinary losses of both Ca²⁺ and Mg²⁺ have also been reported in human subjects given loop diuretics, including furosemide, torasemide and ethacrynic acid (105, 140, 198). In a double-blind placebo controlled study, furosemide significantly increased urinary Ca²⁺ and Mg²⁺ excretion 3 hours post a single oral dose (140). However, subsequent collections for the reminder of 24 hours showed an overall compensatory response, with a decrease in the urinary excretion of divalent cations. Similar finding were documented for urinary NaCl excretion (140). In healthy volunteers, both furosemide and torasemide dose-dependently increased the fractional excretion of Ca²⁺ and Mg²⁺ during 3 consecutive clearance collections of 30 minutes (64). Comparison of a single dose of furosemide (40 mg) with torasemide (20mg) in patients with chronic heart failure showed an increased urinary excretion of Ca²⁺ during the first 4 hours of collection. However, only the effect of
torasemide persisted for the next 8 hours, in comparison to furosemide (205). This can be explained by the longer half-life of torasemide, with both compounds acting on the same tubular site, namely NKCC2-dependent transport in the TAL. Overall loss of Ca\(^{2+}\) and Mg\(^{2+}\) following loop diuretic administration may depend strongly on dosing regimes. Systemic Ca\(^{2+}\) concentrations are in general much more tightly regulated than Mg\(^{2+}\). This may explain why hypomagnesemia occurs in some patients treated with loop diuretics, while a reduction in serum Ca\(^{2+}\) levels are rarely reported (4, 75). However, clinical studies directly comparing these side effects are lacking. The occurrence of alterations in divalent cation homeostasis during prolonged treatment may therefore be related to both the dosing regimen of loop diuretic as well as preexisting deficits in Ca\(^{2+}\) and Mg\(^{2+}\) balance.

Patients with Bartter’s syndrome may be hypotensive, due to NaCl wasting, display hypokalemia, metabolic alkalosis, and hyperaldosteronism, along with an excessive amount of prostaglandin E\(_2\) in the urine (17, 73, 156). Although the clinical phenotype of Bartter syndrome may resemble prolonged administration of a loop diuretic in supraphysiological dosages, variance exists between the different types of Bartter in relation to divalent cation handling. The underlying mechanisms for these discrepancies remain to be investigated in detail, but likely relate to the molecular components mutated, keeping in mind that loop diuretics target only the NKCC2 transporter and full inhibition is unlikely during normal dosing regimes. In fact, while patients with antenatal Bartter’s syndrome (due to mutations in NKCC2 and ROMK) present with significant hypercalciuria and secondary nephrocalcinosis, these patients do not commonly show disturbed Mg\(^{2+}\) balance (156). However, patients suffering from classical Bartter
syndrome type III, due to mutations in the $CLCNKB$ gene often develop hypomagnesemia, but rarely hypercalciuria or secondary nephrocalcinosis. As compared to the antenatal form of Bartter, the absence of $Ca^{2+}$ wasting in classical Bartter patients could stem from less compromised TAL transport pathways as NaCl loss in some patients with $CLCNKB$ defects is often less severe, possibly due to alternative basolateral efflux mechanisms for $Cl^{-}$ in the TAL (92, 130). Recent evidence supports these assumptions. Disruption of the mouse $Clcnk2$ gene encoding the CLC-K2 $Cl^{-}$ channel (equivalent to the human $CLCNKB$ gene encoding CLC-Kb) leads to a Bartter phenotype with renal NaCl wasting and hypokalemic metabolic alkalosis. Importantly, Hennings et al., found expression of the highly homologues CLC-K1 $Cl^{-}$ channel in the medullary portion of the TAL in $Clcnk2$-deficient mice (108), suggesting at least some tubular NaCl transport is retained in that segment. How that would affect the lumen-positive voltages along the length of the TAL remains to be determined. Urinary $Ca^{2+}$/creatinine ratios did not differ between $Clcnk2$-deficient mice and wildtype littermates (108), akin to the findings obtained from Bartter type III patients. Another recently developed $Clcnk2$-deficient mouse model did not show changes in serum $Mg^{2+}$ concentration, however, these mice were severely volume depleted (95). Hennings et al. found increased urinary $Mg^{2+}$/creatinine ratios in their model (108). Importantly, these mice also had altered DCT morphology, with atrophy of the early portion, potentially explaining why $Mg^{2+}$ wasting was present (discussed in detail later).

**ROMK inhibitors**
The recent development of inhibitors of ROMK allows targeting of NKCC2 dependent NaCl transport, by inhibiting K⁺ recycling in the TAL (Figure 2). Furthermore, ROMK is also expressed in more distal portions of the renal tubule, where it allows K⁺ secretion. Here the electrochemical gradient for K⁺ secretion is strongly dependent on the electrogenic uptake of Na⁺, through the epithelial Na⁺ channel, ENaC (Figure 3). Compensatory increases in Na⁺ transport via ENaC occur during inhibition of TAL transport, which leads to depolarization of the cell, and ROMK-dependent K⁺ secretion. Therefore, blocking the ROMK channel would allow inhibition of both Na⁺ and K⁺ transport in the TAL, along with substantial inhibition of K⁺ secretion in more distal parts, potentially minimizing urinary K⁺ losses. In fact, pharmacological inhibition of ROMK leads to significant natriuresis and diuresis, effectively lowering blood pressure, without substantial effects on urinary K⁺ excretion (103). Administration of ROMK inhibitors to dogs for 7 days, did not alter serum levels of either Ca²⁺ or Mg²⁺ (103). However, a single dose elicited an initial increase in urinary Ca²⁺ and Mg²⁺ excretion, when measured initially or after 7 days, of treatment, paralleling the loss of Cl⁻ (103). In spontaneously hypertensive rats, urinary Ca²⁺ dose-dependently increased after ROMK inhibitor administration for 4 days (103). Furthermore, high dose ROMK inhibition increased urinary excretion of Ca²⁺ in Dahl salt-sensitive rats on a high NaCl diet. However, since dietary NaCl supplementation results in markedly increased urinary divalent cation excretion due to inhibition of paracellular transport in kidney, it is difficult to assess the direct effect of ROMK inhibition on Ca²⁺ or Mg²⁺ transport in this experimental setting (251). Overall, it seems that ROMK inhibitors target the TAL in a similar manner as furosemide, thereby dissipating the transepithelial voltage gradient,
which is of upmost importance for driving the paracellular reabsorption of Ca\textsuperscript{2+} or Mg\textsuperscript{2+} across the epithelium and this likely contributes to loss of divalent cations.

**Transcellular transport of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} in the distal convolution and collecting system**

The distal convolution is a microanatomical division of the renal tubule that encompasses several segments of the nephron and collecting system. These include mainly the distal convoluted tubule (DCT), the connecting tubule (CNT), as well as the initial superficial portion of the cortical collecting ducts (CD) (132). Besides differences in the structural appearance of cells, the distal convolution can be identified by the specific expression of key transport proteins (Figure 3). Cells in the DCT express the thiazide-sensitive NaCl cotransporter, NCC (176). The DCT segment can be further subdivided into a DCT1 and DCT2. The latter segment is identified by the coexpression of NCC and ENaC (37, 149). ENaC expression is also observed throughout the remainder of the collecting system. Furthermore, the vasopressin regulated water channel Aquaporin 2 (AQP2) is expressed exclusively in the CNT and CD segments of the tubule (170). In addition to the TAL, ROMK is expressed in the distal nephron and plays a role in K\textsuperscript{+} secretion in the CNT and CD (128, 143, 159) (Figure 3). Along the length of the distal convolution, intercalated cells appear, with highest abundance in the CNT and CD. These cells are involved in acid-base handling and also facilitate electroneutral NaCl reabsorption (99, 144). Species differences are observed in the arrangement of the above-mentioned segments within the distal convolution. As such, the subsegmentation of the DCT as well as the histological division of the individual segments and appearance of various transport proteins, differ
between mice, rat, rabbit and man (147, 149). For instance, the DCT2 segment is pronounced in mouse and rat, shortened in human and absent in rabbit (147). Given the markedly different expression pattern of transporters along the distal convolution, with predominantly electroneutral NaCl transport occurring in the early distal convolution transitioning towards an electrogenic Na\(^+\) uptake in the late convolution, it is not surprising that the transepithelial voltage gradually decreases from between 0 and -5 mV in the DCT to -40 mV in the CNT (84, 153, 195, 223, 247). The transepithelial voltage gradient across the CNT is much larger than in the cortical CD, due to a comparatively higher transport of Na\(^+\) and K\(^+\) in the CNT segment. Consequently, the transepithelial voltage in the CCD may drop to -15 mV (123, 127, 175, 231).

The cells lining the distal convolution give rise to a high resistance epithelium, with values reported of 150 Ω · cm\(^2\) in the early distal convolution, and increasing by almost 6-fold in the cortical CD (107, 153). Although expression of CLDN16 and CLDN19 extends into the DCT, suggesting possible paracellular transport of Ca\(^{2+}\) and Mg\(^{2+}\) (124, 129, 221) in this segment (not depicted in figure 3), divalent cation transport across the distal convolution is thought to occur via a transcellular route. Active transport of Ca\(^{2+}\) and Mg\(^{2+}\) has been described in the distal convolution, with unidirectional divalent transport occurring in the presence of lumen negative transepithelial voltages (137, 192). As estimated by classical micropuncture studies, approximately 3-7% of the Ca\(^{2+}\) filtered by the kidney is reabsorbed in the distal convolution (5, 137, 139). Similar physiological measurements suggest that 5-6% of filtered Mg\(^{2+}\) is reabsorbed in the distal convolution (14, 33, 139).
The transport machinery driving active transcellular Ca\textsuperscript{2+} transport in the distal convolution is well defined. It consists of the Transient Receptor Potential Vanilloid 5 (TRPV5) channel located on the apical membrane, which facilitates the uptake of Ca\textsuperscript{2+} from the filtrate (109, 110) (Figure 3). In mouse, TRPV5 is expressed in the DCT2 segment as well as the CNT and the initial portion of the CD (114, 147, 149). Expression and localization of TRPV5 in the human kidney has not been determined. In mouse, TRPV5 localizes with Calbindin-D\textsubscript{28K}, which may function as an intracellular Ca\textsuperscript{2+} buffer and aid in shuttling Ca\textsuperscript{2+} from apical to basolateral aspects of the cell (131). Extrusion of Ca\textsuperscript{2+} from the cell into renal interstitium, is driven by the Na\textsuperscript{2+}/Ca\textsuperscript{2+} exchanger type 1 (NCX1) and the plasma membrane Ca\textsuperscript{2+}-ATPase proteins (PMCA1 and PMCA4) (8, 197, 239). Both NCX and PMCA4 are highly abundant in the DCT2 and CNT region of the mouse, colocalizing with TRPV5, however weaker expression of the basolateral Ca\textsuperscript{2+} extrusion protein have also been noted in DCT (not depicted in figure 3), although no apical Ca\textsuperscript{2+} channel has been documented in the segment (8, 149). Targeted transgenic strategies have been implemented to understand the contribution of these genes to overall Ca\textsuperscript{2+} transport within the distal convolution. Mice with a targeted deletion of \textit{Trpv5}, show the most pronounced phenotype and display massive renal Ca\textsuperscript{2+} wasting, osteopenia, and compensatory hyperabsorption from the intestine (112). Further, Ca\textsuperscript{2+} concentration was determined in relation to tubular fluid K\textsuperscript{+} along the length of the superficial distal convolution accessible to micropuncture in \textit{Trpv5}-deficient mice. The K\textsuperscript{+} concentration increases in luminal fluid along the length of the distal convolution, due to water removal from the tubular fluid in combination with the onset of electrogenic Na\textsuperscript{+} transport along the tubule. Thus, in basal states, the luminal K\textsuperscript{+} concentration is a good marker for
distance along the distal convolution. In wildtype mice, fractional Ca\(^{2+}\) delivery markedly decreases concomitantly with increased luminal K\(^{+}\) concentration, suggesting that significant reabsorption of Ca\(^{2+}\) takes place along the length of the distal convolution, in line with the expression pattern of TRPV5. In mice lacking *Trpv5*, this relationship between Ca\(^{2+}\) delivery and luminal K\(^{+}\) concentration was no longer apparent, with a markedly elevated fractional Ca\(^{2+}\) delivery found along the distal convolution, indicative of significant wasting within that segment (112). In line with these findings, a rare missense variant in TRPV5 correlates with recurrent kidney stones (177), however no loss-of-function mutations have been described in humans.

Transport of Mg\(^{2+}\) in the distal convolution is predicted to occur via the divalent cation selective Transient Receptor Potential Melastin 6 channel (TRPM6) (241). The role of TRPM6 was determined via elucidation of the causative gene defective in the autosomal recessive disorder of Hypomagnesemia with Secondary Hypocalcemia (HSH). These patients display a severe Mg\(^{2+}\) intestinal absorptive defect, as TRPM6 is expressed in the colonic enterocyte (162, 206, 243). A role for TRPM6 in renal reabsorption of Mg\(^{2+}\) is supported by the observation that HSH patients display an elevated fraction excretion of Mg\(^{2+}\) even in the presence of hypomagnesemia (206, 243). Given a low serum Mg\(^{2+}\) concentration, failure to sufficiently lower urinary fractional Mg\(^{2+}\) excretion is consistent with renal wasting (4). Furthermore, when HSH patients are subjected to intravenous Mg\(^{2+}\) loading, the urinary Mg\(^{2+}\) leak becomes apparent (243). Detailed micropuncture studies utilizing early and late puncture sites from the same distal convolution, demonstrate that Mg\(^{2+}\) transport dominates in the early portion of the convolution, likely the DCT (14). Immunohistochemical localization studies identified
TRPM6 in the apical membrane of cells in the DCT (241), consistent with the micropuncture data reported above. Very recently, Chubanov, Gudermann and colleagues performed a series of elegant experiments utilizing various targeted transgenic strategies in order to understand the contribution of Trpm6 to intestinal and renal Mg$^{2+}$ transport (41). The selective intestinal deletion of Trpm6 resulted in a marked phenotype of severe Mg$^{2+}$ depletion, due to inefficient Mg$^{2+}$ uptake from the intestine. However, specific deletion of Trpm6 from renal epithelial cells, using the Ksp-Cre model did not cause a change in serum levels nor the renal excretion of Mg$^{2+}$, suggesting that, at least in mouse, TRPM6 is not the only apical entry mechanism for Mg$^{2+}$ reabsorption from the distal convolution (41). It still remains to be clarified whether the structurally similar channel homologue TRPM7 also participates in apical uptake of Mg$^{2+}$ from the DCT and hence in renal Mg$^{2+}$ transport (42, 63, 145, 207, 241). Several genes localizing to the distal convolution cause genetic syndromes of hypomagnesemia (51, 63). These gene defects suggest that the resting membrane potential of the DCT cell is critical for Mg$^{2+}$ transport in kidney (26, 51, 63, 87, 158). Consistent with this, the intracellular Mg$^{2+}$ concentration ranges between 0.2 and 1.0 mM (98, 191), while that in the luminal fluid reaching the DCT is estimated to be 0.2 - 0.7 mM (49). Therefore, the membrane potential of the DCT cell likely determines the apical uptake of Mg$^{2+}$ into the DCT cell (43, 104). Aside from TRPM6, the molecular transport machinery involved in transepithelial Mg$^{2+}$ transport in the distal convolution remains poorly defined.

Ca$^{2+}$ and Mg$^{2+}$ transport appears largely restricted to the distal convolution, predominantly the DCT and CNT segments depending on the ion. Little transport of Ca$^{2+}$ and Mg$^{2+}$ is expected to occur within the remainder of the CD, although some studies
found evidence of small yet measurable flux. Micropuncture studies, using the lumen K\(^+\) concentrations for orientation along the distal convolution, found that the fractional delivery of Ca\(^{2+}\) is very small towards the end of the distal convolution, suggesting negligible transport of Ca\(^{2+}\) in downstream segments (112, 150, 173). Consistent with this, Loffing et al, found that NCX1 and TRPV5 expression ends in the CNT and is not detectable in the CCD (149). Conversely, mice expressing green fluorescent protein after the \textit{Trpv5} promoter demonstrate expression of the transgene in cortical CD as well (113).

In rabbit, microperfusion of isolated cortical CD did not reveal significant Ca\(^{2+}\) flux (30, 217). However, other studies report significant permeability for Ca\(^{2+}\) in the microperfused rabbit cortical CD (115, 121). Microcatheterization studies of rat inner medullary CD found a small, albeit significant decrease in Ca\(^{2+}\) over inulin ratio over the length of the inner medullary CD (21). Thus, the contribution of the CD to overall Ca\(^{2+}\) transport appears to be minor, but physiologically relevant Ca\(^{2+}\) transport across the segment cannot be excluded.

Mg\(^{2+}\) transport in the CD appears to be absent. Microperfusion experiments on rabbit cortical CD could not find significant Mg\(^{2+}\) flux (217). Furthermore, the microcatherization studies outlined above, failed to find significant absorption of Mg\(^{2+}\) along the inner medullary CD (21).

\textit{Thiazide-type diuretics}

The class of thiazide-type diuretics includes a range of compounds with a benzothiadiazine structure e.g. hydrochlorothiazide, methyclothiazide, cyclothiazide, bendroflumethiazide. Furthermore, chlorthalidone, metolazone, and indapamide are
thiazide-like diuretics, which do not share the common benzothiadiazine structure. However all the above-mentioned compounds block NaCl transport in the DCT, by inhibiting the NaCl cotransporter, NCC (Figure 3)(195, 237, 240). The mechanism of action for metolazone has been suggested to result from interaction with the Cl– binding site of NCC, thereby blocking transport (237). However, a subsequent study found that the Cl– binding site and the binding site of metolazone might reside in different locations within the cotransporter (164). Furthermore, thiazides likely also target another transport process in intercalated cells, which contributes to electroneutral NaCl reabsorption (144).

In addition to direct diuretic actions on the DCT, effects of thiazides and the thiazide-like diuretic indapamide on vascular function have been noted in experimental animals and humans (reviewed in detail by (68, 234)). Acutely, hydrochlorothiazide can elicit endothelium-dependent vasodilation, which may contribute to the blood pressure lowering effect. However, since thiazides do not seem to affect blood pressure in normotensive patients, it is questionable whether these effects are clinically relevant (68). Indapamide can also elicit a decrease in total peripheral resistance, by acting on the vasculature. Mechanistically, this has been described to result from reductions in membranous Ca2+ influx in the smooth muscle cell, leading to a reduction in vascular reactivity in response to vasoconstrictors (163). Interestingly, indapamide seems to exert its antihypertensive effect in the absence of a strong diuretic activity at standard dosing schemes of 2.5 mg daily, used for the treatment of hypertension (2, 50, 165). Similar findings can be recapitulated in hypertensive patients with severe renal impairment and hence almost no renal function (average creatinine clearance of 16 ml/min) (97),
suggesting that the blood pressure lowering effect of indapamide may in part result from reducing total peripheral resistance.

Among this class of diuretics, hydrochlorothiazide is the most commonly prescribed. The Joint National Committee for the detection, evaluation, and treatment of high blood pressure, recommend thiazide diuretics as first line treatment or in combination with other antihypertensive agents for patients with stage I hypertension (uncomplicated) and stage II hypertension, respectively (39). Furthermore, thiazides are available at a low cost making them important compounds in the treatment of hypertension worldwide. In addition to lowering blood pressure by eliciting renal NaCl loss, thiazides also promote hypocalciuria and may lead to hypercalcemia in some patients (40, 244). Due to these properties, thiazides are frequently used in the treatment of kidney stone disease by reducing urinary Ca^{2+} and to alleviate hypocalcemia by increasing renal Ca^{2+} reabsorption (136, 178). Furthermore, chronic thiazide treatment can cause hypomagnesemia (133, 182), likely due to renal wasting (140). More pronounced perturbations in divalent cation balance can be observed in individuals with Gitelman syndrome. These patients have a defect in the SLC12A3 gene encoding the thiazide-sensitive NaCl cotransporter, NCC (86, 222). Loss of function mutations in SLC12A3 leads to NaCl wasting, hypokalemia, metabolic acidosis, hypomagnesemia and hypocalciuria (86).

Thiazide treatment can cause a robust decrease in the urinary excretion of Ca^{2+} (32, 122). This effect is independent of parathyroid hormone, as similar findings are observed in patients with hypo- and hyperparathyroidism (32). A hypocalciuric effect has also been reported when analyzing 24-hour urinary Ca^{2+} excretion following a single dose of
thiazide to healthy postmenopausal women and volunteers (32, 140). However, when urinary Ca^{2+} excretion was measured more frequently following a single oral dose, the hypocalciuric effect was only observed in the urine collection obtained 12-24 hours post-administration, even though effects on NaCl and fluid excretion were evident earlier (140). Similar effects as seen in humans were reported on divalent cation balance in rodents following chronic thiazide treatment and in mouse models of Gitelman syndrome (142, 150, 172, 211, 249). However, acute administration of thiazides following a bolus injection to animals yielded conflicting results, with respect to the onset of thiazide-induced hypocalciuria, relative to the timing of thiazide-induced NaCl losses (142, 173, 249).

The mechanisms leading to hypocalciuria after thiazide treatment or in Gitelman patients is still not fully delineated and may depend on alterations in both proximal and distal transport mechanisms as reviewed in detail by Reilly and Huang (196). The hypocalciuric effect from alterations in proximal transport likely depends on volume contraction, following administration of thiazides. Specifically it is thought that renal losses of NaCl due to NCC inhibition augment Na^{+} reabsorption in the PT, in order to normalize extracellular circulating volume. Since, Ca^{2+} transport in the PT occurs in parallel with that of Na^{+}, Na^{+} hyperabsorption leads to increased Ca^{2+} transport and hence hypocalciuria. Substantial experimental evidence supports this. For instance, when healthy volunteers had their NaCl losses replaced, the hypocalciuric effect of thiazide administration was absent (32). Detailed physiological measurements using micropuncture in wildtype mice revealed an increased PT reabsorption of Na^{+}, fluid, and Ca^{2+} following administration of thiazides for 6 days, clearly supporting a contribution of
this pathway to thiazide-induced hypocalciuria (173). Similar findings localizing the hypocalciuric effect to the PT have been obtained from micropuncture studies in mice lacking the \textit{Slc12a3} gene (150). Use of NaCl depletion to alter volume status resulted in a robust reduction in Ca\textsuperscript{2+} excretion in wildtype mice, while such dietary manipulations did not affect Ca\textsuperscript{2+} excretion in \textit{Slc12a3}–deficient mice (211). These findings are supportive of an already reduced extracellular volume and hence increased PT transport in mice lacking the \textit{Slc12a3} gene. Micropuncture studies conducted on mouse kidney following chronic thiazide administration did not reveal an additional distal reabsorptive effect. However, the fractional Ca\textsuperscript{2+} delivery to distal puncture sites was measured in relation to K\textsuperscript{+} concentration. This is potentially problematic as NCC inhibition alters K\textsuperscript{+} reabsorption in the distal nephron, by inducing a shift towards electrogenic ENaC-dependent Na\textsuperscript{+} transport and concomitant K\textsuperscript{+} secretion (233). Nonetheless, the thiazide-dependent hypocalciuric effect was still visible in \textit{Trpv5}-deficient mice, suggesting an important contribution of the PT pathway to the mechanism of thiazide-induced Ca\textsuperscript{2+} retention (173).

Indapamide also reduces urinary Ca\textsuperscript{2+} excretion in a number of trials, including in stone-formers (10, 29, 50, 155). In line with a dual mechanism of action for this compound, the blood pressure lowering effect obtained by acting on both the vasculature and by inhibiting NaCl transport in the DCT, could both lead to activation of the renin-angiotensin-aldosterone system (50) and hence stimulate Na\textsuperscript{+} transport in the PT (102). Indapamide may thus promote hypocalciuria by stimulating PT reabsorption, similar to the other thiazide-type diuretics, although the mechanisms of action might partly differ.
A distal mechanism contributing to thiazide-induced hypocalciuria has also been suggested. Some studies found an additional effect of thiazides on \( \text{Ca}^{2+} \) transport in the distal convolution (46, 76, 142, 196). Consistent with this, increased expression of genes involved in transcellular \( \text{Ca}^{2+} \) transport in the distal convolution have been seen after thiazide administration (e.g. TRPV5 and Calbindin-28K), but only when salt supplementation (NaCl and KCl) was given, suggesting that the distal effect may be apparent only when extracellular volume contraction is prevented (142). However, in the same study, salt supplementation alone had similar effects. As such, decreased PT transport in response to volume expansion, which could be envisioned to elicit an increase in the distal transport machinery, to compensate for PT losses of \( \text{Ca}^{2+} \).

Chronic thiazide treatment in hypertensive subjects results in lower than normal serum \( \text{Mg}^{2+} \) concentrations, although the difference compared to patients not receiving diuretics is relatively subtle (133). As such, the appearance of hypomagnesemia was reported to be uncommon in this population of thiazide treated patients. Hypomagnesemia is more commonly observed in the elderly treated with thiazide-diuretics (182). Furthermore, hypomagnesemia remains a hallmark feature of Gitelman syndrome (86). The reason for these differences may relate to incomplete inhibition of the cotransporter during thiazide treatment and preexisting deficits in \( \text{Mg}^{2+} \) stores, which may tend to be more prevalent in the elderly. As such, hypertensive patients receiving the highest dose of thiazides were more prone to developing hypomagnesemia, than patients receiving lower doses (133). Unlike \( \text{Ca}^{2+} \), renal \( \text{Mg}^{2+} \) wasting is evident within 3 hours following thiazide-administration to healthy volunteers (140). The effect of chronic thiazide administration or Gitelman syndrome on renal \( \text{Mg}^{2+} \) handling as understood.
from animal models are complicated somewhat by the substantial alterations that occur in
the morphology of the distal convolution (148, 150, 173, 211, 249). Both mice and rats
treated with thiazides and transgenic models of Gitelman syndrome display marked
atrophy of the early DCT1 segment and compensatory increases in the DCT2 and CNT
(148, 150, 249). Other experimental models using thiazide administration in mice, found
no evidence of significant alterations in DCT volume (142, 173). These findings are
likely dependent on dosage and duration of treatment (148). Since transcellular Mg\textsuperscript{2+}
transport within the distal convolution is primarily localized to the DCT, it is not
surprising that atrophy of the early portion of the segment i.e. DCT1 promotes Mg\textsuperscript{2+}
wasting, possibly by downregulation of TRPM6. However, one study reported
downregulation of TRPM6 in the absence of changes in morphology of the distal
convolution (173). It is also interesting to note that indapamide does not seem to affect
the urinary excretion of Mg\textsuperscript{2+} at therapeutical dosages used in the treatment of
hypertension (1.5-2.5 mg), as does the other thiazide-type diuretics. Electrolyte
measurement in a 24 hour urine collection followin a single dose of 2.5 mg indapamide
showed increased urinary NaCl excretion, reduced urinary Ca\textsuperscript{2+} excretion, but the total
amount of Mg\textsuperscript{2+} excreted in the urine was not altered (199). In patients with stone
disease, 6 or 18 months of treatment with indapamide, 1.5 mg sustained release
formulations, reduced urinary Ca\textsuperscript{2+}, but did not affect urinary Mg\textsuperscript{2+} excretion (10).
Similarly, no effect could be documented on serum Mg\textsuperscript{2+} levels (10, 232). Based on the
limited amount of data, it is difficult to draw firm conclusions on this, however the
available studies do suggest that indapamide may have a Mg\textsuperscript{2+}-sparing effect, by virtue of
its mechanism of action, namely by acting on both the vasculature and the DCT to lower
blood pressure. As such, a comparatively lower inhibition of NCC activity, may allow a lesser inhibition of TRPM6 function, either by preventing atrophy of the DCT or ameliorating direct downregulation of the channel. In conclusion, additional studies are needed to get a clearer understanding of the mechanisms leading to atrophy of the DCT as well as direct inhibition of Mg\(^{2+}\) transport within the distal convolution conferred by thiazide-type diuretics.

\textbf{K\(^+\)-sparing diuretics}

K\(^+\)-sparing diuretics block either ENaC (which is expressed in the DCT2, CNT and CD), or the mineralocorticoid receptor (MR), which has a slightly broader expression pattern along the epithelium of the distal tubule) (3, 37, 149) (Figure 3). MR deletion in a subset of tubular epithelial cells, does not alter NCC expression, while it has marked effects on ENaC and Na\(^+\)/K\(^+\)-ATPase expression in the collecting system (48), suggesting that the primary effect for both types of diuretics on electrolyte transport is the blockade of electrogenic ENaC-mediated Na\(^+\) reabsorption in the distal convolution and CD. Such alterations yield concomitant reductions in tubular K\(^+\)-secretion. The compounds directly blocking ENaC, amiloride and triamterene, as well as the MR antagonists spironolactone and eplerenone, are the most commonly used drugs in this category. K\(^+\)-sparing diuretics are weak diuretics often used in combination with other diuretics to lower blood pressure or to prevent fluid accumulation in edematous conditions such as congestive heart failure ((1, 184)). Their K\(^+\)-sparing ability is important to minimize the risk of hypokalemia, either when combined with other diuretics or alone in conditions with frank hypokalemia.
Both classes of K\(^+\)-sparing diuretics alter renal Ca\(^{2+}\) and Mg\(^{2+}\) transport. Administration of triamterene to healthy subjects resulted in an initial Ca\(^{2+}\) loss, which after 6 hours changed to a decrease in urinary Ca\(^{2+}\) excretion (105). In patients with essential hypertension, spironolactone reduces urinary Ca\(^{2+}\) excretion (83). Other studies found an increase in urinary Ca\(^{2+}\) excretion following spironolactone administration, however as summarized by Prati et al, at least some formulations of spironolactone and amiloride contain significant amounts of Ca\(^{2+}\) (188). Evidence from experimental animals suggests that K\(^+\)-sparing diuretics have a Ca\(^{2+}\) retaining effect. Both triamterene and amiloride lower the Ca\(^{2+}\)/Na\(^{+}\) clearance ratio in dogs, although the effect was much lower than that for thiazides (47). However, only amiloride directly altered the fractional excretion of Ca\(^{2+}\) in this experimental series. In rats, Costanzo observed a reduced fractional excretion of Ca\(^{2+}\) after administration of amiloride (45). Using micropuncture, addition of amiloride to the perfusate augmented reabsorption of Ca\(^{2+}\) from the late portion of the distal convolution, yet both fluid and Na\(^{+}\) reabsorption was inhibited (45). No such effect was present in the early portion, suggesting a direct effect on distal Ca\(^{2+}\) transport machinery. The authors note that the increase in Ca\(^{2+}\) reclamation was tightly correlated to the degree of inhibition of Na\(^{+}\) absorption. Similarly, administration of amiloride to rats treated with furosemide, reduced the fractional excretion of Ca\(^{2+}\), although this was not seen in the absence of furosemide (57). For spironolactone, no Ca\(^{2+}\) sparing effect has been described in experimental animals. Thus K\(^+\)-sparing diuretics may exert a modest effect, if any, upon Ca\(^{2+}\) transport in kidney. The mechanism behind this remains to be fully delineated, but may relate to hyperpolarization of the cell during inhibition of ENaC. In mouse DCT cells, amiloride blocks Na\(^{+}\) uptake, hyperpolarizes the
membrane potential and increases Ca\(^{2+}\) uptake by 39% (80). Furthermore, TRPV5 displays hyperpolarization-dependent activation (111), suggesting that the effect of K\(^+-\) sparing diuretics on Ca\(^{2+}\) transport may occur via increased TRPV5 mediated Ca\(^{2+}\) absorption. An additional proximal effect could be envisioned if these compounds reduce extracellular volume, as they may also relieve the inhibition of PT transport during volume expanded states and thereby enhance paracellular Ca\(^{2+}\) reabsorption in the PT, as occurs for thiazides.

An effect of K\(^+-\) sparing diuretics on urinary excretion of Mg\(^{2+}\) has been documented in both humans and animals. Hypertensive patients treated with triamterene display higher serum Mg\(^{2+}\) levels than untreated patients (133). Furthermore, in healthy subjects, oral administration of triamterene lead to Mg\(^{2+}\) retention, already after the first hour, an effect that lasted for the entire study period of 10 hours (105). In contrast, another study conducted on healthy subjects did not find acute changes in urinary Mg\(^{2+}\) excretion following a single oral dose of amiloride (125). Patients suffering from primary aldosteronism have normal serum Mg\(^{2+}\) concentrations, but display a higher urinary Mg\(^{2+}\) excretion (116). Horton & Biglieri found that adrenalectomy or spironolactone treatment reduced urinary Mg\(^{2+}\) excretion, although it was unclear whether the effect of spironolactone relates to reductions in extracellular fluid volume (116). In healthy volunteers, spironolactone treatment for 5 days decreased urinary Mg\(^{2+}\) excretion (225). Another spironolactone-like compound (Canrenoate potassium) also has Mg\(^{2+}\) sparing properties in liver cirrhosis patients (146). A comparison of amiloride and spironolactone in thiazide-treated healthy subjects, only found a positive effect of amiloride on serum Mg\(^{2+}\) concentrations (166).
In experimental animals, Devane and Ryan found that both amiloride and triamterene reduced urinary excretion of Mg\(^{2+}\) in saline loaded rats. Furthermore, the authors found that amiloride inhibited urinary excretion of Mg\(^{2+}\), even when coadministered with furosemide (56, 57). In another study, the highest dose of amiloride elicited the greatest reduction in the fractional excretion of Mg\(^{2+}\) from furosemide infusion, while no alteration in hematocrit was seen (56), suggesting that the effect was independent of changes in volume status. In another study, the authors found no change in glomerular filtration or serum Mg\(^{2+}\) concentration, during three 30 minute urine collections in clearance experiments in rats undergoing amiloride infusion (58). Importantly, in this study, they were able to control for extracellular volume changes by fluid and electrolyte infusion, which probably yielded an even larger extracellular volume by the end of the study period. Under these conditions, amiloride was still able to reduce urinary fractional excretion of Mg\(^{2+}\) (58). Few animal studies have examined the effect of spironolactone on Mg\(^{2+}\) balance, while studies addressing the direct effect on renal Mg\(^{2+}\) handling is scarce. In dogs with degenerative mitral valve disease, spironolactone stimulated a small yet significant increase in serum Mg\(^{2+}\) concentrations after 20 weeks (235). Injection of spironolactone for 5 days in rabbits also resulted in a higher serum Mg\(^{2+}\) concentration (215). Overall, based on these studies, the underlying mechanism for Mg\(^{2+}\) retention induced by K\(^{+}\)-sparing diuretics seems to occur independent of changes in extracellular volume. An often cited explanation, is the fact that ENaC blockade hyperpolarizes the cell by inhibiting Na\(^{+}\) permeability of the apical membrane (66), thereby increasing the driving force for Mg\(^{2+}\) entry into the cells of the early distal convolution. Indeed, several gene defects highlight the sensitivity of Mg\(^{2+}\) transport in the
DCT to changes in resting membrane potential (26, 51, 63, 87, 158). Structurally, the localization of Mg$^{2+}$ transport along the DCT and the presence of ENaC in only the DCT2 segment of mice (Figure 3), could account for such an effect in this later segment, since various cell types are intermingled along this portion of the nephron (149). However, the DCT2 is very small in humans (147) and it is therefore hard to envision that inhibition of ENaC activity in this more distal portion, would amplify Mg$^{2+}$ uptake in the early distal convolution. Nevertheless, to date, very little information has been published about the precise localization of the transcellular Mg$^{2+}$ transport machinery in human kidney. In conclusion, more studies are required to further delineate the potential effects of K$^+$-sparing diuretics on renal tubular transport of Ca$^{2+}$ and Mg$^{2+}$.

Summary

Many of the proteins involved in tubular transport of Ca$^{2+}$ and Mg$^{2+}$ have been identified and studied within the last decades. These findings have contributed significantly to our understanding of the molecular mechanisms mediating Ca$^{2+}$ and Mg$^{2+}$ transport within the kidney. In contrast, diuretics have long been known to affect Ca$^{2+}$ and Mg$^{2+}$ balance, by changing divalent cation handling in the kidney. The mechanism(s) whereby this occurs has been detailed for some, but not all diuretics. However, an increased understanding of the molecular transport pathways permitting Ca$^{2+}$ and Mg$^{2+}$ reabsorption from the renal tubule aid in clarifying these effects, although our understanding is far from complete. Ongoing studies complemented with targeted transgenic models, to allow specific deletions of transporters that are targeted by
diuretics, within select tubular epithelial cells, will be critical to further our understanding of this topic.
Acknowledgements

The laboratory of H. Dimke is funded by Fabrikant Vilhelm Pedersen og Hustrus Mindelegat (on recommendation from the Novo Nordisk Foundation), the Novo Nordisk Foundation, the Carlsberg Foundation, the A.P. Møller Foundation, the Beckett Foundation, and the Lundbeck Foundation. Dr. R. Todd Alexander is the Canada Research Chair in Renal Tubular Epithelial Transport Physiology and an Alberta Innovates Health Solutions Clinical Investigator. The laboratory of R. Todd Alexander is funded by grants from the Natural Sciences and Engineering Research Council (NSERC) of Canada and the Canadian Institute of Health Research (CIHR).
**Figure legends**

**Figure 1**
Schematic representation of the PT epithelial cell. Please note that this model represents the convoluted S1 and S2 PT segments, where HCO₃⁻ reabsorption predominates. NHE3, Na⁺/H⁺ exchanger 3; AQP1, Aquaporin 1; CA, Carbonic anhydrase; CLDN2, Claudin-2; NBCe1, Electrogenic Na⁺, HCO₃⁻ cotransporter 1; SGLT2, Na⁺/glucose cotransporter type 2; GLUT2, Glucose transporter type 2. Transepithelial potential differences across the convoluted portion of the PT are obtained from (16).

**Figure 2**
Schematic model outlining the molecular transport proteins in the TAL. NKCC2, the furosemide-sensitive Na⁺, K⁺, 2Cl⁻ cotransporter; ROMK, renal outer medullary K⁺ channel; CLC-Kb, Cl⁻ channel Kb; CLC-Ka, Cl⁻ channel Ka, BSND, Barttin; CLDN10-19, Claudin 10-19. The transepithelial voltages are listed. In the TAL, the transepithelial potential difference may be +5 to +10 mV (93, 94), although the transepithelial voltage can increase to values approximating +30 mV in the cortical portion of the segment as outlined in the top of the figure (91, 117, 201).

**Figure 3**
Schematic representation of the cells lining the distal convolution. The epithelial cells representing the DCT1, DCT2 and CNT are depicted. NCC, thiazide-sensitive NaCl cotransporter; ROMK, renal outer medullary K⁺ channel; CLC-Kb, Cl⁻ channel Kb; ENaC, epithelial Na⁺ channel; TRPM6, transient receptor potential Melastin 6 Mg²⁺
channel; TRPV5, transient receptor potential Vanilloid 5 Ca\(^{2+}\) channel; NCX1, Na\(^{2+}\)/Ca\(^{2+}\) exchanger type 1; PMCA, Plasma membrane Ca\(^{2+}\)-ATPase proteins (PMCA1 and PMCA4); MR, Mineralocorticoid receptor. The transepithelial voltage decreases from about 0 to -5 mV in the DCT towards -40 mV in the CNT (84, 153, 195, 223, 247). PMCA4 and NCX1 are highly abundant in the DCT2 and CNT region of the mouse, colocalizing with TRPV5, however weaker expression of the basolateral Ca\(^{2+}\) extrusion protein have also been noted in DCT (not displayed in figure) (8, 149). Although the MR is expressed in the DCT, no effect on NCC has been documented, when the MR is deleted (48). Spironolactone may also inhibit MR in the DCT1 cell – with unknown consequences for Ca\(^{2+}\) and Mg\(^{2+}\) transport (not displayed in figure).
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Channel Not Only Prevented but Also Reversed Development of Hypertension and End-Organ Damage in Dahl Salt-Sensitive Rats. *Hypertension* 2016.
Figure 1

Osmotic Diuretics

CA inhibitors (Azetazolamide)

AQP1

H₂O + CO₂

H₂CO₃

H⁺ + HCO₃⁻

NHE3

Na⁺

H⁺

Na⁺

Mg²⁺

Ca²⁺

CLDN2

CLDN2

CLDN2

AQP1

H₂O

H₂O + CO₂

H₂CO₃

HCO₃⁻

CA

H₂CO₃

Na⁺

Na⁺/K⁺ ATPase

K⁺

GLUT2

Glucose

Na⁺

-1.2mV

0 mV

SGLT2 inhibitors

Na⁺

CLDN2

H₂O

Na⁺

CLDN2

Mg²⁺

Ca²⁺

CLDN2

Glucose
Figure 2