CHAPTER THIRTEEN

Vitamin D-Enhanced Duodenal Calcium Transport

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Abstract

For humans and rodents, duodenum is a very important site of calcium absorption since it is exposed to ionized calcium released from dietary complexes by gastric acid. Calcium traverses the duodenal epithelium via both transcellular and paracellular pathways in a vitamin D-dependent manner. After binding to the nuclear vitamin D receptor, 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] upregulates the expression of several calcium transporter genes, e.g., TRPV5/6, calbindin-D_{9k}, plasma membrane Ca^{2+}-$ATPase_{1b}$, and NCX1, thereby enhancing the transcellular calcium transport. This action has been reported to be under the regulation of parathyroid–kidney–intestinal and bone–kidney–intestinal

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axes, in which the plasma calcium and fibroblast growth factor-23 act as negative feedback regulators, respectively. $1,25(OH)_2D_3$ also modulates the expression of tight junction-related genes and convective water flow, presumably to increase the paracellular calcium permeability and solvent drag-induced calcium transport. However, vitamin D-independent calcium absorption does exist and plays an important role in calcium homeostasis under certain conditions, particularly in neonatal period, pregnancy, and lactation as well as in naturally vitamin D-impoverished subterranean mammals.

1. INTRODUCTION

Calcium is fundamental to a number of cellular and organ functions, such as intracellular signal transduction, neurotransmitter release, muscle contraction, cardiac contractility, and bone metabolism. In mammals, ~99% of total body calcium is stored in bone mainly as hydroxyapatite nanocrystals and to a smaller extent as amorphous calcium phosphate and free-ionized calcium in bone extracellular fluid. Extraosseous calcium (~1% of total body calcium) exists in three major forms, i.e., albumin-bound form, free-ionized calcium, and calcium complexes with phosphate or sulfate. A drastic change in the plasma calcium concentration, either a decrease (hypocalcemia) or increase (hypercalcemia), is lethal; therefore, calcium level is tightly regulated by several hormones, which are three classical calcium-regulating hormones, namely parathyroid hormone (PTH), 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], and calcitonin, as well as some other endocrine or paracrine factors [e.g., estrogen, prolactin, insulin-like growth factor (IGF)-1, and fibroblast growth factor (FGF)-23]. Besides plasma calcium level, body calcium metabolism as a whole is also controlled by these calcium-regulating hormones to assure that dietary calcium is adequately absorbed into the body, properly stored in bone, while the excess is excreted via the kidney. Under certain conditions, for instance pregnancy and lactation, a large amount of calcium is utilized for fetal growth and milk production, respectively; therefore, the intestinal calcium absorption is markedly enhanced to match a high-calcium demand (Charoenphandhu et al., 2009).

Both humans and rodents respond to $1,25(OH)_2D_3$ treatments by raising calcium absorption by 200–300% (Fleet, Eksir, Hance, & Wood, 2002; Kutuzova et al., 2008). However, mild-to-moderate vitamin D insufficiency rarely diminishes intestinal calcium absorption since PTH effectively induces conversion of 25-hydroxyvitamin D_3 [$25(OH)D_3$] into

$1,25(OH)_2D_3$ for stimulation of calcium absorption (Need & Nordin, 2008). It is noteworthy that simple vitamin D_3 supplement [not $1,25(OH)_2D_3$ supplement] cannot increase calcium absorption in either vitamin D-deficient or rachitic volunteers (Gallagher, Jindal, & Smith, 2014; Thacher & Abrams, 2010). Dietary calcium traverses the intestinal epithelium by two major pathways, i.e., transcellular and paracellular pathways in a $1,25(OH)_2D_3$-dependent manner. Both humans and rodents respond to $1,25(OH)_2D_3$ treatments by raising calcium absorption by 200–300% (Fleet et al., 2002; Kutuzova et al., 2008). In the transcellular pathway, free-ionized calcium diffuses across the apical plasma membrane, cytoplasm, and then the basolateral membrane before entering the interstitial fluid and blood circulation. Luminal calcium also moves paracellularly across the tight junction into the lateral intercellular space—also known as paracellular space—before entering the interstitium.

In humans and rodents, although both calcium transport mechanisms take place in all segments of the small intestine, the transcellular calcium transport is predominant in the proximal part, particularly the duodenum, and is of importance during low-calcium intake (Armbrecht, Zenser, Gross, & Davis, 1980). Regular diet without dairy products is generally considered low normal calcium diet, which necessitates the presence of an active uphill transport mechanism. Paracellular calcium transport, on the other hand, is present along the entire length of the small intestine. However, it is believed that a considerable amount of lumen-to-plasma paracellular calcium flux also occurs in the duodenum since it is exposed to high luminal concentration of free-ionized calcium, which is liberated from insoluble complexes by gastric acidic environment (Kopic & Geibel, 2013), especially after high-calcium meal or oral calcium supplementation. Our investigation has provided evidence that the paracellular calcium flux in the rat duodenum is much greater than the transcellular calcium flux (up to 10:1 when luminal calcium >5 mmol/L) (Charoenphandhu, Tudpor, Pulsook, & Krishnamra, 2006).

2. SOURCES OF $1,25(OH)_2D_3$ FOR STIMULATION OF DUODENAL CALCIUM TRANSPORT

There are two possible sources of $1,25(OH)_2D_3$, i.e., from plasma and *de novo* synthesis in the intestinal cells, the latter of which is also known as intracrine $1,25(OH)_2D_3$ (Balesaria, Sangha, & Walters, 2009; Kopic & Geibel, 2013). Plasma $1,25(OH)_2D_3$ is predominantly synthesized in the

renal proximal tubular cells by 25(OH)D_3 1α-hydroxylase (CYP27B1) (Kopic & Geibel, 2013). Regarding the intracrine 1,25$(OH)_2D_3$ synthesis, the duodenal absorptive cells have been shown to express 1α-hydroxylase that is capable of converting 25(OH)D_3 to 1,25$(OH)_2D_3$, which later diffuses from the cytoplasm to activate nuclear vitamin D receptor (VDR) (Balesaria et al., 2009). The *de novo* synthesis of 1α-hydroxylase in human duodenal tissue is upregulated after 25(OH)D_3 exposure. It is noted that an increase in 1α-hydroxylase transcripts shows a positive correlation with the expression of transient receptor potential vanilloid calcium channel (TRPV)-6 (Balesaria et al., 2009). It is, therefore, possible that human duodenal absorptive cells can convert 25(OH)D_3 into 1,25$(OH)_2D_3$, which may subsequently upregulate calcium absorption through TRPV6 (Balesaria et al., 2009; Cui et al., 2009; Koszewski, Horst, & Goff, 2012). Finally, the action of 1,25$(OH)_2D_3$ is terminated by 24-hydroxylase (CYP24), which is also under the regulation of 1,25$(OH)_2D_3$. Kutuzova and DeLuca (2004) reported that, in vitamin D-deficient rats, a single-dose intrajugular injection of 1,25$(OH)_2D_3$ (730 ng/kg body weight) markedly upregulated the duodenal expression of CYP24 transcript within 3 h.

Alternatively, circulating 1,25$(OH)_2D_3$ can exert its biological actions by binding to either plasma membrane receptor 1,25D_3-MARRS (membrane-associated, rapid response steroid-binding) protein or VDR. Activation of 1,25D_3-MARRS is responsible for the 1,25$(OH)_2D_3$-enhanced rapid calcium absorption across the duodenal epithelium (nongenomic action, minute-to-minute regulation) (Nemere, Garbi, Hämmerling, & Khanal, 2010; Nemere & Norman, 1990). However, the underlying cellular mechanism and intracellular signaling of 1,25D_3-MARRS are not completely understood. In contrast, VDR is a nuclear receptor that forms a heterodimer with retinoid X receptor (RXR). Thereafter, the 1,25$(OH)_2D_3$–VDR–RXR complex—now being a transcription factor—binds to the vitamin D-responsive elements (VDREs) to regulate transcription of vitamin D-sensitive genes, including *Trpv6* and *Cyp24a1* (genomic action, long-term regulation) (Meyer, Zella, Nerenz, & Pike, 2007).

3. VITAMIN D-ENHANCED TRANSCELLULAR CALCIUM TRANSPORT

Since ionized calcium cannot freely move across the lipid bilayer of the plasma membrane, the polarized duodenal absorptive cells abundantly express a number of calcium transporters in both apical and basolateral

plasma membranes as well as in the cytoplasm, most of which are under a tight regulation of 1,25(OH)$_2$D$_3$ and VDR signaling (Fig. 1). Thus, the intestinal calcium absorption is dramatically decreased in intestine-specific VDR knockout mice as compared to normal mice (Lieben et al., 2012). The vitamin D-enhanced transcellular calcium transport is an active transport process, which requires cellular energy in the form of ATP. This transport mechanism is also sex and age dependent. For instance, female mice have higher calcium absorption and expression of calcium transporters than male mice, and a single 1,25(OH)$_2$D$_3$ injection in female mice induces a

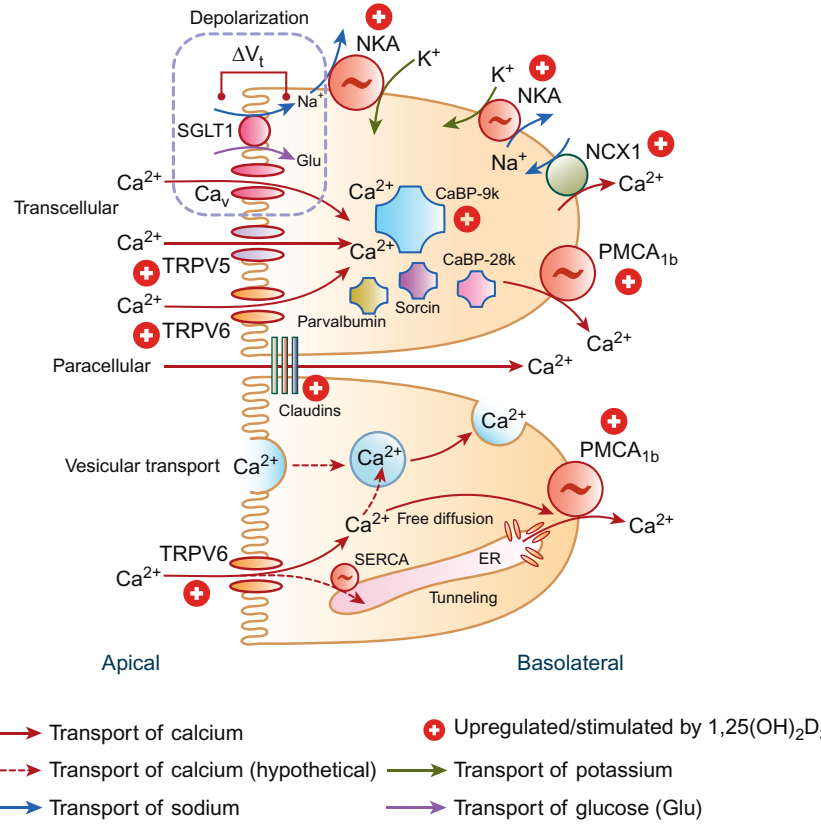

Figure 1 A schematic diagram shows possible mechanisms of transcellular and paracellular calcium transport in the rat duodenum. 1,25(OH)$_2$D$_3$ is capable of stimulating both paracellular and transcellular calcium transport [TRPV5/6-mediated calcium entry, calcium-binding protein (CaBP)-facilitated calcium diffusion, and basolateral active calcium extrusion], as indicated by red plus signs. ΔV_t, change in membrane potential. Please see text for details. Glu, glucose.

greater upregulation of calcium transporter expression than in male mice (Song & Fleet, 2004). As for age dependency, the $1,25(OH)_2D_3$-induced duodenal calcium absorption is relatively high in young adult rodents before gradually decreased with age (Wood, Fleet, Cashman, Bruns, & DeLuca, 1998).

3.1 Apical calcium entry

Free-ionized calcium traverses the duodenal apical membrane via calcium channels by simple diffusion toward electrochemical potential (i.e., luminal calcium concentrations of ~2–6 mmol/L vs. intracellular concentrations of ~0.1–0.3 μmol/L, and more negative potential inside) (Wasserman, 2004). Two families of calcium channels, i.e., TRPV5 and 6 ($P_{Ca}/P_{Na} > 100$) and L-type voltage-dependent calcium channel (Ca_v; conductance 11–25 pS), are expressed in the apical (brush border) membrane of duodenal absorptive cells (Morgan, Mace, Helliwell, Affleck, & Kellett, 2003; van de Graaf et al., 2003), suggesting a redundancy of apical calcium entry mechanisms. In addition, the absence of one apical calcium transporter can lead to a compensatory upregulation of other calcium transporter expression (Gkika et al., 2006). Under normal conditions, TRPV6, and to a lesser extent TRPV5, plays an important role in the duodenal calcium entry in a vitamin D-dependent manner (Kellett, 2011). Opening of apical calcium channels is generally dependent on membrane potential (apical resting potential of −47 mV). Ca_v opens at a depolarizing potential with activation threshold of around −25 mV, whereas TRPV6 fully functions under a relatively hyperpolarized potential of <−50 mV (Kellett, 2011). In renal epithelial cells, the T-type Ca_v has been reported to mediate apical calcium entry. For example, T-type Ca_v opening in response to testosterone was found to enhance calcium reabsorption in the rabbit distal renal tubule, but not proximal tubule, via mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK)- and tyrosine kinase-dependent mechanisms (Couchourel, Leclerc, Filep, & Brunette, 2004).

During postprandial period, L-type Ca_v, particularly the 1.3 subtype ($Ca_v1.3$), helps to enhance apical calcium entry because the $Ca_v1.3$-mediated calcium transport is dependent on luminal glucose (Kellett, 2011; Morgan, Mace, Affleck, & Kellett, 2007). Specifically, glucose together with other substrates of sodium-dependent glucose transporter (SGLT)-1, such as galactose, initially induces sodium entry, thereby depolarizing the apical plasma membrane from −47 to −20 mV (Kellett,

2011). Depolarization then triggers the opening of $Ca_v1.3$ for calcium to diffuse down its concentration gradient into the cytoplasm. This $Ca_v1.3$-mediated calcium transport is markedly diminished by L-type calcium channel blockers, including dihydropyridine (e.g., nifedipine) and phenylalkylamine (e.g., verapamil) (Morgan et al., 2003; Thongon, Nakkrasae, Thongbunchoo, Krishnamra, & Charoenphandhu, 2009).

It has been widely known that 1,25$(OH)_2D_3$ potently enhances the expression of TRPV5 and TRPV6, thus increasing their activities (Khuituan et al., 2012; Okano, Tsugawa, Morishita, & Kato, 2004). A single-dose subcutaneous injection of 2 μg/kg 1,25$(OH)_2D_3$ in mice was found to upregulate the duodenal mRNA expression of both TRPV5 and TRPV6 within 3 h and peaked at 6 h before returning to the baseline levels at 24 h (Khuituan et al., 2012). On the other hand, the $Ca_v1.3$-mediated calcium transport is independent of 1,25$(OH)_2D_3$ (Kellett, 2011). At the molecular level, the regulation of TRPV6 by 1,25$(OH)_2D_3$ has been identified in human intestinal cells. Meyer, Watanuki, Kim, Shevde, and Pike (2006) demonstrated in human intestine-like Caco-2 cells using chromatin immunoprecipitation and found five putative VDREs located upstream to *Trpv6* coding region, and mutagenesis within the VDRE abrogated all responses to the 1,25$(OH)_2D_3$. It was also reported that heterodimer of VDR and RXR could bind to *Trpv6* gene, resulting in both recruitment of chromatin remodeling coactivator steroid receptor coactivator 1 as well as modification of histone acetylation. Hence, *Trpv6* gene was broadly acetylated in response to 1,25$(OH)_2D_3$ specifically at the VDR/RXR-binding sites.

3.2 Cytoplasmic translocation

Theoretically, free-ionized calcium can freely diffuse across cytoplasmic aqueous medium at higher rate than proteins. Specifically, the diffusion coefficient of free-ionized calcium in cytoplasm is 223 $\mu m^2/s$ (Allbritton, Meyer, & Stryer, 1992), whereas the diffusion coefficient of calbindin-D_{9k} is ~1/14 that of calcium. However, cells usually prevent bulk flow of free calcium across the cytoplasm, while utilizing facilitated calcium diffusion in protein-bound form. Since elevated intracellular calcium may trigger undesirable intracellular signal transductions and apoptosis, the duodenal absorptive cells use several mechanisms to keep the cytoplasmic free-ionized calcium level as low as possible. First, excessive free-ionized calcium and certain calcium-laden calcium-binding proteins (e.g., calmodulin) close to

the apical membrane deactivate TRPV6 and perhaps Ca_v, which can, in turn, restrict calcium entry (Derler et al., 2006; Kellett, 2011; Lee et al., 2006; Nilius et al., 2001). Second, most calcium is buffered by cytoplasmic calcium-binding proteins, particularly calbindin-D_{9k} (S100g), and probably also by calbindin-D_{28k}, parvalbumin, and calmodulin (Schröder, Schlumbohm, Kaune, & Breves, 1996; Timmermans, Bindels, & Van Os, 1995; Walters, 1989). Meanwhile, calbindin-D_{9k} with two EF-hand calcium-binding domains indeed elevates the total intracellular concentration of calcium (bound form plus ionized form) by ~50-fold, thereby facilitating translational diffusion (ferry) of calcium across the cytoplasm (Feher, 1983). The unoccupied calcium-binding proteins in close vicinity to apical ion channels prevent channel inactivation, leading to persistent calcium entry (Fig. 2).

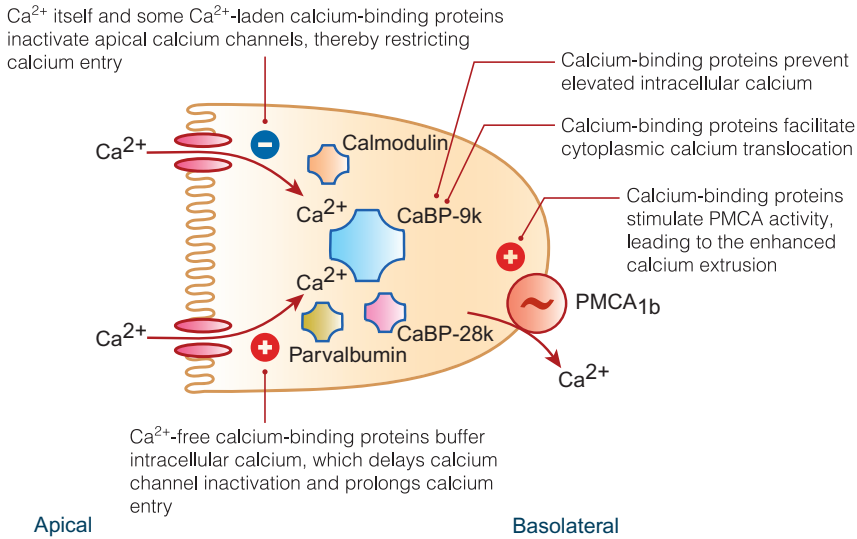

Figure 2 Cytoplasmic calcium-binding proteins (CaBP) in the duodenal enterocytes show a high degree of redundancy and have a number of functions. Calcium-free calcium-binding proteins (e.g., calbindin-D_{9k} and -D_{28k}) buffer the intracellular calcium, which can, in turn, delay calcium channel inactivation and prolong calcium entry. On the other hand, some calcium-laden calcium-binding proteins (e.g., calmodulin and calbindin-D_{28k}) as well as intracellular calcium itself lead to calcium-dependent inactivation of apical calcium channels, thereby restricting calcium entry. Several calcium-binding proteins, especially calbindin-D_{9k}, ferry the intracellular calcium from apical-to-basolateral compartment by a process known as translational diffusion rather than bucket brigade. Finally, they potentiate PMCA activity to accelerate the basolateral calcium extrusion. Ca^{2+}, free-ionized calcium.

The levels of calbindin-D_{9k} mRNA and protein in the duodenal absorptive cells of human and rodents are strongly dependent on $1,25(OH)_2D_3$ (Fleet et al., 2002; Khuituan et al., 2012). Indeed, the expressions of other calcium-binding proteins and/or their transcripts, e.g., calbindin-D_{28k}, parvalbumin, calmodulin, and sorcin, are also $1,25(OH)_2D_3$-dependent (Bindels, Timmermans, Hartog, Coers, & Van Os, 1991; Khanal & Nemere, 2008a; Wood, Tchack, Angelo, Pratt, & Sonna, 2004) although their exact contribution to the cytoplasmic calcium translocation remains enigmatic. The presence of transcellular active calcium transport in the small intestine of calbindin-D_{9k} knockout mice (Benn et al., 2008) indicates functional redundancy of cytoplasmic calcium-binding proteins and thus strengthens the physiological role of other proteins. Upregulation of calbindin-D_{28k} and parvalbumin mRNA expression is evident under certain conditions with high-calcium demand, such as pregnancy and lactation (Charoenphandhu, Wongdee, Teerapornpuntakit, Thongchote, & Krishnamra, 2008; Teerapornpuntakit, Klanchui, Karoonuthaisiri, Wongdee, & Charoenphandhu, 2014), suggesting that they play a role in cytoplasmic facilitated calcium transport. In avian small intestine and mammalian renal tubular cells, calbindin-D_{28k} is the principal protein for intracellular apical-to-basolateral transport (Hall & Norman, 1990).

Cytoplasmic calcium translocation in both avian and mammals may also be mediated by a mechanism called vesicular calcium transport (Fig. 1), in which calcium is captured inside an endocytic vesicle or later pumped into an intracellular membrane-bound vesicle before being extruded at the basolateral membrane by exocytosis (Khanal & Nemere, 2008b; Nemere, Leathers, & Norman, 1986). In the duodenal enterocytes of white Leghorn cockerels, vitamin D-dependent calbindin-D_{28k} is localized in the lysosome-like vesicles, presumably to help buffer intravesicular calcium (Nemere et al., 1986). Vesicular calcium transport underlies the rapid nongenomic $1,25(OH)_2D_3$-enhanced vectorial calcium absorption—previously known as transcaltachia (Nemere & Norman, 1990).

In addition, calcium is hypothesized to translocate across the cytoplasm by tunneling through endoplasmic reticulum (ER) (Fig. 1). This mechanism was identified in pancreatic cells but has never been studied in the duodenal enterocytes. The responsible transporters in the duodenal ER membrane are unknown. In pancreatic acinar cells, Mogami, Nakano, Tepikin, and Petersen (1997) showed recharging of intracellular calcium stores in response to calcium depletion mediated by movement of calcium through a tunnel from the basal to secretory poles. Since VDR has been shown to

interact with sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) in cardiomyocytes (Zhao & Simpson, 2010), it is tempting to speculate that $1,25(OH)_2D_3$ might directly modulate SERCA activity, leading to the enhanced calcium tunneling across the duodenal cytoplasm.

3.3 Basolateral extrusion

Since electrochemical gradient across the duodenal basolateral membrane is thermodynamically unfavorable for simple diffusion [i.e., ~0.1–1 μmol/L cytoplasmic calcium vs. 1.2–1.3 mmol/L plasma calcium, and positive potential difference (PD) of ~58 mV (extracellular positive voltage)], metabolically energized active transport processes are required for basolateral uphill calcium extrusion. The cellular energy of ~9.3 kcal (39 kJ) is required to transport one mole of calcium against this electrochemical gradient (Wasserman & Fullmer, 1995). Although both plasma membrane Ca^{2+}-ATPase (PMCA) subtype 1b and Na^+/Ca^{2+}-exchanger (NCX)-1 play an important role in the basolateral calcium transport, the detailed cellular and molecular mechanisms remain controversial. $PMCA_{1b}$ (*Atp2b1*) is a primary active transporter (P-type ATPase) that can directly hydrolyze ATP to drive its transport activity (K_m ~0.2 μmol/L in the presence of calmodulin), whereas NCX1 (*Slc8a1*; Na^+/Ca^{2+} ratio 3:1) is coupled with Na^+/K^+-ATPase (NKA) that generates sodium gradient for NCX1-mediated calcium efflux (Fig. 3A). In rats, the $PMCA_{1b}$ activity is highest in the duodenum as compared to other small intestinal segments (Ghijsen & Van Os, 1982). $PMCA_{1b}$-mediated calcium transport is approximately fivefold greater than the NCX1-mediated transport (Ghijsen, de Jong, & Van Os, 1983). It was, therefore, previously believed that $PMCA_{1b}$ was the principal active transporter for basolateral calcium extrusion. However, our group recently demonstrated a complete abolishment of the $1,25(OH)_2D_3$-enhanced active calcium transport in the mouse duodenum when inhibited by NCX inhibitor (100 μmol/L KB-R7943) alone and calmodulin-dependent PMCA inhibitor (100 μmol/L trifluoperazine) alone as well as KB-R7943 plus trifluoperazine (Khuituan et al., 2013), indicating that in response to $1,25(OH)_2D_3$, both transporters might function in an interdependent manner. In other words, once $PMCA_{1b}$ is inhibited, calcium transport via NCX1 eventually decreases, and vice versa. Similar finding is also observed when the duodenal active calcium transport is stimulated by prolactin (Dorkkam, Wongdee, Suntornsaratoon, Krishnamra, & Charoenphandhu, 2013).

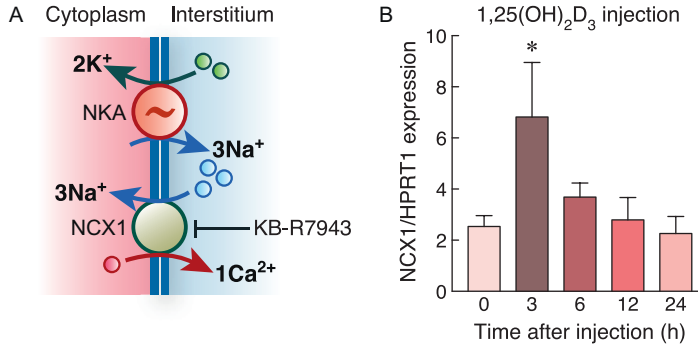

Figure 3 (A) Basolateral calcium extrusion through Na^+/Ca^{2+}-exchanger (NCX)-1. The NCX1 is coupled with Na^+/K^+-ATPase (NKA). NKA first moves three sodium ions out of the cell and moves two potassium ions into the cell, thereby generating sodium gradient for NCX1 function. NCX1 then allows influx of three sodium ions and efflux of one calcium ion. NCX1 activity can be inhibited by KB-R7943. (B) Time-dependent expression of NCX1 in duodenal epithelial cells of mice subcutaneously injected with a single-dose 2 μg/kg 1,25(OH)$_2$D$_3$, as determined by quantitative real-time PCR. The duodenal epithelial cells were collected at 0, 3, 6, 12, and 24 h after 1,25(OH)$_2$D$_3$ injection. Values are means ± SE of NCX1/HPRT1 expression ratios. The results show that, at 3 h postinjection, 1,25(OH)$_2$D$_3$ significantly increases the mRNA level of NCX1 by 2.69-fold (*$P < 0.05$ vs. 0 h; one-way analysis of variance with Dunnett's posttest) before returning to the baseline thereafter. HPRT1, hypoxanthine phosphoribosyltransferase-1 (a housekeeping gene).

Expression of PMCA$_{1b}$ transcripts is significantly upregulated by 1,25(OH)$_2$D$_3$ in subclones of human Caco-2 cells (Fleet et al., 2002), whereas vitamin D deficiency decreases the mRNA level of PMCA (Zelinski, Sykes, & Weiser, 1991). Since 1,25(OH)$_2$D$_3$ supplementation markedly increases the activities of PMCA, but not NCX, in vitamin D-deficient rats (Ghijsen et al., 1983), the NCX1-mediated calcium extrusion is thought to be vitamin D independent. However, in the duodenum of chicks (*Gallus domesticus*), vitamin D deficiency leads to a decrease in NCX activity, which is fully restored by 1,25(OH)$_2$D$_3$ administration (Centeno, Picotto, Pérez, Alisio, & Tolosa de Talamoni, 2011), whereas low-calcium diet enhances both PMCA and NCX activities (Centeno et al., 2004). 1,25(OH)$_2$D$_3$ administration also upregulates the mRNA and protein expression of NCX1 in the chick duodenum (Centeno et al., 2011). Similarly, in the duodenum of vitamin D-replete mouse (Khuituan et al., 2012, 2013), 1,25(OH)$_2$D$_3$ enhances the NCX1-mediated calcium transport presumably by upregulating NCX1 transcription (Fig. 3B). Recently, another family of the basolateral calcium transporters, namely potassium-dependent sodium

calcium exchangers (e.g., NCKX3 and 4), has been suggested to contribute to calcium extrusion (Kopic & Geibel, 2013), but whether $1,25(OH)_2D_3$ is capable of stimulating these transporters in the small intestine is not known. A study in mouse dendritic cells indicated that $1,25(OH)_2D_3$ could increase the membrane abundance of NCKX proteins as well as their activities (Shumilina et al., 2010).

The basolateral calcium extrusion is indeed coupled with apical calcium uptake and cytoplasmic calcium translocation. Claassen, Coetzer, de Winter, Haag, and Kruger (1996) reported a linear relationship between the apical calcium uptake and PMCA activity in the duodenum of male rats. In addition, vitamin D-dependent calbindin-D_{9k} as well as some other calcium-binding proteins, such as calmodulin, calbindin-D_{28k}, and parvalbumin, appears to be salient activators of PMCA activity (Timmermans et al., 1995; Walters, 1989). This coupling mechanism thus ensures that calcium absorption occurs at the highest efficiency without intracellular calcium accumulation, which not only blocks apical calcium uptake but may also induce undesirable responses, e.g., inhibition of sodium absorption and apoptosis.

4. VITAMIN D-ENHANCED PARACELLULAR CALCIUM TRANSPORT

In the leaky epithelia with transepithelial resistance of $<100\ \Omega\ cm^2$ like the small and large intestine, luminal calcium is able to traverse the epithelial sheet through the paracellular pathway—an electrically charged, watery space between the two epithelial cells (also known as the lateral intercellular space). In the rat duodenum, calcium movement is driven by free energy of electrochemical gradient (passive diffusion) or solvent drag (Charoenphandhu & Krishnamra, 2007; Charoenphandhu, Wongdee, & Krishnamra, 2010). Charge- and size-selective properties of the junctional complex, particularly tight junction (effective pore diameter ~0.5–3.5 nm), also affect the paracellular calcium transport (Charoenphandhu et al., 2010).

4.1 Paracellular calcium transport driven by electrochemical gradient

Dostal and Toverud (1984) reported a reduction in the passive nonsaturable component of duodenal calcium absorption in vitamin D-deficient rats, suggesting that the passive paracellular calcium transport is indeed dependent on $1,25(OH)_2D_3$ action. Generally, this mode of calcium transport is

considered unsaturable and increases linearly with luminal free-ionized calcium concentration. It was previously believed that the paracellular space was merely a simple water-filled channel, where transepithelial fluxes in the apical-to-basolateral and basolateral-to-apical directions were identical when the driving transepithelial calcium gradients had the same magnitude. However, our group has demonstrated in human intestinal epithelium-like Caco-2 monolayer that the paracellular space—or more precisely the tight junction—is a rectifying channel (Thongon et al., 2009). Therefore, under certain conditions (e.g., short-circuit condition; transepithelial PD = 0 mV), the apical-to-basolateral paracellular calcium flux is slightly greater than that in the opposite direction (Thongon et al., 2009). In humans and rats fed calcium-replete diet, free-ionized calcium concentration in the duodenal lumen is as high as 2–6 mmol/L, which can diffuse down the concentration gradient via the paracellular pathway into the interstitial fluid or plasma (plasma free-ionized calcium concentration ~1.25 mmol/L) (Duflos, Bellaton, Pansu, & Bronner, 1995; Wasserman, 2004). Our investigation in Ussing chamber showed that a significant amount of the duodenal paracellular passive flux occurred when the apical calcium concentration was 5 mmol/L, but not 2.5 mmol/L, versus the basolateral concentration of 1.25 mmol/L (Charoenphandhu et al., 2006).

In addition to calcium concentration gradient, the voltage gradient or PD can also drive the paracellular calcium flux. Under normal conditions, the duodenal lumen is negative compared to plasma; therefore, plasma calcium diffuses down the voltage gradient into the lumen (i.e., calcium secretion). However, since PD across the duodenal epithelium is relatively low (~2–5 mV, negative luminal potential) and since elimination of PD by short-circuit current does not much affect transepithelial calcium flux, electrodiffusion of calcium may be considered negligible (Charoenphandhu, Limlomwongse, & Krishnamra, 2001). It has been reported that $1,25(OH)_2D_3$ is capable of enhancing the activities of electrogenic transporters, such as NKA and SGLT-1, which, in turn, alters the PD (Elstein & Silver, 1986; Rexhepaj et al., 2011). Hence, $1,25(OH)_2D_3$ might also indirectly modulate the voltage-dependent calcium transport, but the effect would still be relatively small.

4.2 Solvent drag-induced paracellular calcium transport

A correlation between transepithelial paracellular water flow and tracer solutes substantiates physiological significance of solvent drag, in which ions and small hydrophilic molecules traverse the tight junction and the

remaining part of the paracellular space along with the stream of water (Mullen, Muller, & Van Bruggen, 1985). Our group has provided evidence that rat duodenal epithelium is able to absorb calcium via the solvent drag-induced mechanism (Tanrattana, Charoenphandhu, Limlomwongse, & Krishnamra, 2004). Nevertheless, direct visualization of water flow across the tight junction (e.g., using fluorescent marker and confocal microscope) has been unsuccessful (Kovbasnjuk, Leader, Weinstein, & Spring, 1998), and most solvent drag studies rely upon the use of paracellular markers (e.g., mannitol, polyethylene glycol, and inulin)—substances not absorbed transcellularly—and/or measurement of transepithelial $[^3H]_2O$ flux. To obtain a substantial magnitude of solvent drag-induced calcium transport, the duodenal epithelial cells have to activate SGLT-1 and NKA. Specifically, sodium entering through SGLT-1 (and perhaps through some other sodium-coupled apical transports, e.g., amino acid transporters) is pumped into the paracellular space by NKA, thereby increasing the sodium concentration in the space for osmotic water flow. The presence of SGLT-1 substrates, such as glucose and galactose, in the apical compartment thus augments the duodenal calcium absorption (Suntornsaratoon et al., 2014).

In the duodenum of male mice, $1,25(OH)_2D_3$ was found to significantly increase the transepithelial $[^3H]_2O$ flow, but the paracellular transport of mannitol (molecular radius ~0.4 nm) was not observed until there was a widening of tight junction by tumor necrosis factor-α (Khuituan et al., 2013). Such an increase in water flow can be explained by the stimulatory effects of $1,25(OH)_2D_3$ on SGLT-1 and NKA (Elstein & Silver, 1986; Rexhepaj et al., 2011). Indeed, the solvent drag-induced duodenal calcium transport occurs very rapidly within 60 min after a direct exposure to 10–1000 nmol/L $1,25(OH)_2D_3$, which is probably mediated by nongenomic pathways involving $1,25D_3$-MARRS, phosphatidylinositol 3-kinase (PI3K), protein kinase C (PKC), and MEK (Tudpor, Teerapornpuntakit, Jantarajit, Krishnamra, & Charoenphandhu, 2008). In contrast, Nemere et al. (1986) reported no difference in the serum tritium levels after adding $[^3H]_2O$ into the duodenal lumen of vitamin D-deficient and $1,25(OH)_2D_3$-treated chicks, suggesting that $1,25(OH)_2D_3$ might not regulate the lumen-to-plasma water flow in avian. Alternatively, $1,25(OH)_2D_3$ might have used other mechanisms to increase solvent drag-induced calcium transport, for example, by inducing contraction of perijunctional actomyosin ring to widen the tight junction pores (i.e., modulating size selectivity; for review, please see Turner, 2000), or by increasing cation permselectivity of the tight junction (Tudpor et al., 2008).

4.3 Charge- and size-selective properties of tight junction

The duodenum epithelium is basically cation selective with permeability ratio of sodium and chloride (P_{Na}/P_{Cl}) between 1.2 and 2 (Charoenphandhu et al., 2006; Tudpor et al., 2008). It allows paracellular movement of small monovalent and divalent cations, e.g., Na^+ and Ca^{2+}, both of which have ionic radii of ~115 pm. In general, alterations of charge/size-selective properties and/or cation permselectivity of tight junction markedly affect paracellular ion transport via passive diffusion and solvent drag-induced mechanisms. Our group has reported that although a rapid exposure (60 min) to 100 nmol/L $1,25(OH)_2D_3$ did not change the charge-selective property of the rat duodenal epithelium as indicated by no change in P_{Na}/P_{Cl}, the actual values of P_{Na} and P_{Cl} were significantly increased (Tudpor et al., 2008). Therefore, the $1,25(OH)_2D_3$-exposed duodenal epithelium is likely to have higher paracellular permeability to both cations (e.g., calcium) and anions.

At the molecular level, $1,25(OH)_2D_3$ alters tight junction permselectivity of intestinal epithelium by regulating the expression of tight junction-related genes, particularly claudins. Fujita et al. (2008) have demonstrated by using RNA interference and overexpression techniques that claudin-2 and -12 were upregulated by $1,25(OH)_2D_3$ through the VDR, resulting in an increase in paracellular calcium transport in human enterocyte-like Caco-2 cells. *In vivo* experiment also revealed downregulation of claudin-2 and -12 in the duodenum, ileum, and colon of the VDR knockout mice at both transcriptional and translational levels (Fujita et al., 2008). Similarly, Hwang et al. (2013) showed downregulation of claudin-2 as well as claudin-15 mRNAs and proteins in the duodenum of calcium/vitamin D-deficient calbindin-D_{9k} knockout mice. On the other hand, high concentration of $1,25(OH)_2D_3$ conversely suppressed claudin-3 mRNA expression in the rat duodenum as determined by Affymetrix microarrays (Kutuzova & DeLuca, 2004). The aforementioned evidence thus suggested that $1,25(OH)_2D_3$ regulates duodenal paracellular calcium transport through a combination of different claudins, i.e., claudin-2, -3, -12, and/or -15. In addition, other charge-selective claudins, such as claudin-10 and -16, the latter of which has been reported to be calcium-permeable and is expressed in the rat small intestine, may have roles in calcium absorption (Inai et al., 2005; Wongdee, Teerapornpuntakit, Siangpro, Chaipai, & Charoenphandhu, 2013).

$1,25(OH)_2D_3$ also modulates the expression of duodenal nonclaudin tight junction-related proteins, such as *zonula occludens* protein (ZO) and occludin (Hwang et al., 2013). Hwang et al. (2013) found the expressions of occludin and ZO-1 transcripts to be downregulated in calbindin-D_{9k} knockout mice given calcium- and vitamin D-deficient diet, but not normal diet. Western blot and immunohistochemical analyses of ZO-1 in duodenum also showed similar trend as mRNA results. E-cadherin—the critical component of the adherens junction for maintaining integrity of the epithelium—was also upregulated in response to $1,25(OH)_2D_3$ in human intestinal epithelium-like Caco-2 cells via RhoA–Rho-associated coiled-coil kinase (RhoA–ROCK) activation (Ordóñez-Morán et al., 2008).

5. REGULATION OF CALCIUM TRANSPORT BY THE PARATHYROID–KIDNEY–INTESTINAL AXIS

Since dietary calcium intake and daily calcium requirement vary largely from day to day and in different phases of life, the homeostatic system has to adjust calcium storage in bone as well as renal and intestinal calcium transport to meet the body requirements with a minimal change in plasma calcium level (Peacock, 2010). However, plasma calcium fluctuation may occur in several circumstances, e.g., changes in intestinal calcium absorption and renal calcium excretion. Such transient change of plasma calcium is recovered by an integrative response of three calcium-regulating organs organized as the parathyroid–kidney–intestinal axis (Fig. 4) (Kopic & Geibel, 2013; Peacock, 2010). PTH secretion from the parathyroid gland is inhibited after calcium binds to calcium sensing receptor (CaSR) in the plasma membrane of parathyroid chief cells. In a classical loop of regulation, low plasma calcium level curtails this CaSR-induced suppression, thereby increasing PTH secretion. PTH, in turn, raises plasma calcium level by (i) enhancing calcium reabsorption in the thick ascending limb of the Henle's loop and distal renal tubule, which occurs within minutes, and (ii) stimulating osteoblasts to secrete RANKL, which activates osteoclast-mediated bone resorption within minutes to hours (Favus, Bushinsky, & Lemann, 2006; Kopic & Geibel, 2013). PTH can further induce bone calcium release by osteocytes through a poorly defined mechanism, known as osteocytic osteolysis (Tazawa et al., 2004).

PTH and low plasma calcium also independently stimulate 1α-hydroxylase in the proximal renal tubule, thus increasing the production of $1,25(OH)_2D_3$, which potently stimulates intestinal calcium absorption

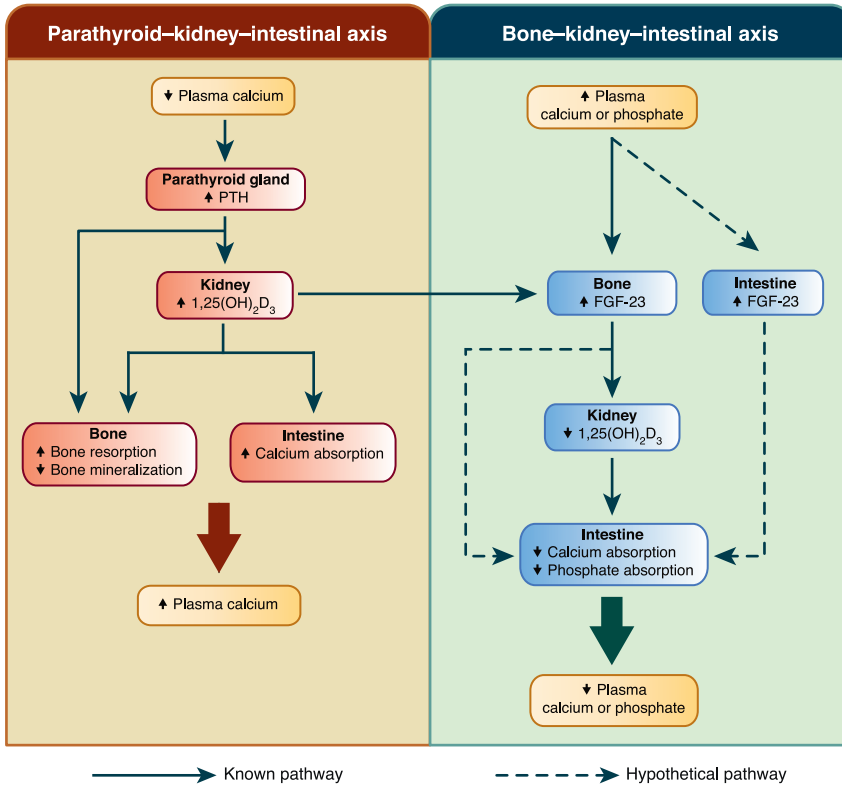

Figure 4 Interrelationship between the parathyroid–kidney–intestinal (left panel) and bone–kidney–intestinal axes (right panel) in regulating calcium homeostasis. In the parathyroid–kidney–intestinal axis, low plasma calcium level is detected by parathyroid glands, thereby stimulating PTH release. PTH stimulates 1α-hydroxylase in the kidney to convert 25(OH)D_3 into 1,25(OH)$_2$D$_3$. An increase in 1,25(OH)$_2$D$_3$ level further enhances bone resorption, inhibits bone mineralization, and promotes intestinal calcium absorption, thus increasing plasma calcium level. In the bone–kidney–intestinal axis, high plasma calcium leads to an increase in circulating level of fibroblast growth factor (FGF)-23 produced by bone and perhaps by the intestine. FGF-23 suppresses renal 1,25(OH)$_2$D$_3$ production by inhibiting 1α-hydroxylase, thereby decreasing intestinal calcium and phosphate absorption, which, in turn, lower plasma calcium and phosphate levels. An increase in circulating 1,25(OH)$_2$D$_3$ level in the parathyroid–kidney–intestinal axis can also increase bone FGF-23 production, which links the two axes together. Furthermore, FGF-23 is hypothesized to exert a direct inhibitory effect on the intestine. Circulating FGF-23 might directly suppress intestinal calcium and phosphate absorption, whereas local FGF-23 production from the enterocytes might serve to prevent excessive intestinal calcium/phosphate absorption.

within 24 h after PTH secretion (Favus et al., 2006). An increase in plasma calcium induces a negative feedback that inhibits PTH secretion from the parathyroid chief cells. At the cellular level, ionized calcium binds to CaSR, which signals through phospholipase C, inositol triphosphate (IP_3), and diacylglycerol (DAG). CaSR can also be stimulated by other cations, e.g., Mg^{2+}, Pb^{2+}, Cd^{2+}, Fe^{2+}, Ba^{2+}, Ni^{2+}, Co^{2+}, and Gd^{3+}, as well as polycationic molecules, e.g., spermine, spermidine, putrescine, and neomycin (Handlogten, Shiraishi, Awata, Huang, & Miller, 2000; Kopic & Geibel, 2013; Quinn et al., 1997). An increase in IP_3 causes calcium release from intracellular store, whereas DAG activates PKC. Both intracellular calcium and PKC then inhibit PTH granule release. Increased intracellular calcium also inhibits PTH synthesis. Thus, plasma calcium itself acts as the principal negative feedback regulator in the parathyroid–kidney–intestinal axis (Kopic & Geibel, 2013). Besides the calcium level, PTH and 1,25$(OH)_2D_3$ themselves take part in the feedback loop; i.e., high PTH levels suppress PTH gene expression while upregulating CaSR. Meanwhile, high 1,25$(OH)_2D_3$ levels also suppress renal CYP27B1 (1α-hydroxylase) and increase CYP24 (24-hydroxylase) activities to lower its own circulating level and thus activity (Favus et al., 2006; Kopic & Geibel, 2013).

PTH not only increases intestinal calcium absorption indirectly by stimulating 1,25$(OH)_2D_3$ production but also has a direct stimulatory effect on the intestinal calcium absorption. Nemere and Szego (1981) demonstrated that 0.01 nM PTH enhanced ^{45}Ca uptake in the rat intestine. Picotto, Massheimer, and Boland (1997) similarly revealed a stimulatory effect of PTH on calcium influx into the rat duodenal cells. This direct effect of PTH on enterocytic calcium influx is potently blocked by L-type calcium channel blockers, verapamil and nitrendipine (Picotto et al., 1997), suggesting that PTH induces calcium entry via L-type Ca_v. The direct stimulatory effect of PTH on intestinal calcium transport was also demonstrated in other species, e.g., chick and sea bream (Fuentes, Figueiredo, Power, & Canário, 2006; Nemere & Norman, 1986).

At the cellular and molecular levels, several lines of evidence have revealed the expression of PTH/PTH-related peptide (PTHrP) receptor transcripts in nonclassical PTH target tissues, e.g., blood vessels, adrenal gland, lung, liver, and intestine (Li et al., 1995; Ureña et al., 1993). Li et al. (1995) by using RT-PCR and Northern blot analysis reported that PTH/PTHrP receptor transcripts were widely expressed in the rat small intestine as well as in rat and human intestinal cell lines, such as IEC-6 and LoVo, respectively. *In situ* hybridization and immunohistochemistry showed nuclear localization of PTHrP and its receptor—which can bind to both PTH and PTHrP—in

the epithelial cells lining villus and crypts, and in smooth muscles surrounding the gut in rat (Watson et al., 2000). The presence of PTH, PTHrP, and their receptors in the same intestinal regions/cell lines, therefore, implicated the possibility of autocrine and paracrine actions of PTH and/or PTHrP (Li et al., 1995; Nemere & Larsson, 2002). Besides calciotropic action, PTH also plays an important role in regulating intestinal transport of other ions, such as phosphate and bicarbonate (Laohapitakworn, Thongbunchoo, Nakkrasae, Krishnamra, & Charoenphandhu, 2011; Nemere, 1996). By using perfused duodenal loop, PTH was found to stimulate phosphate absorption in the chick duodenum (Nemere, 1996), while an *in vitro* experiment in Caco-2 monolayer revealed that PTH rapidly and directly stimulated apical bicarbonate secretion through cystic fibrosis transmembrane conductance regulator in protein kinase A- and PI3K-dependent manner (Charoenphandhu et al., 2011; Laohapitakworn et al., 2011).

As for calcitonin, although it is one of the major calcium-regulating hormones, little is known regarding its effects on intestinal calcium absorption and the available data are controversial. Several investigators have suggested the inhibitory effect of calcitonin on intestinal calcium absorption. For instance, Olson, DeLuca, and Potts (1972) determined calcium absorption in vascularly perfused isolated rat small intestine and found that acute infusion of calcitonin immediately decreased intestinal calcium absorption. Cramer (1973), on the other hand, showed that physiological and pharmacological doses of calcitonin did not alter calcium absorption in rat. A study of the calcitonin effect on intestinal calcium absorption in adult Merino sheep found that intravenous infusion of high-dose calcitonin initially increased calcium absorption associated with hypocalcemia, followed by a marked reduction in calcium absorption after a delay of few days (Swaminathan, Ker, & Care, 1974). Chronic infusion of calcitonin (0.2 U/h for 12 days in rats) paradoxically increased plasma calcium by 50%, which likely resulted from the increased intestinal calcium absorption since this effect disappeared after removal of calcium from the diet (Jaeger, Jones, Clemens, & Hayslett, 1986). However, the exact role(s) and underlying mechanism of calcitonin on intestinal calcium transport are waiting for an extensive systematic investigation.

6. NOVEL CONCEPT OF THE BONE–KIDNEY–INTESTINAL AXIS OF CALCIUM REGULATION

A prolonged increase in the duodenal calcium influx may lead to calcium-dependent inactivation of the transcellular transport process, which

appears to occur from single-channel level (e.g., closure of calcium channel) to systemic level (e.g., release of hypocalcemic hormone) in order to help restrict excessive calcium uptake into the body. The molecular mechanism of this phenomenon in the duodenal enterocytes is not well understood, but can be explained, in part, by inhibition of apical calcium entry through TRPV6 and Ca_v by ionized calcium as well as calcium-laden calcium-binding proteins, particularly calmodulin—known as calcium-dependent inactivation of calcium channels (Derler et al., 2006; Lee et al., 2006). Inhibition of $1,25(OH)_2D_3$ production by elevated plasma ionized calcium (Kopic & Geibel, 2013) after enhanced intestinal calcium absorption certainly contributes to the inactivation of transcellular calcium transport process. Moreover, certain local and systemic humoral factor(s) may negatively regulate the duodenal calcium transport in a calcium and/or $1,25(OH)_2D_3$-dependent manner.

FGF-23 has been known as osteocyte/osteoblast-derived phosphate-regulating hormone although it is also abundantly expressed in other cell types, such as kidney, brain, lung, liver, spleen, and duodenal enterocytes (Khuituan et al., 2012; Kolek et al., 2005). $1,25(OH)_2D_3$ increases FGF-23 expression in both bone and intestine (Khuituan et al., 2012; Kolek et al., 2005). In healthy individuals, an increase in serum phosphate level induces PTH and FGF-23 release. PTH and FGF-23 prevents hyperphosphatemic spikes, which could have undesirable consequences, such as ectopic calcification. The hypophosphatemic action of both hormones is by inducing phosphaturia through inhibition of NaPi-2a and -2c activities in the proximal renal tubules and by suppressing CYP27B1, an enzyme for $1,25(OH)_2D_3$ synthesis (Jüppner, 2011; Kolek et al., 2005). FGF-23 not only controls phosphate homeostasis but also acts as a calcium-regulating hormone. Shimada et al. (2005) have demonstrated using VDR knockout mice that calcium is a potent stimulator of FGF-23 production via a VDR-independent pathway. In normal diet-fed VDR knockout mice, serum FGF-23 levels are very low, whereas calcium supplement significantly increases both serum FGF-23 and mRNA expression in bone. Furthermore, in a study using PTH knockout and PTH/CaSR double knockout mice, FGF-23 level was associated with interactions between serum calcium × phosphorus products and threshold levels for calcium and phosphorus (Quinn et al., 2013). Specifically, the calcium-mediated increase in serum FGF-23 level required a threshold phosphate level of 5 mg/dL (i.e., serum phosphate >5 mg/dL), whereas the phosphate-induced increase

in serum FGF-23 required serum calcium levels of >8 mg/dL. Serum FGF-23 begins to increase exponentially when the calcium × phosphorus products were >50 mg^2/dL^2 (Quinn et al., 2013). The aforementioned findings have indicated that the regulation of FGF-23 by calcium and phosphate is fundamentally important in coordinating the serum levels of both mineral ions, and vice versa, to ensure that the calcium × phosphate product is maintained within a normal range (Quinn et al., 2013).

Regarding the calcium-regulating action of FGF-23, our laboratory has demonstrated, for the first time, a novel role of FGF-23 as a negative feedback regulator for the 1,25(OH)$_2$D$_3$-enhanced duodenal calcium absorption in mice (Khuituan et al., 2012, 2013). This finding provides an alternative explanation of how the duodenal enterocytes restrict excessive calcium transport and thus prevent lethal hypercalcemia. Therein, mice injected with 1 μg/kg 1,25(OH)$_2$D$_3$ once a day for 3 days showed an approximately 1.8-fold increase in duodenal calcium transport, which was completely abolished by concurrent intravenous injection of 140 μg/kg FGF-23 (Khuituan et al., 2012). FGF-23 directly inhibited the 1,25(OH)$_2$D$_3$-induced calcium transport via MAPK/ERK, p38 MAPK, and PKC signaling pathways (Khuituan et al., 2012), and perhaps indirectly via renal inhibition of 1,25(OH)$_2$D$_3$ synthesis (Shimada et al., 2005). The observed action of FGF-23 markedly affected both paracellular and transcellular transport, the latter of which apparently resulted from the downregulation of calcium transporter expression, i.e., TRPV5, TRPV6, and calbindin-D$_{9k}$ (Khuituan et al., 2012). As for the paracellular pathway, FGF-23 modestly but significantly decreased the 1,25(OH)$_2$D$_3$-induced paracellular calcium flux and calcium permeability (Khuituan et al., 2013). FGF-23 is, therefore, recognized as a part of the bone–kidney–intestinal axis, as depicted in Fig. 4.

In addition, the calcium-regulating role of FGF-23 may shed a new light on the search for a biomarker of body calcium status and efficiency of oral calcium supplementation. Specifically, positive calcium balance or efficient calcium supplement should be accompanied by a relatively high circulating level of FGF-23. Up until now, clinicians often use plasma levels of calcium, inorganic phosphate, PTH, 25(OH)D$_3$, 1,25(OH)$_2$D$_3$, and bone turnover markers (e.g., C-telopeptide of type I collagen) to represent body calcium status. Other promising candidates as indicators of calcium deficiency and calcium intake in humans include urinary concentrations of pseudouridine and citrate as revealed by a metabonomics analysis (Wang et al., 2013).

7. VITAMIN D-INDEPENDENT INTESTINAL CALCIUM TRANSPORT

Although $1,25(OH)_2D_3$ is of the utmost importance for avian and mammalian intestinal calcium absorption, the vitamin D-independent calcium transport does have considerable impact under certain conditions (e.g., young growing period) as well as in some mammalian species (e.g., underground mole-rats). In other words, the duodenal calcium absorption sometimes requires a different set of calciotropic hormones independent of $1,25(OH)_2D_3$. In some circumstances, such as pregnancy and lactation, $1,25(OH)_2D_3$ is not the main calciotropic hormone, but it exerts a permissive action or works cooperatively with other hormones to regulate calcium metabolism.

7.1 Calcium absorption in neonatal period

In 14-day-old suckling rats, the duodenal calcium absorption that mostly occurs through the paracellular (nonsaturable) pathway is similar under vitamin D-replete and vitamin D-deficient conditions, indicating the vitamin D-independent nature of neonatal calcium transport (Dostal & Toverud, 1984). The vitamin D-dependent calcium absorption is usually seen after weaning (~18- to 21-day-old rats) (Dostal & Toverud, 1984; Halloran & DeLuca, 1980). Interestingly, Halloran and DeLuca (1980) demonstrated that intrajugular injection of $1,25(OH)_2D_3$ enhanced neither passive nor active calcium absorption in vitamin D-deficient suckling rats (14 days postpartum), but markedly stimulated active calcium absorption in weaning rats (28 days postpartum). An investigation on vitamin D-deficient newborn piglets (<6 days postpartum) showed that duodenal active calcium transport was not different from that in vitamin D-replete piglets, and 2-day $1,25(OH)_2D_3$ supplementation could not enhance calcium absorption (Schroeder, Dahl, & Breves, 1998). Since a number of nutrients (e.g., casein phosphopeptides, lactose, glucose, and galactose) and bioactive peptides (e.g., prolactin, IGF-1, calcitonin, and PTHrP)—all of which are also present in milk—have been reported to augment calcium transport (Kocián, Skála, & Bakos, 1973; Pahuja & DeLuca, 1981; Schuette, Knowles, & Ford, 1989; Zhou, Nemere, & Norman, 1992), these milk ingredients may be alternative factors that stimulate duodenal calcium absorption in the neonates. Certain milk peptides, especially prolactin, may be able to exert its calciotropic action from the blood side after being

absorbed as a whole peptide in newborn animals (Gonnella, Harmatz, & Walker, 1989; Whitworth & Grosvenor, 1978).

7.2 Calcium absorption in pregnant and lactating periods

During pregnancy, the duodenal calcium absorption is markedly stimulated to supply calcium for the development of fetus and mineral accretion in maternal bone, the latter of which serves to expand the calcium storage pool in bone that is later resorbed during lactation, a period of high-calcium demand for milk production (Charoenphandhu et al., 2010). Calcium hyperabsorption persists until the end of lactation (Charoenphandhu et al., 2010). Bone is also an important source of calcium for lactogenesis, particularly when dietary calcium intake is inadequate (Bowman & Miller, 2001; Kovacs, 2005). It has been known for several decades that the intestinal calcium absorption during pregnancy and lactations are independent of vitamin D (Pahuja & DeLuca, 1981). The duodenal TRPV6 mRNA expression and calcium transport are significantly increased in pregnant VDR-null mice, similar to those found in wild-type mice (Fudge & Kovacs, 2010). Although the transcellular active calcium transport does occur in vitamin D-deficient dams, lactating rodents sometimes show an elevated 1,25(OH)$_2$D$_3$ level (Douard et al., 2012), which is capable of potentiating the action of other calciotropic hormone (e.g., prolactin), and may also be essential for calcium mobilization into milk (Ajibade et al., 2010). Brommage, Baxter, and Gierke (1990) estimated ~23% of milk calcium transfer being vitamin D-dependent.

Our group has elaborated that the pituitary hormone prolactin is the salient calciotropic hormone for pregnancy and lactation (for reviews, please see Charoenphandhu et al., 2010; Wongdee & Charoenphandhu, 2013). Long-term hyperprolactinemia (~75–200 ng/mL; baseline level in nonpregnant rat ~7–10 ng/mL), which occurs physiologically during these reproductive periods, leads to upregulation of transcellular calcium transporter expression (e.g., TRPV5, TRPV6, calbindin-D$_{9k}$, and PMCA$_{1b}$) as well as the expression of tight junction genes, thereby increasing both transcellular and paracellular duodenal calcium transport (Charoenphandhu et al., 2009; Teerapornpuntakit et al., 2014). During lactation, the suckling-induced prolactin surge (~400–800 ng/mL) further increases the duodenal calcium flux to compensate for calcium loss in milk, possibly by sensitizing the Ca$_v$1.3-mediated transcellular calcium transport (Charoenphandhu et al., 2009; Nakkrasae, Thongon, Thongbunchoo,

Krishnamra, & Charoenphandhu, 2010; Suntornsaratoon et al., 2014). Besides $1,25(OH)_2D_3$ and prolactin, other endocrine factors with elevated levels during pregnancy and/or lactation, especially placental lactogen, estrogen, calcitonin, and PTHrP, may also contribute to the enhanced calcium absorption (Colin et al., 1999; Fuentes et al., 2006; Jaeger et al., 1986; Takeuchi, Morikawa, Ueda, & Mochizuki, 1988).

7.3 Calcium absorption in naturally vitamin D-impoverished mammals

Not all adult mammals require vitamin D for intestinal calcium absorption. For example, the subterranean mole-rats (such as *Cryptomys damarensis* and *Heterocephalus glaber*) live in an underground maze without exposure to sunlight and usually consume vitamin D-depleted herbivorous diet. Therefore, they are naturally vitamin D-deficient with undetectable $25(OH)D_3$ and remarkably low $1,25(OH)_2D_3$ levels (Pitcher & Buffenstein, 1995). However, the mole-rats have efficient calcium absorption despite unclear cellular and molecular mechanisms. The duodenal calcium absorption is paracellular, whereas the transcellular active calcium absorption is found only in the cecum and proximal colon (Pitcher & Buffenstein, 1994). The 3-day oral vitamin D supplementation is not able to enhance calcium absorption in either foregut or hindgut of mole-rats (Pitcher & Buffenstein, 1995). The subterranean mole-rats appear to compensate for the vitamin D-impoverished state by depositing extra calcium in the chisel tooth and consuming calcium-rich plants (Buffenstein, Laundy, Pitcher, & Pettifor, 1995).

8. CONCLUSION AND PERSPECTIVES

Duodenal calcium transport exhibits redundancy in term of calcium transporter expression, possibly to ensure that dietary calcium uptake is always adequate to meet calcium demand. For example, a number of duodenal transporters, e.g., TRPV5, TRPV6, and $Ca_v1.3$, work cooperatively and complementarily for apical calcium uptake. The redundancy thus explains as to why the transcellular active calcium transport is not completely abolished by TRPV6 knockout, calbindin-D_{9k} knockout, or TRPV6/calbindin-D_{9k} double knockout (Benn et al., 2008). Our group has also reported that TRPV5/TRPV6 double knockdown is unable to nullify apical-to-basolateral calcium transport across human intestinal epithelium-like Caco-2 monolayer (Nakkrasae et al., 2010). Calbindin-D_{28k}-null mice manifest normal bone and teeth development, suggesting no disturbance to

calcium transport in the absence of calbindin-D_{28k} (Turnbull et al., 2004). Therefore, further investigation is required to demonstrate contribution of each transporter to the total calcium absorption.

The expressions and/or activities of most proteins related to intestinal calcium absorption in humans and rodents (i.e., TRPV5, TRPV6, calbindin-D_{9k}, $PMCA_{1b}$, NCX1, and several claudins) are dependent on 1,25$(OH)_2D_3$. Both transcellular (saturable) and paracellular (nonsaturable) calcium transport are thus markedly stimulated by 1,25$(OH)_2D_3$. Unlike the well-established 1,25$(OH)_2D_3$-induced transcellular mechanism, little is known regarding the cellular and molecular mechanisms of the 1,25 $(OH)_2D_3$-induced paracellular calcium transport, especially in humans and nonhuman primates. Furthermore, the vitamin D-independent calcium absorption is evident under certain conditions, such as neonatal period, but responsible endocrine and paracrine regulators remain to be investigated. Species bias—i.e., information is mainly from human, rodent, and chick—precludes drawing a solid conclusion that 1,25$(OH)_2D_3$ is the utmost important regulator of intestinal calcium transport in avian and mammalian species. Data from foregut fermenters (e.g., dairy cow), horses, and rabbits are scant although some species (e.g., rabbits) have been postulated to possess similar calcium metabolism and 1,25$(OH)_2D_3$-dependent intestinal calcium absorption as in humans and rodents (Norris, Pettifor, Gray, & Buffenstein, 2001).

Indeed, duodenum is not the only site of calcium absorption, but it has been intensively studied due to its vigorous active calcium transport, calcium transport efficiency, and vitamin D responsiveness as well as abundance of calcium transporter expression (Lee et al., 1981; Teerapornpuntakit, Dorkkam, Wongdee, Krishnamra, & Charoenphandhu, 2009; Wasserman, 2004). Most absorbable dietary calcium (>80% of absorbed calcium) is transported by the jejunum and ileum (lower transport rate but greater sojourn time of 40–50 min compared to the duodenal sojourn time of 2–3 min) using the paracellular passive transport rather than the transcellular active transport (Duflos et al., 1995; Wasserman, 2004). In normal rats, the cecum—the most proximal part of the large intestine—absorbs calcium at a higher rate than the duodenum (>2-fold), and surgical removal of cecum (cecectomy) leads to fecal calcium wasting, compensatory increase in colonic calcium transport, and osteopenia (Charoenphandhu, Suntornsaratoon, Jongwattanapisan, Wongdee, & Krishnamra, 2012; Jongwattanapisan et al., 2012). Calcium transport in the distal small intestine and large intestine (cecum and colon) is also 1,25$(OH)_2D_3$-dependent (Grinstead, Pak, &

Krejs, 1984; Karbach, 1991; Karbach & Feldmeier, 1993; Lee et al., 1981). However, exogenous $1,25(OH)_2D_3$ cannot further increase cecal calcium transport (Karbach & Feldmeier, 1993). Intraperitoneal $1,25(OH)_2D_3$ injection was found to similarly increase dietary calcium uptake in intact and cecectomized vitamin D-replete male rats (Brommage, Binacua, & Carrié, 1995), suggesting that $1,25(OH)_2D_3$ is not the main regulator of cecal calcium absorption. Hence, the underlying molecular mechanisms and endocrine regulatory axes as well as paracrine/autocrine regulation of calcium absorption in the intestinal segments distal to the duodenum are important issues worth exploring.

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