Apical Entry Channels in Calcium-Transporting Epithelia

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The identification of the apical calcium channels CaT1 and ECaC revealed the key molecular mechanisms underlying apical calcium entry in calcium-transporting epithelia. These channels are regulated directly or indirectly by vitamin D and dietary calcium and undergo feedback control by intracellular calcium, suggesting their rate-limiting roles in transcellular calcium transport.

Calcium is the fifth most abundant element in the earth’s crust and the most abundant cation in the human body. A 70-kg person possesses roughly 1 kg of calcium. Of this, 99% is in the mineral phase of the bones and teeth and 1% is in the extracellular and intracellular fluids. The ionic form of calcium serves as a universal intracellular messenger to modulate many processes, such as neurotransmission, muscle contraction, and secretion. Since all of the calcium in our bodies is ultimately absorbed from the diet, intestinal calcium absorption is an important determinant of calcium homeostasis.

Two pathways are responsible for calcium entry into the body: the paracellular and the transcellular pathways. In the paracellular pathway, calcium enters through tight junctions located between the epithelial cells, whereas in the transcellular pathway, calcium enters across the apical and basolateral membranes of a cell, a process that requires an apical calcium entry channel, an intracellular calcium binding protein called calbindin (calbindin D28k), and calcium pump (plasma membrane calcium ATPase (PMCA1b)). Similar mechanisms of transcellular calcium transport exist in the renal distal tubules and the syncytium of the placenta. Recently, our understanding of the paracellular and transcellular pathways has been advanced by the identification of the channels for both pathways. With respect to the paracellular pathway, claudins are thought to form part of a paracellular channel-like structure. In the present review, we will focus on two recently identified apical calcium channels, calcium transport protein subtype 1 (CaT1) and epithelial calcium channel (ECaC), in the transcellular pathway. (Basic information about these two channels is available in Table 1.) CaT1 was first identified in the rat intestine (12) and ECaC in the rabbit kidney (4). Several other names (CaT-L/ECaC2 and CaT2/ECaC1) were also used for CaT1 and ECaC, respectively (Table 1). Recently, a human gene nomenclature was introduced for cation channels related to the Drosophila transient receptor potential (TRP) channels. Since CaT1 and ECaC are distally related to the TRPs, the human genes for CaT1 and ECaC were termed TRPV6 and TRPV5, respectively (“V” denotes “vanilloid receptor (VR1)-related”).

The human CaT1 and ECaC share 75% amino acid sequence identity. They are encoded by two genes juxtaposed on human chromosome 7q33–35. One is likely duplicated from the other during the course of evolution. CaT1 and ECaC are calcium-selective members of a cation channel subfamily consisting of six members (10). The other four members of the family encode nonselective cation channels that serve as sensors. Three of them (VR1, VRL-1, and TRPV3) are heat sensors that are expressed in sensory neurons and skin, and another one (OTRPC4/VR-OAC/VRL-2/TRP12) is an osmoreceptor expressed in the central nervous system and the kidney. In contrast, CaT1 and ECaC, which share ~30% amino acid identity with the other channels of the family, serve as apical transporters in calcium-transporting epithelia.

Apical localization in calcium-transporting epithelia

The intestine, kidney, and placenta are three major organs involved in calcium transport by calcium absorption from the diet, renal tubular calcium reabsorption, and calcium transport from the maternal to the fetal circulation, respectively. CaT1 appears to be the major apical calcium entry channel in the intestine and placenta, whereas ECaC is largely kidney specific (Fig. 1). In addition, the distribution of CaT1 is not restricted to calcium-transporting epithelia. CaT1 has been detected in many other tissues, especially in exocrine tissues (see Table 1).

The expression of CaT1 in the apical membrane of both mouse and human intestine has recently been demonstrated using an anti-CaT1 antibody (20). In the intestine, CaT1 is most abundantly expressed in the duodenum, although staining for CaT1 can be detected in the colon as well. In the duodenal villus, robust mRNA and protein levels were found in the well-differentiated absorptive cells in the top half of the villus (12, 20). Apical localization of ECaC was observed in rabbit, rat (3), and mouse in the distal segment of the nephron. In all of the animal models tested, ECaC is expressed in the distal segment of the distal convoluted tubule and connecting tubule (DCT-2), in the same cells that contain other machinery for cytosolic calcium transport, including calbindin D28k and the basolateral sodium/calcium exchanger 1 (NCX1). Information about the localization of ECaC protein in the human kidney has not been available so far.
In the human placenta, CaT1 expression is very high, at least 50 times higher than in duodenum and kidney. We found that CaT1 mRNA levels are 1,000 times higher than those of CaT2 (ECaC) in the human placenta (10). Expression of CaT1 in trophoblasts and syncytiotrophoblasts has been shown by in situ hybridization (17). The subcellular localization of CaT1 protein in the placenta has not been reported thus far.

Since CaT1 and ECaC share ~75% amino acid sequence identity and even higher nucleotide sequence identity (90% in some regions), artifacts in terms of their specific detection can be expected when antibodies or primers are used that have not been properly selected. Indeed, discrepancies exist in the literature regarding the distributions of CaT1 and ECaC. In rat, Northern blot analysis using CaT2/ECaC probes under stringent conditions picked up CaT1 signals in the duodenum, whereas only CaT1 and not ECaC is abundantly expressed in the intestine (11). The mRNA signal of CaT1 is comparable in human kidney cortex and outer medulla, whereas the level of ECaC in the cortex is about three times that in the outer medulla (unpublished observations). We obtained four clones each for CaT1 and ECaC (CaT2) from the screening of a human kidney library. Northern blot also showed that CaT1 is abundantly expressed and ECaC levels are undetectable (16) or very low (15). We hardly detected any ECaC signals using <40 cycles in real-time PCR in mouse duodenal samples. In mouse kidney, on the other hand, ECaC and CaT1 are both expressed (15, 16) and the CaT1 mRNA level is ~10% of that of ECaC (unpublished data). In the rat, CaT1 and ECaC are exclusively expressed in the intestine and kidney, respectively (11). In the rabbit, ECaC was reported to be expressed in both duodenum and kidney (4). Currently, there are no data available about CaT1 expression in the rabbit. In human small intestine, CaT1 but not ECaC can be detected; to our surprise, CaT1 mRNA levels are ~10 times those of ECaC in human kidney, as determined by quantitative PCR (10). We also found that the mRNA signal of CaT1 is comparable in human kidney cortex and outer medulla, whereas the level of ECaC in the cortex is about three times that in the outer medulla (unpublished observations). We obtained four clones each for CaT1 and ECaC (CaT2) from the screening of a human kidney library. Northern blot also showed that CaT1
expression is higher in human kidney than in small intestine. In the intestinal specimen, however, the CaT1 mRNA level might have been decreased by a high-calcium diet or other factors, or the specimen might have been from a more distal portion that expresses less CaT1. It is conceivable that CaT1 is much more important in renal calcium handling in humans than in mice.

Function as facilitative transporter

In the intestinal lumen, calcium concentration varies but is often in the millimolar range. Inside the absorptive cell, the calcium concentration is ~100 nM. This gives an ~10,000-fold concentration gradient across the apical membrane, and therefore transport of calcium into the cell does not require the consumption of metabolic energy. Since calcium absorption in the intestine and reabsorption in the kidney are continuous processes, the channels at the apical membranes will be continuously active without physical stimuli, such as a change in membrane potential or channel agonists. Therefore, unlike the ligand-gated or voltage-gated calcium channels in excitable cells such as neurons and myocytes, the protein expected to mediate apical entry of calcium in the duodenum or distal tubules should have the following properties: 1) ability to mediate passive transport of calcium down the electrochemical gradient, not coupled to energy consumption; 2) being constitutively active, not voltage- or ligand-gated; 3) being selective for calcium over magnesium, because the two pathways for calcium and magnesium absorption are different; 4) having a $K_m$ value in the millimolar or submillimolar range; and 5) having a feedback mechanism to prevent toxic accumulation of free cytosolic calcium in the cell.

The properties predicted above have all been observed for the CaT1 and ECaC channels. When expressed in the Xenopus laevis oocytes, these channels show constitutive activity and saturation kinetics. The $K_m$ values are 0.44 and 0.25 mM, respectively, for rat and human CaT1 (12, 13) and 0.66 and 0.2 mM, respectively, for rat and rabbit ECaC (4, 11). Calcium influx mediated by CaT1 does not appear to be coupled to sodium, chloride, or protons. The transport activities of both proteins are sensitive to pH, and calcium uptake activity increases at alkaline pH. Like most electrogenic transport processes, the current-voltage relationships of both channels suggest that a hyperpolarizing potential favors calcium influx through both channels. Both CaT1 and ECaC are permeable to Ba$^{2+}$ and Sr$^{2+}$ but not Mg$^{2+}$. The macroscopic properties of these channels expressed in X. laevis oocytes indicate that these proteins work as facilitative transporters that constantly transport substrate down the concentration gradient with saturation kinetics but without obvious gating mechanisms.
activity of CaT1 was upregulated when the oocytes were injected with EGTA to buffer the intracellular calcium, indicating that intracellular calcium inhibits CaT1 activity.

As members of the family of six membrane-spanning channels, both CaT1 and ECaC show single-channel activity in the absence of divalent cations in the extracellular solution. Using sodium as a charge carrier, single-channel conductances are 42 pS for rat CaT1 (19) and 78 pS for rabbit ECaC (9). However, single-channel activity has not been reported for both channels using divalent cations as charge carriers. Both channels show high selectivity for calcium, with calcium-to-sodium permeability ratios of >100.

**Regulation by vitamin D and calcium intake**

In our first set of studies (12), we failed to observe an upregulation of CaT1 mRNA in rat duodenum when a single dose of 1,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$] was administered to normal rats. In a study using human duodenal biopsies from 20 normal subjects (Barley et al., 2001), CaT1 mRNA levels were not significantly correlated with vitamin D metabolites but were moderately correlated with calbindin D$_{9k}$ and more strongly with PMCA$_1$ mRNA levels. It was noted that the CaT1 mRNA level in the duodenal biopsies varied considerably; the results may have been affected considerably by uncontrolled calcium intake, a factor that was later found to have a significant impact on CaT1 expression (15). The significance of the effects of 1,25(OH)$_2$D$_3$ on CaT1 expression was first explored in a human intestinal cell line Caco-2, which was used for calcium transport studies by R. J. Wood and colleagues (18). Caco-2 cells express low but detectable levels of CaT1 mRNA in the absence of 1,25(OH)$_2$D$_3$ treatment. CaT1 mRNA expression was rapidly upregulated, with a 4-fold increase at 4 h and a 10-fold increase at 24 h after treatment with 1,25(OH)$_2$D$_3$ at $10^{-7}$ M. An interesting finding is that the increase in CaT1 mRNA expression preceded by several hours the 1,25(OH)$_2$D$_3$-mediated induction of calbindin D$_{9k}$ mRNA. Using Caco-2 clonal cell lines, Fleet et al. (1) showed that a high level of calbindin D$_{9k}$ was not sufficient to produce high net apical-to-basolateral calcium transport. Rather, the induction of CaT1, and to a lesser extent PMCA$_1$ levels, correlated with net apical-to-basolateral calcium transport. The observation that CaT1 mRNA is subject to vitamin D regulation was soon confirmed in vivo by a study of Van Cromphaut et al. (15) using vitamin D receptor (VDR)-null mice. CaT1 mRNA in the duodenum was reduced by >90% in two VDR-null strains on a normal-calcium diet; calbindin D$_{9k}$ expression was decreased only in one strain, whereas PMCA$_{1b}$ mRNA expression was normal in both strains. All of the studies (1, 15, 18) show that the apical entry channel CaT1 mRNA is most robustly regulated by vitamin D among the mRNAs for the proteins involved in transcellular transport, arguing that the apical entry step is the rate-limiting step of calcium transport instead of the intracellular diffusion step, which was previously considered to be the critical step for vitamin D-regulated calcium transport.

Vitamin D depletion reduced the expression of ECaC in rat kidney, and repletion of vitamin D restored its expression at both mRNA and protein levels (3). However, in the VDR-null mice, ECaC mRNA expression was not decreased; as a matter of fact, it was slightly increased at the mRNA level in the two strains of VDR-null mice on a normal diet (15). In mice lacking 25-hydroxyvitamin D$_3$-1$_{α}$-hydroxylase (1$_{α}$-OHase), a key enzyme for 1,25(OH)$_2$D$_3$ production, substantial decreases in serum calcium level as well as the mRNA and protein levels of ECaC, calbindins, and NCX1 were found (2). High calcium intake restored the serum calcium level as well as levels of ECaC, calbindin D$_{28k}$, and NCX1, with the exception of calbindin D$_{9k}$ (2). It appears that the level of expression of ECaC is positively regulated by the calcium load reflected by the serum calcium level (2). Together, the observation that ECaC is regulated by vitamin D might be due to a decrease in the serum calcium level or through its nongenomic effects. Unlike the duodenum in the VDR-null mouse, in which only CaT1 was severely reduced compared with calbindin D$_{9k}$ and PMCA$_{1b}$, in the 1$_{α}$-OHase-null mouse on a normal diet, coordinated changes of ECaC, calbindins D$_{9k}$ and D$_{28k}$, and NCX1 were observed. This indicates that the transcellular calcium transport in the distal tubules is a coordinated process involving all of the participating proteins.

It seems that the level of intestinal CaT1 depends on the body's need for calcium, which is reflected by the serum calcium level and 1,25(OH)$_2$D$_3$ production. Therefore, both low calcium intake and 1,25(OH)$_2$D$_3$ administration increase the level of intestinal CaT1. In contrast, the renal ECaC level is largely dependent on the filtered calcium load or serum calcium level. ECaC’s role is to make sure that calcium is not wasted, so the more calcium reaches the distal nephron, the more ECaC is expressed to reabsorb the calcium. This mechanism may prevent the formation of kidney stones. When the serum calcium level is low, increased ECaC expression would also be expected to preserve more calcium. The level of ECaC expression may be differentially regulated in different ranges of the serum calcium level. The apparent increase in ECaC induced by 1,25(OH)$_2$D$_3$ may, in part, reflect the increase in the calcium load in the kidney as a result of increased expression of intestinal CaT1.

**Association of calcium transport activity with the CaT1 expression level**

In the VDR-null mouse on a normal diet, a threefold decrease in calcium absorption was found in both the Leuven and Tokyo strains (15) compared with control mice, although it should be noted that part of the absorption may be through the paracellular pathway, presumably independent of VDR. Among the three proteins that mediate calcium transport, a threefold decrease in the calbindin D$_{9k}$ protein was seen in the

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Tokyo strain but not in the other strain, and the PMCA1b mRNA was not significantly altered. In contrast, the CaT1 mRNA level was decreased to <10% in both strains. Therefore, it appears that the decreases in calcium absorption are a result of the decreases of CaT1 expression rather than in calbindin D9k and PMCA1b. This suggests once again that CaT1-mediated calcium entry is the rate-limiting step of transcellular calcium transport in the duodenum.

In another study (14), using mice with disruption of the Na+/P,-cotransporter gene Npt2, which exhibit increases in their levels of 1,25(OH)2D3 and serum calcium as well as in intestinal calcium absorption, the CaT1 mRNA level was increased, as was calbindin D9k. In mice with L-type Ca-channel modulators Bay K 8644, nitrendipine but was inhibited by ruthenium red with an IC50 of 70 (Moreau et al., 2001). The above observations indicate that CaT1 is the major calcium uptake channel in trophoblasts.

**Regulation of channel activity by intracellular calcium**

Calcium is an important intracellular messenger, and a sustained increase of intracellular calcium may cause cell death. Calcium-transporting epithelial cells have developed mechanisms for avoiding toxic levels of calcium while maintaining an efficient calcium flow from the apical to the basolateral membrane. One way is to express high levels of calcium-binding proteins (calbindin D9k, calbindin D28k, or both) to buffer the intracellular calcium; another is to turn off the apical calcium entry channel when a dangerous local rise of calcium is occurring—a calcium feedback inhibition mechanism. Both CaT1 and ECaC exhibit calcium-dependent inactivation (7). CaT1 shows a fast phase (within 50 ms) and a slow phase of inactivation (over a period of ~1 s). The slow phase involves direct binding of calmodulin to the CaT1 COOH-terminal region (7). CaT1's calmodulin binding site is conserved among different species but is not present in the same region of ECaC (7). Calcium-dependent binding of calmodulin to CaT1 inactivates the CaT1 channel. A protein kinase C site is present in the calmodulin binding site in human CaT1 but not in other species studied so far. The phosphorylation of this protein kinase C site prevents calmodulin binding, thereby maintaining the activity of CaT1 so as to allow more calcium to enter the cell (7). ECaC shows essentially no fast phase of inactivation compared with CaT1. The first intracellular loop of CaT1 is involved in the fast phase of inactivation (8), and this sequence is not conserved between CaT1 and ECaC. Shifting the first intracellular loop of CaT1, which accounts for the fast phase of inactivation of CaT1, with the same loop of ECaC transfers the fast inactivation kinetics from CaT1 to ECaC (8). Of note, the first intracellular loop is entirely encoded by one exon—an example of a single exon endowing a distinct, functional feature.

It is interesting that CaT1 but not ECaC has a fast inactivation phase. The calcium concentration in the intestinal lumen can vary greatly. The sudden appearance of a high calcium concentration can occur as a result of calcium entry in response to 1,25-dihydroxy vitamin D3, as seen in the presence of 1,25(OH)2D3) (left), CaT1 and calbindin D9k levels are low. When CaT1 is open, calcium entry will increase the local calcium concentration beneath the membrane. Increased local calcium concentration results in calmodulin binding to CaT1 and shutting off the channel. In the presence of 1,25(OH)2D3 (right), however, the levels of both CaT1 and calbindin D9k are increased. Although the amount of calcium entry is increased, the local free calcium level is kept low as calbindin D9k binds and buffers calcium. Thus calcium flow through the apical membrane is increased in the presence of 1,25(OH)2D3 because the number of CaT1 channels increases and the feedback inhibition by intracellular calcium is released by the calcium-buffering effect of calbindin D9k.

**FIGURE 2.** Coordinated model of the mechanism by which CaT1 and calbindin D9k increase calcium entry in response to 1,25-dihydroxy vitamin D3 (1,25(OH)2D3). In the absence of 1,25(OH)2D3, CaT1 and calbindin D9k levels are low. When CaT1 is open, calcium entry will increase the local calcium concentration beneath the membrane. Increased local calcium concentration results in calmodulin binding to CaT1 and shutting off the channel.

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level in the intestinal lumen could be disastrous if there were no fast inactivation mechanism in CaT1. In contrast, in the renal distal tubule, the calcium concentration is relatively constant and will never reach a level as high as that faced by the intestinal CaT1 from time to time. Therefore, the absence of the fast inactivation mechanism in ECaC does not affect its function as a renal calcium entry channel.

The feedback inhibition mechanism ensures that CaT1-expressing cells are protected from calcium overload; however, this mechanism will also limit the calcium entry step because an increase in calcium influx will increase the local calcium concentration beneath the apical membrane, which then inactivates the channel. CaT1 activity is inversely proportional to the intracellular calcium level in cells whose intracellular calcium concentration is controlled with a calcium-EGTA buffer (Bödding et al., 2002). To increase the calcium flow across the cell, the calcium binding protein calbindin D$_{9k}$ serves to buffer the local calcium and in turn reduces the calcium feedback inhibition of the channel. Thus a coordinated increase in the expression of both the apical channel and calbindin is necessary to achieve maximal calcium influx from the apical side (Fig. 2). The observation of a lag period in the calbindin response to vitamin D relative to that of CaT1 (18) suggests that the increase in calbindin is an amplifying mechanism that increases calcium influx both by relieving feedback inhibition of the apical entry step and by facilitating diffusion of calcium from the apical to the basolateral membrane. The lag in calbindin production in response to vitamin D administration suggests that the increase in intracellular calcium as a result of CaT1 expression could contribute to calbindin induction.

In summary, studies on the localization, function, and regulation of CaT1 and ECaC indicate that they are the major players in the apical entry step of transcellular calcium transport for intestinal absorption and renal reabsorption of calcium, respectively. The calcium entry step is a critical step in the transport process, and the proteins have intrinsic feedback mechanisms as well as that caused by CaT1’s interaction with calmodulin. This step is also subject to exquisite control at levels of expression of the proteins in response to the body’s calcium status. The intestinal expression of CaT1 is responsive to levels of vitamin D and calcium intake, whereas the renal expression of ECaC appears to be regulated by the level of calcium but not directly through a genomic action of vitamin D.

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