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Topical Review

Epithelial Ca^{2+} entry channels: transcellular Ca^{2+} transport and beyond

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The recently discovered apical calcium channels CaT1 (TRPV6) and ECaC (TRPV5) belong to a family of six members called the 'TRPV family'. Unlike the other four members which are nonselective cation channels functioning as heat or osmolarity sensors in the body, CaT1 and ECaC are remarkably calcium-selective channels which serve as apical calcium entry mechanisms in absorptive and secretory tissues. CaT1 is highly expressed in the proximal intestine, placenta and exocrine tissues, whereas ECaC expression is most prominent in the distal convoluted and connecting tubules of the kidney. CaT1 in the intestine is highly responsive to 1,25-dihydroxyvitamin D_3 and shows both fast and slow calcium-dependent feedback inhibition to prevent calcium overload. In contrast, ECaC only shows slow inactivation kinetics and appears to be mostly regulated by the calcium load in the kidney. Outside the calcium-transporting epithelia, CaT1 is highly expressed in exocrine tissues such as pancreas, prostate and salivary gland. In these tissues it probably mediates re-uptake of calcium following its release by secretory vesicles. CaT1 also contributes to store-operated calcium entry in Jurkat T-lymphocytes and prostate cancer LNCaP cells, possibly in conjunction with other cellular components which link CaT1 activity to the filling state of the calcium stores. Finally, CaT1 expression is upregulated in prostate cancer and other cancers of epithelial origin, highlighting its potential as a target for cancer therapy.

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As the most abundant cation in the human body, calcium repesents a key component of the mineral phase of teeth and bones and is maintained nearly constant in the blood and extracellular fluids (Brown, 1991). In its ionized form, calcium (Ca^{2+}) serves as an intracellular messenger that participates in muscle contraction, neurotransmission, enzyme and hormone secretion, as well as many other biological processes, e.g. cell cycle regulation and programmed cell death (Clapham, 1995; Berridge *et al.* 2000). In spite of the fact that all of the calcium in our bodies is derived from our diet, over the past two decades, the detailed molecular mechanism of Ca^{2+} entry into the body has received little attention compared with that directed at understanding Ca^{2+} signalling. Nevertheless, our understanding of Ca^{2+} absorption has been advanced by the identification of the apical Ca^{2+} entry channels in Ca^{2+}-transporting epithelia, CaT1 from the intestine (Peng *et al.* 1999) and ECaC from the kidney (Hoenderop *et al.* 1999). In addition to mediating luminal calcium uptake into epithelial cells, CaT1 also exhibits properties similar to those of the Ca^{2+} release-activated Ca^{2+} channels (Yue *et al.* 2001), and contributes to the store-operated calcium entry in the human Jurkat T-lymphocytes (Cui *et al.* 2002) and LNCaP prostate cancer cells (Vanden Abeele *et al.* 2003). Thus, CaT1 is a link between Ca^{2+} transport and Ca^{2+} signalling. CaT1 expression is up-regulated in prostate carcinoma (Peng *et al.* 2001*b*; Wissenbach *et al.* 2001) and other cancers of epithelial origin (Zhuang *et al.* 2002), suggesting an important pathological role for CaT1. In this review, we will summarize the biology of the recently identified Ca^{2+} entry channels CaT1 and ECaC.

The cation channels of the *TRPV* family function as sensors as well as Ca^{2+} transporters

CaT1 and ECaC are TRP-related, six-membrane-spanning channels, belonging to a gene subfamily comprising six members (Peng *et al.* 2001*a*). The first TRP protein was identified in *Drosophila* photoreceptor cells and the name TRP was based on the gene locus *trp*, which, when mutated, causes a transient receptor potential (light response) rather than a sustained response, thereby disrupting visual excitation downstream of IP_3 production

(Montell & Rubin, 1989). This gene subfamily was recently termed *TRPV*, with the *V* denoting 'vanilloid' because the first mammalian member of the family identified was the vanilloid receptor VR1 (Montell *et al.* 2002). The TRPV ion channels are much more similar to the *Caenorhabditis elegans* OSM-9 channel, which is required for olfaction, mechanosensation, and olfactory adaptation (Colbert *et al.* 1997). VR1 (TRPV1) (Caterina *et al.* 1997), as well as CaT1 (TRPV6) (Peng *et al.* 1999) and ECaC (TRPV5) (Hoenderop *et al.* 1999), were identified using an expression cloning approach based on their capacity to mediate an increase in intracellular Ca^{2+} in transfected cells (VR1) or Ca^{2+} influx into *Xenopus laevis* oocytes (CaT1, ECaC). The other members of the family, VRL-1/GRC (*TRPV2*) (Caterina *et al.* 1999; Kanzaki *et al.* 1999), OTRPC4/VR-OAC/VRL-2/Trp12 (*TRPV4*) (Strotmann *et al.* 2000; Liedtke *et al.* 2000; Wissenbach *et al.* 2000; Delany *et al.* 2001) and TRPV3/VRL3 (Smith *et al.* 2002; Xu *et al.* 2002; Peier *et al.* 2002), were identified based on sequence homology. All of the six human genes in the family have now been identified (Peng *et al.* 2001*a*). The *TRPV* family has two branches (Fig. 1): CaT1 and ECaC with 75.6 % amino acid identity and the other four channels with ~30 % identity to CaT1 and ECaC. CaT1 and ECaC are Ca^{2+}-selective channels and serve as apical Ca^{2+} entry mechanisms in Ca^{2+}-transporting epithelia; the other four members are nonselective channels and serve as sensors. VR1, VRL-1 and TRPV3 are heat sensors and are activated over different temperature ranges (Clapham, 2002*a*). VR1 is also ligand gated and can be activated by capsaicin (Caterina *et al.* 1997). OTRPC4 is activated under hypotonic conditions and serves as an osmoreceptor (Strotmann *et al.* 2000; Liedtke *et al.* 2000). The CaT1 and ECaC genes, *TRPV6* and *TRPV5*, respectively, are localized on chromosome 7q33–35; the genes for the three heat sensors (*TRPV1–3*) are localized in 17p11.2–13.3, while the OTRPC4 gene (*TRPV4*) is on a different chromosome (12q24.1). Thus there is a good relationship between the chromosomal localization and the functions of the genes in this family (Table 1), suggesting that gene duplication events occurred during the development of the gene family.

This group of ion channels and the other channels in the TRP supperfamily, the *TRPC* (canonical) and *TRPM* (melastatin) products (Montell *et al.* 2002), together with polycystin 2 and related proteins (products of *PKD2*,

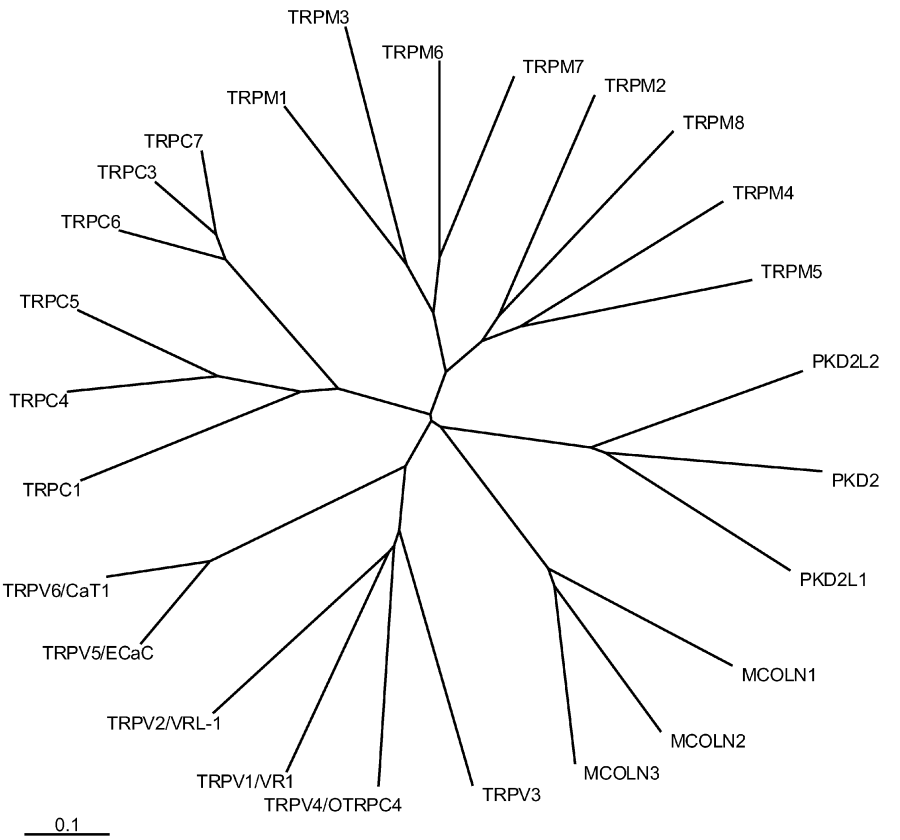

Figure 1. Phylogenetic tree of the TRP supper family and related channels

The tree was generated with the TREEVIEW program (Page, 1996) using sequence alignment produced by ClustalW at http://www.ebi.ac.uk/clustalw/#. The bar indicates 0.1 amino-acid substitutions per site. Human protein sequences were used for the alignment. Each sequence was obtained by input individual gene name in the UniGene web site: http://www.ncbi.nlm.nih.gov/UniGene/

Table 1. A family of Ca^{2+}-permeable channels encoded by *TRPV* genes

Gene	Locus #	Protein	Function	Distribution *	P_{Ca}/P_{Na}	Activation	Inhibition
TRPV1	17p13.3	VR1	Heat sensor	Trigeminal and dorsal root ganglia	9.6 (capsaicin-activated current); 3.8 (heat-activated current)	Heat (threshold 42 °C), capsaicin, resiniferatoxin, anandamide, acidic pH	Ruthenium red (IC_{50} ~0.15 μM), capsazepine, iodo-resiniferatoxin, SB-366791
TRPV2	17p11.2	VRL-1, GRC	Heat sensor	Brain, spinal cord, lung, spleen, intestine, kidney	2.9 (heat-activated current)	Heat (threshold 53 °C)	Ruthenium red (IC_{50} < 1 μM), La^{3+}, SKF 96365
TRPV3	17p13.3	VRL3, TRPV3	Heat sensor	Central nervous system, spinal cord, skin, testis	12.1 (heat-activated current)	Heat (threshold 37 °C)	Ruthenium red (IC_{50} < 10 μM)
TRPV4	12q24.1	OTRPC4 (VR-OAC, VRL2,TRP12)	Osmoreceptor	Kidney, trachea, salivary gland, fat, testis	6	Hypotonic osmolarity, 4α-phorbol didecanoate,	Ruthenium red (IC_{50} < 1 μM), La^{3+}, Gd^{3+}
TRPV5	7q35	ECaC (ECaC1, CaT2)	Apical Ca^{2+} transporter	Kidney	107	Constitutive active, low $[Ca^{2+}]_I$, alkaline pH	Ruthenium red (IC_{50} ~0.1 μM), Gd^{3+}, La^{3+}, Cd^{2+}, econazole, miconazole
TRPV6	7q33-34	CaT1 (ECaC2, CaT-L)	Apical Ca^{2+} transporter	Intestine, placenta, pancreas, prostate, salivary gland, testis, kidney	130	Constitutive active, low $[Ca^{2+}]_i$, Ca^{2+} store depletion, alkaline pH	Ruthenium red, (IC_{50} ~9 μM), Gd^{3+}, La^{3+}, Cd^{2+}, Pb^{2+}, econazole, miconazole

* Only tissues with high expression levels are listed. # Gene mapping data are based on that provided in the UniGene web site at http://www.ncbi.nlm.nih.gov/UniGene/

PKD2L and *PKD2L2* genes), the mucolipins (products of *MCOLN1-3*), and sperm-associated cation channels (products of *CATSPER1-2*), share a common six-membrane-spanning molecular architecture with hyperpolarization-activated and cyclic nucleotide-gated K^+ channels (*HCN* and *CNG* products) as well as voltage-gated and Ca^{2+}-activated K^+ channels (some *KCN* products). The *TRPV* and *TRPC* family members also have three to four ankyrin repeats in their N-terminal region. However, very little sequence identity is shared between the *TRPV* and the 'canonical' *TRPC* channels. When CaT1 is aligned with the *TRPC* family members using the BLAST engine for local alignment with the default setting, there is no significant homology between CaT1 and TRPC1 or TRPC6, and very low homology (18–24 % identities) between CaT1 and TRPC3, TRPC4, TRPC5, and TRPC7 over a stretch of 136–147 amino acids covering the putative pore region and transmembrane domain 6. Using the same approach, CaT1 shows 23 % identity to polycystin 2 over a stretch of 227 amino acids, 24 % to melastatin 1 (*TRPM1*) over a stretch of 143 amino acids and no significant similarity to the recently identified mucolipins (*MCOLN1-3*). The phylogenetic tree (Fig. 1) for the human *TRP*, *PKD2* and *MCOLN* families shows the sequence diversity of this group of functional channels.

Functional and pharmacological properties

When expressed in *X. laevis* oocytes, CaT1 and ECaC show constitutive activity and saturation kinetics with apparent $K_{0.5}$ values ranging from 0.2 to 0.66 mM (Peng *et al.* 1999, 2000*a,b*; Hoenderop *et al.* 1999). Ca^{2+} influx is not coupled to Na^+, Cl^- or H^+ gradients, though transport activities are sensitive to pH, with increased activities at alkaline pH. Like most electrogenic transport processes, the current–voltage relationships of both channels suggest that a hyperpolarizing potential favours Ca^{2+} influx. Both CaT1 and ECaC are permeable to Ba^{2+} and Sr^{2+} but not to Mg^{2+}. The macroscopic properties of the channels indicate that they function as facilitative transporters, mediating cellular uptake of calcium down its electrochemical gradient, with saturation kinetics but no obvious gating mechanisms (e.g. by ligand or voltage).

As members of the family of six-membrane-spanning channels, both CaT1 and ECaC exhibit characteristic pore and single channel properties (Nilius *et al.* 2000; Yue *et al.* 2001; Vassilev *et al.* 2001). In the nominal absence of extracellular divalent cations, when using Na^+ as charge carrier, single channel conductances of 42 and 78 pS were obtained for rat CaT1 (Yue *et al.* 2001) and rabbit ECaC, respectively (Nilius *et al.* 2000). Single channel activities

for these channels using divalent cations as charge carriers have not yet been reported. Both channels show high selectivity for Ca^{2+}, with Ca^{2+}-to-Na^{+} permeability ratios (P_{Ca}/P_{Na}) of over 100. In the presence of monovalent cations and Ca^{2+} these channels exhibit the so-called anomalous mole fraction behaviour – currents decrease initially as extracellular Ca^{2+} levels are reduced, but at very low extracellular Ca^{2+} levels, currents then increase beyond the amplitude seen in the presence of high Ca^{2+} levels, due to the increasing permeability to monovalent cations. This behaviour is thought to be related to the affinity difference between monovalent and divalent cations in the channel pore. The presence of extracellular Mg^{2+} was shown to block the monovalent cation currents of CaT1 (Yue *et al.* 2001) and ECaC (Nilius *et al.* 2000). Mg^{2+} blockage may contribute to the inward rectification of the monovalent cation currents, possibly preventing the exit of Ca^{2+} under depolarizing conditions. However, CaT1 also has intrinsic inward rectification, independent of Mg^{2+} blockage (Voets *et al.* 2003).

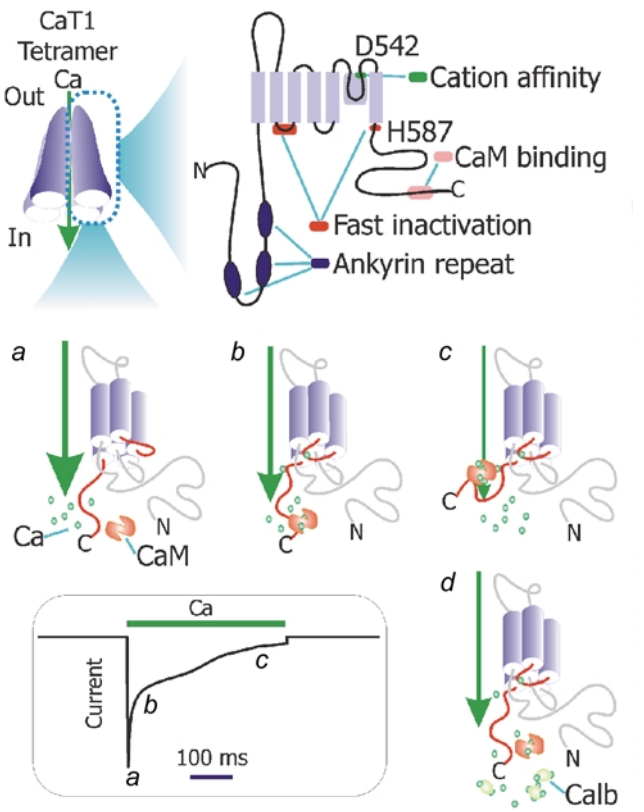

Figure 2. Molecular architecture and feedback inactivation of CaT1

Upper panel: key domains and residues in CaT1. A CaT1 tetramer, the functional unit of the channel, is shown on the left. Ankyrin repeats may mediate protein–protein interaction; D542 is a key residue cation affinity or permeation. The first intracellular loop and H587 are involved in the fast phase of inactivation. The calmodulin (CaM) binding site close to the carboxyl terminus is involved in the slow phase of Ca^{2+}-dependent inactivation. Lower panel: fast and slow phases of inactivation and involved residues and domains in CaT1. A typical Ca^{2+}-dependent inactivation process of Ca^{2+} current of CaT1 is shown in the box based on that described by Niemeyer *et al.* (2001) and Nilius *et al.* (2002). The currents and the presumed corresponding CaT1 states are labelled *a*, *b*, and *c*; *d* shows the putative role of calbindin D_{9k} (Calb) on Ca^{2+} influx: buffering the Ca^{2+} underneath the channel thereby releases the channel from Ca^{2+} feedback inhibition.

CaT1 and ECaC can be blocked by trivalent and divalent cations. La^{3+}, Gd^{3+}, Pb^{2+}, Cd^{2+} and Cu^{2+} are among the most effective blockers of CaT1 and ECaC (Peng *et al.* 1999, 2000*a*,*b*; Nilius *et al.* 2001*a*). The difference in the IC_{50} for inhibition by Cd^{2+} (~5 μM) and the $K_{0.5}$ for the Cd^{2+} current (1.3 mM) suggest that two binding sites are present in the ECaC (CaT2) channel (Peng *et al.* 2000*a*). The CaT1 and ECaC channels are relatively insensitive to L-type voltage-gated channels blockers (Peng *et al.* 1999, 2000*a*; Hoenderop *et al.* 1999). The classical L-type channel blockers nifedipine, diltiazem and verapamil (100 μM) inhibited CaT1-mediated Ca^{2+} uptake by 10–15 % (Peng *et al.* 1999) and ECaC-mediated Ca^{2+} currents by less than 10 % (Peng *et al.* 2000*a*). Preincubation with verapamil increased the inhibition of ECaC-mediated Ca^{2+} current to ~ 50 % (Peng *et al.* 2000*a*). The imidazole derivatives econazole and miconazole have been found to be among the most effective organic blockers of ECaC with IC_{50} values of 1.27 μM and 1.77 μM for the Na^{+} current, respectively (Nilius *et al.* 2001*a*). Another imidazole derivative, SKF96365, is rather ineffective in blocking ECaC (Nilius *et al.* 2001*a*). Econazole, miconazole and SKF96365 have also been shown to inhibit Ca^{2+} uptake in oocytes expressing CaT1 and ECaC, with econazole being the most effective inhibitor (~50 % inhibition at 50 μM). SKF96365 was almost ineffective at this concentration (J.-B. Peng & M. A. Hediger, unpublished observations). Ruthenium red is currently the most effective blocker of ECaC (IC_{50} = 111 nM) (Nilius *et al.* 2001*a*), but it is a much less effective blocker of CaT1 (IC_{50} = 9 μM) (Hoenderop *et al.* 2001*b*). None of the above blockers are specific enough to discriminate these channels from others, and the mechanisms of inhibition are unclear.

A single negatively charged residue, Asp542, was found to determine Ca^{2+} permeation and Mg^{2+} blockade in ECaC (Nilius *et al.* 2001*b*). However, another study indicated that Asp542 plays a critical role in Ca^{2+} and Mg^{2+} affinity but that it is not a major determinant of Ca^{2+} permeation (Jean *et al.* 2002). The corresponding residue in CaT1 is also Asp542 and it is critical for Ca^{2+} transport mediated by CaT1 (Fig. 2; J.-B. Peng & M. A. Hediger, unpublished observation). The corresponding aspartate residue in VR1 was reported to determine the pore properties of VR1, including the permeability to Mg^{2+} and sensitivity to ruthenium red (Garcia-Martinez *et al.* 2000). The residue was suggested to form a ring of negative charges that produces a high affinity binding site for cationic molecules

at the extracellular entryway of VR1 (Garcia-Martinez *et al.* 2000). As ECaC, CaT1 and VR1 share significant sequence similarity, and yet VR1 is a nonselective cation channel, it therefore appears that additional residues in CaT1 and ECaC are required for the unique high selectivity of these channels for Ca^{2+} over monovalent cations and Mg^{2+}.

Auto-regulatory mechanism by intracellular Ca^{2+}

As sustained increases in intracellular Ca^{2+} may cause cell death, Ca^{2+}-transporting cells express calbindins to buffer the increases in intracellular Ca^{2+} resulting from calcium entry through these channels. To avoid elevations in intracellular Ca^{2+} to toxic levels that are beyond the buffering capacity of the calbindins, both CaT1 and ECaC exhibit Ca^{2+}-dependent inactivation (Niemeyer *et al.* 2001; Nilius *et al.* 2002). CaT1 shows both 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 C-terminal region (Niemeyer *et al.* 2001) (Fig. 2). CaT1's calmodulin binding site is conserved among different species, but is not present in the same region of ECaC (Niemeyer *et al.* 2001). Ca^{2+}-dependent binding of calmodulin to CaT1 inactivates the CaT1 channel. A protein kinase C site is present within the calmodulin binding site in human CaT1 but not in the 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 Ca^{2+} to enter the cell (Niemeyer *et al.* 2001). ECaC shows essentially no fast phase of inactivation compared with CaT1. The first intracellular loop of CaT1 determines the fast phase of inactivation (Nilius *et al.* 2002) and its sequence is not conserved between CaT1 and ECaC (Fig. 2). Of note, the first intracellular loop is entirely encoded by one exon – an example of a single exon endowing a distinct functional feature. In addition to the first intracellular loop, H587, a positively charged amino acid residue downstream of the last transmembrane domain of CaT1 has also been identified as being involved in fast inactivation of CaT1 (Suzuki *et al.* 2002).

The feedback inhibition is a two-edged sword: it protects the cell from Ca^{2+} overload but it also limits the flow of Ca^{2+} into the cell. CaT1 activity is inversely related to the intracellular Ca^{2+} level on its cytoplasmic side (Bodding *et al.* 2002). Thus, the availability of calbindin D_{9k} to buffer the local increase in Ca^{2+} will reduce the Ca^{2+} feedback inhibition and in turn, increase the flow of Ca^{2+} into the cell. Therefore, a coordinated increase in the expression of both the apical channel and intracellular calbindin is necessary to achieve maximal Ca^{2+} influx at the apical side (Fig. 2*d*).

Roles in Ca^{2+}-transporting epithelia

The intestine, kidney and placenta are three major organs involved in Ca^{2+} transport that participate in Ca^{2+} absorption from the diet, renal tubular Ca^{2+} reabsorption, and Ca^{2+} transport from the maternal to the fetal circulation, respectively. The transcellular pathway which allows uphill Ca^{2+} transport against an electrochemical gradient occurs mostly in the proximal intestine, the distal tubule of the nephron and the placental syncytium. In this pathway, Ca^{2+} crosses the polarized epithelium by entering the epithelial cells across the apical membrane and exiting across the basolateral membrane. The extracellular Ca^{2+} concentration is in the millimolar range and the intracellular Ca^{2+} concentration is around 100 nM; thus an energy-consuming process, mediated by the Ca^{2+} pump and/or the Na^{+}–Ca^{2+} exchanger, is used to overcome the ~10 000 times Ca^{2+} concentration difference and the ~60 mV membrane potential difference, allowing Ca^{2+} to exit the cell. In contrast to the basolateral side, there is a favourable electrochemical gradient for Ca^{2+} entry at the apical membrane. The proteins that mediate this step should work like facilitative transporters with saturation kinetics in the millimolar range but without any ion coupling or gating mechanisms. Both CaT1 and ECaC possess these properties and are well suited as apical Ca^{2+} entry mechanisms. As the Ca^{2+} concentration in the intestinal lumen can vary greatly, the sudden appearance of a high level of Ca^{2+} in the intestinal lumen could be disastrous. Therefore, a fast inactivation mechanism is needed for the intestinal channel. In contrast, a fast inactivation mechanism is not necessary in the renal distal tubule, where the Ca^{2+} concentration is relatively constant. Thus, the fast inactivation property of CaT1 makes it more suitable for the intestinal environment.

Indeed, CaT1 is the major apical Ca^{2+} entry channel in the mouse (Weber *et al.* 2001; Van Cromphaut *et al.* 2001), rat (Peng *et al.* 2000*a*) and human intestine (Barley *et al.* 2001; Peng *et al.* 2000*b*, 2001*a*). CaT1 protein localizes in the apical membrane of the small and large intestines of both mouse and human (Zhuang *et al.* 2002). In the kidney, however, ECaC is the major apical channel in the mouse (Van Cromphaut *et al.* 2001) and rat (Peng *et al.* 2000*a*); in human kidney, in contrast, we found that the abundance of CaT1 mRNA is about 10 times greater than that of ECaC (Peng *et al.* 2001*a*). An apical localization of ECaC has been observed in rabbit (Hoenderop *et al.* 2000), rat (Hoenderop *et al.* 2001*a*) and mouse kidney (Loffing *et al.* 2001) in the distal segment of the nephron. In all of the animal models examined to date, ECaC is expressed in the distal segment (DCT-2) of the distal convoluted tubule and connecting tubule (CNT), in the same cells that contain additional machinery for cytosolic Ca^{2+} transport, including calbindin D_{28k} and the basolateral Na^{+}–Ca^{2+} exchanger 1 (NCX1). The localization of CaT1 in the kidney is less clear. It has been reported to be expressed in the thick ascending limb (Suzuki *et al.* 2000), and more recently, to be co-expressed with ECaC in the distal tubules, where it has been suggested to form a

heterotetramer with the latter (Hoenderop *et al.* 2003). Using mRNA samples from human kidney cortex and outer medulla, we found that the level of ECaC mRNA in the cortex is 3 times that in the outer medulla. In contrast, CaT1 mRNA appears to be higher in the outer medulla than in the cortex (J.-B. Peng & M. A. Hediger, unpublished observation). Given the great differences in the localization and levels of expression of the two channels reported to date, the likelihood and significance of heterotetramerization of the two proteins *in vivo* requires further evaluation.

In the human placenta, the level of CaT1 mRNA is about 1000 times that of ECaC (Peng *et al.* 2001*a*). *In situ* hybridization demonstrated the presence of CaT1 transcript in trophoblasts and syncytiotrophoblasts (Wissenbach *et al.* 2001). In cultured cytotrophoblast cells isolated from human term placenta, Ca^{2+} uptake activity was correlated with the secretion of human chorionic gonadotrophin and the mRNA levels of CaT1 and ECaC (Moreau *et al.* 2002*b*). The uptake of Ca^{2+} by trophoblast cells was insensitive to L-type Ca^{2+} channel modulators but was inhibited by ruthenium red with an IC_{50} of 9 μM, indicating that CaT1 is the major channel. This is consistent with the report that CaT1 but not ECaC was detected by Northern blot in these cells (Moreau *et al.* 2002*a*).

Calcium absorption is regulated by 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the active form of vitamin D. Studies on the action of $1,25(OH)_2D_3$ on Ca^{2+} transport have focused on the calbindins (Wasserman & Fullmer, 1995; Christakos *et al.* 1992). The possible significance of the effects of $1,25(OH)_2D_3$ on CaT1 expression was first explored in a human intestinal cell line, Caco-2, which has been used for studies of Ca^{2+} transport (Wood *et al.* 2001). The induction of CaT1 by $1,25(OH)_2D_3$ in Caco-2 cells was more robust than that of calbindin D_{9k} and preceded the latter by several hours (Wood *et al.* 2001). Regulation of CaT1 by vitamin D was soon confirmed in a study using vitamin D receptor-null mice (Van Cromphaut *et al.* 2001). Duodenal CaT1 mRNA was reduced more than 90 % with a 3-fold decrease in calcium absorption in two VDR-KO strains on a normal calcium diet (Van Cromphaut *et al.* 2001). Calbindin D_{9k} was decreased only in one strain and plasma membrane Ca^{2+}-ATPase PMCA1b expression was normal in both VDR-null strains, indicating that the decrease in CaT1 expression is responsible for the decrease in calcium absorption (Van Cromphaut *et al.* 2001). These two studies both show that among the proteins involved in transcellular Ca^{2+} transport the apical entry channel CaT1 is most robustly regulated by vitamin D (Wood *et al.* 2001; Van Cromphaut *et al.* 2001). CaT1 mRNA expression level, as well as its induction by $1,25(OH)_2D_3$ correlate better to transcellular calcium transport in Caco-2 cell lines than calbindin D_{9k} and $PMCA_1$ (Fleet *et al.* 2002). These studies suggest that instead of the intracellular diffusion step mediated by calbindin D_{9k}, the CaT1-mediated apical entry step is the rate-limiting step for vitamin D-regulated Ca^{2+} transport.

In a study using human duodenal biopsies from 20 normal subjects, CaT1 mRNA levels were not significantly correlated with vitamin D metabolites, but were moderately correlated with calbindin D_{9k} and more strongly with the $PMCA_1$ (Barley *et al.* 2001). However, the results of this study may have been affected considerably by the uncontrolled calcium intake of the subjects, a factor that was later found to have a significant impact on CaT1 expression (Van Cromphaut *et al.* 2001). CaT1 expression was greatly reduced on a high calcium diet and greatly increased on a low calcium diet (Van Cromphaut *et al.* 2001), and the extent of the reduction was independent of the vitamin D receptor (Song *et al.* 2003).

Vitamin D regulation of ECaC in the kidney is controversial. It has been reported that vitamin D depletion reduces the expression of ECaC in rat kidney and that repletion of vitamin D restores its expression (Hoenderop *et al.* 2001*a*); however, in the VDR-null mice, ECaC expression was not decreased in two strains of VDR-null mice on a normal diet (Van Cromphaut *et al.* 2001). In mice lacking 25-hydroxyvitamin D_3-1α-hydroxylase (1α-OHase), a key enzyme for $1,25(OH)_2D_3$ production, substantial decreases in the levels of expression of ECaC, calbindins and the Na^+–Ca^{2+} exchanger 1 (NCX1) were found, along with a decrease in serum calcium level (Hoenderop *et al.* 2002). High calcium intake restored the levels of ECaC, calbindin D_{28k} (but not the calbindin D_{9k}) and NCX1, as well as the serum calcium level (Hoenderop *et al.* 2002). It appears that the level of expression of ECaC is positively regulated by the calcium load indicated by the serum calcium level (Hoenderop *et al.* 2002). The apparent increase in ECaC induced by $1,25(OH)_2D_3$ may reflect the increase in the calcium load in the kidney as a result of increased expression of intestinal CaT1. In contrast to the observation that only duodenal CaT1 in the VDR-null mouse was severely reduced compared to calbindin D_{9k} and $PMCA_{1b}$, in the 1α-OHase null mice on a normal diet, coordinated changes of ECaC, calbindins D_{9k} and D_{28k} and NCX1 were observed. This indicates that transcellular Ca^{2+} transport in the distal tubules is a coordinated process involving all of the participating proteins.

CaT1 and the Ca^{2+} release-activated Ca^{2+} (CRAC) channel

Ca^{2+} entry in Ca^{2+}-transporting cells does not require a specific signal; in other nonexcitable cells, however, Ca^{2+} enters in response to the release of Ca^{2+} from intracellular calcium stores – a process termed 'capacitative Ca^{2+} entry' or 'store-operated Ca^{2+} entry' (Putney, 1997; Parekh & Penner, 1997). In this process, plasma membrane Ca^{2+} channels are activated in response to calcium store

depletion, often upon stimulation by a G protein-coupled receptor. The Ca^{2+} release-activated Ca^{2+} (CRAC) channel refers to the store-operated channel in the rat basophilic leukemia (RBL) and Jurkat T-lymphocyte cell lines. Its biophysical properties have been extensively studied compared to other store-operated channels described in a variety of cell types. The molecular identity of the CRAC channel is still unknown and its identification is of significant importance.

When using Na^+ as a charge carrier, in the absence of divalent cations, single channel recordings in Jurkat T-lymphocytes uncovered a channel activity that was attributed to CRAC channels (Kerschbaum & Cahalan, 1999). Interestingly, CaT1, when expressed in Chinese hamster ovary cells, exhibited pore properties indistinguishable in many respects from those of CRAC (Yue *et al.* 2001). These include high selectivity for Ca^{2+} ($P_{Ca}/P_{Na} > 100$), an order of selectivity for divalent cations ($Ca^{2+} \gg Ba^{2+} > Sr^{2+} > Mn^{2+}$), loss of selectivity in the absence of divalent cations, block by La^{3+}, the anomalous mole fraction effect, whole-cell current kinetics and single channel conductance to Na^+ in divalent ion-free conditions (Yue *et al.* 2001). In addition, when CaT1 is expressed at low levels, it can be activated by depletion of calcium stores. Thus CaT1 was proposed to constitute part or all of the CRAC channel pore, with the assumption that other cellular components are necessary for controlling CaT1 via calcium stores (Yue *et al.* 2001).

Despite the similarity of the CaT1 and CRAC channel currents, a comparison of CaT1 expressed in human embryonic kidney (HEK 293) cells and CRAC in rat basophilic leukaemia (RBL) cells revealed certain differences between the CaT1 and CRAC channel properties (Voets *et al.* 2001). These include the following: (1) CRAC (but not CaT1) can be activated by ionomycin-mediated store depletion and can be blocked by 2-aminoethoxydiphenyl borate (2-APB); (2) CaT1 (but not CRAC) can be blocked in a voltage-dependent manner by an increase in intracellular Mg^{2+}; and (3) the channels display a difference in relative permeability to Na^+ and Cs^+ (Voets *et al.* 2001). Further comparison of the properties of CaT1 exogenously expressed in HEK 293 cells and RBL cells revealed that CaT1 current was constitutively active in HEK 293 cells, whereas in RBL cells it was either constitutively active at a high current density or was activated by calcium store depletion – a matter which depends on the expression levels of CaT1 (and perhaps also on the matched up-regulation of other cellular components) (Schindl *et al.* 2002). Both constitutively active and store depletion-activated CaT1 currents were distinguishable from endogenous I_{CRAC} in RBL cells by their current–voltage relationship in divalent cation-free conditions. The store depletion-activated CaT1 current was blocked by 2-APB, as was the endogenous I_{CRAC}, indicating that a common 2-APB-sensitive regulatory component controls the activation of both CRAC and CaT1 in the RBL (Schindl *et al.* 2002). Another study showed that exogenously expressed human CaT1 in RBL and HEK 293 cells was not activated by store depletion; rather, the activity of CaT1 is affected by the local Ca^{2+} concentration close to the pore (Bodding *et al.* 2002). The authors suggested that CaT1 activity is inversely related to the intracellular Ca^{2+} levels and that it serves as an intracellular Ca^{2+} sensor in these cells (Bodding *et al.* 2002).

In spite of the studies showing that the CaT1 current may not be I_{CRAC}, studies in Jurkat T-lymphocytes (Cui *et al.* 2002) and in the prostate cancer epithelial cell line LNCaP (Vanden Abeele *et al.* 2003) suggest that CaT1 contributes significantly to the endogenous store-operated Ca^{2+} entry in both cells. The CaT1 mRNA level in Jurkat T-lymphocytes is 2.5 % of that detected in placenta (Cui *et al.* 2002) – a level that is comparable to CaT1 expression in epithelial cells of the small intestine. CaT1 contributed to the store-operated currents when expressed in Jurkat T-lymphocytes, and over-expression of a dominant negative CaT1 mutant in the same cells resulted in suppression of endogenous I_{CRAC} (Cui *et al.* 2002). In LNCaP cells, in which CaT1 is endogenously expressed at a considerable level and is negatively regulated by androgen (Peng *et al.* 2001*b*), anti-sense hybrid depletion of CaT1 decreased store-operated current by approximately 50 %, whereas up-regulation of CaT1 mRNA by 60 % through anti-androgen treatment enhanced the current by 30 % (Vanden Abeele *et al.* 2003). The CaT1 contribution may have been underestimated, given that the anti-sense approach generally cannot completely eliminate CaT1 expression. Thus, CaT1 appears to represent a major component of the store-operated Ca^{2+} entry in LNCaP cells.

Recently, the biophysical properties of the CRAC channel have been redefined since recent studies using Jurkat T-lymphocytes revealed that the monovalent cation current previously attributed to I_{CRAC} (Kerschbaum & Cahalan, 1999) corresponds to the Mg^{2+}-inhibited cation current (I_{MIC}) (Prakriya & Lewis, 2002) (also known as the Mg-nucleotide-regulated metal ion currents (MagNuM); Hermosura *et al.* 2002). A similar conclusion was reached based on studies using RBL cells (Kozak *et al.* 2002); Bakowski & Parekh, 2002*a*). The I_{MIC} is likely to be mediated by TRPM7 (also known as ChaK1; Ryazanov *et al.* 1999), LTRPC7 (Nadler *et al.* 2001) and TRP-PLIK (Runnels *et al.* 2001)). The following properties of I_{CRAC} differ from I_{MIC}: activation by store depletion, resistance to suppression by 8 mM intracellular Mg^{2+}, inhibition by SKF 96365 and low Cs^+ permeability (Prakriya & Lewis, 2002). The single channel openings of the CRAC channel in Jurkat T-lymphocyte were not detectable, even under

divalent cation-free conditions, when using a monovalent cation as a charge carrier, and a 0.2 pS conductance was estimated from noise analysis (Prakriya & Lewis, 2002), much lower than that estimated for CaT1 (42 pS). In contrast, I_{MIC} (TRPM7?) has a ~44 pS single channel conductance (Prakriya & Lewis, 2002). Thus, the newly described biophysical properties of the CRAC channel differ significantly from those of CaT1, calling into question whether and/or to what extent CaT1 contributes to the CRAC channel (Clapham, 2002*b*) (for a detailed discussion see Bakowski & Parekh, 2002*b*).

The CRAC channel is one of the store-operated Ca^{2+} channels whose properties have been well defined in RBL cells and Jurkat T-lymphocytes using biophysical approaches (see Clapham, 2002*b*). Store-operated Ca^{2+} entry or capacitative Ca^{2+} entry has been described in many cell types (Parekh & Penner, 1997). CRAC and/or other Ca^{2+}-permeable channels contribute to Ca^{2+} entry in a variety of cells such as prostate cancer LNCaP cells (Skryma *et al.* 2000) and pancreatic acinar cells (Raraty *et al.* 2000). Whether or not CaT1 is a part of the CRAC pore, it probably plays an important role in store-operated Ca^{2+} entry in cells where it is highly expressed such as in Jurkat T-lymphocytes (Cui *et al.* 2002), LNCaP cells (Vanden Abeele *et al.* 2003) and probably also pancreatic acinar and other secretory cells. Given that CaT1 is a constitutively active calcium entry channel, one question that arises is how CaT1 can play a role as a store-operated channel. CaT1 is constitutively active on its own, consistent with its role in Ca^{2+} transporting epithelia (Fig. 3; see constitutively active mode). We propose that, in other cells, including those not involved in Ca^{2+} transport, CaT1 is inactive until it is stimulated. As previously suggested (Yue *et al.* 2001) and further supported by another study (Schindl *et al.* 2002), other cellular components may govern the store-operated property of CaT1. These components may function as a molecular switch, turning off CaT1 when it is bound to the channel (Fig. 3, molecular switch mode). The component may be present in cells with significant store-operated Ca^{2+} entry pathways but absent in Ca^{2+} transporting epithelia. It is likely that 2-APB acts on this component rather than on the channel itself (Schindl *et al.* 2002).

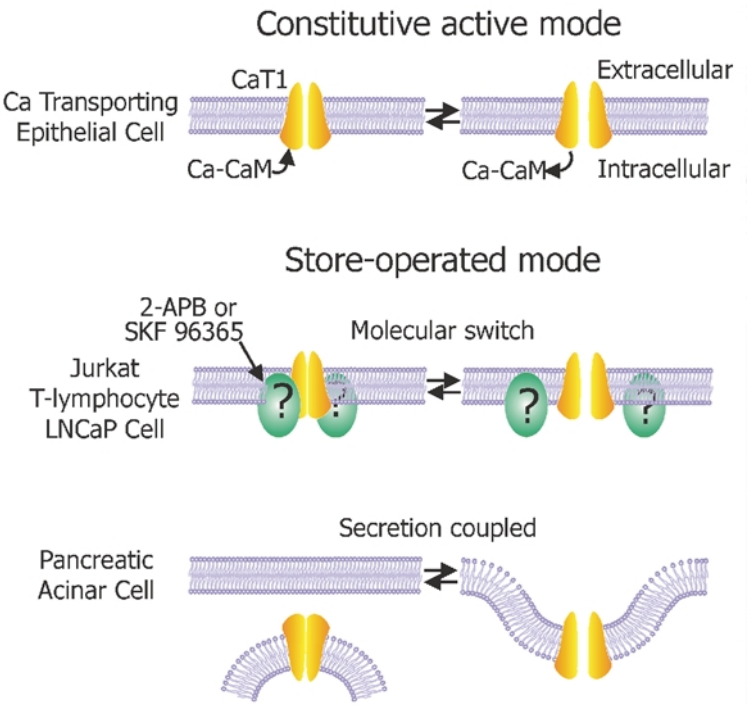

Figure 3. Modes of CaT1 activation

Constitutive active mode: CaT1 alone is constitutively active and works in this mode in Ca^{2+}-transporting epithelia such as the brush-border membrane of small intestine. The channels are subject to Ca^{2+}-dependent feedback regulation and this process is partially mediated by calmodulin (CaM). Store-operated mode: CaT1 is not active in unstimulated cells and is activated by calcium store depletion. Two putative mechanisms are shown. Molecular switch mode: another cellular component serves as a molecular switch to shut CaT1 off when it is associated with CaT1 or to activate CaT1 upon its dissociation. This component might be the target of 2-APB or SKF 96365, which may promote the association state, as suggested by studies in Jurkat T-lymphocytes or LNCaP cells. Secretion-coupled mode: inactivated CaT1 may localize in the membrane of intracellular vesicles. Upon exocytosis, CaT1 is incorporated into plasma membrane. The plasma membrane-associated CaT1 takes up Ca^{2+} released from the vesicle to refill the calcium store or to trigger further exocytosis.

CaT1 function in exocrine tissues

The tissues that express CaT1 at the highest levels, as assessed by Northern analysis or *in situ* hybridization, are those with exocrine functions, including pancreas, salivary gland and prostate (Peng *et al.* 2000*b*; Wissenbach *et al.* 2001). The use of immunocytochemistry confirmed CaT1 expression in these tissues and also revealed the expression of CaT1 in stomach, mammary gland and sweat gland (Zhuang *et al.* 2002).

The roles of CaT1 in these glandular tissues have not yet been studied, but it is possible that it serves some common function. If CaT1 is present in the membrane of zymogen granules, it would have to be in an inactive state to prevent release of the calcium from the zymogen granules. Once it is incorporated into the plasma membrane, it would then become active to take up Ca^{2+} released from the granule. In the pancreas, CaT1 staining was mostly restricted to the apical secretory pole, with both intracellular and apical membrane staining (Zhuang *et al.* 2002). This pattern of immunostaining suggests the possibility that CaT1 is localized in zymogen granules, although further studies are needed for verification. The proteins for cellular signalling, including the IP_3 receptors (Nathanson *et al.* 1994; Yule *et al.* 1997) and G protein-coupled receptors such as the muscarinic type 3 receptor and cholecystokinin receptors (Shin *et al.* 2001), are localized in the apical region. The localization of CaT1 in the granular area overlaps with the small trigger zone where Ca^{2+} waves and oscillations originate in a pancreatic acinar cell (Kasai *et al.* 1993). As CaT1 is a Ca^{2+}-selective Ca^{2+} channel, its involvement in Ca^{2+} signalling in pancreatic acinar cells is likely. However, the role of CaT1 in the process has not been shown. CaT1 may play a role in the clearance of Ca^{2+} released from the zymogen granules by re-uptake of Ca^{2+} into the cells, thereby replenishing the Ca^{2+} pool – equivalent to store-operated Ca^{2+} entry (Fig. 3; secretion-coupled mode). Hyperstimulation of pancreatic acinar cells by cholecystokinin evokes a sustained increase of intracellular Ca^{2+} due to store-operated Ca^{2+} entry which in turn causes trypsin activation and vacuole formation in the apical granular pole similar to what happens in acute pancreatitis (Raraty *et al.* 2000). The possible role of CaT1 in this process has yet to be investigated.

The reuptake mechanism is necessary for exocrine function as secretory vesicles have high calcium content (e.g. 10–100 mM). The coincidence of the apical localization of the Ca^{2+} selective channel CaT1 in exocrine glandular cells in the pancreas, prostate, stomach and mammary gland (Zhuang *et al.* 2002) and the high calcium content in the secretory vesicles suggest a common reuptake mechanism in these cells. While CaT1 is highly expressed in pancreatic acinar cells, ECaC has been reported to be present in the pancreatic islets (Janssen *et al.* 2002), suggesting distinct roles of these two channels in exocrine and endocrine function, respectively.

Up-regulation of CaT1 expression in cancers

Up-regulation of CaT1 in prostate cancer was reported at the mRNA level (Peng *et al.* 2001*b*; Wissenbach *et al.* 2001) and later confirmed at the protein level (Zhuang *et al.* 2002). We found that CaT1 was expressed in both benign prostatic hyperplastic (BPH) tissues and prostate cancers in the epithelial cells. However, the CaT1 transcript was significantly increased in cancerous prostate tissues. The expression level of CaT1 mRNA increased with the degree of malignancy of prostate cancer (Peng *et al.* 2001*b*). Wissenbach *et al.* (2001) did not detect CaT1 expression in BPH samples or in the organ-confined primary Gleason grade 3 tumour. In contrast, the highest levels of CaT1 mRNA were detected in high-grade tumours with extraprostatic extension. Even higher levels of CaT1 expression were detected in prostate cancer with lymph node metastases and in recurrent lesions (Wissenbach *et al.* 2001).

In the androgen-sensitive prostatic cancer cell line LNCaP, which expresses CaT1 endogenously, the CaT1 transcript level is negatively regulated by androgen (Peng *et al.* 2001*b*). CaT1 expression levels in the LNCaP cells is positively related to the store-operated Ca^{2+} entry in the cells (Vanden Abeele *et al.* 2003). If this is also true *in vivo*, then androgen withdrawal therapy could increase CaT1 expression, which might be a signal for proliferation of the tumour cells. Conversely, strategies that reduced CaT1 expression could be beneficial in the treatment of prostate cancer.

Pathological up-regulation of CaT1 does not appear to be restricted to prostate cancer but seems to be a general phenomenon of cancers of epithelial origin. Zhuang *et al.* (2002) found CaT1 protein to be present at elevated levels in comparison with normal tissues in a series of prostate, breast, thyroid, colon and ovarian carcinomas, consistent with previous reports of up-regulation of CaT1 mRNA in prostate cancer tissues. Increased CaT1 expression might mean an increase in the level of intracellular Ca^{2+} (if CaT1 is in the constitutively active mode), or increased Ca^{2+} influx in response to stimuli (if CaT1 is in store-operated mode) or both. Ca^{2+} is involved in cell differentiation and proliferation, as well as apoptosis (Berridge *et al.* 2000). If CaT1 expression is linked to cell proliferation and/or apoptosis, altered CaT1 function might have significant implications for tumour growth. Thus, inhibition of CaT1 might be a therapeutic strategy to prevent uncontrolled growth of cancers of epithelial origin.

Concluding remarks

Recent studies of the Ca^{2+} entry channels CaT1 and ECaC have provided us with a better understanding of the

transcellular pathway of Ca^{2+} transport and its regulation. Although rapid progress has been made in this new field, there are still many issues to be addressed. Neither of the channels has been directly shown to mediate apical calcium entry in the transcellular pathway of calcium transport. The channels have been proposed to serve as gatekeepers of transcellular calcium transport, but the roles of all of the participating proteins have not yet been carefully evaluated in a functional setting, although this will now become possible given the identification of the major players in calcium transport. The roles of CaT1 beyond Ca^{2+} transport are emerging but are yet to be investigated in further detail. CaT1's possible role in exocrine function is uncharacterized to date. The cause and the consequence of CaT1 overexpression in cancers may help identify CaT1 as a target for cancer therapy.

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