Molecular Cloning and Characterization of a Channel-like Transporter Mediating Intestinal Calcium Absorption*

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Calcium is a major component of the mineral phase of bone and serves as a key intracellular second messenger. Postnatally, all bodily calcium must be absorbed from the diet through the intestine. Here we report the properties of a calcium transport protein (CaT1) cloned from rat duodenum using an expression cloning strategy in Xenopus laevis oocytes, which likely plays a key role in the intestinal uptake of calcium. CaT1 shows homology (75% amino acid sequence identity) to the apical calcium channel ECaC recently cloned from vitamin D-responsive cells of rabbit kidney and is structurally related to the capsaicin receptor and the TRP family of ion channels. Based on Northern analysis of rat tissues, a 3-kilobase CaT1 transcript is present in rat duodenum, proximal jejunum, cecum, and colon, and a 6.5-kilobase transcript is present in brain, thymus, and adrenal gland. In situ hybridization revealed strong CaT1 mRNA expression in enterocytes of duodenum, proximal jejunal, and cecum. No signals were detected in kidney, heart, liver, lung, spleen, and skeletal muscle. When expressed in Xenopus oocytes, CaT1 mediates saturable Ca2+ uptake with a Michaelis constant of 0.44 mM. Transport of Ca2+ by CaT1 is electrogenic, voltage-dependent, and exhibits a charge/Ca2+ uptake ratio close to 2:1, indicating that CaT1-mediated Ca2+ influx is not coupled to other ions. CaT1 activity is pH-sensitive, exhibiting significant inhibition by low pH. CaT1 is also permeant to Sr2+ and Ba2+ (but not Mg2+), although the currents evoked by Sr2+ and Ba2+ are much smaller than those evoked by Ca2+. The trivalent cations Gd3+ and La3+ and the divalent cations Cu2+, Pb2+, Cd2+, Co2+, and Ni2+ (each at 100 μM) do not evoke currents themselves, but inhibit CaT1-mediated Ca2+ transport. Fe3+, Fe2+, Mn2+, and Zn2+ have no significant effects at 100 μM on CaT1-mediated Ca2+ transport. CaT1 mRNA levels are not responsive to 1,25-dihydroxyvitamin D3 administration or to calcium deficiency. Our studies strongly suggest that CaT1 provides the principal mechanism for Ca2+ entry into enterocytes as part of the transepithelial pathway of calcium absorption in the intestine.

Calcium is the most abundant cation and the fifth most common inorganic element in the human body. It is a well known first and second messenger in signal transduction and an essential component of bone mineral (1, 2). Calcium homeostasis in blood and other extracellular fluids is tightly controlled through the actions of calcitropic hormones on bone, kidneys, and intestine (2). The availability of dietary calcium is a critical determinant of calcium homeostasis (3). In humans, dietary intake of calcium approximates 500–1000 mg/day, and obligatory endogenous losses in stool and urine total ~250 mg/day. On the order of 30% of calcium in the diet must be absorbed to sustain bone growth in children and to prevent postmenopausal bone loss in aging women (2, 4). To meet the body’s need for calcium, the intestines of most vertebrates evolved specialized vitamin D-dependent and -independent mechanisms for ensuring adequate intestinal calcium uptake. Intestinal absorption of Ca2+ occurs by a saturable, transcellular process and a nonsaturable, paracellular pathway (5). When dietary calcium is abundant, the passive paracellular pathway is thought to be predominant. In contrast, when dietary calcium is limited, the active, vitamin D-dependent transcellular pathway plays a major role in calcium absorption (5–7). The transcellular pathway is a multistep process, consisting of entry of luminal Ca2+ into the enterocyte and translocation of Ca2+ from its point of entry (the microvillus border of the apical plasma membrane) to the basolateral membrane, followed by active extrusion from the cell (8–10). Intracellular Ca2+ diffusion is thought to be facilitated by a calcium-binding protein, calbindin D9K (11), whose biosynthesis is dependent on vitamin D (12). The extrusion of Ca2+ takes place against an electrochemical gradient and is mainly mediated by Ca-ATPase (13, 14). The entry of Ca2+ across the apical membrane of the enterocyte is strongly favored electrochemically because the concentration of Ca2+ within the cell (10−7 to 10−6 M) is considerably lower than that in the intestinal lumen (10−3 M), and the cell is electronegative relative to the intestinal lumen (15). Therefore, the movement of Ca2+ across the apical membrane does not require the expenditure of energy (10, 16). However, it has been controversial as to whether a transporter or a channel is responsible for this process, although previous studies indicated that Ca2+ entry is voltage-independent and largely insensitive to classic L-type calcium channel blockers (17, 18).

A more detailed understanding of the mechanisms underlying the transcellular calcium absorptive pathway requires the cloning of the gene(s) encoding the relevant calcium carrier(s). To achieve this goal, we took advantage of an expression cloning strategy by using Xenopus laevis oocytes as the expression system (19). Functional screening of a rat duodenal library by measuring ΔGCa2+ uptake resulted in the isolation of a cDNA clone encoding a calcium transport protein (CaT1). Interestingly, CaT1 is structurally related to the recently reported renal apical calcium channel ECaC (20). Our data indicate that
CaT1 plays a key role in mediating Ca\(^{2+}\) entry into the enterocytes as the first step of transcellular intestinal calcium absorption.

**EXPERIMENTAL PROCEDURES**

\(4^{4}\text{Ca}^{2+}\) Uptake Assay—Defolliculated X. laevis oocytes were injected with 50 nl of either water or RNA. \(4^{4}\text{Ca}^{2+}\) uptake was assayed 3 days after injection of poly(A)\(^{+}\) or 1–3 days after injection of synthetic cRNA. For expression cloning, oocytes were incubated in modified Barth’s solution (19) supplemented with 1 mM SrCl\(_2\) (to avoid excessive loading of oocytes with Ca\(^{2+}\)) as well as penicillin, streptomycin, and gentamycin at 1 mg/ml. Standard uptake solution contained the following components: 100 mM NaCl, 2 mM KCl, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\) (including \(4^{4}\text{Ca}^{2+}\), NEN Life Science Products), and 10 mM Hepes, pH 7.5. Uptake was performed at room temperature for 20 min (for the expression-cloning procedure, 2-h uptakes were employed), and oocytes were washed six times with ice-cold uptake solution plus 20 mM MgCl\(_2\). The effects of capsaicin or L-type channel blockers on Ca\(^{2+}\) uptake were studied in uptake solution by addition of 50 \(\mu\)M capsaicin (in ethanol solution, 0.05% final concentration) or 10–100 \(\mu\)M calcium channel blockers in water (nifedipine was diluted with uptake solution from 100 mM Me\(_2\)SO stock solution). Control experiments were performed with the appropriate ethanol and Me\(_2\)SO concentrations. Unless specified, data are presented as means obtained from at least three experiments with 7–10 oocytes/group with S.E. as the index of dispersion. Statistical significance was defined as having a \(p\) value of <0.05 as determined by Student’s t test.

Expression Cloning—Expression cloning using Xenopus oocytes was performed essentially as described (19). Briefly, duodenal poly(A)\(^{+}\) RNA from rats fed a calcium-deficient diet (ICN Pharmaceuticals, Inc., Costa Mesa, CA) for 2 weeks was size-fractionated. A cDNA library was then constructed from the fractions of 2.5–3 kilobases (kb) that stimulated \(4^{4}\text{Ca}^{2+}\) uptake activity when expressed in oocytes. The RNAs synthesized in vitro from pools of ~500 clones were injected into oocytes, and the abilities of the pools to stimulate Ca\(^{2+}\) uptake were assayed. A positive pool was sequentially subdivided and assayed in the same manner until a single clone was obtained. The cDNA clone was sequenced bidirectionally in the W. M. Keck facility at Yale University.

Northern Analysis—Poly(A)\(^{+}\) RNA (3 \(\mu\)g) from rat tissues was electrophoresed in formaldehyde-agarose gels and transferred to nitrocellulose membranes. The filters were probed with \(3^{2}\)P-labeled full-length CaT1 cDNA; hybridized at 42 °C with a solution containing 50% formamide, 5\% saline/sodium phosphate/EDTA, 2\% Denhardt’s solution, 0.1% SDS, and 100 \(\mu\)g/ml denatured salmon sperm DNA; and washed with 5\% SSC and 0.1% SDS at 50 °C for 2 \(\times\) 30 min and with 0.1\% SSC at 65 °C for 3 \(\times\) 30 min. Autoradiography was performed at ~80 °C for 1–2 days.

In Situ Hybridization—Digoxigenin-labeled sense and antisense runoff transcripts were synthesized using the Genius kit (Roche Molecular Biochemicals). CaT1 cDNA probes were transcribed from a polycistronic chain reaction fragment that contains ~2.7 kb of CaT1 cDNA (nucleotides 126–2894) flanked at either end by promoter sequences for an \(\alpha\)erase chain reaction fragment that contains the respective Biochemicals). CaT1 cRNA probes were transcribed from a polycloning procedure, 2-h uptakes were employed, and oocytes were washed six times with ice-cold uptake solution plus 20 mM MgCl\(_2\). The effects of capsaicin or L-type channel blockers on Ca\(^{2+}\) uptake were studied in uptake solution by addition of 50 \(\mu\)M capsaicin (in ethanol solution, 0.05% final concentration) or 10–100 \(\mu\)M calcium channel blockers in water (nifedipine was diluted with uptake solution from 100 mM Me\(_2\)SO stock solution). Control experiments were performed with the appropriate ethanol and Me\(_2\)SO concentrations. Unless specified, data are presented as means obtained from at least three experiments with 7–10 oocytes/group with S.E. as the index of dispersion. Statistical significance was defined as having a \(p\) value of <0.05 as determined by Student’s t test.

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In Vitro Transcription and Translation—In vitro transcription was performed with the Megascript \(\alpha\)RNAiMAX kit (Ambion Inc., Austin, TX). In vitro translation of the CaT1 protein was performed with the rabbit reticulocyte lysate system (Promega, Madison, WI) according to the manufacturer’s instructions.

Two-microelectrode Voltage Clamp—The two-microelectrode voltage clamp experiments were performed following the method described previously (21) using a commercial amplifier (Clampator One, Model CA-1B, Dagan Corp., Minneapolis, MN) and the pCLAMP software (Ver.

\(^{1}\)The abbreviations used are: kb, kilobase(s); TM, transmembrane domain.

**Fig. 1.** Expression cloning of a calcium transport protein (CaT1) cDNA. Results are shown for \(4^{4}\text{Ca}^{2+}\) uptake by Xenopus oocytes injected with water, rat duodenal poly(A)\(^{+}\) RNA, size-fractionated poly(A)\(^{+}\) RNA fraction 18 (2.5–3 kb), or synthetic cRNA prepared from the cloned CaT1 cDNA. Data represent the means ± S.E. for groups of 8–10 oocytes.

**RESULTS**

Expression Cloning of CaT1—The lack of information on the molecular structure of the intestinal apical calcium transport protein(s) prompted us to employ the X. laevis oocyte expression cloning procedure (19) for isolation of cDNA clones. Oocytes injected with mRNA from rat duodenum or cecum exhibited reproducible increases in Ca\(^{2+}\) uptake over water-injected control oocytes. After size fractionation of rat duodenal poly(A)\(^{+}\) RNA, we detected a substantial increase in \(4^{4}\text{Ca}^{2+}\) uptake by injection of RNA from a 2.5–3-kb pool (Fig. 1). A library was constructed using this RNA pool, and a single clone was isolated from this size-fractionated cDNA library by screening progressively smaller pools of clones for their ability to induce \(4^{4}\text{Ca}^{2+}\) uptake in CaT1-expressing oocytes. The resultant 3-kb cDNA produced large increases in Ca\(^{2+}\) uptake (~30-fold) when expressed in oocytes (Fig. 1). Based on the properties of the encoded protein, including its saturation kinetics, we named this protein CaT1, for Ca\(^{2+}\) transport protein, subtype 1.
Fig. 2. Primary amino acid sequence of CaT1 and its predicted secondary structure. **A**, amino acid sequence of CaT1 encoded by its cDNA. Ankyrin repeat domains are in gray boxes; transmembrane segments are in black boxes; and the potential pore region is in the open box. Putative protein kinase A and C phosphorylation sites and N-linked glycosylation sites are underlined and indicated by the star, diamond, and club, respectively. **B**, predicted membrane topology and domain structure of CaT1. Ankyrin repeats are in gray, and the N-glycosylation site (branched chains) and the putative kinase A (star) and C (arrows) phosphorylation sites are marked. Outer and inner leaflets of plasma membrane are indicated. AA, amino acids.

**Primary Structure of CaT1**—The 2995-base pair CaT1 cDNA contains an open reading frame of 2181 base pairs that encodes a protein of 727 amino acid residues with a predicted relative molecular mass of 83,245 Da (Fig. 2A), which is consistent with the molecular mass obtained by in vitro translation without microsomes (84 kDa; data not shown). Hydrophathy analysis suggests that CaT1 is a polytopic protein containing six transmembrane domains (TMs) with an additional short hydrophobic stretch between TM5 and TM6 (Fig. 2, A and B). Consistent with the molecular mass of the protein obtained by in vitro translation in the presence of microsomes (89 kDa; data not shown), an N-glycosylation site is predicted in the first extracellular loop of the protein. The amino-terminal hydrophilic segment (326 amino acid residues) of CaT1 contains four ankyrin repeat domains, suggesting that the protein may somehow associate with the spectrin-based membrane cytoskeleton (24). The carboxyl terminus (150 amino acid residues) contains no recognizable motifs. Putative phosphorylation sites for protein kinases A and C are present in the cytoplasmic domains (Fig. 2, A and B), suggesting that transport activity could be regulated by phosphorylation.

CaT1 shows 75% amino acid sequence identity to the recently cloned rabbit apical epithelial calcium channel ECaC (20) when using the BESTFIT sequence alignment program. The amino- and carboxyl-terminal cytoplasmic domains of CaT1 from rat and ECaC from rabbit exhibit a lower degree of similarity than the equivalent regions of rat CaT1 and partial sequences obtained from human small intestine (data not shown) by homology screening using the CaT1 cDNA as a probe. Comparisons of sequences of 150 amino acids in the amino- and carboxyl-terminal cytoplasmic domains revealed 90 and 74% identities respectively, between rat and human CaT1, but only 61 and 50% identities, respectively, between CaT1 and ECaC. CaT1 has four ankyrin repeats and one protein kinase A phosphorylation site in its amino-terminal segment, whereas ECaC contains three ankyrin repeats and no protein kinase A site in the same region. In contrast, ECaC possesses three protein kinase C sites and two protein kinase A sites in its carboxyl terminus, whereas CaT1 has only one protein kinase C site and no protein kinase A sites in the same region. In addition, CaT1 lacks the putative N-glycosylation site found in ECaC between the pore region and TM6.

Additional homology searches of available protein data bases revealed significant similarities between CaT1 and the capsaicin receptor, VR1 (25), and OSM-9, a Caenorhabditis elegans membrane protein involved in olfaction, mechanosensation, and olfactory adaptation (26). These proteins are structurally related to the family of putative store-operated calcium channels (27), among which the first two identified were the Drosophila retinal proteins TRP (28) and TRPL (29). Based on the program BESTFIT, CaT1 shows 33.7 and 26.7% identities to VR1 and OSM-9, respectively, over a stretch of at least 500 residues, as well as 26.2% and 28.9% identities to TRP and TRPL, respectively, in more restricted regions (residues 552–593 for TRP and residues 556–593 for TRPL). The latter region covers part of the pore region and the last transmembrane domain. A common feature of all of these proteins is the presence of six TMs with a hydrophobic stretch between TM5 and TM6, resembling one of the four repeated motifs of six TMs in the voltage-gated channels. Another common feature is the presence of three to four ankyrin repeat domains in the cytoplasmic N-terminal region. Of note, members of the polycystin family also possess six transmembrane segments (30–32) and show a modest degree of homology to CaT1 in small regions of the predicted amino acid sequences (residues 596–687 in PKD2, 23% identity; and residues 381–483 in PKD2L, 26% identity), but the polycystins contain no ankyrin repeats.

A homology search using the CaT1 sequence in expressed sequence tag data bases revealed the following GenBank™ sequences with high degrees of similarity to CaT1 (percent identities refer to nucleotide identities): GenBank™ accession number AI101583 from rat brain (99%); AI007094 from mouse thymus (96%); AA447311, AA469437, and AA579526 from human prostate (87, 85, and 84%, respectively), W88570 from human fetal liver (91%); AA078617 from human brain (85%); and T92755 from human lung (92%).

**Tissue Distribution of CaT1**—Northern analysis of rat tissues revealed a strong 3.0-kb band in rat small intestine and a weaker 6.5-kb band in brain, thymus, and adrenal gland (Fig. 3A). No CaT1 transcripts were detected in heart, kidney, liver, lung, spleen, and skeletal muscle. Northern analysis of the
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The gastrointestinal tract revealed that the 3-kb CaT1 transcript is expressed in duodenum, proximal jejunum, cecum, and colon, but not in stomach, distal jejunum, or ileum (Fig. 3B). The CaT1 mRNA in rat duodenum was not regulated by 1,25-dihydroxyvitamin D3 or calcium deficiency in duodenum. Lane 1, 2 µg of poly(A)+ RNA from normal rats (pooled RNA from five rats); lane 2, pooled poly(A)+ RNA from 1,25-dihydroxyvitamin D3-treated rats (duodenal mRNA was isolated from five rats 15 h after injection with 1 µg of 1,25-dihydroxyvitamin D3); lane 3, pooled poly(A)+ RNA from calcium-deficient rats. 10 rats were fed a calcium-deficient diet and MilliQ water for 2 weeks before sacrifice. The same blot was also hybridized with a 32P-labeled rat glyceroldehyde-3-phosphate dehydrogenase (GAPDH) cDNA fragment as a control.

In situ hybridization revealed expression of CaT1 mRNA in the absorptive epithelial cells of duodenum, proximal jejunum, cecum, and colon, but not in ileum (Fig. 4). CaT1 mRNA is expressed at high levels in duodenum and cecum, at lower levels in proximal jejenum, and at very low levels in colon. In all CaT1-expressing intestinal segments, mRNA levels are higher at the villi tips than in the villi crypts (Fig. 4, A, B, and D). No signals were detected in kidney under the same experimental conditions or in sense controls.

Characterization of Functional Properties of CaT1 by 45Ca2+ Uptake Assay—Since CaT1 shares similarity in its structure with the capsaiacin receptor (VR1) (25) and TRP (transient receptor potential) and TRPL (TRP-like) channels (26, 29, 33), we tested the possibility that the activity of CaT1 could be stimulated by capsaiacin or calcium store depletion. Capsaiacin (up to 50 µM) did not stimulate CaT1-mediated 45Ca2+ uptake in oocytes (data not shown). Instead of stimulating Ca2+ entry, depletion of calcium stores by thapsigargin treatment decreased CaT1-mediated Ca2+ activity to ~20% of its base-line activity (Fig. 5A). Based on these data, it is unlikely that CaT1 is another subtype of capsaiacin-gated or store-operated ion channels.

When expressed in oocytes, CaT1-mediated 45Ca2+ uptake was linear for up to 2 h (data not shown). Ca2+ uptake was concentration-dependent and saturable, with an apparent Michaelis constant (Km) of 0.44 ± 0.07 mM (Fig. 5B). This Km is appropriate for absorbing Ca2+ from the intestine, which is normally 1–5 mM after a calcium-containing meal. Consistent with the prediction from early studies that apical Ca2+ uptake is not energy-dependent, CaT1-mediated transport did not appear to be coupled to Na+, Cl−, or H+ (Fig. 5, C and D). To study the substrate specificity of CaT1, we initially performed inhibition studies of 45Ca2+ uptake by various di- and trivalent cations (100 µM) (Fig. 5E). Gd3+, La3+, Cu2+, Pb2+, Cd2+, Co2+, and Ni2+ produced marked to moderate inhibition, whereas Fe3+, Sr2+, and Mn2+ had no significant effects. In contrast, Ba2+ and Sr2+ had only slight inhibitory effects, even at a concentration of 10 mM, whereas Mg2+ (10 mM) produced no significant inhibition (Fig. 5E).

Ca2+ entry into enterocytes has, in general, been reported to be insensitive to classic voltage-dependent calcium channel blockers and to be only slightly inhibited by verapamil (17, 18). Among the three classes of L-type calcium channel blockers that we tested (nifedipine, diltiazem, and verapamil), only the latter two modestly inhibited CaT1-mediated Ca2+ uptake (by 10–15%) at relatively high concentrations (10–100 µM) (data not shown).

Electrophysiological Properties of CaT1-mediated Transport—External application of Ca2+ to oocytes expressing CaT1 generated inward currents at a holding potential of −50 mV (Fig. 6A, left panel), which were absent in control oocytes (data not shown).

Fig. 3. Northern analysis of tissue distribution of CaT1 mRNA and effects of 1,25-dihydroxyvitamin D3 and calcium deficiency on duodenal mRNA levels. A, evaluation of tissue distribution of CaT1 mRNA by Northern analysis. Each lane was loaded with 3 µg of poly(A)+ RNA from the indicated adult rat tissues. B, Northern analysis of CaT1 mRNA in gastrointestinal tract. Each lane was loaded with 3 µg of poly(A)+ RNA from stomach (lane 1), duodenum and proximal jejunum (~20 cm from stomach) (lane 2), the rest of the jejunum (lane 3), ileum (lane 4), cecum (lane 5), and colon (lane 6). C, CaT1 mRNA expression is not regulated by 1,25-dihydroxyvitamin D3 or calcium deficiency in duodenum. Lane 1, 2 µg of poly(A)+ RNA from normal rats (pooled RNA from five rats); lane 2, pooled poly(A)+ RNA from 1,25-dihydroxyvitamin D3-treated rats (duodenal mRNA was isolated from five rats 15 h after injection with 1 µg of 1,25-dihydroxyvitamin D3); lane 3, pooled poly(A)+ RNA from calcium-deficient rats. 10 rats were fed a calcium-deficient diet and MilliQ water for 2 weeks before sacrifice. The same blot was also hybridized with a 32P-labeled rat glyceroldehyde-3-phosphate dehydrogenase (GAPDH) cDNA fragment as a control.

Fig. 4. CaT1 mRNA distribution in rat intestine detected by in situ hybridization. Shown are bright-field micrographs of cryosections hybridized to a digoxigenin-labeled CaT1 antisense cRNA probe. A, duodenum (M, muscle layer; V, villi; C, crypt; L, lumen). CaT1 is expressed in enterocytes lining the villi, with the highest mRNA concentrations at the villi tips. B, jejunum (proximal). CaT1 is expressed only at the villi tips (arrows). C, ileum. D, cecum. CaT1 is strongly expressed in enterocytes. E, colon. Weak CaT1 labeling is present in the surface epithelial cells (arrows). The bar denotes 100 µm.
Chelating intracellular Ca\textsuperscript{2+} activated chloride channels remained active during this phase. Uptakes were performed at varying Ca\textsuperscript{2+} concentrations (0.02–5 mM) with CaT1-mediated Ca\textsuperscript{2+} uptake mediated by CaT1. Uptakes were performed in standard solution without thapsigargin. Black bars, CaT1-injected oocytes; gray bars, water-injected oocytes; white bars, difference between the two. B, concentration-dependent Ca\textsuperscript{2+} uptake mediated by CaT1. Uptakes were performed at varying Ca\textsuperscript{2+} concentrations (0.02–5 mM) with CaT1 cRNA- and water-injected oocytes. C, Na\textsuperscript{+} and Cl\textsuperscript{−} dependence of CaT1-mediated Ca\textsuperscript{2+} uptake. Uptakes were performed with standard medium, sodium-free medium in which NaCl was substituted by choline chloride (Cho-Cl), or low Cl\textsuperscript{−} (4 mM) medium in which NaCl was substituted by sodium cyclamate (Na-Cyc). D, pH effect on CaT1-mediated Ca\textsuperscript{2+} uptake. Uptake was performed in standard uptake solution with varying pH. Data shown were obtained by subtracting the uptake of Ca\textsuperscript{2+} by water-injected oocytes under the same experimental condition. E, CaT1-mediated Ca\textsuperscript{2+} uptake is inhibitable by divalent and trivalent metal ions. Uptakes were performed in the presence of Mg\textsuperscript{2+}, Sr\textsuperscript{2+}, or Ba\textsuperscript{2+} at 10 mM or other metal ions at 100 mM in standard uptake solution with 1 mM Ca\textsuperscript{2+}. Fe\textsuperscript{2+} was maintained in solution with 1 mM L-ascorbic acid, which had no effect on Ca\textsuperscript{2+} uptake. Fe\textsuperscript{II} and Fe\textsuperscript{III} represent Fe\textsuperscript{2+} and Fe\textsuperscript{3+}, respectively. Gadolinium and lanthanum are in their trivalent forms, and other metals are in their divalent forms. All metal ions were prepared from their chloride salts. 

Addition of 5 mM Ca\textsuperscript{2+} evoked an overshoot of inward current to several hundred nA, followed by a rapid reduction to a plateau value of 20–50 nA (Fig. 6A, left panel). CaT1-mediated current was also voltage-dependent, as revealed by current-voltage (I-V) curves (Fig. 6A, right panel). The peak current (curve 2) is due to endogenous Ca\textsuperscript{2+}-activated chloride channel currents (34) because it could be blocked by chloride channel blockers such as flufenamate (data not shown). The plateau also contained flufenamate-inhibitable currents, suggesting that some endogenous, Ca\textsuperscript{2+}-activated chloride channels remained active during this phase.

Chelating intracellular Ca\textsuperscript{2+} by injection of EGTA into oocytes expressing CaT1 to a final concentration of 1–2 mM resulted in a 3–5-fold increase in Ca\textsuperscript{2+} uptake (data not shown) and abolished the overshoot of the current (Fig. 6B, left panel). Under the same condition, EGTA-injected control oocytes produced no detectable currents. Therefore, CaT1 likely mediates the observed Ca\textsuperscript{2+}-evoked currents in EGTA-injected oocytes (Fig. 6B).

In the absence of Ca\textsuperscript{2+}, oocytes expressing CaT1 exhibited a significant permeability to Na\textsuperscript{+} at hyperpolarized potentials (Fig. 7A). Similar conductances were observed for K\textsuperscript{+}, Rb\textsuperscript{+}, and Li\textsuperscript{+} (K\textsuperscript{+} > Rb\textsuperscript{+} > Na\textsuperscript{+} > Li\textsuperscript{+}) (data not shown). CaT1-mediated permeation of monovalent cations exhibited inward rectification because the sum of endogenous K\textsuperscript{+} and Na\textsuperscript{+} concentrations is high in *Xenopus* oocytes. In addition, Ca\textsuperscript{2+}-evoked currents were slightly lower in the presence of 100 mM Na\textsuperscript{+} than in its absence (Fig. 7B), presumably due to the presence of modest competition between Ca\textsuperscript{2+} and Na\textsuperscript{+} for permeation via CaT1. Interestingly, with prolonged application of Ca\textsuperscript{2+} (30 min) to non-clamped oocytes expressing CaT1, Ca\textsuperscript{2+} entry was enhanced by extracellular Na\textsuperscript{+} (Fig. 5C). Further studies are needed to fully elucidate the mechanisms underlying the effects of Na\textsuperscript{+} on Ca\textsuperscript{2+} transport.

To determine whether Ca\textsuperscript{2+} entry via CaT1 is associated with influx or efflux of other ions, the charge\textsuperscript{2+}/Ca\textsuperscript{2+} influx ratio was determined in voltage-clamped oocytes pre-injected with EGTA (Fig. 8A). In the absence of external Na\textsuperscript{+}, the calculated ratio was not significantly different from 2 (Fig. 8B), indicating that permeation of Ca\textsuperscript{2+} alone accounts for the observed inward current.

Despite their weak inhibitory potencies, Ba\textsuperscript{2+} and Sr\textsuperscript{2+} (but not Mg\textsuperscript{2+}) evoked CaT1-specific currents, albeit with much smaller amplitudes (Fig. 9). In EGTA-injected oocytes expressing CaT1 that were clamped at −50 mV, currents due to addition of 5 mM Ba\textsuperscript{2+} and Sr\textsuperscript{2+} represented 12 ± 2 and 20 ± 4% (n = 17), respectively, of the current evoked by 5 mM Ca\textsuperscript{2+}. No significant Sr\textsuperscript{2+}- or Ba\textsuperscript{2+}-evoked currents were observed in control oocytes under similar conditions. Other divalent metal ions, including Fe\textsuperscript{2+}, Mn\textsuperscript{2+}, Zn\textsuperscript{2+}, Co\textsuperscript{2+}, Ni\textsuperscript{2+}, Cu\textsuperscript{2+}, Pb\textsuperscript{2+}, and Pb\textsuperscript{2+}, and

FIG. 5. Characterization of CaT1 by \textsuperscript{45}Ca\textsuperscript{2+} uptake assay. A, CaT1-mediated Ca\textsuperscript{2+} uptake is not stimulated by calcium store depletion. Calcium store depletion by thapsigargin treatment (2 μM in 0.025% Me\textsubscript{2}SO for >3 h in Ca\textsuperscript{2+}-free modified Barth’s solution) was performed as described (51). Control experiments were performed in the same solution without thapsigargin. Black bars, CaT1-injected oocytes; gray bars, water-injected oocytes; white bars, difference between the two. B, concentration-dependent Ca\textsuperscript{2+} uptake mediated by CaT1. Uptakes were performed at varying Ca\textsuperscript{2+} concentrations (0.02–5 mM) with CaT1 cRNA- and water-injected oocytes. C, Na\textsuperscript{+} and Cl\textsuperscript{−} dependence of CaT1-mediated Ca\textsuperscript{2+} uptake. Uptakes were performed with standard medium, sodium-free medium in which NaCl was substituted by choline chloride (Cho-Cl), or low Cl\textsuperscript{−} (4 mM) medium in which NaCl was substituted by sodium cyclamate (Na-Cyc). D, pH effect on CaT1-mediated Ca\textsuperscript{2+} uptake. Uptake was performed in standard uptake solution with varying pH. Data shown were obtained by subtracting the uptake of Ca\textsuperscript{2+} by water-injected oocytes under the same experimental condition. E, CaT1-mediated Ca\textsuperscript{2+} uptake is inhibitable by divalent and trivalent metal ions. Uptakes were performed in the presence of Mg\textsuperscript{2+}, Sr\textsuperscript{2+}, or Ba\textsuperscript{2+} at 10 mM or other metal ions at 100 mM in standard uptake solution with 1 mM Ca\textsuperscript{2+}. Fe\textsuperscript{2+} was maintained in solution with 1 mM L-ascorbic acid, which had no effect on Ca\textsuperscript{2+} uptake. Fe\textsuperscript{II} and Fe\textsuperscript{III} represent Fe\textsuperscript{2+} and Fe\textsuperscript{3+}, respectively. Gadolinium and lanthanum are in their trivalent forms, and other metals are in their divalent forms. All metal ions were prepared from their chloride salts.
Calli in the presence or absence of extracellular Na\(^{+}\) at \(-50\) mV. CaT1-expressing oocytes were pre-injected with EGTA. Cho, choline chloride.

**Fig. 7. Na\(^{+}\) effects on oocytes expressing CaT1.** A, comparison of total conductance between water-injected oocytes and oocytes expressing CaT1 in the presence of standard uptake solution and in the absence of extracellular Ca\(^{2+}\). Shown are average I-V curves obtained from control (n = 12) or CaT1-expressing (n = 28) oocytes following voltage jumps from the holding level of \(-50\) mV to the final potentials, which ranged between \(-140\) and \(+60\) mV. B, currents due to addition of 5 mM Ca\(^{2+}\) in the presence or absence of extracellular Na\(^{+}\) at \(-50\) mV. CaT1-expressing oocytes were pre-injected with EGTA. Cho, choline chloride.

**Fig. 8. Charge/Ca\(^{2+}\) uptake ratio of CaT1-mediated Ca\(^{2+}\) transport.** Ca\(^{2+}\)-elicited inward current and \(^{45}\)Ca\(^{2+}\) influx were measured simultaneously under voltage clamp (V\(_{h} = -50\) mV). A, shown is a representative example of currents generated by 2 mM Ca\(^{2+}\) (cold + hot) in an EGTA-injected, CaT1-expressing oocyte in the presence of an external solution containing 100 mM choline chloride. The charge moved was calculated by integrating the Ca\(^{2+}\) current at 100 mV. B, a mean charge/Ca\(^{2+}\) uptake ratio of 1.90 \pm 0.15 (n = 5) was obtained.

**Fig. 9. Sr\(^{2+}\) and Ba\(^{2+}\) (but not Mg\(^{2+}\)) are substrates of CaT1.** Shown is a comparison of currents evoked by Ca\(^{2+}\), Ba\(^{2+}\), Sr\(^{2+}\), and Mg\(^{2+}\) (each at 5 mM) at \(-50\) mV in an oocyte expressing CaT1 (top) and in a water-injected oocyte (bottom). Oocytes were injected with EGTA 2 h before measurements.

Cd\(^{2+}\), and the trivalent metal ions Fe\(^{3+}\), La\(^{3+}\), and Gd\(^{3+}\) (each at 100 \(\mu\)M) did not evoke measurable currents when applied to oocytes expressing CaT1 (data not shown). In agreement with their inhibitory effects on \(^{45}\)Ca\(^{2+}\) uptake (Fig. 4E), Gd\(^{3+}\), La\(^{3+}\), Cu\(^{2+}\), Pb\(^{2+}\), Cd\(^{2+}\), Co\(^{2+}\), and Ni\(^{2+}\) (each at 100 \(\mu\)M) all inhibited the Ca\(^{2+}\)-evoked currents, whereas the same concentration of Fe\(^{3+}\), Mn\(^{2+}\), and Zn\(^{2+}\) had no observable effects (data not shown). Magnesium is neither a substrate (up to 20 mM) nor an effective blocker of CaT1.

Single channel activities were sought in CaT1-expressing oocytes using the cell-attached and excised patch configurations of the patch clamp technique. No CaT1-specific channel activities could be identified that were clearly distinguishable from the endogenous channels present in control oocytes, based on studies of 52 patches from 46 oocytes (EGTA- or non-EGTA-injected) obtained from seven frogs. Further studies are needed to determine whether CaT1-specific single channel activity can be identified under appropriate experimental conditions.

**DISCUSSION**

Despite extensive studies of intestinal Ca\(^{2+}\) absorption, the protein that mediates Ca\(^{2+}\) entry in the transcellular pathway has remained elusive. A calcium channel with a structure similar to that of CaT1 has recently been isolated from rabbit kidney that exhibits an apical localization in the epithelial cells of the distal tubule (20). CaT1, which was cloned from rat duodenum, exhibits the characteristic expression pattern and functional properties of the previously described intestinal Ca\(^{2+}\) uptake mechanism. By measuring mucosa-to-serosa calcium transport across short-circuited rat intestinal segments, a saturable pathway has been identified in duodenum and jejunum (35) and cecum (36). The latter is the site with the highest transcellular calcium absorption in rat intestine (36). The mRNA distribution pattern of CaT1 in rat intestine (Figs. 3 and 4) shows the highest levels in cecum and duodenum and thereby closely matches the results describing segmental heterogeneity of intestinal transcellular calcium absorption (35–39). The ankyrin repeats predicted from the deduced amino acid sequence of CaT1 suggest that it may be associated with cytoskeletal proteins supporting the microvilli at the apical poles of the absorptive cells in the intestine (24). The 6.5-kb CaT1 transcript detected in brain, thymus, and adrenal gland and the presence of expressed sequence tags in rat and human brain and human prostate, fetal liver spleen, placenta, and lung suggest that CaT1 or a similar protein(s) plays additional roles beyond intestinal calcium absorption.

The apparent affinity constant for CaT1-mediated Ca\(^{2+}\) uptake in Xenopus oocytes of 0.44 mM is in the range of the values previously reported by other investigators in physiological studies of calcium absorption in rat (37–39), hamster (39), pig (40), and human (41, 42) intestines. In agreement with previous predictions (10, 15, 16), CaT1-mediated Ca\(^{2+}\) transport is driven by the electrochemical gradient of Ca\(^{2+}\). There is no evidence for coupling of Ca\(^{2+}\) uptake to other ions or to metabolic energy. Although CaT1-mediated Ca\(^{2+}\) transport is electrogenic and voltage-dependent, its kinetic behavior is distinct from that of the voltage-dependent calcium channels, which are operated by membrane voltage. At a macroscopic level, the kinetic properties of CaT1 resemble those of a facilitated transporter, and our patch clamp studies have not as yet provided any evidence for distinct single channel activity. CaT1 may represent an evolutionary transition between a channel and a facilitated transporter.

CaT1 is rather specific for Ca\(^{2+}\), showing only moderate abilities to transport Sr\(^{2+}\) and Ba\(^{2+}\). Our results provide a molecular basis for the widespread use of stable isotopes of strontium as a surrogate for monitoring intestinal absorption of calcium, as performed recently in human subjects (43, 44). Mg\(^{2+}\) does not appear to be a substrate for CaT1, in agreement with results reported previously (45). The inhibitory effects on CaT1-mediated Ca\(^{2+}\) uptake exerted by other metal ions, such as Cd\(^{2+}\) and Pb\(^{2+}\), provide a potential mechanistic basis for understanding the interactions of various metal ions during intestinal absorption (46).

It is a puzzle why CaT1 shows higher activities at alkaline than at acidic pH, despite being exposed to an acidic environment in the upper duodenal lumen \(\text{in vivo}\). However, a decrease in calcium absorption in rat duodenum at reduced levels of pH has been reported previously (47). It is well known that the...
milk-alkali syndrome was once a relatively common cause of hypercalcemia as a result of aggressive treatment of peptic ulcer disease with milk and antacids (3, 4, 48), perhaps due to increased CaT1-mediated Ca$^{2+}$ uptake at alkaline pH. Ca$^{2+}$ is transported bidirectionally (mucosa-to-serosa and serosa-to-mucosa) in the small intestine. Secreted Ca$^{2+}$ can be absorbed more distally in the large intestine, which has a higher luminal pH. Considering the length, sojourn time, and intraluminal pH in rat duodenum (8 cm, 3 min, and pH 6.6, respectively) (49), cecum (4 cm, 92 min, and pH 7.6) (50), and colon (14 cm, 92 min, and pH 7.0) (50), it is likely that the increased luminal pH in cecum and colon enhances the ability of these segments in Ca$^{2+}$ absorption. A study using human colonic apical membrane vesicles has provided evidence that a similar mechanism for Ca$^{2+}$ uptake exists in human colon (42), which exhibits apparent Michaelis constants of 0.51 ± 0.05 and 0.42 ± 0.04 mM in the proximal and distal colon, respectively, which are close to that for CaT1 (0.44 ± 0.07 mM).

The duodenal mRNA level of CaT1 was not responsive to 1,25-dihydroxyvitamin D$_3$ administration or to a calcium-deficient diet in rats in vivo, which is in agreement with previous studies demonstrating that apical entry of Ca$^{2+}$ is not substantially regulated by vitamin D (17, 37). However, because the overall transepithelial absorption of calcium is regulated by vitamin D (and this includes regulation of the synthesis of calbindin D$_{9K}$ (10–12), CaT1 activity would be expected to be regulated directly or indirectly by the vitamin D and/or calcium status of the organism to prevent toxic accumulation of intracellular Ca$^{2+}$. The presence of multiple putative protein kinase A and C phosphorylation sites in CaT1 may suggest phosphorylation-dependent regulation. In addition, the findings that EGTA injection increases CaT1 activity and that the calcium-evoked current decays upon prolonged calcium application (Fig. 8) suggest that CaT1 is controlled by a feedback regulatory mechanism, possibly through interaction of intracellular calcium with the transporter. The mechanisms underlying these phenomena need to be studied further.

CaT1 shares structural similarity (75% identity) with the epithelial calcium channel ECaC from rabbit kidney (20). There are numerous differences between the two proteins, in particular with respect to the amino- and carboxyl-terminal cytoplasmic domains (which are considerably more conserved between rat and human CaT1 than between CaT1 and ECaC), the number of ankyrin repeats, the number and distribution of protein kinase A and C phosphorylation sites, and their N-glycosylation sites. CaT1 and ECaC exhibit functional similarities with respect to saturation kinetics ($K_m$ for Ca$^{2+}$ = 0.44 and 0.2 mM, respectively) and pH sensitivity. However, more studies on ECaC are required to fully evaluate the differences between the two proteins in terms of the substrate specificities, the effects of Na$^+$ on their activities, and regulation by intracellular Ca$^{2+}$, etc. Moreover, additional investigation is needed to document that CaT1 and ECaC are the products of different genes as opposed to being splice variants in a given species.

A striking difference between CaT1 and ECaC is that ECaC is abundant in the distal tubules and cortical collecting duct of rabbit kidney (20), whereas the CaT1 mRNA was undetectable in rat kidney, based on Northern analysis and in situ hybridization. Although the transepithelial absorption of calcium in the intestine and its reabsorption in kidney share certain similarities (9), many differences also exist. For instance, different 1,25-dihydroxyvitamin D$_3$-regulated calcium-binding proteins (calbindins D$_{9K}$ and D$_{28K}$, respectively) are present in the intestine and kidney. Therefore, it is not too surprising that different calcium-absorptive proteins exist in the intestine and kidney.

CaT1 also shows moderate similarity to the capsaicin receptor, VR1, which is a ligand-gated, nonselective cation channel (25) and is structurally related to members of the TRP family of channels, some of which are operated by calcium store depletion (27, 33). CaT1 does not appear to be a ligand-gated channel since its open state does not require ligand binding. In contrast to channels opened by calcium store depletion, CaT1 activity is down-regulated in association with calcium store depletion.

Alterations in calcium absorption are present in many physiological and pathological states (3). Increased absorption occurs during pregnancy and lactation as well as in pathological states such as sarcoidosis and other granulomatous disorders, primary hyperparathyroidism, diabetes, idiopathic hypercalciuric syndromes, and phosphorus depletion. Increased calcium absorption can also be induced pharmacologically as in milk-alkali syndrome and by vitamin D intoxication, estrogens, and non-absorbable antacids. It will be of considerable interest to determine whether CaT1 contributes to the excessive absorption of calcium present in one or more of these states. Malabsorption of calcium is a common feature of aging and can contribute to osteoporosis. Moreover, pathological states such as intrinsic bowel disease, hepatobiliary disease, renal disease, hyperthyroidism, and hypoparathyroidism are also associated with calcium malabsorption. The cloning and characterization of the calcium transporter CaT1 may provide a molecular basis for achieving a better understanding of calcium malabsorption in such states as well as the regulation of intestinal calcium absorption under more normal physiological conditions.

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