

Molecular Identification of the Apical Ca^{2+} Channel in 1,25-Dihydroxyvitamin D_3 -responsive Epithelia*

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In mammals, the extracellular calcium concentration is maintained within a narrow range despite large variations in daily dietary input and body demand. The small intestine and kidney constitute the influx pathways into the extracellular Ca^{2+} pool and, therefore, play a primary role in Ca^{2+} homeostasis. We identified an apical Ca^{2+} influx channel, which is expressed in proximal small intestine, the distal part of the nephron and placenta. This novel epithelial Ca^{2+} channel (ECaC) of 730 amino acids contains six putative membrane-spanning domains with an additional hydrophobic stretch predicted to be the pore region. ECaC resembles the recently cloned capsaicin receptor and the transient receptor potential-related ion channels with respect to its predicted topology but shares less than 30% sequence homology with these channels. In kidney, ECaC is abundantly present in the apical membrane of Ca^{2+} transporting cells and colocalizes with 1,25-dihydroxyvitamin D_3 -dependent calbindin- $\text{D}_{28\text{K}}$. ECaC expression in *Xenopus* oocytes confers Ca^{2+} influx with properties identical to those observed in distal renal cells. Thus, ECaC has the expected properties for being the gatekeeper of 1,25-dihydroxyvitamin D_3 -dependent active transepithelial Ca^{2+} transport.

Calcium is the most abundant cation in the human body, but less than 1% is present in ionic form in the extracellular compartment (1). The extracellular Ca^{2+} concentration is precisely controlled by parathyroid hormone (PTH)¹ and 1,25-dihydroxyvitamin D_3 (1,25-(OH) $_2\text{D}_3$). Daily dietary intake is less than 1000 mg of which only 30% is absorbed in the intestinal

tract. This percentage is significantly enhanced during growth, pregnancy, and lactation by increased levels of circulating 1,25-(OH) $_2\text{D}_3$. Although there is a continuous turnover of bone mass, there is no net gain or loss of Ca^{2+} from bone in a young and healthy individual. This implicates that healthy adults excrete maximally 300 mg Ca^{2+} in the urine to balance intestinal Ca^{2+} uptake and that the remaining filtered load of Ca^{2+} has to be reabsorbed by the kidney. Recently, the mechanism by which extracellular Ca^{2+} is sensed by the parathyroid gland was elucidated by cloning of the Ca^{2+} -sensing receptor (2), and mutations in this receptor gene explained familial hypocalcemic hypercalcemia (3). The importance of 1,25-(OH) $_2\text{D}_3$ in Ca^{2+} homeostasis of the body is reflected by mutations in the genes coding for 1 α -hydroxylase (4), a renal enzyme controlling its synthesis, and the 1,25-(OH) $_2\text{D}_3$ -receptor (5). Transepithelial Ca^{2+} transport is a three-step process consisting of passive entry across the apical membrane, cytosolic diffusion facilitated by 1,25-(OH) $_2\text{D}_3$ -dependent calcium-binding proteins (calbindins), and active extrusion across the opposing basolateral membrane mediated by a high affinity Ca^{2+} -ATPase and Na^+ - Ca^{2+} exchanger (6). Until now, the molecular mechanism responsible for Ca^{2+} entry into small intestinal and renal cells, which serve as the influx pathways into the extracellular Ca^{2+} pool, is still elusive (6).

EXPERIMENTAL PROCEDURES

Primary Cultures of Kidney Cells—Rabbit connecting tubule (CNT) and cortical collecting duct (CCD) cells were immunodissected from New Zealand White rabbits (~0.5 kg) with monoclonal antibody R2G9, set in primary culture on permeable filter supports (0.33 cm 2 , Costar), and grown to confluence for 5 days, as described previously (7).

Expression Cloning and DNA Analysis—Poly(A)⁺ RNA, which induced $^{45}\text{Ca}^{2+}$ uptake in *Xenopus laevis* oocytes, was isolated from primary cultures of rabbit CNT and CCD cells and used to construct a directional cDNA library using a SuperScriptTM cDNA synthesis system (Life Technologies, Inc.). cDNA was ligated into the pSPORT1 vector, and ElectroMax DH10B cells were transformed using a Bio-Rad Gene Pulser. cRNA synthesized *in vitro* from pools of ~30,000 independent bacterial clones from this cDNA library was injected in oocytes. A pool expressing highest $^{45}\text{Ca}^{2+}$ uptake rates was sequentially subdivided and analyzed until a single clone (ECaC) was identified that was double-stranded sequenced using an automatic sequencer (ABI Prism 310 Genetic Analyzer). The mean hydrophobicity index was computed according to the algorithm of Kyte and Doolittle (8) with a window of 9 residues. Homology searches were performed against the nonredundant GenBankTM data base.

Radioactive Ion Uptake in Oocytes—Collagenase-treated *X. laevis* oocytes were injected with 20 ng of *in vitro* synthesized cRNA transcribed from pooled bacterial clones or 2 ng of *in vitro* synthesized cRNA from ECaC cDNA. Ca^{2+} and Na^+ uptake was determined 3 days after injection by incubating 10–15 oocytes in 500 μl of medium (in mM: 90 NaCl, 0.1 CaCl_2 , 1 $\mu\text{Ci}\cdot\text{ml}^{-1}$ $^{45}\text{Ca}^{2+}$ or 0.4 $\mu\text{Ci}\cdot\text{ml}^{-1}$ $^{22}\text{Na}^+$, 5 HEPES-Tris, pH 7.4) for 2 h at 18 °C. In the expression cloning experiments this medium was supplemented with 10 μM flodipine, 10 μM methoxyverapamil, 1 mM MgCl_2 , and 1 mM BaCl_2 . Each oocyte was washed three times in stop buffer (in mM: 90 NaCl, 1 MgCl_2 , 0.5 CaCl_2 , 1.5 LaCl_3 , 5 HEPES-Tris, pH 7.4, 4 °C), solubilized with 10% (w/v) SDS, dissolved in scintillation fluid, and counted for radioactivity.

Northern Analysis—Poly(A)⁺ RNA (2.5 $\mu\text{g}/\text{lane}$) was separated on a 18% (v/v) formaldehyde-1% (w/v) agarose gel and blotted onto a nitrocellulose filter (Amersham Pharmacia Biotech). The ECaC insert was excised from pSPORT1 and labeled with ^{32}P using a T7 QuickPrime kit (Amersham Pharmacia Biotech). Hybridization was for 16 h at 65 °C in 250 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.2, 7% (w/v) SDS, 1 mM EDTA, and filters were washed in 40 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.2, 0.1% (w/v) SDS, 1 mM EDTA for 20 min at 65 °C.

Immunohistochemistry—Rabbit kidney slices were fixed in 1% (v/v)

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¹ The abbreviations used are: PTH, parathyroid hormone; 1,25-(OH) $_2\text{D}_3$, 1,25-dihydroxyvitamin D_3 ; CNT, connecting tubule; CCD, cortical collecting duct; ECaC, epithelial Ca^{2+} channel; TRP, transient receptor potential.

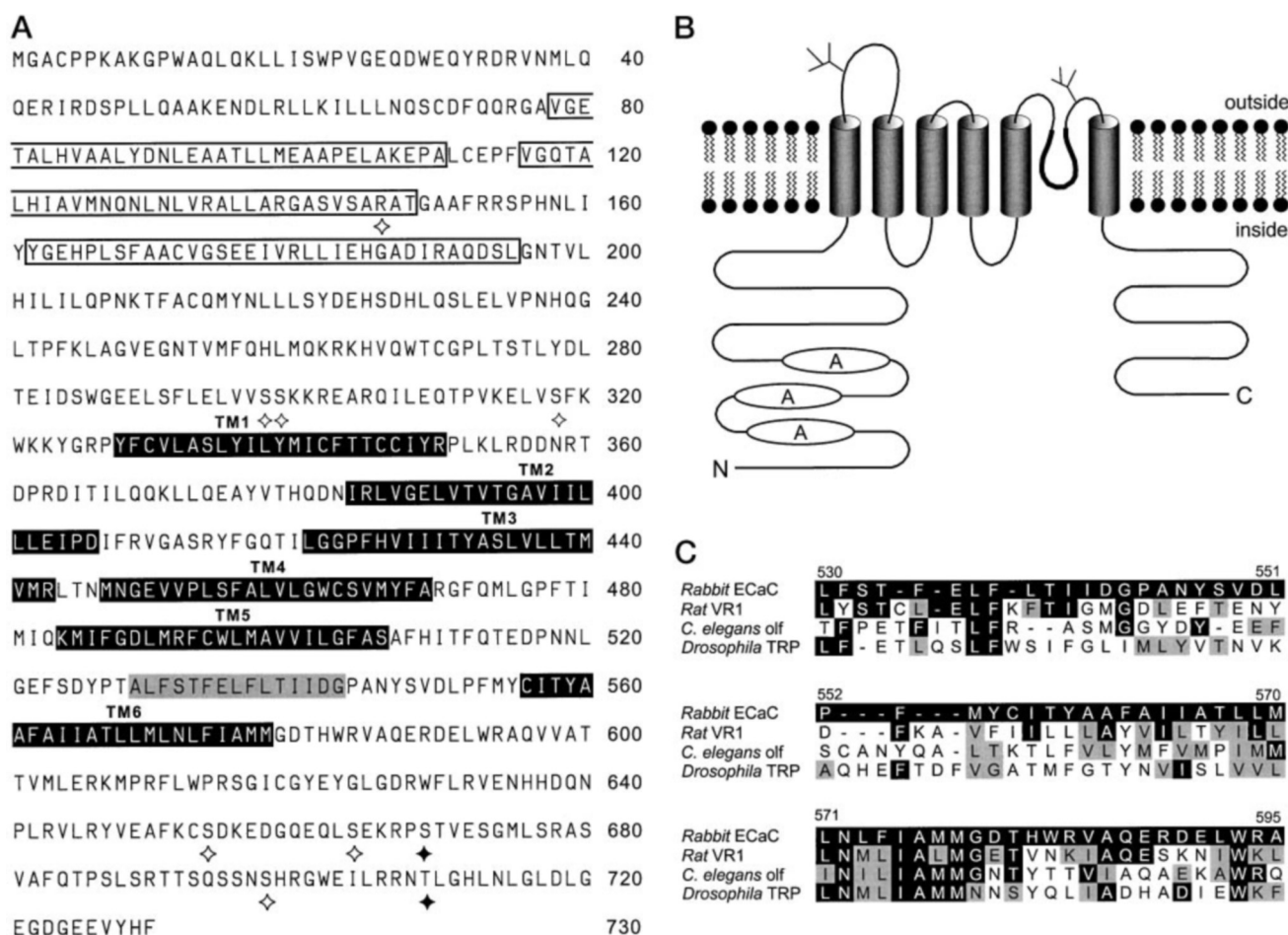


FIG. 1. Molecular structure of epithelial Ca^{2+} channel (ECaC). A, predicted amino acid sequence encoded by the ECaC cDNA. Open boxes delineate ankyrin repeat domains, black boxes predicted transmembrane domains, the gray box a possible pore region, and filled and open diamonds putative protein kinase A and C phosphorylation sites, respectively. B, predicted membrane topology and domain structure of ECaC. Outer and inner plasma membrane leaflets are indicated. C, alignment of ECaC pore region with that of related sequences. Identical residues are in black boxes, and conservative substitutions are in gray boxes. The GenBankTM accession numbers of the rabbit ECaC, rat capsaicin receptor, *Drosophila* TRP protein, and *Caenorhabditis elegans* olfactory channel are AJ133128, AF029310, P19334, and AF031408, respectively.

periodate-lysine-paraformaldehyde fixative for 2 h, washed with 20% (w/v) sucrose in phosphate-buffered saline, and subsequently frozen in liquid N_2 . Sections (7 μm) were blocked with 5% (w/v) blocking reagent (NEN Life Science Products) in phosphate-buffered saline for 15 min. Sections were washed three times with Tris-buffered saline (TBS; 150 mM NaCl, 100 mM Tris-HCl, pH 7.5) and incubated with affinity-purified guinea pig antiserum raised against the ECaC C-tail (amino acids 580–706) and rabbit anti-calbindin- $\text{D}_{28\text{K}}$ antiserum (9) for 16 h at 4 °C. After thorough washing with TBS, the sections were incubated with the corresponding fluorescein isothiocyanate- or tetramethylrhodamine isothiocyanate-conjugated anti-immunoglobulin G for 60 min. Subsequently, sections were washed with TBS, distilled water, and methanol and finally mounted in Mowiol (Hoechst). All controls, including sections treated with preimmune serum or with conjugated antibodies only, were devoid of any staining.

Transcellular Ca^{2+} Transport.—Confluent monolayers of rabbit CNT and CCD cells were washed twice and preincubated in medium (in mM: 140 NaCl, 2 KCl, 1 K_2HPO_4 , 1 MgCl_2 , 5 glucose, 5 L-alanine, 0.005 indomethacin, 0.0001 bovine PTH-(1–34), 10 HEPES-Tris, pH 7.4) containing 0.1 mM and 1 mM CaCl_2 in the apical and basolateral compartment, respectively, for 15 min at 37 °C. Subsequently, the apical fluid was replaced with medium containing 1 $\mu\text{Ci}\cdot\text{ml}^{-1}$ $^{45}\text{Ca}^{2+}$, and transcellular Ca^{2+} transport was determined following removal of a 20- μl sample from the basolateral medium at 30 min. The basolateral-to-apical flux was negligible under all experimental conditions.

RESULTS AND DISCUSSION

Here, we report the expression cloning, tissue distribution, immunolocalization, and functional characterization of the apical Ca^{2+} influx channel, which is expressed solely in proximal

small intestine, the distal part of the nephron, and placenta. In analogy to the recently cloned amiloride-sensitive and aldosterone-dependent epithelial Na^+ channel (ENaC) (10), present in the apical membrane of sodium-transporting epithelia, this novel epithelial Ca^{2+} channel was named ECaC. By screening for maximal $^{45}\text{Ca}^{2+}$ influx activity in oocytes a single 2.8-kilobase pair cDNA was isolated from a directional cDNA library prepared from poly(A)⁺ RNA of rabbit distal tubular cells. The ECaC cDNA contains an open reading frame of 2190 nucleotides that encodes a protein of 730 amino acids with a predicted relative molecular mass of 83 kDa (M_r 83,000) (Fig. 1A). Hydropathy analysis suggests that ECaC contains three structural domains: a large hydrophilic amino-terminal domain of 327 amino acids containing three ankyrin binding repeats and several potential protein kinase C phosphorylation sites, suggesting an intracellular location; a six transmembrane-spanning domain with two potential *N*-linked glycosylation sites and an additional hydrophobic stretch between transmembrane segments 5 and 6 indicative of an ion pore region; and a hydrophilic 151-amino acid carboxyl terminus containing potential protein kinase A and C phosphorylation sites (Fig. 1B).

A protein data base search revealed only a significant homology of less than 30% between ECaC and the recently cloned capsaicin receptor (VR1) (11), the transient receptor potential (TRP)-related ion channels (12) and olfactory channels (13).

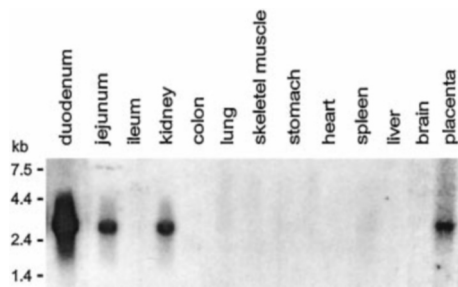


FIG. 2. **ECaC expression in small intestine, kidney, and placenta.** High-stringency Northern blot analysis of RNA from a range of tissues probed with ^{32}P -labeled ECaC cDNA. Lanes were loaded with 2.5 μg of poly(A) $^{+}$ RNA from rabbit duodenum, jejunum, ileum, kidney, distal colon, lung, skeletal muscle, stomach, heart, spleen, liver, brain, and human placenta, respectively. Molecular mass standards (in kilobases) are indicated on the left.

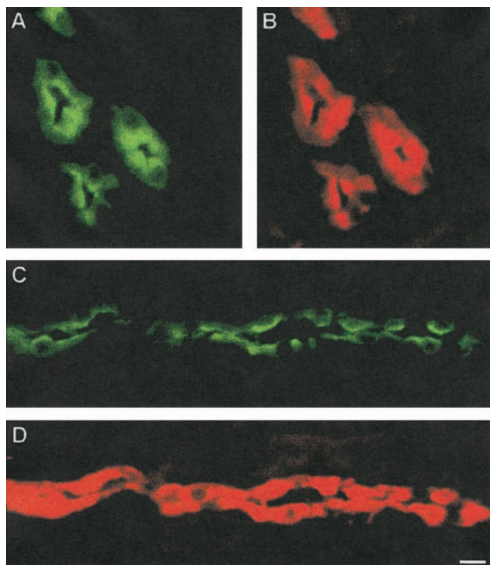


FIG. 3. **Immunohistochemistry of ECaC in rabbit kidney.** Kidney cortex sections were double stained with anti-ECaC antiserum (A, C) and anti-calbindin- $\text{D}_{28\text{K}}$ (B, D). Localization of ECaC in distal convoluted tubules (A) and cortical collecting ducts (C) is restricted to the apical region of the cell and colocalizes with calbindin- $\text{D}_{28\text{K}}$. The cells in the collecting duct lacking ECaC were also calbindin- $\text{D}_{28\text{K}}$ -negative and are, therefore, most likely intercalated cells. Note the absence of immunopositive staining of ECaC in the surrounding glomeruli, proximal convoluted tubules, and thick ascending limb of Henle's loop. Bar denotes 10 μm .

The capsaicin receptor is a nonselective cation channel and functions as a transducer of painful thermal stimuli (11). Members of the TRP family have been proposed to mediate the entry of extracellular Ca^{2+} into cells in response to depletion of intracellular Ca^{2+} stores (12). These proteins resemble ECaC with respect to their predicted topological organization and the presence of multiple NH_2 -terminal ankyrin repeats (14). There was also striking amino acid sequence similarity between ECaC, VR1, and TRP-related proteins within and adjacent to the sixth transmembrane segment, including the predicted area that may contribute to the ion permeation path (Fig. 1C) (15). Outside these regions, however, ECaC shares only 20% sequence similarity with VR1 and TRP family members, suggesting a distant evolutionary relationship among these channels.

High stringency Northern blot analysis of ECaC transcripts revealed prominent bands of ~ 3 kb in small intestine, kidney, and placenta (Fig. 2). We found that in the intestine ECaC mRNA expression was highest in duodenum, decreased in jejunum, and absent in ileum and colon. ECaC mRNA expression

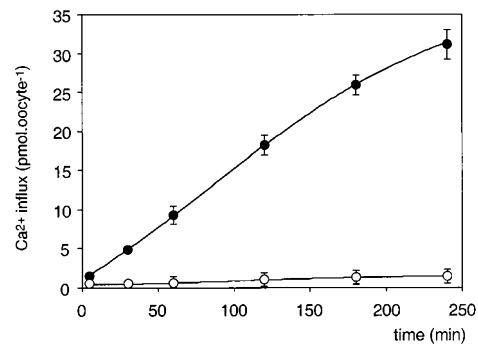


FIG. 4. **$^{45}\text{Ca}^{2+}$ uptake in oocytes expressing ECaC as a function of time.** Measurements were performed at the indicated time points in water-injected (\circ) and ECaC-injected (\bullet) oocytes. Values are means \pm S.E. of three experiments.

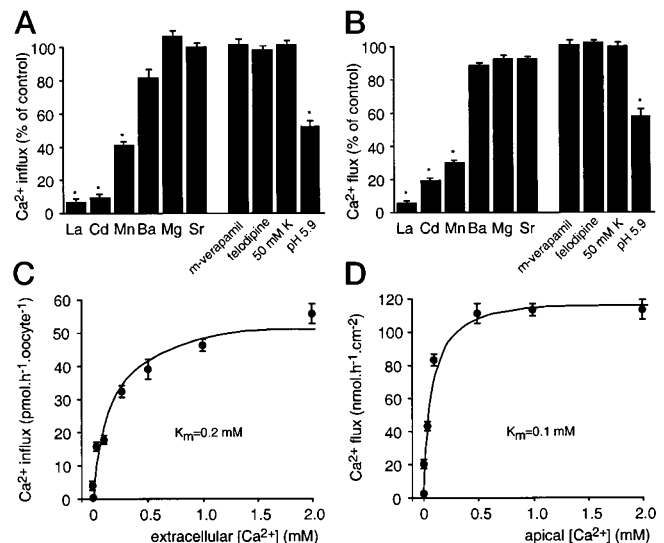


FIG. 5. **Functional characterization of ECaC.** A and B, substrate specificity, pH dependence, and voltage dependence of $^{45}\text{Ca}^{2+}$ uptake in oocytes expressing ECaC (A) and transcellular $^{45}\text{Ca}^{2+}$ transport across rabbit kidney cells (B). Cations were added at a final concentration of 0.5 mM, pH was lowered from 7.4 to 5.9, 50 mM NaCl was replaced by 50 mM KCl to depolarize the membrane, methoxyverapamil (*m-verapamil*) and felodipine were added at 10 μM . In transcellular Ca^{2+} transport measurements across renal cells these additions were applied to the apical compartment only. C and D, concentration dependence of $^{45}\text{Ca}^{2+}$ uptake in oocytes expressing ECaC (C) and transcellular $^{45}\text{Ca}^{2+}$ transport in rabbit kidney cells (D). Measurements were performed at the indicated Ca^{2+} concentrations in extracellular and apical medium of oocytes and confluent monolayers, respectively. Water-injected oocytes exhibited a Ca^{2+} uptake of less than 6% of the ECaC-injected oocytes. Values are means \pm S.E. of five experiments. Statistical significance (*, $p < 0.05$) was determined by analysis of variance.

in kidney and placenta was comparable with jejunum. In addition, ECaC transcripts in lung, skeletal muscle, stomach, heart, liver, spleen, and brain were undetectable. Most important is that expression of ECaC coincides with that of calbindin- $\text{D}_{9\text{K}}$ in intestine and placenta and calbindin- $\text{D}_{28\text{K}}$ in kidney (16, 17).

Immunofluorescence staining revealed that in kidney ECaC is abundantly present along the apical membrane in the majority of cells lining the distal part of the nephron including distal convoluted tubule, connecting tubule and cortical collecting duct, where it colocalizes with calbindin- $\text{D}_{28\text{K}}$ (Fig. 3). This part of the nephron is the site of PTH- and $1,25\text{-(OH)}_2\text{D}_3$ -regulated transcellular Ca^{2+} reabsorption (18).

By functional expression of ECaC in *Xenopus* oocytes we observed that the $^{45}\text{Ca}^{2+}$ uptake was linear for at least 2 h (Fig. 4) and increased with increasing extracellular Ca^{2+} concentra-

tions between 0.01 and 2.0 mM with an apparent affinity for Ca^{2+} of ~ 0.2 mM (Fig. 5C). This is well within the range of physiologically relevant extracellular calcium concentrations. As shown in Fig. 5A, trivalent and divalent cations inhibited $^{45}\text{Ca}^{2+}$ influx in the following rank order of potency: $\text{La}^{3+} > \text{Cd}^{2+} > \text{Mn}^{2+}$, while Ba^{2+} , Mg^{2+} , and Sr^{2+} had no effect. It is striking that Ba^{2+} and Sr^{2+} , which are highly permeant in voltage-gated Ca^{2+} channels (19), do not interfere with ECaC. In addition, the L-type Ca^{2+} channel antagonists and depolarization with 50 mM KCl were without effect. VR1, to which ECaC has the highest homology, shows a relative low permeability to monovalent ions such as Na^+ (11). In a double labeling experiment, ECaC-injected oocytes did not exhibit a significant Na^+ influx (0.88 ± 0.05 and 0.95 ± 0.05 nmol·h $^{-1}$ ·oocyte $^{-1}$ for ECaC and water-injected oocytes, respectively; $n = 29$ oocytes; $p > 0.2$), while displaying a markedly increased Ca^{2+} influx. In humans metabolic acidosis induces hypercalciuria (18, 20), and here we demonstrate that acidification of the extracellular medium to pH 5.9 significantly inhibits $^{45}\text{Ca}^{2+}$ influx (Fig. 5, A and B). If extrapolatable to the *in vivo* situation this effect could well be the molecular explanation of acidosis-induced calciuresis. Taken together, these characteristics indicate that ECaC is distinct from previously described Ca^{2+} channels. Furthermore, the above described pharmacological and functional properties of ECaC are identical to those of Ca^{2+} transport across the monolayers (Fig. 5, C and D), providing evidence that the protein is a major constituent of the transcellular Ca^{2+} transport system in renal cells. Together with the previous finding that the Ca^{2+} influx rate at the apical membrane of renal distal cells is tightly coupled to transepithelial Ca^{2+} flux over a wide range of transport rates (21), this suggests that the apical Ca^{2+} influx is the rate-limiting step in transcellular Ca^{2+} transport. Moreover, this implicates that hormonal regulation of a single influx pathway, *i.e.* ECaC, may control the rate of transcellular Ca^{2+} transport.

In humans, approximately 4% of the population suffers from idiopathic hypercalciuria with the characteristics of autosomal dominant transmission (22). In some of the affected individuals, hypercalciuria is secondary to hyperabsorption of Ca^{2+}

(22). Gain of function mutations in ECaC or its dysregulation may well be the cause of absorptive hypercalciuria. The present elucidation of ECaC allows to study these possibilities with molecular genetic approaches.

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