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-D\(_{28K}\). 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) and 1,25-dihydroxyvitamin D\(_3\) (1,25-(OH)\(_2\))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)\(_2\))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 hypoparathyroidism (3). The importance of 1,25-(OH)\(_2\))D\(_{3}\) in Ca\(^{2+}\) homeostasis of the body is reflected by mutations in the genes coding for 1α-hydroxylase (4), a renal enzyme controlling its synthesis, and the 1,25-(OH)\(_2\))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)\(_2\))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 RZ20, 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 45Ca\(^{2+}\) 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 SuperScript\textsuperscript{TM} 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 45Ca\(^{2+}\) 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 of the coding region was calculated according to the algorithm of Kyte and Doolittle (8) with a window of 9 residues. Homology searches were performed against the nonredundant GenBank\textsuperscript{TM} data base.

Radioactive Ion Uptake in Oocytes—Collagenase-treated X. laevis oocytes were injected with 20 ng of in vitro synthesized cRNA transcript 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 µM Ciml\(^{-1}\) Ca\(^{2+}\) or 0.4 µM Ciml\(^{-1}\) 2Na\(^{+}\), 5 HEPES-Tris, pH 7.4) for 2 h at 18°C. In the expression cloning experiments this medium was supplemented with 10 µM felodipine, 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, 0.5 MgCl\(_2\), 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 µg/lane) was separated on a 18% (w/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 32P using a T7 Quick Prime kit (Amersham Pharmacia Biotech). Hybridization was for 16 h at 65°C in 250 µM Na\(_2\)HPO\(_4\), NaH\(_2\)PO\(_4\), pH 7.2, 7% (w/v) SDS, 1 mM EDTA, and filters were washed in 40× Na\(_2\)HPO\(_4\), NaH\(_2\)PO\(_4\), pH 7.2, 0.1% (w/v) SDS, 1× EDTA for 20 min at 65°C.
periodate-lysine-paraformaldehyde fixative for 2 h, washed with 20% (w/v) sucrose in phosphate-buffered saline, and subsequently frozen in liquid N₂. 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-D₂₈K 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.

**Apical Ca²⁺ Transport—Confluent monolayers of rabbit CNT and CCD cells were washed twice and preincubated in medium (in mM): 140 NaCl, 2 KCl, 1 K₂HPO₄, 1 MgCl₂, 5 glucose, 5 l-alanine, 0.005 indomethacine, 0.0001 bovine PTH-(1–34), 10 HEPES-Tris, pH 7.4) containing 0.1 mM and 1 mM CaCl₂ in the apical and basolateral compartment, respectively, for 15 min at 37 °C. Subsequently, the apical fluid was replaced with medium containing 1 µMml⁻¹ ⁴⁵Ca²⁺, and transcellular Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ channel was named ECaC. By screening for maximal ⁴⁵Ca²⁺ 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 (M₉83,000) (Fig. 1 A). 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. 1 B).

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).
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 in kidney and placenta was comparable with jejenum. 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-D\(_{28K}\) in intestine and placenta and calbindin-D\(_{28K}\) 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. In addition, ECaC mRNA expression was highest in duodenum, decreased in jejunum, and absent in ileum and colon. ECaC mRNA expression in kidney and placenta was comparable with jejenum. 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-D\(_{28K}\) in intestine and placenta and calbindin-D\(_{28K}\) in kidney (16, 17).

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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: La\(^{3+}\) > Cd\(^{2+}\) > 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 calciresis. 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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