Epithelial calcium transporter expression in human duodenum

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Barley, Natalie F., Alison Howard, David O’Callaghan, Stephen Legon, and Julian R. F. Walters. Epithelial calcium transporter expression in human duodenum. Am J Physiol Gastrointest Liver Physiol 280: G285–G290, 2001.—Calcium absorption in intestine and kidney involves transport through the apical membrane, cytoplasm, and basolateral membrane of the epithelial cells. Apical membrane calcium influx channels have recently been described in rabbit (epithelial calcium channel, ECaC) and rat (calcium transport protein, CaT1). We amplified from human duodenum a 446-base partial cDNA probe (ECAC2) having a predicted amino acid similarity of 97% to rat CaT1. Duodenum, but not ileum, colon, or kidney, expressed a 3-kb transcript. A larger transcript was also found in placenta and pancreas, and a different, faint transcript was found in brain. In duodenal biopsies from 20 normal volunteers, expression varied considerably but was not significantly correlated with vitamin D metabolites. This signal correlated with calbindin-D9k (r = 0.48, P < 0.05) and more strongly with the plasma membrane calcium ATPase PMCA1b (r = 0.83, P < 0.001). These data show that although individual variations in calcium channel transcripts are not vitamin D dependent, expression of genes governing apical entry and basolateral extrusion are tightly linked. This may account for some of the unexplained variability in calcium absorption.

DIETARY CALCIUM IS ABSORBED in the intestine and is necessary for the development and maintenance of bone mineralization. Studies in humans have indicated that there is considerable variation in fractional calcium absorption among individuals (9). Although the active metabolite of vitamin D, 1,25-dihydroxycholecalciferol [1,25(OH)2D3], is clearly involved in calcium absorption, studies have suggested that much of the variability in human fractional calcium absorption is not caused by this or other known factors (1).

Active absorption of dietary calcium occurs principally in the proximal small intestine and necessitates separate steps of transport, first across the apical (brush border) membrane, then through the cytoplasm, and finally extrusion at the basolateral membrane of the enterocyte. Transepithelial transport of calcium also takes place in the placenta and in the kidney. In the intestine, the vitamin D-dependent calcium binding protein calbindin-D9k and the plasma membrane calcium-pumping ATPase 1b (PMCA1b) are thought to be the major molecules involved in the cytoplasm and basolateral membrane. We (13, 15) have characterized the molecular nature of both of these in human duodenum. Recently, we (25) studied the range and variability of their expression in duodenum, showing that there was significant, although weak, correlation of 1,25(OH)2D3 with calbindin-D9k expression but not with PMCA1b.

The molecular nature of the apical calcium influx channel has been unclear until recently. In 1999, Hoenderop and colleagues (11) identified a strong candidate for this channel in the rabbit. The RNA for this epithelial calcium channel (ECaC) was expressed in proximal small intestine, in the distal part of the nephron, and in placenta. Peng and colleagues (20) described a homologous channel in the rat. This was expressed principally in duodenum and cecum but not in kidney and was named CaT1 (for calcium transport protein). These two molecules, which have 75% amino acid identity, are unlike previously described calcium channels.

In this paper we report the amplification of a partial sequence of an apical calcium transport channel in humans using a strategy of mixed-primer PCR from duodenal cDNA. We have investigated the expression of this transcript in duodenum and its relationship with other critical factors involved in intestinal calcium absorption.

EXPERIMENTAL PROCEDURES

Amplification of cDNA. PCR primers were based on regions of conserved amino acids in the rabbit ECaC (accession no. AJ133128) and rat CaT1 (accession no. AF160798) sequence. The forward primer, coding for GGPFHV between positions 1553 and 1568 of rat CaT1 cDNA, had the sequence

G285
performed in buffer containing 125 mM NaH$_2$PO$_4$, pH 7.2, and washed as previously described (14). Hybridization was with the ECAC1 sequence reported by Müller et al. (17), which also has this degree of similarity with the rabbit sequence. These highly conserved amino acids form part of the predicted transmembrane regions 3, 4, 5, and 6 and the pore (11, 20). The CaT1-related sequence reported here has been given the approved gene symbol ECAC2, for epithelial calcium channel 2.

**Expression studies.** Northern blotting was performed using total RNA samples prepared from duodenum, ileum, colon, and placenta (14, 26) and with poly(A)-enriched RNA on a multitissue Northern blot (Clontech, Palo Alto, CA). The cDNA probe amplified above was $^{32}$P-labeled, hybridized, and washed as previously described (14). Hybridization was performed in buffer containing 125 mM NaH$_2$PO$_4$, pH 7.2, 10% dextran sulfate, 5% SDS, 0.5% (wt/vol) dried milk powder, 25 μM aurin tricarboxylic acid, and 2.5 mM EDTA overnight at 60°C. Blots were washed for 1 h in 2% SDS, 0.25 M NaH$_2$PO$_4$, pH 7.2, and 1 mM EDTA and for 1 h in 30 mM NaCl, 2 mM NaH$_2$PO$_4$, and 0.2 mM EDTA with 0.2% SDS, also at 60°C.

To determine the range of expression in duodenum, we used total RNA on blots previously prepared for the study of calbindin-D$_{28k}$ and PMCA1 (25). Blots of duodenal RNA were available for study from 20 subjects who had undergone normal endoscopy approved by the local Research Ethics Committee. RNA signals were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression, and 1,25(OH)$_2$D and 25(OH)D levels in plasma had been determined. Results were quantified by phosphorimaging. Statistical analysis used the data analysis package of Microsoft Excel.

**RESULTS**

**Human calcium transporter cDNA probe.** Amplification of human duodenal cDNA with the primers based on conserved regions of rabbit ECaC and rat CaT1 resulted in a single band on electrophoresis that had the expected size of ~479 bp. This was eluted, cloned, and sequenced. This sequence was obtained in at least 10 different clones, with no variation apart from a single nucleotide difference, probably the result of fidelity in PCR. The sequence matched several expressed sequence tags in the human database, including W88570, AA447311, H18519, and T92755, but did not detect any previously known human genes, although subsequently, a related human epithelial calcium channel sequence, AJ271207, has been reported and named ECAC1 (17). As shown in Fig. 1A, the human nucleotide sequence amplified had high identity (90%) to the corresponding region of rat CaT1. It had lower identity with rabbit ECaC (86%) and is clearly distinct from the other reported human sequence (ECAC1), with which it has 91% identity in this region.

The derived amino acid translation of this cDNA, shown in Fig. 1B, had much greater similarity to rat CaT1 than to rabbit ECaC (97% and 86%, respectively). This high degree of similarity indicates that this partial sequence comes from the human homologue of CaT1. There is 92% amino acid similarity in this region with the ECAC1 sequence reported by Müller et al. (17), which also has this degree of similarity with the rabbit sequence. These highly conserved amino acids form part of the predicted transmembrane regions 3, 4, 5, and 6 and the pore (11, 20). The CaT1-related sequence reported here has been given the approved gene symbol ECAC2, for epithelial calcium channel 2.

**Tissue expression.** The expression of human ECAC2 (CaT1) was investigated in the intestine and other tissues by Northern blotting (Fig. 2). A single signal ~3 kb in size was readily detectable in duodenum but not in ileum or colon, although the quality of the placental total RNA was poor. On a multitissue blot of poly(A)-enriched RNA, two bands, one the same size and another larger transcript (~9 kb), were demonstrated in human placenta and pancreas. Another faint transcript (~7.5 kb) could be detected in brain. No expression could be found in kidney using this probe.

**Variation in duodenal expression.** To investigate vitamin D dependence and other correlates of expression, we quantified levels of transcripts detected by this calcium channel probe on Northern blots of RNA from the normal duodenum of 20 subjects. Their details are described in Table 1. Signals were obvious in all of these duodenal samples but varied considerably (Fig. 3). After correction for differences in RNA loading by measuring GAPDH expression, there was a 10-fold range in expression between the highest and lowest values.

We investigated whether there was any correlation between these values and the previously determined values for calbindin-D$_{28k}$ and PMCA1 expression or for the vitamin D metabolites 1,25(OH)$_2$D and 25(OH)D (Table 2). Calcium channel expression showed no significant relationship with either vitamin D metabolite; the data for 1,25(OH)$_2$D are shown in Fig. 4A. Calcium channel and calbindin-D$_{28k}$ transcripts showed a significant correlation ($r = 0.48, P < 0.05$; Fig. 4B).

An unexpectedly strong relationship was found between calcium channel and PMCA1 transcripts ($r = 0.83, P < 0.001$; Fig. 4C). Significant correlation ($r = 0.81$) was also present before normalization of values with GAPDH, thus ruling out an artifactual association produced by this GAPDH correction. After further adjustment for the associations of the channel with PMCA1 or of calbindin-D$_{28k}$ with the channel and 1,25(OH)$_2$D, no other significant correlations were apparent.

**DISCUSSION**

The sequence we amplified by RT-PCR from human duodenal RNA appears to be part of the human homologue of rat CaT1, which has been shown to have the expected properties of an apical membrane calcium channel implicated in calcium entry to the enterocyte during calcium absorption (20). In humans, as in rats, we have shown expression of the transcript in proximal small intestine and also in the placenta and pancreas,
where related roles in calcium transport can be envisaged. In these latter tissues, a second, larger-sized transcript can be detected, raising the possibilities of alternative splicing or control of RNA stability. Low-level expression of a different-sized transcript in brain will also require further exploration to determine any role in this tissue.

The inability to detect this transcript in human kidney RNA suggests that, despite their high degree of identity in this region, the conditions used in the
pression studies do not result in cross-hybridization with human ECAC1, the other member of this epithelial apical membrane calcium channel/transporter family. ECAC expression has been detected in the rabbit distal nephron as well as the duodenum (11) and, with the use of specific primers, has been shown in kidney, duodenum, and other tissues in humans (17). Our PCR data with mixed primers suggests that the CaT1-related ECAC2 sequence described here is of greater abundance than ECAC1 in duodenum, although this method is subject to considerable error. To determine accurately the relative abundance of these two genes in duodenum and elsewhere will require parallel Northern blots with unequivocally different probes, perhaps from the 3′-end of the cDNA. Further data regarding the human sequence and information about tissue and cellular expression in humans will clarify the relationships between these genes and how the functions of their various products might differ according to species or tissues.

We were able to study the variability of expression of the transcripts detected with our probe in normal duodenal RNA from a range of subjects to test the hypothesis that expression was affected by 1,25(OH)2D levels. Our data suggest that there is no vitamin D dependence of expression, at least at the population level. Using the same experimental samples, we (25) were previously able to show that calbindin-D9k transcripts correlate with 1,25(OH)2D. It is interesting to note that the strength of this relationship in humans is weak, at both the RNA and protein levels (23), and that the presence of a vitamin D-responsive element in the calbindin-D9k gene remains debatable (2, 22). However, expression of the calcium channel signal and calbindin-D9k are also significantly correlated in our samples. It will be of interest to see whether this relates to similar transcriptional control of these genes by factors expressed in duodenum or whether calcium entering the cell through apical channels may be affecting calbindin-D9k transcript levels independently of 1,25(OH)2D. In the vitamin D receptor knockout mouse, there is still some expression of intestinal calbindin-D9k (27), and posttranscriptional effects of calcium on calbindin-D9k RNA stability have been described (6).

The lack of vitamin D dependence of expression of the human apical calcium channel is also similar to that suggested for rat CaT1 by the results of feeding different levels of dietary calcium (20). In the chicken, using ion microscopic imaging, Chandra and colleagues (4) showed that calcium entry at the apical membrane was unchanged in vitamin D-deficient chicks but that transport away from this site was impaired in this species because of lower vitamin D-dependent calbindin-D9k concentrations. In contrast, preliminary data in the vitamin D knockout mouse (24) reported greatly reduced expression of ECaC, indicating that, in this species at least, one of the apical calcium channel genes may be vitamin D dependent.

Probably the most interesting finding in the present study is the strength of the correlation of the expression of calcium channel and PMCA1 transcripts. First, this shows that there is sufficient precision in our experimental methods to detect physiologically relevant associations in variations in gene expression in

![Fig. 1. Expression of transcripts detected with the CaT1 homologue ECAC2 probe in various human tissues. The Northern blots show total RNA (25 μg) prepared from duodenum, ileum, colon, and placenta (lanes 1–4, respectively) and poly-A enriched RNA (2 μg) from heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas (lanes 5–12, respectively). Size markers are indicated (kb).](http://ajpgi.physiology.org/)

![Fig. 2. Expression of transcripts detected with the CaT1 homologue ECAC2 probe in various human tissues. The Northern blots show total RNA (25 μg) prepared from duodenum, ileum, colon, and placenta (lanes 1–4, respectively) and poly-A enriched RNA (2 μg) from heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas (lanes 5–12, respectively). Size markers are indicated (kb).](http://ajpgi.physiology.org/)

![Fig. 3. Representative Northern blot of duodenal RNA showing variations in the signals detected by the ECAC2, PMCA1, calbindin-D9k (Cb9k), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probes from 12 subjects. One blot with total RNA samples (25 μg) was hybridized, stripped, and reprobed in turn with each of the four cDNAs. The approximate sizes of the bands detected were 3 (ECAC2), 7.5 and 5.5 (PMCA1), 0.6 (Cb9k), and 1.5 (GAPDH) kb.](http://ajpgi.physiology.org/)

### Table 1. Characteristics of patients studied

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean ± SD</th>
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<tr>
<td>Age, yr</td>
<td>47.1 ± 16.7</td>
</tr>
<tr>
<td>Range</td>
<td>25–71</td>
</tr>
<tr>
<td>Sex, male:female</td>
<td>14:6</td>
</tr>
<tr>
<td>25-(OH)D, ng/ml (mean ± SD)</td>
<td>13.3 ± 8.2</td>
</tr>
<tr>
<td>1,25-(OH)2D, pg/ml (mean ± SD)</td>
<td>37.0 ± 11.5</td>
</tr>
<tr>
<td>Vitamin D receptor genotype</td>
<td>TT:10, Tt:9, tt:2</td>
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</table>

endoscopic biopsies. More importantly, this points to fundamental mechanisms controlling the activities of calcium channels and PMCA1. It makes considerable sense for the expression of genes regulating calcium entry and exit from the cell to be closely regulated. PMCA1 appears to have the properties expected of the major basolateral membrane calcium pump involved in calcium absorption. Although it is expressed in most if not all tissues, it is highly expressed in duodenum in humans as in other species (8, 14, 15). The structure of the PMCA1 gene ATP2B1 has been studied (10), but the control of its tissue-specific expression is not yet understood. Identification of the promoter of the human ECAC2 and further studies of PMCA1 will be needed to show how the expression of these genes is tightly regulated.

The large variation in expression of epithelial calcium channel transcripts may be an important factor explaining some of the considerable variability in calcium absorption among individuals. Low fractional calcium absorption has recently been shown to be related to an increase in the frequency of osteoporotic hip fractures in subjects on low-calcium diets (7); calcium supplementation can improve bone mineralization (5). The reasons for much of the variability in fractional calcium absorption have remained unclear (1). The identification of these human calcium channels, and subsequently the control of their expression, may help resolve the differences in calcium absorption and help target therapy.

High intestinal expression of apical calcium channels could result in absorptive hypercalciuria, leading to renal calculi (19). The lack of expression of this CaT1 homologue, ECAC2, in renal tissues could be important in this. Several steps in the pathophysiology of absorptive hypercalciuria have been suggested. It is likely that multiple genetic changes can produce this phenotype, but it is intriguing to note that a relationship of hypercalciuria and high red blood cell ATPase activity has been reported (3). How this finding is linked to our discovery of closely correlated calcium channel and PMCA1 expression remains to be determined.

Recently, a locus for absorptive hypercalciuria has been mapped in three families by linkage studies to chromosome 1q23.3-q24 (21). Another locus at 4q33-qter has been suggested in two unrelated patients (16). PMCA1 is located at chromosome 12q21–23 (18), and mapping of a genomic clone derived from our cDNA sequence by fluorescent in situ hybridization has suggested a locus for ECAC2 at chromosome 7q34–7q35 (Barley et al., manuscript in preparation). Human ECAC1 has been mapped to 7q31.1–7q31.2 (17). Thus it seems unlikely that genetic variation of apical calcium channels is directly responsible for hypercalciuria in these families. However, the factor(s) regulating

Table 2. Expression in human duodenum of calcium transport genes: correlation coefficients

<table>
<thead>
<tr>
<th></th>
<th>ECAC2 signal</th>
<th>Age</th>
<th>25-(OH)D</th>
<th>1,25-(OH)2D</th>
<th>Cb signal</th>
<th>PMCA1 signal</th>
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<tbody>
<tr>
<td>ECAC2 signal</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Age</td>
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<td>1.00</td>
<td></td>
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<tr>
<td>25-(OH)D</td>
<td>0.20</td>
<td>−0.37</td>
<td>1.00</td>
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<tr>
<td>1,25-(OH)2D</td>
<td>0.14</td>
<td>0.07</td>
<td>0.18</td>
<td>1.00</td>
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<td></td>
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<tr>
<td>Cb signal</td>
<td>0.48*</td>
<td>0.11</td>
<td>0.13</td>
<td>0.40</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>PMCA1 signal</td>
<td>0.83†</td>
<td>−0.10</td>
<td>0.05</td>
<td>−0.24</td>
<td>0.27</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Simple correlation coefficients are shown for the results from 20 subjects. The RNA signals detected with the epithelial calcium channel 2 (ECAC2) probe, calbindin-D9k (Cb), and plasma membrane calcium-pumping ATPase1 (PMCA1) were corrected for glyceraldehyde-3-phosphate dehydrogenase expression. *P < 0.05; †P < 0.001.

Fig. 4. Correlates of calcium transporter expression in human duodenum. Bands detected by the ECAC2 probe were quantified in arbitrary units (AU) by phosphorimaging Northern blots of duodenal RNA from 20 subjects. The signal was corrected for loading differences by the GAPDH signal. The relationships of the corrected signal with 1,25-dihydroxycholecalciferol (A), calbindin-D9k (B), and PMCA1 (C) expression are shown. The regression lines are included in B and C, where the correlations were significant.
expression of these calcium channels and PMCA1 could be found at these loci.

The identification of the partial sequence of a human homologue of a gene that is likely to be responsible for calcium entry at the apical membrane in intestine and the associations with its expression have suggested several fruitful areas for further study. In particular, the lack of association with vitamin D in a healthy population, the wide variability, and the closely regulated coexpression with PMCA1 may help explain many of the gaps in our current knowledge relating to the physiology of calcium absorption.

We acknowledge the contributions to the original study of Lisa Lowery and Barbara Mawer. We thank James Scott for providing the physiology of calcium absorption.

REFERENCES

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