Luminal calcium concentration controls intestinal calcium absorption by modification of intestinal alkaline phosphatase activity

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Abstract
Intestinal alkaline phosphatase (IAP) is a brush-border phosphomonoesterase. Its location suggests an involvement in the uptake of nutrients, but its role has not yet been defined. IAP expression parallels that of other proteins involved in Ca absorption under vitamin D stimulation. Experiments carried out in vitro with purified IAP have demonstrated an interaction between Ca and IAP. The gut is prepared to face different levels of Ca intake over time, but high Ca intake in a situation of a low-Ca diet over time would cause excessive entry of Ca into the enterocytes. The presence of a mechanism to block Ca entry and to avoid possible adverse effects is thus predictable. Thus, in the present study, Sprague–Dawley rats were fed with different amounts of Ca in the diet (0.2, 1 and 2 g%), and the percentage of Ca absorption (%Ca) in the presence and absence of l-phenylalanine (Phe) was calculated. The presence of Phe caused a significant increase in %Ca (52.3 (SEM 8.9) % in the absence of Phe, regardless of the amount of Ca intake; paired t test, P=0.02). When data were analysed with respect to Ca intake, a significant difference was found only in the group with low Ca intake (paired t test, P=0.03). Additionally, IAP activity increased significantly (ANOVA, P<0.05) as Ca concentrations increased in the duodenal lumen. The present study provides in vitro evidence that luminal Ca concentration increases the activity of IAP and simultaneously decreases %Ca, acting as a minute-to-minute regulatory mechanism of Ca entry.

Key words: Calcium: Calcium absorption: Intestinal alkaline phosphatase

Intestinal alkaline phosphatase (IAP, EC 3.1.3.1) is a brush-border phosphomonoesterase that causes the hydrolysis of non-specific phosphate ester bonds at alkaline pH. Its contribution to total plasma alkaline phosphatase activity is less than 10%, even under pathological conditions that increase this isoenzyme11). Its maximum expression is observed in the duodenum, with a gradual decrease throughout the intestine2) and its activity is higher in the tip of the villus than in the crypt, being a marker of mature enterocytes3). Its location suggests an involvement in the uptake of nutrients, but its role has not yet been defined. A relationship with the process of fat absorption4) and preservation of the normal homeostasis of the gut microbiota has been described previously5,6).

IAP expression parallels that of other intestinal proteins involved in Ca absorption under vitamin D stimulation: Ca channel TRPV6, calbindin D9k, calcium sodium exchanger NCX1 and Ca pump PMCA1b15,6,7). Experiments carried out in vitro with purified IAP from the intestine of rats have demonstrated an interaction between Ca and IAP8). Ca binds to IAP and causes changes in its activity and molecular mass9). The effect of Ca appears to be the consequence of Mg replacement and seems to be important in the stability of the dimeric active structure of IAP10).

As mentioned before, the expression of proteins involved in the mechanism of Ca absorption is regulated by vitamin D11), in a process involving protein synthesis12). Therefore, this system is prepared to face different levels of Ca intake over time. However, in a study in patients, it has been demonstrated that high Ca intake (>1500 mg/d) is not related to high Ca absorption13,14).

High Ca intake in a situation of a low-Ca diet over time would cause excessive entry of Ca to the enterocytes with toxic effects. The presence of a mechanism to block Ca entry when Ca concentration in the lumen is high is thus predictable. We thus hypothesise that a regulatory mechanism of Ca entry, independent of vitamin D, exists in the apical membrane.

The present study provides in vivo evidence that luminal Ca concentration increases the activity of IAP bound to the brush border and simultaneously decreases the percentage of Ca absorption (%Ca), acting as a minute-to-minute regulatory

Abbreviations: %Ca, percentage of calcium absorption; IAP, intestinal alkaline phosphatase; IOD, integrated optical density; Phe, l-phenylalanine.

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mechanism of Ca entry. L-Phenylalanine (Phe), which is an inhibitor of IAP, was used in the present study as a tool to modify IAP activity and to reverse the effect of Ca.

Materials and methods

Animals and reagents

Male Sprague–Dawley inbred rats (7 weeks old, 300 (SD 30) g body weight) fed with balanced food (Gepsa, Pilar-Córdoba, Argentina) and tap water ad libitum were used in the experiments. All the experiments were carried out according to international rules of animal care.

In vivo calcium absorption

The animals were placed in individual metabolism cages for 3 d and fed with different amounts of Ca in the diet (0.2, 1 and 2 g%, n 6 per group). During day 1, rats drank tap water and on days 2 and 3, they were provided with 16% Phe in the drinking-water. Each day and for 24 h, the amount of food eaten was measured and faeces were collected and incinerated at 550°C. The amount of Ca in food and faeces was determined by atomic absorption spectroscopy (Arolab MK II, Buenos Aires, Argentina), and 24 h Ca intake and 24 h faecal Ca were calculated. The %Ca for each animal was calculated as %Ca = (24 h Ca intake − 24 h faecal Ca) × 100/24 h Ca intake, on day 1 (%Ca1) and on day 3 (%Ca3).

The effect of Phe on Ca absorption was analysed by the comparison of %Ca1 – (in the absence of Phe) and %Ca3 – (in the presence of Phe) for each group with different amounts of Ca in the diet. Day 2 was not included in the analyses because Ca in the faeces is a mixture of the period with and without Phe.

Changes in IAP activity and expression as a function of Ca concentration were studied using in situ isolated duodenal loops because (1) the mechanisms of Ca absorption in vivo are active, therefore it is not easy to know the exact concentration of Ca in the duodenal lumen, and (2) it is not possible to obtain a sample of the duodenal mucosa during a 3-d experiment.

In situ isolated duodenal loops

Rats were anaesthetised intraperitoneally with urethane (120 mg/100 g body weight) and kept in thermostated stretchers. During the experiment, the room temperature was kept between 21 and 22°C, and the body temperature of rats was kept at 35 ± 1°C with an IR lamp.

A 6 cm portion from the distal duodenum to the pylorus was isolated and a catheter was placed at the distal end. A volume of 2 ml of a filling solution was introduced in the in situ isolated duodenal loops through the catheter. The filling solution of the control group (n 6) consisted of 25 mM-Tris, 160 mM-glucose (pH 7.5), whereas that of the treated groups (n 6 each) had the same composition plus CaCl2 (10, 50 or 100 mM). Ca concentrations were chosen based on in vivo Ca experiments and the intake of drinking-water. It is assumed that 10, 50 and 100 mM are close to the in vivo Ca concentration reached in the experiment described above after Ca intake.

After 20 min, biopsies of the duodenum were obtained and processed to measure IAP activity by histochemistry and IAP expression by Western blot and immunohistochemical analysis.

In the control group, samples of the duodenum were obtained immediately after filling with the solution and after 20 min of incubation. The evaluation of these results confirmed that changes in IAP activity induced by Ca are not the consequence of the surgical procedure.

Tissue preparation

The isolated duodenum was removed from rats, immersed in 10% formaldehyde in PBS, dehydrated through ascending ethanol and embedded in paraffin. The paraffin blocks were cut into 5 µm sections using a microtome (Minot-Mikromet Typ 1212; Leitz, Wetzlar, Germany).

Histochemical measurement of intestinal alkaline phosphatase activity

Tissue samples were deparaffinised with xylene and rehydrated through graded ethanol and water. IAP activity in the sections was detected by incubation for 30 min with 5-bromo-4-chloro-3-indolyl phosphate, as the substrate, in a buffer with the following composition: 100 mM-Tris, 100 mM-NaCl, 5 mM-MgCl2, pH 9.5. The sections were stained with 1% eosin, and finally, the slides were treated with graded ethanol and xylene, and mounted in Canada balsam for permanent slide preparation. The specificity of the reaction was confirmed by significant inhibition of enzyme activity in the presence of Phe.

The slides were then examined using a light microscope (Olympus, Tokyo, Japan) and photographed with a digital camera (Olympus S-350, Beijing, China). A digital image was obtained and brush-border integrated optical density (IOD) was measured with ImageJ 1.40 (National Institutes of Health, Bethesda, MD, USA). Thereafter, five sites of the stained section were randomly selected. A parallel line was drawn coincident with the brush-border membrane and IOD was recorded. Background correction was made with IOD of the intestinal lumen.

Immunohistochemical analysis

Sections were deparaffinised with xylene and rehydrated through graded ethanol and water. After the treatment with methanol for 2 min, slides were immersed for 30 min at 20°C in a 5% H2O2 solution in absolute methanol to inhibit endogenous peroxidase activity. Subsequently, excess of H2O2 was removed by washing the slides three times with 50 mM-Tris at pH 7.6 (Tris buffer). Antigen retrieval was carried out by boiling tissue sections in 1% sodium citrate buffer (pH 6.0) for 3 min in a microwave oven. The slides were then rinsed three times with Tris buffer and the sections incubated with guinea pig anti-rat IAP polyclonal primary antibody for
post hoc linear trend analysis. Comparison of the in vitro effect of Phe on Ca absorption between the values of Ca intake of each group was performed with paired Student’s t test and comparison of Ca absorption between the groups with different amounts of Ca in the diet was performed with ANOVA. Data are expressed as means with their standard errors and differences were considered significant when \( P<0.05 \).

### Results

#### In vivo calcium absorption experiments

The presence of Phe caused a significant increase in %Ca (0%Ca1 = 31·1 (SEM 8·9) % v. %Ca2 = 52·3 (SEM 6·5) %), regardless of Ca intake (paired \( t \) test, \( P=0.02 \)), and no differences in food intake were observed between the groups.

When data were analysed with respect to Ca intake (Fig. 1), an increase in all the groups was observed after Phe addition, but significant differences were found only in the group with a low-Ca diet (0·2 g%; paired \( t \) test, \( P=0.03 \)).

Additionally, we observed that as the amount of Ca in the diet increased (Table 1), %Ca decreased significantly both in the absence (ANOVA, \( P=0.02 \)) and presence of Phe (ANOVA, \( P=0.0001 \)). The results of %Ca in the absence of Phe are consistent with previous data\(^{13,14}\), which confirm the existence of a local regulatory mechanism because the expression of proteins involved in Ca transport was identical. This mechanism would block the entry of high Ca concentration into the enterocytes when luminal Ca content is increased.

### Intestinal alkaline phosphatase activity

IAP activity in the brush border increased significantly (ANOVA, post hoc linear trend test; \( P=0.005 \)) after the exposure to different Ca concentrations for 20 min (Fig. 2). These results cannot be the consequence of the surgical process because IAP activity did not display significant changes after 20 min of exposure to the buffer without Ca (IOD units: 0 min – 0·44 (SEM 0·04); 20 min – 0·45 (SEM 0·04); unpaired \( t \) test, \( P>0.05 \)).

### Intestinal alkaline phosphatase expression

Western blot analysis did not show changes in IAP expression after the exposure to different Ca concentrations for 20 min (relative abundance: 0 mm – 0·41 (SEM 0·03); 10 mm – 0·39)

#### Table 1. Percentage of calcium absorption (%Ca) for each amount of calcium added to the diet (0·2, 1 and 2 g%) in the presence and absence of Phe (+Phe and – Phe, respectively) (Mean values with their standard errors)

<table>
<thead>
<tr>
<th>g(^{%})</th>
<th>– Phe Mean</th>
<th>SEM</th>
<th>+Phe Mean</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0·2</td>
<td>63·89(^a)</td>
<td>11·26</td>
<td>88·00(^a)</td>
<td>4·80</td>
</tr>
<tr>
<td>1</td>
<td>39·22</td>
<td>8·48</td>
<td>47·80(^a)</td>
<td>7·26</td>
</tr>
<tr>
<td>2</td>
<td>8·48(^a)</td>
<td>14·90</td>
<td>24·12(^b)</td>
<td>7·11</td>
</tr>
</tbody>
</table>

\(^a,b\) Mean values with unlike superscript letters were significantly different in %Ca for each amount of Ca added to the diet (\( P<0.05 \); ANOVA, post hoc Bonferroni test).
Discussion

It has been previously demonstrated in vitro that the activity of purified IAP is modified by the presence of Ca\textsuperscript{2+}(19). In those experiments, it has been demonstrated that this enzyme binds Ca and that its molecular mass increases, probably as a consequence of molecular aggregation. With regard to aggregation, the process is reversible when Ca is removed by the addition of ethylene glycol tetraacetic acid (EGTA)(10). It has been previously demonstrated in vivo that luminal Ca concentration increases the activity of IAP and simultaneously decreases %Ca, acting as a minute-to-minute regulatory mechanism of Ca entry. Therefore, Phe could improve Ca absorption in cases of therapeutic administration of the cation.

TRPV6 and IAP activity could be related to duodenal surface pH since it is known that the channel is inhibited by decreases in pH(23,24). The hydrolysis of phosphoric esters by the enzyme produces phosphoric acid and reduces bicarbonate secretion by the enterocytes, and regulates surface microclimate pH in the duodenum of rats(25,26). Experiments with intestinal everted sacs and cell cultures are being carried out to analyse the relationship between TRPV6 and IAP.

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