

## 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 6.5)% in the presence of Phe *v.* 31.1 (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 vivo* 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 isoenzyme<sup>(1)</sup>. Its maximum expression is observed in the duodenum, with a gradual decrease throughout the intestine<sup>(2)</sup>, and its activity is higher in the tip of the villus than in the crypt, being a marker of mature enterocytes<sup>(3)</sup>. 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 absorption<sup>(4)</sup> and preservation of the normal homeostasis of the gut microbiota has been described previously<sup>(5)</sup>.

IAP expression parallels that of other intestinal proteins involved in Ca absorption under vitamin D stimulation: Ca channel TRPV6, calbindin CD<sub>9k</sub>, calcium sodium exchanger NCX<sub>1</sub> and Ca pump PMCAb1<sup>(3,6,7)</sup>. Experiments carried out *in vitro* with purified IAP from the intestine of rats have demonstrated an interaction between Ca and IAP<sup>(8)</sup>. Ca binds to IAP and causes changes in its activity and molecular

mass<sup>(9)</sup>. 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 IAP<sup>(10)</sup>.

As mentioned before, the expression of proteins involved in the mechanism of Ca absorption is regulated by vitamin D<sup>(11)</sup>, in a process involving protein synthesis<sup>(12)</sup>. 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 absorption<sup>(13,14)</sup>.

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<sup>(15)</sup>, 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<sup>(16)</sup>.

### 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 mM-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 (%Ca<sub>1</sub>) and on day 3 (%Ca<sub>3</sub>).

The effect of Phe on Ca absorption was analysed by the comparison of %Ca<sub>1</sub> – (in the absence of Phe) and %Ca<sub>3</sub> – (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<sup>(17)</sup> 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 duodenum 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 CaCl<sub>2</sub> (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-Mikrotm Typ 1212; Leitz, Wetzlar, Germany).

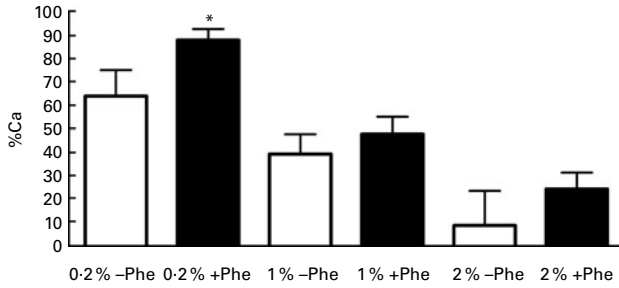
### Histochemical measurement of intestinal alkaline phosphatase activity

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<sup>(18)</sup>, in a buffer with the following composition: 100 mM-Tris, 100 mM-NaCl, 5 mM-MgCl<sub>2</sub>, 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% H<sub>2</sub>O<sub>2</sub> solution in absolute methanol to inhibit endogenous peroxidase activity. Subsequently, excess of H<sub>2</sub>O<sub>2</sub> 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<sup>(19)</sup>. The slides were then rinsed three times with Tris buffer and the sections incubated with guinea pig anti-rat IAP polyclonal primary antibody<sup>(9)</sup> for



**Fig. 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 L-phenylalanine (Phe; +Phe and -Phe, respectively). Values are means, with their standard errors represented by vertical bars. \* Mean value was significantly different from that of the 0.2% -Phe condition ( $P < 0.05$ ; paired  $t$  test).

60 min at 20°C (1:100 dilution). The sections were rinsed three times with Tris buffer and then incubated at 20°C for 60 min with a 1:5000 dilution of rabbit anti-guinea pig IgG (whole molecule) peroxidase-conjugated secondary antibody (Sigma Company, St Louis, MO, USA). The antigen-antibody reaction sites were made visible by incubation with 3-amino-9-ethyl-carbazole in acetate buffer (pH 5) and 15% H<sub>2</sub>O<sub>2</sub>. Finally, tissues were mounted in glycerol jelly and the slides were examined as described below. Semi-quantitative analysis of IAP expression was estimated as IOD, which was obtained as explained for the histochemical measurement.

#### Western blot

PAGE<sup>(20)</sup> was performed using a power supply (EPS 3500; Pharmacia Biotech, Uppsala, Sweden). The following molecular-weight markers were used: apoferritin (443 kDa), fibrinogen (341 kDa), equine  $\gamma$ -globulin (158 kDa), bovine serum albumin (dimer of 132 kDa) and bovine serum albumin (monomer of 66 kDa; Sigma Company). Thereafter, proteins were electrophoretically transferred (Multiphor II, Novablot electrode; Pharmacia Biotech) to a nitrocellulose membrane (Hybond TM-C extra; Amersham Life Science, Piscataway, NJ, USA). Then, the filters were washed with PBS (80 mM-Na<sub>2</sub>HPO<sub>4</sub>, 20 mM-NaH<sub>2</sub>PO<sub>4</sub>, 100 mM-NaCl, 1 g/l Tween, pH 7.5), and then the nitrocellulose membrane was incubated overnight with PBS containing 50 g/l of defatted milk powder. After washing twice with PBS for 10 min, the membranes were incubated with guinea pig anti-rat IAP polyclonal primary antibody<sup>(9)</sup> (1:100 dilution) for 60 min at 20°C with constant shaking. After washing twice with PBS for 10 min, filters were incubated with rabbit anti-guinea pig IgG (whole molecule) peroxidase-conjugated secondary antibody (1:5000 dilution; Sigma Company). The filters were washed with PBS and developed with diaminobenzidine substrate solution (Sigma Company) and H<sub>2</sub>O<sub>2</sub> in PBS. The amount of protein was expressed as the amount in each band, in relation to the amount loaded in each lane (relative abundance).

#### Statistical analysis

Comparison of means from *in vitro* experiments with different Ca concentrations was performed with one-way ANOVA and

*post hoc* linear trend analysis. Comparison of the *in vivo* 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 (%Ca<sub>1</sub> = 31.1 (SEM 8.9)% *v.* %Ca<sub>3</sub> = 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<sup>(13,14)</sup>, 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 0.03); 20 min - 0.45 (SEM 0.04); unpaired  $t$  test,  $P > 0.05$ ).

### Intestinal alkaline phosphatase expression

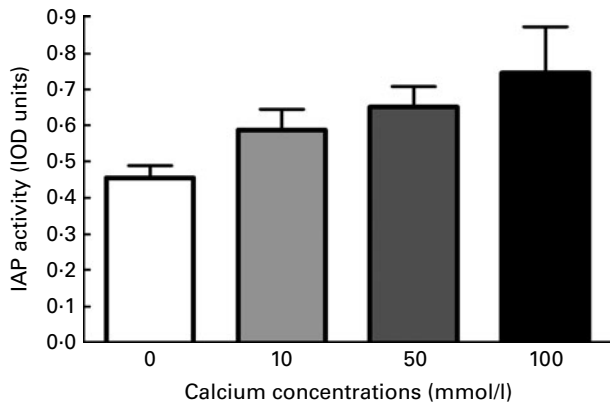
Western blot analysis did not show changes in IAP expression after the exposure to different Ca concentrations for 20 min (relative abundance: 0 mM - 0.41 (SEM 0.03); 10 mM - 0.39

**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)

| g%  | -Phe               |       | +Phe                 |      |
|-----|--------------------|-------|----------------------|------|
|     | Mean               | SEM   | Mean                 | SEM  |
| 0.2 | 63.89 <sup>a</sup> | 11.26 | 88.00 <sup>a,b</sup> | 4.80 |
| 1   | 39.22              | 8.48  | 47.80 <sup>a</sup>   | 7.26 |
| 2   | 8.48 <sup>a</sup>  | 14.90 | 24.12 <sup>b</sup>   | 7.11 |

<sup>a,b</sup> 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).



**Fig. 2.** Intestinal alkaline phosphatase (IAP) activity in the brush-border membrane of the duodenum in the presence of different luminal calcium concentrations ( $P < 0.05$ ; ANOVA, *post hoc* linear trend test). Values are means, with their standard errors represented by vertical bars. IOD, integrated optical density.

(SEM 0.04); 50 mM – 0.40 (SEM 0.02); 100 mM – 0.42 (SEM 0.03); ANOVA,  $P > 0.05$ ). Consistently, immunohistochemical analysis confirmed these results (IOD units: 0 mM – 0.54 (SEM 0.08); 10 mM – 0.49 (SEM 0.07); 50 mM – 0.54 (SEM 0.12); 100 mM – 0.52 (SEM 0.10); ANOVA,  $P > 0.05$ ).

## Discussion

It has been previously demonstrated *in vitro* that the activity of purified IAP is modified by the presence of Ca<sup>(9)</sup>. 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)<sup>(10)</sup>.

The results of the experiments described in the present study confirm that %Ca decreases as Ca intake increases. These results are consistent with previous data<sup>(13,14)</sup>, and seem to confirm the existence of a local regulatory mechanism because of the fact that the analysis of %Ca in the absence of Phe, regardless of Ca intake, showed identical expression of the proteins involved in Ca transport. We hypothesise that when Ca transport is stimulated by vitamin D, high Ca intake could be dangerous for the cells. As a consequence, there should be a mechanism to stop Ca entry at high luminal concentrations of Ca.

We here demonstrated that Ca absorption would be controlled by luminal Ca concentration. Ca showed a stimulatory effect on IAP activity *in vivo*, and this effect was found to be dependent on Ca concentration ( $P = 0.005$ ). Western blot and immunohistochemical analysis confirmed the absence of changes in IAP expression, ensuring that changes in IAP activity were not the consequence of different expressions of this enzyme. To confirm IAP involvement in Ca absorption, *in vivo* experiments were carried out in the presence of Phe, an inhibitor of IAP, in the drinking-water. Although a previous study<sup>(21)</sup> has indicated that Phe would have no effect on Ca absorption, we found that the presence of Phe increased %Ca. An increase in %Ca in all the groups was observed

after Phe addition but significant differences were found only in the group with low Ca intake ( $P = 0.03$ ). The lack of statistical differences in Phe effect on %Ca with normal or high Ca intake could be explained, as in this situation, the enzyme would be, at least in part, stimulated by the luminal Ca concentration because Ca and Phe act on IAP activity in an opposite way; therefore, the inhibitory effect of Phe would not be as effective as in the group with low Ca intake. The increase in %Ca in the group with low Ca intake cannot be explained by the increase in protein expression as an adaptation to dietary Ca content, as demonstrated in a previous study<sup>(22)</sup>. Previously, it has been demonstrated that although calbindin expression and calcitriol levels were modified after 3 d of low Ca intake, the active duodenal Ca transport remained unchanged, which is consistent with our experiment. It is known that at low Ca intake, Ca is absorbed mainly by an active transcellular transport, but that at higher intakes, an increasing proportion of Ca is absorbed by simple diffusion<sup>(6)</sup>. Thus, Phe could only modify transcellular transport of Ca.

In conclusion, the present study provides *in vivo* 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. 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<sup>(23,24)</sup>. 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<sup>(25,26)</sup>. Experiments with intestinal everted sacs and cell cultures are being carried out to analyse the relationship between TRPV6 and IAP.

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