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RESEARCH ARTICLE

Regulation of intestinal calcium absorption by luminal calcium content: Role of intestinal alkaline phosphatase

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Scope: Intestinal alkaline phosphatase is a brush border enzyme that is stimulated by calcium. Inhibition of intestinal alkaline phosphatase increases intestinal calcium absorption. We hypothesized that intestinal alkaline phosphatase acts as a minute-to-minute regulatory mechanism of calcium entry. The aim of this study was to evaluate the mechanism by which intestinal luminal calcium controls intestinal calcium absorption.

Methods and results: We performed kinetic studies with purified intestinal alkaline phosphatase and everted duodenal sacs and showed that intestinal alkaline phosphatase modifies the luminal pH as a function of enzyme concentration and calcium luminal content. A decrease in pH occurred simultaneously with a decrease in calcium absorption. The inhibition of intestinal alkaline phosphatase by L-phenylalanine caused an increase in calcium absorption. This effect was also confirmed in calcium uptake experiments with isolated duodenal cells.

Conclusion: Changes in luminal pH arising from intestinal alkaline phosphatase activity induced by luminal calcium concentration modulate intestinal calcium absorption.

Keywords:
Calcium / Calcium uptake / Intestinal alkaline phosphatase / Luminal calcium content

1 Introduction

Calcium (Ca) is a cation essential not only for calcified tissue formation, but also for excitability and permeability of the plasma membrane, exocrine secretion, enzyme regulation, neurotransmission, muscle contraction, and signal transduction pathways. Ca homeostasis is conserved by different mechanisms, including changes in the efficiency of intestinal Ca absorption, bone remodeling and urinary Ca excretion. In the intestine, $1,25(OH)_2$ vitamin D affects the transcellular Ca transport, which comprises the apical Ca channel, the transient receptor potential vanilloid type 5 and 6 (TRPV5/6), the intracellular calbindin-D_{9k}, the basolateral membrane pump (PMCA1b) and the Na^+/Ca exchanger (NCX1) [1].

The mechanisms of adaptation to dietary Ca depend on $1,25(OH)_2$ vitamin D [2] and there is an inverse relationship between Ca intake and absorption efficiency. In humans, it has been demonstrated that when Ca concentrations in food exceed 1000 mg/day, net Ca absorption is not higher than 500–600 mg/day [3]. Similar results have been found in rats [4]. Calcium uptake and responses to $1,25(OH)_2$ vitamin D depend on the degree of maturation of enterocytes.

Intestinal alkaline phosphatase (IAP) is a brush border enzyme of the intestinal epithelium with highest expression in the duodenum [5]. IAP is a phosphomonoesterase that catalyses the hydrolysis of nonspecific phosphate ester bonds, whose activity has a positive correlation with pH.

Previous studies have shown that IAP expression is controlled by $1,25(OH)_2$ vitamin D [6–8] and that the interaction between IAP and Ca results in changes in the activity of this enzyme [9, 10].

It has been demonstrated that the inhibition of IAP with the uncompetitive inhibitor L-phenylalanine (Phe) [11] in vivo increases the percentage of Ca absorption, an effect that is more evident when rats are fed a low-Ca diet [4]. Ca absorption would be controlled by intestinal luminal Ca content because there is a stimulatory effect on IAP activity depending on luminal Ca concentration and IAP would act as a minute-to-minute regulatory mechanism of Ca entry [4].

It is known that Ca channel TRPV5/6 is inhibited by a decrease in pH [12, 13]. The hydrolysis of phosphoric esters

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Abbreviations: Ca, calcium; **IAP**, intestinal alkaline phosphatase; **IOD**, integrated optical density; **NCX1**, Na^+/Ca exchanger; **Phe**, L-phenylalanine; **PMCA1b**, basolateral membrane pump; **TRPV5/6**, transient receptor potential vanilloid type 5 and 6

by IAP produces phosphoric acid and reduces bicarbonate secretion by the enterocyte [14, 15]. Since IAP activity is modified by luminal Ca concentration [4], IAP could have a role in the regulation of TRPV5/6 channel activity through luminal pH changes in function of luminal Ca concentration. Therefore, the aim of this work was to study the mechanism by which intestinal luminal Ca concentration controls intestinal Ca absorption.

2 Materials and methods

2.1 Animals

Experiments were carried out in female Sprague Dawley rats of 180–220 g of body weight. Rats, provided by the School of Medicine, Rosario National University (Argentina), were fed with balanced food for rodents (Gepsa, Argentina) and water *ad libitum*. All the experiments were conducted in accordance with international guidelines for animal care [16] and has been approved by the bioethics committee of School of Medicine, Rosario National University.

2.2 In vitro effect of IAP on pH

The effect of IAP on pH was evaluated through two different experiments: (i) *Effect of enzyme concentration*: the pH of the solution was measured (pH meter HANNA HI 9017, Woonsocket, RI, USA) for 20 min after the addition of 3 or 15 μg ($n = 4$ per group) of purified rat IAP (obtained as described previously [10]) to a solution containing 1 mM Tris, 1 mM $MgCl_2$, 160 mM glucose, and 100 mM β-glycerophosphate, in the absence of Ca. The results were compared with the pH of the same solution without the addition of IAP. (ii) *After enzyme inhibition*: To confirm that the changes in pH were due to the action of IAP, 16 mM Phe was used to inhibit IAP. Glycine 16 mM was used as negative control after verifying that it is not a rat IAP inhibitor. The pH of the solution was measured for 5 min without the enzyme or Phe to obtain the basal level. Then, 15 μg of purified IAP was added to the solution and the pH was recorded for another 20 min and finally, Phe was added and the pH was recorded for 5 min ($n = 4$).

2.3 Calcium absorption and pH changes with different Ca concentrations

The effect of luminal Ca on IAP activity and luminal pH was investigated with everted duodenal sacs. After euthanasia by CO_2 asphyxiation, the duodenum was removed and everted, rinsed with 9 g/L NaCl solution and divided in three segments of similar size [17].

The mucosal surfaces of everted duodenal sacs were exposed for 20 min to the following solution: 1 mM Tris, 1 mM $MgCl_2$, 160 mM glucose, 100 mM β-glycerophosphate, 1, 10, 50 or 100 mM $CaCl_2$, pH 9. The range of Ca concentrations employed can be found in the small intestine in human beings after the ingestion of a tablet containing 500–1000 mg calcium as salts or in experimental animals fed with a hypercalcic diet (2 g Ca/100 g diet).

The serosal surfaces were exposed to the same solution without Ca ($n = 4$ per group). The experiments were carried out with constant stirring and temperature (37°C). Samples were taken to measure Ca and phosphate. The percentage of Ca absorbed was determined with mucosal Ca concentration, which was spectrophotometrically measured with a commercial kit (Ca Color, Wiener Lab, Rosario, Argentina). Phosphate was measured (Fosfatemia UV, Wiener Lab, Rosario, Argentina) to calculate the percentage of Ca not absorbed by formation of insoluble calcium phosphate salts. The pH was determined in the mucosal solution every 1 min throughout the experiment. This experiment was also carried out in the presence of 16 mM Phe as IAP inhibitor.

2.3.1 PAGE and Western blot

Four percent stacking and 5% separating gels were used [9]. Electrical supply was controlled with an EPS 3500 power supply (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) 132 kDa, bovine serum albumin (monomer) 66 kDa, (Sigma Co, St. Louis, MI, USA).

After electrophoresis, proteins were electrophoretically transferred (Multiphor II; Novablot electrode; Pharmacia Biothec; Uppsala S75184-Sweden) to a nitrocellulose membrane (Hybond TM-C extra, Amersham Life Science). The membranes were washed with PBS (80 mmole/L Na_2HPO_4, 20 mmole/L NaH_2PO_4, 100 mmole/L NaCl, 1 g/L Tween, pH 7.5), and then, incubated overnight with PBS containing defatted milk powder 50 g/L. After washing twice with PBS for 10 min, membranes were incubated with Guinea Pig anti-rat IAP primary antibody (prepared in the laboratory [10]) 1/100 in PBS, for 60 min at room temperature with constant shaking. After washing twice with PBS, a rabbit anti-Guinea Pig IgG [whole molecule] peroxidase conjugate secondary antibody (Sigma, St. Louis, Missouri, USA) was used. Finally, diaminobenzidine substrate solution (Sigma, St. Louis, Missouri, USA) and hydrogen peroxide in PBS were used. The integrated optical density (IOD) of the bands was measured with the GelPro Analyzer 3.0 software (Media Cybernetics, Silver Spring MD, USA).

2.4 In vitro calcium uptake

Duodenal cells were isolated as described previously [18]. Cell viability was assessed by Trypan Blue exclusion. Exclusion of

the dye in >90% of the cells was observed for at least 90 min after isolation.

A total of 200 000 isolated cells were incubated for 10 min at 37°C with the uptake buffer (1 mM-Tris, 1 mM $MgCl_2$, 50 mM $CaCl_2$, ^{45}Ca (1 μCi/mL, New England Nuclear, Research products, Boston, MA, USA)) with or without 16 mM Phe as IAP inhibitor ($n = 10$). After 10 min, the cell suspension was diluted 25-fold in ice-cold medium (phosphate buffered saline (PBS) + 0.02 mM $CaCl_2$, 4°C). The cells were sedimented by centrifugation at 3500 rpm for 1 min and resuspended in 1 M NaOH. Aliquots were taken to measure radioactivity in a scintillation counter (Beckman LS 100C, USA). pH was also measured in the cell supernatant and correlated with the value of Ca uptake.

2.5 Statistical analysis

Data are expressed as mean ± SEM and differences were considered significant if $p < 0.05$. When the effect of Ca and time on pH was evaluated, differences among groups were analyzed with two-way analysis of variance (ANOVA) and Bonferroni post test. Unpaired Student´s *t*-test was used to compare two groups. Linear regression and Pearson correlation was used to analyze the values of Ca uptake as a function of pH. Statistical analyses were performed with the software GraphPad Prism 2.0 (GraphPad Software, San Diego, CA, USA).

3 Results

3.1 In vitro effect of IAP on pH

The pH of the solution depended on IAP concentration and time (Fig. 1, Two-way ANOVA, Bonferroni post test, $p < 0.05$). The decrease in pH was a function of IAP concentration and followed a one-phase exponential decay function of time.

To confirm that the decrease in pH was due to the action of IAP, we performed another experiment where we recorded the pH in the presence of 15 μg of IAP and after the addition of 16 mM Phe as IAP inhibitor or glycine as negative control. The rate constant of the one-phase exponential decay of pH as a function of time was significantly different from cero in the solution without glycine or Phe 0.0060 ± 0.0002 min^{-1} and in the presence of glycine: 0.0037 ± 0.0003 min^{-1}. After Phe addition the rate constant was not significantly different from cero 0.0005 ± 0.0001 min^{-1}.

3.2 pH changes with different Ca concentrations

pH changes in the solution in contact with the duodenal mucosa as a function of luminal Ca concentration (1, 10, 50, and 100 mM) was investigated with duodenal everted sacs. The decrease in pH showed a one-phase exponential decay as a function of time in all groups (Fig. 2). Differences were observed between pH values for each time within the same concentration (Two-way ANOVA, Bonferroni post test, $p < 0.05$). Moreover, the decrease was dependent on luminal Ca concentration, reaching the lowest values at concentrations of 50 and 100 mM Ca.

We found a significant decrease in mucosal pH in the solution after 5 min in groups with Ca 10, 50, and 100 mM compared to 1 mM and in groups with Ca 50 and 100 mM compared to 10 mM. No differences were found between groups with Ca 50 and 100 mM throughout the experiment.

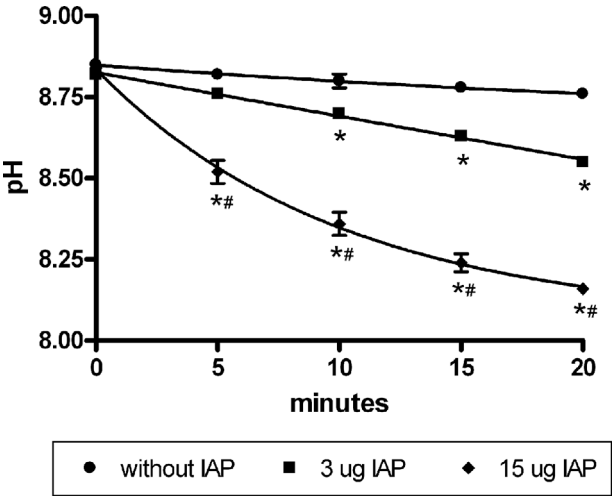

Figure 1. pH of solution as a function of time and amount of intestinal alkaline phosphatase. * indicates significant difference compared with the data without IAP at the same time; # indicates significant difference compared with 3 μg IAP at the same time. Two-way ANOVA, Bonferroni post test, $p < 0.05$.

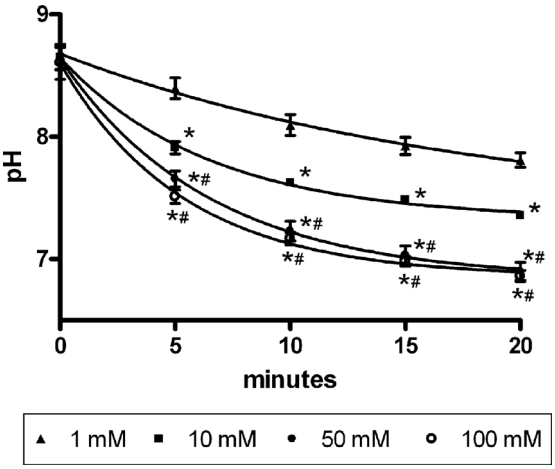

Figure 2. pH of mucosal solution of everted duodenal sac as a function of time and Ca. * indicates significant difference compared with 1 mM at the same time. # indicates significant difference compared with 10 mM at the same time. Two-way ANOVA, Bonferroni post test, $p < 0.05$.

Table 1. Rate constant of the one phase exponential decay of pH as a function of luminal Ca concentration with and without the addition of Phe as IAP inhibitor

Ca (mM)	Rate constant (k)	
	IAP	IAP + Phe
1	0.059 ± 0.020	0.026 ± 0.033
10	0.151 ± 0.011	0.020 ± 0.017[a)]
50	0.155 ± 0.013	0.023 ± 0.021[a)]
100	0.184 ± 0.012	0.028 ± 0.048[a)]

IAP, intestinal alkaline phosphatase; Phe, L-phenylalanine 16 mM.
a) Indicates significant difference compared with IAP without Phe. Two-way ANOVA, Bonferroni post test, $p < 0.05$.

pH as a function of time was fitted by a one-phase exponential decay function. The rate constant of the function for each Ca concentration was compared with the rate constant of the experiments carried out in the presence of Phe as IAP inhibitor. A statistical decrease in the rate constant was found in the presence of Phe for Ca concentrations of 10, 50, and 100 mM (Table 1, Two-way ANOVA, Bonferroni post test, $p < 0.05$). Moreover, the rate constant between different times at the same Ca concentration showed differences only in experiments without Phe.

3.2.1 Ca absorption

The percentage of Ca absorption (Fig. 3) in everted duodenal sacs showed a decreasing function of luminal Ca concentration. The theoretical minimum of Ca absorbed according to this function was 34.17 ± 7.07 μmoles. A significant decrease in the percentage of Ca absorption was observed with higher luminal Ca concentration. After IAP inhibition with 16 mM Phe all groups showed higher percentage of Ca absorption (Fig. 1) (Two-way ANOVA, Bonferroni post test, $p < 0.05$).

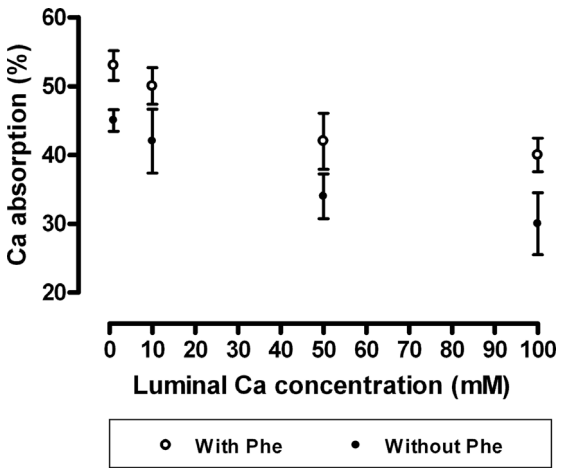

Figure 3. Ca absorption (%) as a function of luminal Ca concentration in everted duodenal sacs with and without the addition of L-phenylalanine as IAP inhibitor.

Table 2. Western blot of IAP from mucosal solution in everted duodenal sacs experiments

Ca (mM)	Integrated optical density		
	0 min	10 min	20 min
1	4.8 ± 2.7	94.0 ± 46.9	156.9 ± 45.9
10	6.4 ± 5.1	42.2 ± 22.1	94.7 ± 23.1
50	2.5 ± 0.5	29.7 ± 13.7	54.5 ± 5.5
100	2.1 ± 0.1	8.1 ± 1.2	9.6 ± 0.4

Phosphate in the luminal solution was determined to evaluate Ca precipitation as calcium phosphate of various chemical compositions. The value of pH, phosphate, and Ca concentration and the solubility product of calcium phosphate were used to obtain the amount of Ca that would precipitate. The results indicated that the percentage of Ca involved in the formation of insoluble compounds is negligible with respect to the amount of Ca absorbed: Ca 1 mM = 0.08 ± 0.02%; Ca 10 mM = 0.003 ± 0.002%; Ca 50 mM = 0.0004 ± 0.0002%; Ca 100 mM = 0.0003 ± 0.0001%.

3.2.2 Western blot

IAP secretion into the lumen was analyzed by Western blot analysis in mucosal solution. As the concentration of Ca is increased, a lower release of the enzyme to mucosal solution was observed (Table 2; ANOVA, the linear trend test, $p < 0.05$). Although IAP is less released to the mucosal solution and it possibly contributes to the increase in the activity of IAP in the brush border, it was previously demonstrated that the amount of IAP in brush border of enterocytes remains constant [4].

3.3 Calcium uptake

Calcium uptake experiments were carried out with isolated duodenal cells in the presence of 50 mM-Ca. The addition of Phe as IAP inhibitor caused an increase in Ca uptake: without Phe = 265.4 ± 44.80 kBq/200 000 cells; with Phe = 438.8 ± 46.53 kBq/200 000 cells (unpaired Student's *t*-test, $p < 0.05$). No difference was found in the pH of the cell supernatant: without Phe = 5.45 ± 0.02; with Phe = 5.59 ± 0.06 (unpaired Student's *t*-test, $p > 0.05$). However, Ca uptake followed a linear function of the pH of the solution: Ca uptake (kBq/200 000 cells) = −8888 + 1979 * pH (Pearson correlation, R-squared = 0.54, $p < 0.05$).

4 Discussion

We found that IAP modified the luminal pH as a function of IAP concentration and Ca luminal content. This was partially

reversed by IAP inhibition by Phe, which confirms that the pH changes are caused by IAP activity.

The decrease in pH was simultaneous with a decrease in Ca absorption in everted duodenal sacs, which was not due to a precipitation as calcium phosphate. Lower values of luminal pH were coincident with a lower percentage of Ca absorption. This can be explained by an activation of IAP activity by high luminal Ca content as describe previously [4]. IAP inhibition with Phe was consistent with higher luminal pH and an increase in the percentage of Ca absorption in duodenal everted sacs. This effect was also confirmed in Ca uptake experiments in isolated duodenal cells, where Phe caused an increase in Ca uptake. Additionally, Ca uptake followed a linear function of pH. This indicates that a higher pH increased Ca uptake. There were no differences in the slopes and intercepts with and without Phe. This result would confirm that pH controls Ca absorption and that pH is modified by IAP as a function of luminal Ca content.

A low-Ca diet leads to an increase in the expression of proteins involved in Ca absorption to increase the efficiency of Ca absorption. However, this may change abruptly by eating foods with high Ca content which could produce a high Ca entry to the enterocyte, with potential toxic effects. Since an increase in intracellular Ca may cause cellular damage, Ca transport should have a protective mechanism. This protective mechanism is exerted by calbindins, which buffer the increases in the intracellular Ca resulting from Ca entry through apical channels as TRPV5 and TRPV6. To avoid increases in intracellular Ca to toxic levels beyond the buffering capacity of calbindins, both TRPV5 and TRPV6 exhibit Ca-dependent inactivation [19].

TRPV6 is the main apical Ca entry channel in the mouse, rat, and human intestine [19]. TRPV6 expression is regulated by $1,25(OH)_2$ vitamin D [20] but, as the experiments carried out in this study were a few minutes long, we can assume that $1,25(OH)_2$ vitamin D did not modify its expression. Moreover, previous studies demonstrated that $1,25(HO)_2$ vitamin D increase the expression of IAP [6–8] and that IAP activity is related to calcium absorption [21, 22]. We have demonstrated that IAP and Ca interaction results in changes on enzyme activity and calcium absorption [9, 10]. However, the relationship among calcium concentration, IAP activity, and calcium absorption demonstrated in this paper is not as a consequence of changes in $1,25(OH)_2$ vitamin D, as the experiment were carried out in vitro with isolated intestinal loop and within isolated duodenal cells. The experimental design used in this paper demonstrates that, at least in part, luminal duodenal calcium concentration regulates intestinal calcium absorption without the action of $1,25(OH)_2$ vitamin D. TRPV6 activity is increased by low luminal Ca content, alkaline pH, and depletion of intracellular Ca [12, 13]. The transcellular transport of Ca is inhibited by acidification of the apical medium of primary cultures of connecting tubules and rabbit cortical collecting duct cells [13]. A decrease in Ca absorption in the rat duodenum at reduced levels of pH has been reported previously [23]. It is well known that the milk-alkali syndrome was once a relatively common cause of hypercalcemia perhaps due to increased TRPV6-mediated Ca uptake at alkaline pH. Therefore, a minute-to-minute mechanism to control the Ca entry in the apical membrane of the enterocyte is expected.

We have recently provided in vivo evidence [4] that luminal Ca content increases the activity of membrane-bound IAP, without changes in its expression, and simultaneously decreases the percentage of Ca absorption, acting as a minute-to-minute regulatory mechanism of Ca entry.

We concluded that the changes in luminal pH arising from IAP activity induced by luminal Ca content modulate intestinal Ca absorption. Hence, we propose the following mechanism: IAP activity parallels luminal Ca concentration by decreasing pH and decreasing Ca absorption. The decrease in pH would act as a negative feedback system on IAP activity when IAP induces values of pH far from optimal, and therefore would decrease Ca absorption. Even though we did not measure TRPV5/6 activity, it is known that this activity is regulated by luminal pH [12, 13]. Therefore, IAP could affect TRPV5/6 activity and act on Ca absorption. This role of IAP may be a protective mechanism to inhibit the entry of high Ca content into enterocytes and thus avoid potential toxic effects.

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The authors have declared no conflict of interest.

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