Reprod. Nutr. Develop. (1989) 29, 227-233

Original article

Indirect effect of casein phosphopeptides on calcium absorption in rat ileum *in vitro*

Y. Li, D. Tomé * et J.F. Desjeux

INSERM U 290, Fonctions intestinales, métabolisme et nutrition, hôpital Saint-Lazare, 75010 Paris, France

(received 26-10-1988, accepted 3-2-1989)

Summary — Casein phosphopeptides (CPP) are the phosphorylated fragments of bovine milk casein. They are believed to enhance intestinal absorption of calcium by their ability to form soluble complexes with calcium thereby inhibiting the precipitation of phosphate—calcium salts. In order to evaluate whether they also act in an additional direct manner on the intestinal mucosa, these peptides were added in a phosphate-free medium at a concentration of 1, 2, or 4 mg/ml on the mucosal side of rat ileum mounted in an Ussing chamber *in vitro*. No effect on the electrical parameters of the tissue was observed. The unidirectional mucosal-to-serosal flux of calcium was significantly reduced in the presence of the peptides, without alteration in the serosal-to-mucosal flux. Jms was 51.71 ± 2.67 μEq/h.cm² for control *vs.* 19.23 ± 3.95 in the presence of 4 mg/ml CPP. This effect was associated with a reduction in free calcium in the mucosal reservoir of the Ussing chamber, without modification of mucosal total calcium or of serosal total and free calcium. These results indicate that CPP did not directly act on rat ileum to enhance calcium absorption. These peptides bind calcium, and the CPP—calcium complex which was not efficiently absorbed remained on the mucosal side of the tissue. In these conditions, the physiological role of CPP on intestinal calcium absorption could be only an indirect luminal inhibition of the precipitation of phosphate—calcium salts. This effect remains to be clearly established.

milk — intestine — peptide — transport

Résumé — Action indirecte des phosphopeptides de caséines sur l'absorption du calcium par l'iléon de rat *in vitro*. *Les phosphopeptides (CPP) de caséines sont des fragments phosphorylés extraits des caséines du lait de vache. Il a été montré que ces peptides qui fixent le calcium étaient susceptibles de stimuler l'absorption du calcium dans l'iléon de rat* in vivo, *probablement en inhibant la précipitation de phosphates de calcium. Afin de vérifier si ces peptides n'ont pas aussi une action directe sur la muqueuse intestinale, ils ont été introduits à la concentration de 1, 2 ou 4 mg/ml sur le versant muqueux de l'iléon de rat monté en chambre de Ussing* in vitro *dans un milieu sans phosphate. Aucun effet sur les paramètres électriques du tissu n'a pu être observé. L'addition des CPP provoque, en revanche, une réduction significative du flux unidirectionnel de calcium muqueux-séreux, sans modification du flux inverse séreux-muqueux. Le flux Jms est de 51,71 ± 2,67 μEq/h.cm² dans une chambre témoin, et de 19,23 ± 3,95 en présence*

* Author to whom correspondence should be addressed.

de CPP 4 mg/ml. Cet effet est associé à une réduction du calcium libre dans le compartiment muqueux de la chambre de Ussing, sans modification ni du calcium total muqueux, ni du calcium total et du calcium libre séreux. Ces résultats indiquent que les CPP n'agissent pas directement sur l'iléon de rat pour stimuler l'absorption du calcium. Ces peptides fixent le calcium et le complexe CPP—calcium n'est pas efficacement absorbé et reste sur le versant luminal de l'intestin. Une action éventuelle des CPP sur le transport du calcium serait uniquement liée à l'inhibition intraluminale de la précipitation de phosphates de calcium.

lait — intestin — peptide — transport

Introduction

Milk and milk products are believed to enhance the assimilation of calcium. The calcium in milk is mostly associated with the protein fraction and especially with casein (Fransson and Lonnerdal, 1983). Bovine casein is a mixture of αs_1, αs_2, ß- and κ-caseins, phosphoproteins that contain 8—9, 11—13, 5, and 1—2 phosphate groups, respectively, for the common genetic variants (Mercier, 1981). An important fraction of the casein-bound calcium is associated with the phosphate groups. In addition, the phosphorylated fragments of casein, the casein phosphopeptides (CPP), were demonstrated to be released from casein by peptic—tryptic digestion *in vitro* or in the gastrointestinal tract during digestion *in vivo* (Mellander, 1950; Naito *et al.*, 1972; Naito and Suzuki, 1974). These peptides have a high calcium binding capacity and are believed to enhance calcium absorption and utilization (Mellander, 1950). During luminal digestion of casein in rat *in vivo*, they are formed in the distal small intestine where both soluble calcium and calcium absorption are higher than in rats fed other dietary proteins (Lee *et al.*, 1979, 1980, 1983; Sato *et al.*, 1983, 1986). This effect was interpreted as an inhibition of the precipitation of calcium phosphate salts by CPP that promotes the passive diffusion part of calcium absorption in the ileum (Mellander, 1950; Sato *et al.*, 1983, 1986). It was not clear, however, if CPP only indirectly promote calcium absorption by an increase in soluble calcium in the lumen, or if they also directly modify intestinal calcium uptake by an additional mucosal action or by the absorption of a peptide-bound form of calcium (Sato *et al.*, 1986; Blake and Henning, 1988).

The aim of the present study was to determine whether CPP only act on the passive diffusion part of calcium absorption in the distal intestinal mucosa, or if they would also modify the transport of calcium by an additional direct action on the epithelium. For that purpose, bidirectional fluxes of calcium were measured in rat ileum *in vitro* in the absence and in the presence of CPP added on the mucosal side of the tissue in a phosphate-free medium. Results showed that CPP did not directly act on intestinal mucosa but could indirectly modify calcium transport by their calcium-binding capacity.

Materials and Methods

Chemicals

A mixture of CPP extracted from tryptic digest of whole bovine casein was obtained from Sopharga (Creuilly, France). It contained 6.6% phosphorus. $^{45}CaCl_2$ (869.5 kBq/µg of Ca) was purchased from Du Pont de Nemours (Dreich, FRG).

Ussing chamber apparatus

Tissues of rat ileum were studied with the Ussing chamber apparatus (Powell *et al.*, 1972). Male Wistar rats were fed with a standard diet (ref. A 04, UAR, Villemoisson, France) that was free of casein and contained : 5 900 mg/kg phosphorus, 6 000 mg/kg calcium, and 1 500 IU/kg vitamin D_3. Non fasting rats weighing 100—200 g were killed by intracardiac injection of sodium pentobarbital. Segments of ileum were removed and rinsed of intestinal content. The tissue with intact musculature was opened along the mesenteric border, mounted as a flat sheet between the two halves of a lucite chamber (exposed area, 0.5 cm^2), and bathed on each side at a temperature of 37°C with 10 ml of isotonic phosphate-free Krebs—Ringer bicarbonate solution containing (mM) 142.4 Na^+, 4.2 K^+, 1.5 Ca^{++}, 1.2 Mg^{++}, 126.5 Cl^-, and 25 HCO_3 (pH 7.85 with 95% O_2, 5% CO_2). The spontaneous transmucosal electrical potential difference (PD) was measured *via* 4% agar bridges placed on each side of the tissue and adapted to calomel half-cells connected to an automatic voltage clamp system (WPI, New Haven, CT, USA). PD was continuously short-circuited by a short-circuit current (Isc) *via* 4% agar bridges placed in each reservoir and adapted to Ag/AgCl electrodes connected to the voltage clamp system. Delivered Isc was corrected for fluid resistance. The electrical conductance (G) of the tissue was calculated according to Ohm's law.

Calcium flux measurement

Eight tissues were mounted. After the stability of the electrical parameters had been checked for at least 15 min, tissues were paired according to conductance (G) which did not differ by more than 25%. $^{45}CaCl_2$ (148 kBq) was then added to either the mucosal or the serosal side of the paired tissues. Twenty min later the appropriate quantity of a concentrated solution of CPP (20 mg/ml) in Ringer's, or no CPP for control, was introduced in the mucosal compartment only. After 20 min, 0.5-ml aliquots were removed at 10-min intervals during 40 min from the reservoir opposite to that to which ^{45}Ca had been added. The mucosal-to-serosal (Jms) and serosal-to-mucosal (Jsm) unidirectional fluxes of calcium were calculated from the radioactivity and expressed in nanomoles of calcium transported per hour and per square centimeter of serosal surface area. Net flux (Jnet) was determined for each pair of tissues according to Jnet = Jms—Jsm (Nellans and Kimberg, 1978; Favus, 1985).

Analytical techniques

Total calcium was determined by the *ortho*cresolphthalein complex (OCPC) method and free calcium with an ionized calcium analyzer (Radiometer). Osmolarity was measured with an osmometer (Fiske).

High—pressure liquid chromatography (HPLC)

Samples were injected into a Waters gradient system equipped with a Waters C18 μ-Bondapack column (350 x 4.6 mm) and a UV detector at 214 nm. The column was eluted at 40 °C with a gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min.

Calculations

Results were expressed as means ± SE. Statistical analysis was performed by analysis of variance.

Results

The addition of CPP at a concentration of 1, 2 or 4 mg/ml to the mucosal side of the rat ileum *in vitro* did not modify Isc, PD, and G, but significantly reduced net Ca absorption in comparison to a control chamber without CPP (Table I). In the absence of CPP, the two unidirectional fluxes Jms and Jsm were not different. Addition of CPP resulted in a reduction in the unidirectional mucosal-to-serosal flux (Jms), whereas the unidirectional serosal-to-mucosal flux (Jsm) remained unchanged.

Mucosal addition of CPP in the Ussing chamber significantly reduced mucosal free Ca without alteration in mucosal total Ca, whereas both free and total Ca remained unchanged in the serosal

Table I. Effect of mucosal addition of casein phosphopeptides (CPP) on electrical parameters and calcium transport in rat ileum *in vitro*.

	Electrical parameters			*Calcium fluxes*		
	Isc	*PD*	*G*	*Jms*	*Jsm*	*Jnet*
Control	1.38 ± 0.10	− 1.47 ± 0.23	24.90 ± 2.36	51.71 ± 2.67	46.02 ± 5.03	5.49 ± 6.22
CCP (1 mg/ml)	0.94 ± 0.12	− 1.17 ± 0.26	22.03 ± 2.21	34.51 ± 5.39	45.82 ± 4.02	− 11.29 ± 5.13
P	NS	NS	NS	< 0.05	NS	< 0.05
CPP (2 mg/ml)	1.33 ± 0.066	− 1.43 ± 0.12	25.35 ± 1.78	26.92 ± 3.32	46.89 ± 12.23	− 20.48 ± 7.62
P	NS	NS	NS	< 0.05	NS	< 0.05
CPP (4 mg/ml)	1.16 ± 0.24	− 1.35 ± 0.46	22.02 ± 2.05	19.23 ± 3.95	45.34 ± 9.78	− 25.92 ± 9.52
P	NS	NS	NS	< 0.01	NS	< 0.01

Values are means ± SE of results for 6 animals. Isc, short-circuit current; PD, potential difference; G, conductance; Jms, mucosal-to-serosal flux; Jsm, serosal-to-mucosal flux; Jnet = Jms—Jsm; units for Isc and calcium fluxes are $\mu Eq \cdot h^{-1} \cdot cm^{-2}$; units for G are $mS \cdot cm^{-2}$. CPP were added in the mucosal reservoir of the Ussing chamber and fluxes measured 20 min later.

reservoir (Fig. 1). In no case was there any modification in osmolarity in either the mucosal or the serosal reservoir (not shown).

There was a linear relationship between calcium Jms and free calcium in the mucosal reservoir (Jms = −6.43 + 35.99 [free Ca]; $r = 0.95$; $P < 0.05$).

RP-HPLC analysis showed that CPP was a complex mixture composed of about 20 different peptides (Fig. 2A). This mixture was not modified after 50 min of incubation on the mucosal side of the rat ileum *in vitro* (Fig. 2B, C).

Discussion

It is known that the most important part of the protein-bound calcium in milk is associated with phosphate groups of casein (Fransson and Lonnerdal, 1983). The phosphorylated fragments of casein (CPP) were demonstrated to enhance the absorption of calcium in the distal small intestine of rat *in vivo* (Sato *et al.*, 1986). The present *in vitro* study indicates that CPP indirectly modified the passive transport of calcium in the rat ileal mucosa by their calcium-binding capacity, without any direct action on the epithelium.

According to current studies on intestinal calcium absorption, it is generally considered that the duodenum and the upper jejunum are the sites of active, carrier-mediated transport, whereas passive transport is the primary mechanism in the distal intestine where the largest part of calcium is probably absorbed (Nellans and Kimberg, 1978; Pansu *et al.*, 1984; Favus, 1985). A passive diffusion transport of calcium in the rat ileum was confirmed by the present results, which show no difference between Jms and Jsm in the absence of CPP.

It was originally suggested that CPP stimulate calcium absorption by their capacity to form a soluble complex with calcium (Mellander, 1950, 1963). Our results demonstrate that CPP bound calcium but remained intact in the mucosal reservoir of the Ussing chamber. No effect on the electrical parameters of the tissue and no stimulation of calcium absorption were observed. CPP *per se* did not directly stimulate mucosal calcium absorption, and calcium was not efficiently absorbed from the CPP-bound form.

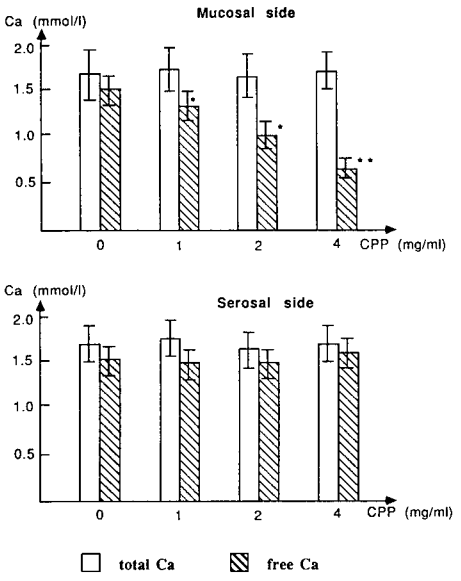

Fig. 1. Effect of mucosal addition of 1, 2 or 4 mg/ml CPP, or no CPP for control, on total and free calcium in the mucosal and serosal reservoirs of the Ussing chamber in the presence of rat ileum (exposed area, 0.5 cm^2). Significance of the difference *versus* control : * $P < 0.05$; ** $P < 0.01$.

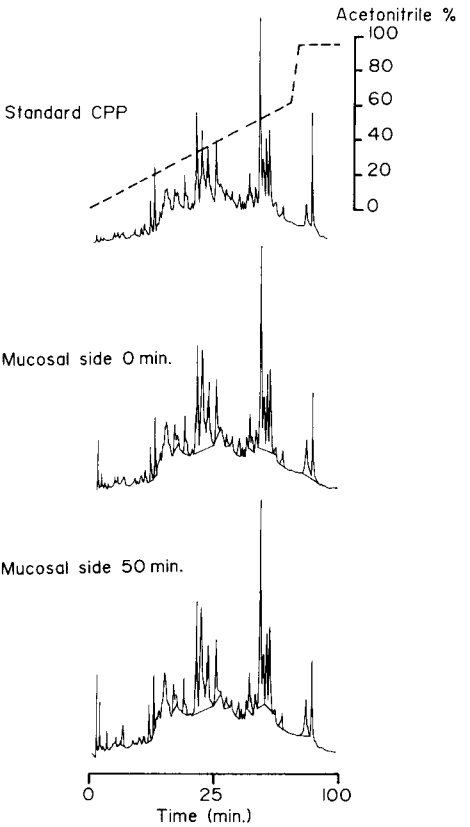

Fig. 2. Reverse-phase high-pressure liquid chromatography elution profile of standard solution of CPP (4 mg/ml), and of the Ussing Chamber mucosal reservoir content at time 0 and 50 min after mucosal addition of 4 mg/ml CPP. Exposed area of rat ileum was 0.5 cm^2. 60-µl samples were injected into the chromatograph and eluted at a flow rate of 1 ml/min with a linear gradient of acetonitrile in 0.1% TFA.

It is also generally accepted that calcium must be in a soluble, ionized form before it can be absorbed. In fact, CPP bound calcium and consequently reduced its free form. The reduction in free calcium on the mucosal side of the tissue was significantly correlated to a reduction in the unidirectional mucosal-to-serosal flux of calcium (Jms). Thus, the observed reduction in Jms in the presence of CPP was probably associated with a reduced liberation of the ionized form of calcium from its CPP-bound form.

On the other hand, it is known that calcium absorption can be inhibited by the precipitation of calcium phosphate salts during digestion *in vivo* and that CPP could probably prevent this precipitation (Mellander, 1950). The CPP-bound form of calcium that remains soluble could then progressively liberate the ionized form which is absorbed. However, no difference was recently observed in calcium absorption from milk and calcium salts in healthy young subjects (Sheikh *et al.*, 1987).

In conclusion, CPP did not directly act on the intestinal mucosa *in vitro* to enhance the absorption of calcium, and the CPP—calcium complex formed was not efficiently absorbed and remained on the mucosal side of the ileum. In that condition, the only effect of CPP was to bind calcium in the lumen of the intestine. This effect could be of interest in inhibiting the precipitation of insoluble calcium salts *in vivo*. The efficiency of this effect remains to be clearly established.

References

Blake H.H. & Henning S.J. (1988) Absorption and transport of milk calcium by infant rats. *Am. J. Physiol.* 254 (*Gastrointest. Liver Physiol.* 17), G12-G19

Favus M.J. (1985) Factors that influence absorption and secretion of calcium in the small intestine and colon. *Am. J. Physiol.*, 248 (*Gastrointest. Liver Physiol.* 16), G147-G157

Fransson G. & Lonnerdal B. (1983) Distribution of trace elements and minerals in human and cow's milk. *Pediatr. Res.* 17, 912-915

Lee Y.S., Noguchi T. & Naito H. (1979) An enhanced intestinal absorption of calcium in the rat directly attributed to dietary casein. *Agric. Biol. Chem.* 43, 2009-2011

Lee Y.S., Noguchi T. & Naito H. (1980) Phosphopeptides and soluble calcium in the small intestine of rats given a casein diet. *Br. J. Nutr.* 43, 457-467

Lee Y.S., Noguchi T. & Naito H. (1983) Intestinal absorption of calcium in rats given diets containing casein or amino acid mixture : the role of casein phosphopeptides. *Br. J. Nutr.* 49, 67-76

Mellander O. (1950) The physiological importance of the casein phosphopeptide calcium salts. *Acta Soc. Med. Upsaliensis* 55, 247-255

Mellander O. (1963) *In: The Transfer of Calcium and Strontium Across Biological Membranes* (R.H. Wasserman, ed.), Academic Press, Inc., New York, p. 265

Mercier J.C. (1981) Phosphorylation of caseins, present evidence for an amino acid triplet code posttranslationally recognized by specific kinases. *Biochimie* 63, 1-17

Naito H., Kawakami A. & Imamura T. (1972) *In vivo* formation of phosphopeptide with calcium-binding property in the small intestinal tract of the rat fed on casein. *Agric. Biol. Chem.* 36, 409-415

Naito H. & Suzuki H. (1974) Further evidence for the formation of phosphopeptide in the intestinal lumen from dietary ß-casein. *Agric. Biol. Chem.* 38, 1534-1545

Nellans H.N. & Goldsmith R.S. (1981) Transepithelial calcium transport by rat cecum : high-efficiency absorptive site. *Am. J. Physiol.* 240 (*Gastrointest. Liver Physiol.* 16), G424-G431

Nellans H.N. & Kimberg D.V. (1978) Cellular and paracellular calcium transport in rat ileum : effects of dietary calcium. *Am. J. Physiol.* 235, E726-E737

Pansu D., Bellaton C. & Roche C. (1984) L'Absorption intestinale du calcium et sa régulation. Phénomènes tissulaires, membranaires et moléculaires. *Diabete Métabol.* 10, 106-120

Powell D.W., Binder H.J. & Curran P.F. (1972) Electrolyte secretion by the guinea pig ileum *in vitro*. *Am. J. Physiol.* 223 (*Gastrointest. Liver Physiol.* 16), G531-G537

Sato R., Noguchi H. & Naito H. (1983) The necessity for the phosphate portion of casein molecule to enhance calcium absorption from the small intestine. *Agric. Biol. Chem.* 47, 2415-2417

Sato R., Noguchi T. & Naito H. (1986) Casein phosphopeptide (CPP) enhances calcium absorption from the ligated segment of rat small intestine. *J. Nutr. Sci. Vitaminol.* 32, 67-76

Sheikh M.S., Santa Ana C.A., Nicar M.J., Schiller L.R. & Fordtran J.S. (1987) Gastrointestinal absorption of calcium from milk and calcium salts. *N. Engl. J. Med.* 317, 532-536