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Influence of casein phosphopeptides and lactulose on intestinal calcium absorption in adult female rats

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Summary — Fractional intestinal Ca absorption was measured in female rats by mixing $^{47}$Ca and the nonabsorbable marker $^{47}$Sc into purified diets and measuring the decrease in the ratio of $^{47}$Ca to $^{47}$Sc in the diet. Ca absorption in 28-wk-old rats consuming a 0.5% Ca diet was not significantly influenced by replacing whey protein with casein, casein phosphopeptides or phosphoserine. Ca absorption was doubled by adding 4% lactulose in both the presence and absence of casein phosphopeptides. Ca absorption in 56-wk-old rats consuming a 0.2% Ca diet was elevated a modest 26% by increasing the level of dietary casein from 9 to 27%.

INTRODUCTION

Milk and dairy products are the most important dietary source of Ca in human life and the absorption of Ca from these products is high compared with other foods. The high lactose content of milk is usually considered to be a major factor responsible for the high Ca absorption from milk and dairy products. As reviewed by Miller (1989), lactose has repeatedly been shown to stimulate intestinal Ca absorption in rats. This beneficial effect of lactose was also observed in infants receiving a soy protein-based formula with either lactose or corn syrup as the carbohydrate source (Ziegler and Fomon, 1983). A cow’s milk-based infant formula was shown to give higher bone mineralization in infants in comparison with a soy-based, lactose-free formula (Steichen and Tsang, 1987). In adult humans the effect
of lactose on Ca absorption is still a matter of controversy (Miller, 1989).

In addition to lactose, casein has been suggested to further enhance Ca absorba-
bility. The phosphorylated regions of the various bovine caseins are involved in sta-
bilizing the colloidal Ca phosphate com-
plex of cow's milk (Holt and Sawyer, 1988). Casein derived phosphopeptides bind metal ions (Oesterberg, 1966) and stabilize supersaturated Ca phosphate systems (Reeves and Latour, 1958; Ger-
ber and Jost, 1986; Berrocal et al, 1989).

In vivo digestion of casein produces phosphopeptides which enhance the intes-
tinal solubility of Ca according to Naito et al (1972). The amounts of soluble Ca and P in rat intestine were higher after a meal with casein than other dietary proteins (Lee et al, 1980). The in vivo formation of a defined phosphopeptide was recently demonstrated in cannulated minipigs fed casein, and the casein fragment αs1 [66–74] isolated by preparative HPLC (Meisel and Frister, 1988). These observations are compatible with a role of casein phospho-
peptides on the intestinal solubility and absorption of Ca but they provide no direct evidence for a stimulation of Ca absorp-
tion.

A balance study with young pigs fed a 0.8% Ca diet for 52 d showed no influence of 5% casein phosphopeptides on intesti-
 nal Ca absorption (Pointillart and Guégen, 1989). Despite the absence of a long-term effect, a transient stimulation of Ca ab-
sorption might have occurred. Thus, a short-term assay might be a more sensi-
tive test of the ability of casein phospho-
peptides to enhance Ca absorption. This approach was chosen in our study in which intestinal Ca absorption from CaCO₃ was compared in rats fed diets containing whey protein, casein, casein phosphopeptides or phosphoserine as ni-
trogen sources.

MATERIALS AND METHODS

Materials

Lactulose and phosphoserine were purchased from Fluka, Switzerland. The whey protein iso-
late (BiPRO) was purchased from Bio-Isolates, UK and vitamin-free casein was purchased from ICN Biomedicals, USA. Casein phospho-
peptides were produced as already described (Juillerat et al, 1989) by tryptic hydrolysis of whole bovine casein, followed by separation of the pH 4.5 soluble peptides on an anion ex-
change column. Protein values were calculated from 6.38 times their nitrogen contents. Further details on these proteins are given in table I.

\[ ^{47}\text{Ca} \text{ and } [^{3}\text{H}]\text{poly-ethyleneglycol were purchased from Amersham, England.} \]

In vitro stability of phosphopeptides

The small intestine of an adult rat was dissected into 3 segments corresponding approximately to duodenum, jejunum and ileum. Each segment, including its contents, was immediately extract-
ed with 5 ml of ice-cold 0.02 M pH 8.0 Tris–HCl buffer. Following removal of insoluble material by centrifugation, phosphatase activity was measured and found highest in the ileum. Ca-
sein phosphopeptides (10 mg/ml) were incubat-
ed with the Tris-buffered ileal extract for up to 3 h at 25 °C. Samples were chromatographed on a Mono-Q anion exchange column (Juillerat et
al, 1989) and, from the decrease in the respective peak areas, the rate of dephosphorylation of the phosphopeptides was calculated. In order to measure the liberation of inorganic P from the peptides, an aliquot of the incubation mixture was treated with 2 N H₂SO₄ and assayed for in-
organic P according to the procedure of Van Veldhoven and Mannaerts (1987).

In vivo stability of phosphopeptides

1.5 ml of a 40 mg/ml casein phosphopeptide solution in 0.1 M CaCl₂ was administered to-
gether with 2 μCi of \([^{3}\text{H}]\text{polyethyleneglycol} \text{ by stomach gavage to a 250-g rat. One h later the} \]
rat was sacrificed and the small intestine divided into the 3 segments described above which were then extracted with ice-cold pH 7.0 Tris buffer. The extracts were centrifuged and supernatants assayed for radioactivity and chromatographed on the Mono-Q column.

**Intestinal Ca absorption**

Female rats of the Fischer 344 strain were obtained from IFFA-CREDO (Les Oncins, France) at 15 wk of age. Intestinal Ca absorption from diets consumed during a single night was measured during wk 28 and 56 of age. For the first study examining the effect of casein phosphopeptides, the rats were fed a pelleted diet containing 0.53% Ca as CaC0$_3$, 0.35% P, 5% corn oil and 11.2% whey protein for 3 wk preceding the experiment. For the second study examining the influence of different levels of dietary casein, the rats were fed a pelleted diet containing 0.2% Ca as CaC0$_3$, 0.5% P, 7% corn oil and 18% casein for 3 wk preceding the test. In both studies, the diets contained 15% sucrose, 3% cellulose, the AIN-76A concentrations of dl-methionine (0.2%), choline bitartrate (0.3%), vitamins and minerals (except Ca and P), with corn starch as the major carbohydrate source.

Fractional Ca absorption was determined from the decrease in the ratio of $^{47}$Ca to $^{47}$Sc in feces relative to diet using the method of McCre- die et al. (1984) modified to permit inclusion of $^{47}$Ca in the diet rather than giving the isotope by stomach gavage. The gamma-emitting $^{47}$Ca ($t_{1/2} = 4.53$ d; energies of 490, 810 and 1 290 keV) decays to the gamma-emitting $^{47}$Sc ($t_{1/2} = 3.43$ d; energy of 160 keV). Both isotopes were counted simultaneously by discrimination of their gamma energies. Since $^{47}$Sc is not absorbed by the intestine, it serves as a nonabsorbable marker and thus only representative samples of both diet and feces need to be counted.

The test diets were identical to the adaptation diets except for the specific changes described in the legends to figures 3 and 4 and the addition of the radioactive tracers and food coloring. $^{47}$Ca (50 μCi) and $^{47}$Sc (= 30 μCi) were added to a powdered diet containing 0.01% Sc as ScCl$_3$ (a carrier for the $^{47}$Sc) and the food dye Fast Green FCF (0.03%). The dye permitted easy identification of the radioactive feces. The diets were thoroughly mixed using a kit-chen-type blender and water was added to produce a thick paste that could be formed into pellets by hand. The formation of these pellets was facilitated by using cornstarch as the major carbohydrate source.

On the day of each experiment, the adaptation diet was removed during the morning and replaced with the radiolabeled diet in the evening just prior to darkness. The radiolabeled diet was removed the following morning and replaced with the normal diet. An advantage of this assay is that the rats consume the tests diets under normal feeding conditions. Feces were collected during the following 4 d with care taken to count only the green-colored radioac-
tive feces. Throughout the experiment the rats remained in their individual plastic cages with wood chips as bedding material. Demineralized water was provided during the experiment.

A Packard Auto-Gamma counter was employed with the pre-set window for $^{57}$Co/$^{75}$Se used to detect $^{47}$Sc and an energy range of 360 to 1560 keV for $^{47}$Ca. Diet and fecal samples were counted in plastic tubes (11 mm internal diameter and 75 mm height) with care taken to fill each tube to a height of 56 mm in order to maintain counting efficiencies constant. The sample depth control was set to 2.5 cm. Appropriate corrections were made for the 21% spill-over of $^{47}$Ca counts into the $^{47}$Sc channel. From the ratios of $^{47}$Ca/$^{47}$Sc in diets and feces, fractional Ca absorption was calculated as described by McCredie et al (1984).

**RESULTS**

**In vitro and in vivo stabilities of casein phosphopeptides**

During *in vitro* incubation of the casein phosphopeptides with extracts from rat ileum, there was a progressive rise in inorganic P which reached a level of ~50% of the total initial organic P content of the peptides after 3 h (fig 1). Chromatographic analysis showed the cumulated decrease

![Graph](image)

**Fig 1. In vitro dephosphorylation of casein phosphopeptides by rat ileal extracts.** Samples were analyzed by FPLC chromatography using a Mono-Q resin as described by Juillerat et al (1989). All data are presented as percentages of initial concentrations. The solid line represents the total of all phosphopeptide peaks, the dashed line the $\alpha_{s1}$ [59–79] fragment, and the dotted line the release of inorganic P.

**Fig 2. In vivo stability of casein phosphopeptides.** FPLC profiles of the phosphopeptide preparation given to the rats (lower profile) and the ileal extract 1 h after stomach gavage (upper profile). The phosphopeptides are identified as follows: $\beta$-cas [33–48]; $\alpha_{s1}$-cas [43–58]; $\beta$-cas [1–28]; $\alpha_{s1}$-cas [59–79] and $\beta$-cas [1–25]; and $\alpha_{s2}$-cas [46–70] for peaks 1 through 5, respectively.

Stabilité in vitro des phosphopeptides de caséine. Le chromatogramme (FPLC) du bas représente la fraction de phosphopeptides administrée aux rats et le profil du haut représente l'extrait de l'iléum 1 h après gavage. Les peptides suivants ont été identifiés : 1) $\beta$-cas [33–48]; 2) $\alpha_{s1}$-cas [43–58]; 3) $\beta$-cas [1–28]; 4) $\alpha_{s1}$-cas [59–79] et $\beta$-cas [1–25]; 5) $\alpha_{s2}$-cas [46–70].
in the peak areas corresponding to all phosphopeptides. The highest rate of dephosphorylation was observed during the first 15 min of incubation. One of the casein fragments, \( \alpha S_1 \) [59-79], was more slowly dephosphorylated than the other fragments.

One h after administration of the peptide–polyethyleneglycol mixture by stomach gavage, the main fraction of radioactivity was recovered in the ileum. Extracts of all 3 segments of the small intestine showed the duodenal and jejunal segments to be essentially devoid of phosphopeptides, while phosphopeptides were found in the ileum. Chromatographic analysis (fig 2) showed that the fragment \( \alpha S_1 \) [59-79] was the most abundant phosphopeptide present. This observation is interesting in view of the presence of a similar fragment, \( \alpha S_1 \) [66-74], found by Meisel and Frister (1988) in minipigs fed a casein meal.

**Fig 3.** Intestinal Ca absorption in rats fed diets containing 0.53% Ca and 0.35% P. There were 8 rats in each group and data are presented as means ± SEM. The groups were (A) 11.2% whey protein; (B) 11.2% casein; (C) 11.2% whey protein and 4% lactulose; (D) 10.3% whey protein and 1.8% phosphoserine; (E) 7.4% whey protein and 3.8% phosphopeptides; (F) 3.6% whey protein and 7.6% phosphopeptides; and (G) 3.6% whey protein, 7.6% phosphopeptides and 4% lactulose. Each diet contained 11.2% total protein. CaCl\(_2\) was used to correct for the small differences in endogenous Ca contents of each diet. Dietary P was provided by the endogenous P of the protein sources and KH\(_2\)PO\(_4\). Phosphoserine was added to provide an equivalent quantity of organic P as the 7.6% phosphopeptide group and lactulose replaced an equivalent quantity of cornstarch. An analysis of variance (\( F \) value = 0.21; \( P > 0.2 \)) demonstrated that there were no significant differences in Ca absorption among groups A, B, D, E and F. Ca absorption was elevated in both groups receiving lactulose at \( P < 0.01 \) as these values are outside the 99% confidence limits of the other 5 groups.

Absorption du Ca chez le rat soumis à des diètes contenant 0.53% Ca et 0.35% P. Chaque groupe est composé de 8 rats et les valeurs moyennes ± SEM sont représentées dans la figure. Les diètes sont composées de : (A) 11.2% de protéines de sérum laitier; (B) 11.2% de caséine; (C) 11.2% de protéines sèches et 4% de lactulose; (D) 10.3% de protéines sèches et 1.8% de phosphoserine; (E) 7.4% de protéines sèches et 3.8% de phosphopeptides; (F) 3.6% de protéines sèches et 7.6% de phosphopeptides; (G) 3.6% de protéines sèches et 4% de lactulose. Le taux de protéine totale a été maintenu à 11.2%. La concentration des diètes en Ca et en P a été maintenue à niveau constant par l'adjonction de CaCl\(_2\) et KH\(_2\)PO\(_4\). La phosphosérine a été ajoutée en quantité correspondante à la quantité de phosphore présent dans 7.6% de phosphopeptides. Dans les diètes contenant du lactulose, ce dernier remplace en quantité équivalente l'amidon de maïs. Aucune différence significative n'a pu être mise en évidence entre les groupes A, B, D, E et F en utilisant une analyse de variance (\( F = 0.21; \ P > 0.2 \)). Seules les diètes contenant du lactulose ont provoqué une augmentation significative de l'absorption de Ca.
**Intestinal Ca absorption**

Ca absorption from a 0.5% Ca diet as influenced by different proteins and lactulose is showed in figure 3. Ca absorption from a whey protein-based diet was not significantly different from that of a casein-based diet nor from diets in which part of the whey protein had been replaced by casein, casein phosphopeptides or phosphoserine, while keeping the total protein level of the diets constant at 11.2%. In contrast, addition of 4% lactulose doubled Ca absorption. This strong enhancement of Ca absorption by lactulose was not affected by the presence of the casein phosphopeptides. Ca absorption from a 0.2% Ca diet was slightly enhanced by increasing the casein content of the diet from 9 to 27% (fig 4).

**DISCUSSION**

Several authors have suggested that casein phosphopeptides enhance intestinal Ca absorption. However, Pointillart and Guégen (1989) found no evidence for such an enhancement in young pigs fed chymotryptic casein phosphopeptides for 52 days. In addition, Li et al (1989) could find no evidence for a direct stimulatory effect of casein phosphopeptides on intestinal Ca transport in the rat ileum as these peptides inhibited the mucosal to serosal transfer of Ca in direct relationship to the reduction in mucosal ionized Ca concentrations.

In the present study, intestinal Ca absorption in adult female rats was measured over a period of one night while the rats maintained their normal eating patterns. Our assay readily detected the stimulatory effect of lactulose, and in other experiments (data not shown), the inhibiting effects of phytate and oxalate. Our failure to find any significant stimulation of Ca absorption indicates that phosphopeptides do not play a decisive role in the absorption process. This lack of stimulatory activity cannot be explained by an excessively rapid dephosphorylation or hydrolysis in the intestine as we have provided chromatographic evidence for the presence of at least part of the phosphorylated species in the ileum 1 h after stomach gavage.

The mechanism by which increasing levels of dietary casein enhance Ca ab-
sorption is unclear but unlikely to be related to the formation of phosphopeptides since tripling the dietary casein level resulted in only a minor elevation of Ca absorption. This modest enhancement of Ca absorption (26%) is not specific for casein as it is also observed with soy protein (R Brommage, unpublished results).

In light of these recent studies, a role for casein phosphopeptides in intestinal Ca absorption appears to be questionable.

The mechanism by which lactulose stimulates intestinal Ca absorption is unclear but this analogue of lactose may act in a similar manner to lactose. However, in contrast to lactose, lactulose is not metabolized in the small intestine and thus may be effective at lower dietary levels than lactose. Further experiments are underway to understand the mechanism of the stimulation of intestinal Ca absorption by lactulose.

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