Calcium absorption is not increased by caseinophosphopeptides1–3

Birgit Teucher, Gosia Majsak-Newman, Jack R Dainty, David McDonagh, Richard J FitzGerald, and Susan J Fairweather-Tait

ABSTRACT
Background: One of the suggested health benefits of caseinophosphopeptides (CPPs) is their ability to enhance calcium absorption. This possibility is based on the assumption that they resist proteolysis in the upper gastrointestinal tract and maintain calcium in a soluble form at alkaline pH in the distal ileum.

Objective: The effects of CPP-enriched preparations (containing candidate functional food ingredients) on calcium absorption from a calcium lactate drink were tested.

Design: A randomized crossover trial was undertaken in 15 adults in whom we measured the absorption of calcium from a calcium lactate drink (drink A: 400 mg Ca as lactate) and 2 preparations enriched with forms of CPP (1.7 g each; drinks B and C). Both drinks B and C contained 400 mg Ca as calcium lactate plus 100 mg CPP-derived calcium. Each volunteer received the 3 drinks in random order. Absorption was measured by the dual-label calcium stable-isotope technique.

Results: The quantity of calcium absorbed was significantly lower from drink A (103 mg) than from drink B (117 mg; P = 0.012) or drink C (121 mg; P = 0.002), which indicated a positive effect of the CPPs. However, because the CPP preparations contributed additional calcium besides that found in the calcium lactate (drink A), fractional absorption of calcium from drink B (23%) was slightly but significantly (P = 0.015) lower than that from drink A (26%).

Conclusions: The differences in calcium absorption are unlikely to have any biological significance. CPPs are unsuitable as candidate ingredients for functional foods that are designed to deliver improved calcium nutrition.

INTRODUCTION
Milk contains numerous physiologically active peptides under consideration as dietary supplements or nutraceuticals. Caseinophosphopeptides (CPPs), a group of peptides derived from the enzymatic hydrolysis of casein, have been reported to enhance calcium absorption through their ability to maintain calcium in a soluble form at alkaline pH in the distal small intestine (4–6). Published data on the effect of CPPs on calcium absorption in model systems support this hypothesis (7) and indicate that CPPs increase passive calcium transport in the distal small intestine of the rat (8). However, the capacity for calcium absorption in the distal small intestine and colon differs significantly between rats and humans (9), and, therefore, the CPP effect in rats may not accurately predict the effect in humans. Nevertheless, several studies of the absorption in humans of calcium in the presence of CPP isolates suggest a trend toward greater bioavailability (10–13), although there are differences in the CPP isolate used, the ratio of calcium to CPP isolate, test meal composition, and experimental approach. We (14) recently reported that calcium absorption from semi-skimmed milk was not significantly higher with low concentrations of a commercially available CPP (2 g phosphopeptide/L, CE90 CPP; DMV International, Veghel, Netherlands) than without the CPP. To fully explore the potential of CPPs as a functional food ingredient, we examined the effect of larger doses of fully characterized CPPs on calcium absorption.

The aim of the current study was to measure the effect of high doses of 2 well-defined CPP-enriched preparations (15), generated under reproducible pilot plant conditions, on calcium absorption. The ratio of CPP protein to calcium administered was ≈ 3:1 (wt:wt). True fractional absorption was ascertained by using the dual-label stable-isotope technique.

SUBJECTS AND METHODS

Subjects
Nonsmokers between the ages of 18 and 45 y who were free of any medical condition that might affect the outcome of this study were eligible to volunteer. Ten females and 5 males were successfully recruited from the database of the Human Nutrition Unit at the Institute of Food Research.

The objectives and procedures of the study were explained, and written informed consent was obtained from each subject before his or her participation. The study protocol was approved by the Norwich District Ethics Committee.

Caseinophosphopeptide-enriched preparations
The CPP-enriched fractions were specially prepared for this study by using PTN 3.0S (CPP1; used in drink B) and Proteinase

1 From the Institute of Food Research, Norwich, United Kingdom (BT, GM-N, JDR, and SJF-T); Glanbia Ingredients, Kilkenny, Ireland (DM); and the Life Sciences Department, University of Limerick, Limerick, Ireland (RJF).
2 Supported by the Commission of the European Communities Agriculture and Fisheries (FAIR) specific RTD programme (CT98-3077).
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DS (CPP2; used in drink C) food-grade proteinase activities (Teagasc; Dairy Products Research Centre, Cork, Ireland). The CPPs were precipitated with ethanol after aggregation with calcium lactate. These spray-dried CPP-enriched preparations had nitrogen-to-phosphate ratios ranging from 3.23 to 4.07 (wt:wt) and were >98% soluble at pH values between 2.0 and 8.0; >75% of peptides were < 3 kDa. The maximum calcium-binding capacity was 2.49 mmol Ca/mg peptide.

Test drink composition

The CPP preparations used in drink B (derived from hydrolysis with PTN3.0 S), called CPP1, and in drink C (derived from hydrolysis with Proteinase DS), called CPP2, were accurately weighed into each test drink. The average weight of CPP1 and CPP2 was 1740 mg powder (ie, 1200 mg protein) and 1823 mg powder (ie, 1262 mg protein), respectively. The calcium content of the CPP preparations was 116.7 mg (6.71%) in CPP1 and 113.6 mg (6.23%) in CPP2, respectively. An average composition of the test drinks is shown in Table 1.

Preparation and labeling of test drinks and the intravenous dose

CaCO₃ enriched with ⁴²Ca (79%) or ⁴⁴Ca (93.8%) was purchased from Chemgas (Boulogne, France). All oral and intravenous doses given to subjects were precisely weighed on an electronic scale (A&D Instruments, Abingdon, United Kingdom) that was accurate to 0.001 mg.

The ⁴²Ca tracer for intravenous injection was prepared by the Ipswich Hospital Pharmacy (Ipswich, United Kingdom). Briefly, ⁴²Ca (as CaCO₃) was dissolved in concentrated HCl (in a molar ratio of 3:1; Aristar Grade; Merck Ltd, Poole, United Kingdom), and the solution was adjusted to pH 6 and made up to 1 L with deionized water. Aliquots of 19 mL containing ≈36 mg ⁴⁴Ca as calcium lactate were stored at −18 °C until use.

All test drinks were freshly prepared immediately before consumption. A weighed aliquot of 360 mg calcium as calcium lactate (food grade; Ocon Chemicals, Ovens, Ireland) was dissolved in deionized water and mixed with a 36-mg aliquot of the labeled calcium lactate. For the drinks containing a CPP preparation, 1700 mg CPP powder was dissolved in 20 mL deionized water, and the mixture was combined with the calcium lactate. To improve the palatability of the drinks, 13 g apple juice concentrate (Kelkin, Dublin, Ireland) was added. The pH of the drink as consumed was 4.8. The serving glasses were rinsed with deionized water, and all the washings were also consumed.

Study design

A randomized crossover study design was used to test the effect of 2 CPP-enriched preparations on calcium absorption. Each volunteer completed 3 absorption tests, consuming one of the following test drinks in random order with an interval of ≥2 wk: 400 mg calcium as lactate (drink A), 400 mg calcium as lactate and 1200 mg CPP1 protein (drink B), and 400 mg calcium as lactate and 1200 mg CPP2 protein (drink C).

Each test phase lasted a total of 5 d (Figure 1). On the experimental days, subjects attended the Human Nutrition Unit after an overnight fast (minimum: 10 h). A cannula was inserted into the antecubital vein, and a single baseline venous blood sample (5 mL) was taken via the indwelling cannula on each of the 3 test days to measure vitamin D [25-hydroxyvitamin D; 25(OH)D].

Subjects then received the test drink containing the ⁴⁴Ca-labeled lactate with or without CPPs.

One hour after the administration of the oral dose, a solution of ⁴²Ca (≈7 mg as calcium chloride) was infused slowly (over 17–20 min) via the cannula. After the intravenous dose, the cannula was flushed with saline and removed. Subjects were not allowed any further food or drink (except water) for 4 h after

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**TABLE 1**

<table>
<thead>
<tr>
<th>Components</th>
<th>Drink A (control)</th>
<th>Drink B (CPP1)</th>
<th>Drink C (CPP2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total calcium lactate (mg)</td>
<td>397.5 ± 0.4</td>
<td>397.4 ± 0.9</td>
<td>397.7 ± 0.9</td>
</tr>
<tr>
<td>Labeled ⁴⁴Ca (mg)</td>
<td>36.3 ± 0.01</td>
<td>36.2 ± 0.7</td>
<td>36.3 ± 0.01</td>
</tr>
<tr>
<td>Unlabeled ⁴⁴Ca (mg)</td>
<td>361.3 ± 0.4</td>
<td>361.3 ± 0.4</td>
<td>361.4 ± 0.9</td>
</tr>
<tr>
<td>CPP1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (mg)</td>
<td>—</td>
<td>1200 ± 0.5</td>
<td>—</td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>—</td>
<td>117 ± 0.1</td>
<td>—</td>
</tr>
<tr>
<td>CPP2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (mg)</td>
<td>—</td>
<td>1262 ± 1.9</td>
<td>—</td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>—</td>
<td>114 ± 0.2</td>
<td>—</td>
</tr>
<tr>
<td>Apple juice (mL)</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td>Deionized water (mL)</td>
<td>130</td>
<td>130</td>
<td>130</td>
</tr>
</tbody>
</table>

1 CPP, caseinophosphopeptide.
2 x ± SD (all such values).
3 Median (all such values).

**FIGURE 1.** Overview of the bioavailability study test phase.
dosing. Volunteers continued with a milk protein–free diet according to the protocol.

Calcium absorption was measured by using the double-label stable-isotope technique. Each volunteer provided a 24-h urine sample (baseline) before the experimental day and 24-h urine collections taken on the 3 d immediately after the administration of the isotopes. Urine collection began on the day before the absorption test after the first void and continued through to the next morning, including the first morning urine. After the isotope administration, urine was collected through to the next day, including the first morning urine. The same procedure applied for the following 2 d.

To exclude any confounding effect of hydrolyzed milk proteins in the diet consumed during the course of the investigation, a milk protein–free diet was supplied to volunteers for 4 d during each experimental period—ie, 2 d before the experiment day, on the experiment day, and 1 d after the experiment day. Subjects were offered a choice between several milk protein–free dinner options and were given a list of milk protein–containing foods that were not allowed during the experimental period.

Habitual calcium intake

The habitual calcium intake was assessed by using a validated, computerized food-frequency questionnaire (16). Norfolk is a hard-water area, and the calcium intake from tap water was calculated by using a typical value for the Norwich area, ie, 113 mg/L.

Analysis of urine samples

Aliquots of the urine were analyzed for total calcium by using atomic absorption spectroscopy and calcium isotope ratios as described previously (14). All samples were analyzed in duplicate for both $^{42}$Ca/$^{40}$Ca and $^{44}$Ca/$^{40}$Ca. Each sample was bracketed by the NIST915 calcium carbonate standard (17) of the same concentration. Relative external precision for $^{42}$Ca/$^{40}$Ca and $^{44}$Ca/$^{40}$Ca was 0.07% and 0.1%, respectively ($n = 6$). The limit of detection for the 2 enriched isotope tracers in these samples—$^{42}$Ca and $^{44}$Ca—was estimated to be 0.2% and 0.3%, respectively. None of the samples analyzed was $<$2% enriched.

25-hydroxyvitamin D

Serum was separated from whole blood and stored at $−80 \, ^\circ\text{C}$ before analysis. All samples were analyzed in one batch by using an ether or hexanol extraction followed by a competitive protein–binding assay for 25(OH)D. The analysis was carried out by the Chemical Pathology Department at Charing Cross Hospital (London, United Kingdom).

Calculations

True fractional calcium absorption (TFCA) is the proportion of calcium absorbed from the diet, and that measure may be used as an index of bioavailability (18, 19). TFCA is given by the ratio of the 2 stable isotopes measured in urine, expressed as the fraction of the administered dose. This technique assumes that the oral tracer, once absorbed, follows the same kinetics as do the intravenous tracer and natural calcium. By using matrix techniques as described previously (14), we calculated TFCA from the isotope enrichment of urine samples collected from all three 24-h collections after dosing.

Statistical analysis

Repeated-measures one-factor analysis of variance was used to evaluate the calcium absorption data from the 3 drinks. To obtain $P$ values for each pairwise comparison, it was necessary to fit each contrast (eg, drink A compared with drink B), to obtain the $P$ value and then to adjust by using the Holm correction to allow for multiple testing. Repeated-measures one-factor analysis of variance was also used to evaluate the effect of 25(OH)D on calcium absorption from the 3 test drinks. The time of the year is one of the potential explanatory variables for vitamin D, and, as is standard practice for such modeling problems, time of year was converted to Julian Day, and the sin and cos of this continuous variable were included in the analysis. Data are expressed as means $\pm$ SEMs; $P$ values $<0.05$ were considered significant. The statistical analysis was performed by using R data analysis software (version 2.3.0; R Foundation for Statistical Computing, Vienna, Austria; 20).

RESULTS

Subject characteristics

The mean ($\pm$ SD) age and BMI (in kg/m$^2$) of the subjects were 37.7 $\pm$ 5.1 y and 25.6 $\pm$ 5.1, respectively. Routine biochemical screening found no clinically relevant deviations from the reference ranges for healthy subjects. None of the subjects dropped out of the study or reported taking any medicine or supplements known to influence calcium metabolism.

25-Hydroxyvitamin D

All calcium absorption tests were carried out between May and December. Three tests for each subject were completed within 16 wk of the first test, and there was no significant time-of-year effect on 25(OH)D concentrations between the 3 test periods. Moreover, there was no significant difference ($P = 0.59$) in the mean 25(OH)D concentrations between the 3 absorption test periods (drinks A, B, and C); therefore, the mean 25(OH)D concentration were presented as the overall mean ($\pm$ SD) of the 3 test periods—ie, 68.2 $\pm$ 16.3 nmol/L. The between-subject 25(OH)D concentration ranged from 38 to 90 nmol/L.

Habitual calcium intake

The habitual calcium intake of this group of volunteers was assessed by using a validated food-frequency questionnaire (16) for each test period. The calcium intake did not differ significantly between the 3 test periods ($P = 0.38$). The mean calcium intake was 1288 $\pm$ 360 mg/d.

Calcium absorption

The TFCA from the calcium-lactate (drink A), CPP1 (drink B), and CPP2 (drink C) drinks was 26.0 $\pm$ 8.6%, 22.7 $\pm$ 6.6%, and 23.5 $\pm$ 6.8%, respectively (Table 2). TFCA from drink B was found to be significantly different from than from drink A ($P = 0.010$). The difference in TFCA between the test drinks containing either CPP1 or CPP2 was not significant ($P = 0.411$). In absolute amounts (ie, mg Ca absorbed), TFCA from drink A (control) was significantly lower (103 mg) than from drink B (117 mg; $P = 0.012$) and drink C (121 mg; $P = 0.002$).
Although CPP is believed to enhance absorption by maintaining calcium in a soluble form, when higher quantities of CPP preparations as functional food ingredients by measuring their bioavailability, we investigated the effect of a CPP preparation on TCFA from a rice-based cereal and reported that the quantity of calcium absorbed was significantly greater than that after the control meal without CPP. However, there was no significant difference in TCFA, and the greater quantity of calcium absorbed from the rice-based cereal could possibly be attributed to the difference in calcium dose between the control (481 mg) and the CPP-containing (an additional 69 or 138 mg when 1 or 2 g CPP, respectively, was added) drinks. The same group reported that CPP did not increase absorption from bread meals (13).

In the current study, assuming that the CPP-derived calcium was available for absorption, a comparison between the control drink and the 2 CPP test drinks should take into account the difference in calcium doses, i.e., 400 compared with 500 mg Ca. The difference in dose may explain why, in terms of absolute absorption (mg calcium absorbed), the difference between the control drink and the CPP test drinks was significant. However, in terms of fractional absorption, the difference was significant only between the control drink A (26.0%) and drink B (22.7%). The observed difference is unlikely to have any nutritional significance, bearing in mind the variability surrounding any given absorption value and the small effect of difference in dose. In summary, the results of this study confirm those of an earlier study using a lower dose of CPPs (15)—i.e., relatively high doses of CPPs do not increase calcium absorption in humans, and CPPs therefore are not effective candidate compounds for functional foods.

We thank Jurian Hoogewerff for performing the inductively coupled plasma mass spectrometry and for data management; John Eagles for technical assistance; Rob Foxall for statistical analysis; the staff at the Human Nutrition Unit, Institute of Food Research, for their clinical expertise; and the volunteers for participating in this study.

BT coordinated the human study, contributed to the study design and data analysis and interpretation, and prepared the manuscript draft; GM-N was responsible for the human trial and prepared the samples for mass spectrometric analysis; JRD undertook the isotope calculations and contributed to

### DISCUSSION

TFCAs may be affected by intraindividual variations in 25(OH)D (21). Therefore, all 3 absorption tests for each subject were carried out within 16 wk of the first test to avoid any effect of seasonal variation. The concentrations of 25(OH)D in the drinks did not differ significantly between each test period, and the overall mean (68 nmol/L) compares well with values reported by others in a population in the United Kingdom (22). The habitual calcium intake of 1288 mg/d was high, which confirms previous findings in the population of Norfolk (16).

The aim of this study was to assess the potential use of 2 CPP preparations as functional food ingredients by measuring their effect on TCFA from a calcium lactate drink. The test drink contained 400 mg calcium and 1200 mg CPP protein, which represents a calcium-to-CPP ratio of 1:3 (w:w). There is no agreement, to date, on what constitutes a dose of CPP isolates that is effective in enhancing calcium absorption. Various ratios have been used, ranging from 1:0.35 (11) to 1:3 (13). In addition to the ratio, the absolute amount of CPP and its binding properties are central to any modulating effect on calcium uptake in the gut. Although CPP is believed to enhance absorption by maintaining calcium in a soluble form, when higher quantities of CPP preparations with strong binding characteristics are given to rats, the absorption of calcium is significantly reduced (23); however, it is not obvious from the literature what be the equivalent effect would be in humans.

We were unable to show a calcium absorption—enhancing effect of either CPP1 or CPP2. However, in interpreting our results, 2 well-established facts about calcium absorption should be taken into consideration. First, previously published data suggest that the variability surrounding any given absorption value within a subject is ≈10%, and the CV tends to increase with the length of the interim period (24). This means that, with an absorption value of 25%, the chances are ≈2 out of 3 that, when the test is repeated 1 mo later, the absorption value would be between 22.5% and 27.5%. Second, it is well documented that an inverse correlation exists between TCFA and a calcium dose of 15 to 500 mg (25). Both CPP preparations used in this study contributed ≈100 mg additional calcium to the test drinks already containing 400 mg Ca (as calcium lactate), for a total dose of ≈500 mg. Studies of the availability of CPP-derived calcium in minipigs suggest that the CPP calcium may freely exchange with other sources of calcium in the stomach during digestion (26). However, the extent of this exchange is not known, nor have any studies in humans explored the bioavailability of CPP-derived calcium. It is most likely that calcium-replete CPP isolates would be used as functional food ingredients because their calcium-binding capacity is higher than that of calcium-deplete CPP preparations. If we were to be able to answer the question of whether the CPP-derived calcium is as available for absorption as any other calcium source in the test meal, the CPP-derived calcium would have to be intrinsically labeled with a stable calcium isotope different from the isotope being used to label the other calcium source—in this case, calcium lactate. An absorption test from a drink containing the labeled calcium lactate as well as the labeled CPP preparation would then ascertain whether the fractional absorption from the 2 sources of calcium is identical.

### TABLE 2

<table>
<thead>
<tr>
<th>Quantity of calcium absorbed by 15 adults (5 men, 10 women) from calcium lactate (control), calcium lactate with caseinophosphopeptide 1 (CPP1), and calcium lactate with caseinophosphopeptide 2 (CPP2)</th>
<th>Drink A (control)</th>
<th>Drink B (CPP1)</th>
<th>Drink C (CPP2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drink</td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>mg</td>
<td>103 ± 34</td>
<td>117 ± 34</td>
<td>120.5 ± 35</td>
</tr>
<tr>
<td>%</td>
<td>26.0 ± 8.6</td>
<td>22.7 ± 6.6</td>
<td>23.5 ± 6.8</td>
</tr>
<tr>
<td>Range (mg)</td>
<td>57–173</td>
<td>47–173</td>
<td>63–201</td>
</tr>
<tr>
<td>Range (%)</td>
<td>14.2–43.6</td>
<td>9.2–33.7</td>
<td>12.3–39.3</td>
</tr>
</tbody>
</table>

1 The nonsignificant differences are also given for completeness: the quantity absorbed from drink B compared with the quantity absorbed from Drink C, \( P = 0.402 \); the percentage absorbed from drink A compared with the percentage absorbed from drink C, \( P = 0.050 \); the percentage absorbed from drink B compared with the percentage absorbed from drink C, \( P = 0.411 \).

2 Test drink B contains on average 117 mg more calcium than does drink A.

3 Test drink C contains on average 114 mg more calcium than does drink A.

4 \( X ± SD \) (all such values).

5 \( p \leq 0.05 \) significantly different from drink A: \( 5P = 0.012 \), \( 4P = 0.002 \), \( 3P = 0.016 \).
data analysis and interpretation; DM developed and supplied the caseinophosphopeptide preparations; RJF contributed to the study design and interpretation of the results; SJF-T contributed to the study design and interpretation of results and edited the manuscript. None of the authors had any personal or financial conflict of interest.

REFERENCES