# Effect of casein, casein phosphopeptides and calcium intake on ileal $^{45}Ca$ disappearance and temporal systolic blood pressure in spontaneously hypertensive rats*

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**Paracellular $^{45}Ca$ absorption and temporal systolic blood pressure (SBP) measurements were recorded in spontaneously hypertensive (SHR) and normotensive Wistar-Kyoto (WKY) rats fed on casein (C) and soya-bean-protein isolate (S) diets, containing 20 (H), 5 (M) and 0·5 (L) g Ca/kg. Similar measurements were also taken in SHR rats only fed on C-M and S-M diets supplemented with 30 g caseinophosphopeptides (CPP)/kg. Absorption of $^{45}Ca$ from the ileal loop was equivalent in both SHR and WKY animals and largely affected by the level of dietary Ca. In addition, animals fed on C diets exhibited significantly ($P < 0·05$) greater ileal absorption of $^{45}Ca$ compared with S-fed animals. This result was attributed to the presence of CPP and a greater ($P < 0·05$) proportion of soluble $^{45}Ca$ in the contents of the ileum. Animals fed on S diets supplemented with CPP confirmed this finding. The SBP of SHR rats was higher ($P < 0·01$) than WKY controls after 9–10 weeks of age. The temporal pattern of observed hypertension was independent of dietary influence in the SHR. The severity of hypertension in SHR rats was affected only by dietary Ca deficiency, and not by Ca supplementation or CPP enhancement of Ca bioavailability. These findings suggest that tryptic digestion products of casein in milk can enhance Ca bioavailability by increasing Ca solubility; however, this action had no effect in reducing hypertension in SHR.**

**Calcium bioavailability: Systolic blood pressure: Caseinophosphopeptides**

Evidence derived from numerous epidemiological (McCarron *et al.* 1982; Garcia-Palmieri *et al.* 1984; Reed *et al.* 1985), clinical (Addison, 1924; Ackley *et al.* 1983; McCarron & Morris, 1985) and experimental animal studies (Ayachi, 1979; McCarron *et al.* 1985; Hatton *et al.* 1989; Blakeborough *et al.* 1990) have demonstrated a potential beneficial anti-hypertensive effect of dietary calcium, although the issue remains a topic of controversy (Resnick, 1987; Heagerty, 1990). The $Ca^{2+}$ ion has an important biological function in the regulation of vascular smooth muscle tone and, thus, blood pressure in the spontaneously hypertensive rat (SHR) by altering the activity of the sympathetic nervous system, or the renin–aldosterone system. These associations have been demonstrated in SHR where low dietary Ca intakes accelerate, whilst oral Ca supplementation can attenuate, the development of hypertension (McCarron *et al.* 1981; Hatton *et al.* 1989).

Milk and milk products have been reported to be good sources of Ca; further, the

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reputation of milk as an excellent source of Ca is due not only to its high Ca content, but also to the excellent bioavailability of this mineral. In particular, the exchange of Ca between soluble and colloidal forms has been shown to correlate well with bioavailability (Buchowski *et al.* 1989). An important component of colloidal Ca is the Ca-binding phosphoseryl groups of casein which, following tryptic digestion, produce oligo-caseinophosphopeptides (CPP) within the small intestine (Naito & Suzuki, 1974; Meisel & Frister, 1989; Nagasawa *et al.* 1991). CPP have been shown to form complexes with Ca and inhibit precipitation of calcium phosphate, thereby increasing soluble Ca in the lower small intestine (Lee *et al.* 1980, 1983). Mykkanen & Wasserman (1980) also reported that macropeptides, derived from in vitro tryptic digestion of whole casein, increased intestinal transport of Ca and enhanced total utilization of Ca in chicks.

Despite the potential importance of casein bioactive peptides in enhancing Ca absorption from the small intestine, there have been very few attempts to characterize this effect with varying Ca intakes and, furthermore, to determine a potential physiological significance. We have recently observed little effect of casein-derived CPP enhanced Ca absorption on blood pressure variables of normotensive rats with normal Ca intakes (Nagasawa *et al.* 1991). The objectives of the present study were first, to characterize the effect of dietary casein and soya-bean proteins on paracellular Ca absorption in the SHR with varying Ca intakes. Second, the relative effectiveness of increased level of Ca intake, or enhanced Ca bioavailability rendered by casein and its phosphopeptides (CPP), on the development of hypertension was examined. The SHR were used for their susceptibility to genetic hypertension, noted to be similar to the development of human hypertension (Okamoto & Aoki, 1963).

## MATERIALS AND METHODS

### *Materials*

$^{45}CaCl_2$ (specific activity 18·2 mCi/mg Ca) was obtained from ICN Biomedical Inc., Irvine, CA, USA). NCS tissue solubilizer and ACS scintillation cocktail were purchased from Amersham (Oakville, ON). Blood pressure tail cuffs for the indirect measurement of rat systolic blood pressure were obtained from Harvard Apparatus Ltd (South Natick, MA, USA). CPP were obtained from Meiji Seika Kaisha Inc., Japan. Mineral salts and other chemicals were purchased from BDH Chemicals (Vancouver, BC).

### *Animals and diets*

Male SHR and WKY rats (4 weeks old; Charles River Inc., Montreal, PQ), initially weighing 60–70 g, were used in all experiments. All animals were individually caged under controlled temperatures (25°) and lighting (14 h light-10 h dark cycle) conditions. SHR and WKY rats were matched for body-weight and randomly segregated into different dietary treatment groups. Dietary proteins (vitamin-free casein and soya-bean-protein isolate) were obtained from ICN Biochemicals (Cleveland, OH). All diets were adjusted to a specific Ca level by the addition of calcium carbonate. Animals were fed *ad lib.* until they reached 100 g body-weight, after which they were trained to a meal-feeding pattern. A meal-feeding protocol was used to standardize the amount of intestinal contents before performing the daily systolic blood pressure measurements and, in particular, the *in situ* Ca absorption experiments. Bioactive peptides, generated from the digestion of casein, have been shown to be influenced strongly by meal patterns (Lee *et al.* 1980; Yvon & Pelissier, 1987). Animals were trained to consume diets within a 6 h period each day (09.00–15.00 hours) over a 2-week period before starting the study. All animals were given deionized water *ad lib.* Weekly body-weights and daily animal feed intakes, corrected for food spilled,

were routinely recorded. Animals were cared for in accordance with the principles of the *Guide to the Care and Use of Experimental Animals* of the Canadian Council of Animal Care (1984).

*Expt 1*

Forty-eight SHR and forty-eight WKY control rats were used in this experiment. Animals were randomly segregated into six experimental groups, which included a high level of Ca (20 g/kg); a medium level of Ca (5 g/kg) and a low level of Ca (0·5 g/kg) in 200 g casein and soya-bean-protein-isolate/kg diets respectively. The diet compositions are presented in Table 1.

The amount of radioactivity ($^{45}CaCl_2$) administered to rats fed on the different levels of Ca was adjusted to 15 $\mu$Ci for animals fed on the high-Ca diet, 5·4 $\mu$Ci for the medium-Ca diet-fed animals and 0·375 $\mu$Ci for animals fed on the low-Ca diet in an attempt to normalize the dose to intestinal Ca contents and, thus, yield similar ileal loop $^{45}Ca$ specific activities.

*Expt 2*

The composition of CPP was determined before addition to the diets. Thirty-two SHR rats only were used in this experiment. Animals were segregated into four experimental groups, which included diets containing casein (200 g protein/kg), casein supplemented with 30 g CPP/kg, soya-bean-protein isolate (200 g protein/kg) and soya-bean-protein isolate supplemented with 30 g CPP/kg. All diets were balanced for Ca content (5 g/kg) but not phosphorus, and contained polyethylene glycol (PEG; molecular weight 4000) as a non-absorbable marker at a level of 2 g/kg (Table 1). PEG was added to diets to determine whether differences in intestinal Ca absorption were due to transit time. All animals received 5·4 $\mu$Ci $^{45}CaCl_2$ into the ligated ileal loop.

*Analytical methods*

Systolic blood pressure (SBP) recordings were made weekly, starting after 6 weeks of age. Experimental data recording was initiated at 8 weeks after a 2-week training period and continued to 13 weeks of age. Measurements were performed between 13.00 and 15.00 hours in conscious rats using the indirect tail cuff method. Each recorded value represents the mean of three successive determinations over a period of 10–15 min. Coefficients of variation in SBP measurements were 4·3%.

Ca absorption from the small intestine was measured by an *in situ* ligated intestinal loop technique, based on the procedure reported by Lee *et al.* (1983) and Sato *et al.* (1986), on the final day of the experiment, at 14 weeks of age. All animals were allowed free access to diets for a 1·5 h period, following which they were anaesthetized by halothane vapour (25 ml halothane/l in oxygen at a flow-rate of 2 l/min), 1·5 h after test diet withdrawal. A longitudinal abdominal incision was made to expose the small intestine; the ileum was isolated and ligated at points 80 and 200 mm from the ileocaecal junction to make a closed sac, leaving the intestinal contents intact. $^{45}Ca$ in a 0·15 M-saline vehicle (300 $\mu$l) was injected directly into the ileal loop and the loop carefully manipulated to ensure uniform mixing. The small intestine was returned to the abdomen and both abdominal and skin layers were sutured closed. Animals were placed on a warm surface and allowed to recover without further handling. At 1 h after injecting the radiolabelled dose the intestinal contents of the ligated loop were flushed with 5 ml of 0·15 M-saline and homogenized using a Polytron homogenizer. A portion (500 $\mu$l) was removed and digested with NCS tissue solubilizer. The digested sample was mixed with 15 ml ACS scintillation cocktail and

Table 1 *Expts 1 and 2. Composition of diets*

| | Casein diets | | | | | Soya-bean-protein diets | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Expt... | 1 | | | 2 | | 1 | | | 2 | |
| Calcium supplement (g/kg)... Dietary component (g/kg) | 20 | 5 | 0·5 | Casein | Casein + CPP | 20 | 5 | 0·5 | Soya-bean protein | Soya-bean protein + CPP |
| Casein | 200 | 200 | 200 | 200 | 200 | — | — | — | — | — |
| Soya-bean protein isolate | — | — | — | — | — | 200 | 200 | 200 | 200 | 200 |
| DL-methionine | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Maize starch | 150 | 150 | 150 | 150 | 150 | 150 | 150 | 150 | 150 | 150 |
| Sucrose | 450·2 | 487·7 | 498·9 | 468·2 | 441·2 | 450·8 | 488·3 | 499·6 | 468·2 | 441·2 |
| Fibre | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| Vegetable oil | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| Ca-free mineral mix | 35 | 35 | 35 | 35 | 35 | 35 | 35 | 35 | 35 | 35 |
| Vitamin mixture | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Choline bitartrate | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Calcium carbonate | 49·8 | 12·3 | 1·1 | 12·2 | 9·2 | 49·2 | 11·7 | 0·4 | 11·8 | 8·8 |
| Polyethylene glycol | — | — | — | 20 | 20 | — | — | — | 20 | 20 |
| CPP | — | — | — | — | 30 | — | — | — | — | 30 |
| Final Ca concentration (g/kg) | 20 | 5·0 | 0·5 | 5·0 | 5·0 | 20 | 5·0 | 0·5 | 5·0 | 5·0 |
| Final phosphorus concentration (g/kg) | 5·4 | 5·4 | 5·4 | 5·6 | 6·8 | 5·0 | 5·0 | 5·0 | 6·0 | 6·9 |

CPP, caseinophosphopeptides.

analysed for radioactivity by liquid-scintillation spectrophotometry. The absorption of Ca was estimated by measuring the proportion of $^{45}Ca$ remaining in the loop after the 1 h period and expressing it as a percentage of the original dose injected into the ligated intestine. A further portion of the intestinal homogenate was centrifuged at 10000 ***g*** for 20 min and analysed for radioactivity. The radioactivity in this supernatant fraction represented soluble $^{45}Ca$. The remainder of the intestinal homogenate was used for the measurement of $^{40}Ca$ by wet ashing samples with hydrochloric acid–nitric acid according to the method of Mauer (1977). After ashing, samples were diluted with lanthanum chloride (5 g/l) and the Ca measured using a Perkin Elmer atomic absorption spectrophotometer.

Plasma minerals were measured from blood samples that were taken by heart puncture immediately before flushing the intestinal contents. Ca, Na and K were determined in plasma by atomic absorption spectrophotometry in the presence of $LaCl_3$ (5 g/l). Ionized Ca was calculated from plasma total Ca and protein concentrations by the equation of Zeisler (1954) used by Reeves & O'Dell (1988) for rats. Plasma protein was measured by a modified Lowry method (Markwell *et al.* 1978).

Contents of the ileal wash were also analysed for PEG (Malawer & Powell, 1967) and CPP contents. In quantifying ileal CPP content, intestinal flush samples were deproteinized with 500 g trichloroacetic acid (TCA)/l and centrifuged (10000 ***g***) for 20 min to collect TCA-soluble material. The TCA was removed by diethyl ether extraction and the extract applied to a Sephadex G-25 column, which was washed with 50 mM-Tris–HCl buffer (pH 8·0) at a flow-rate of 0·3 ml/min. Fractions were collected and analysed for P (Chen *et al.* 1956), peptide content (Markwell *et al.* 1978) and $^{45}Ca$ radioactivity. A single peak testing positive for both P and peptide content was referred to as the CPP fraction.

### *Statistical analysis*

All values are expressed as means with their standard errors. The difference between treatment means was tested by paired *t* test or one-way analysis of variance (ANOVA; SPSS Inc., Chicago, IL). Differences between means were identified by the Newman-Keuls multiple-range test at $P < 0{\cdot}05$ (SPSS). Treatment interactions were identified by two-way ANOVA (MANOVA; SPSS). Correlation coefficients were determined by the method of least squares.

## RESULTS

### *Expt 1*

Body-weight gain and daily food intake of animals over the course of the experimental period are presented in Table 2. SHR and WKY rats fed on the same diets showed comparable daily intakes and body-weight gains. Rats fed on the high-Ca–casein diet exhibited a significantly ($P < 0{\cdot}05$) greater body-weight gain and food intake than animals fed on the high-Ca–soya-bean diet. A significant ($F(2,61) = 3{\cdot}26$, $P = 0{\cdot}045$) interaction between overall Ca intake and protein source was determined for food intake.

Plasma electrolyte values obtained at 14 weeks of age for both SHR and WKY rats are summarized in Table 3. There were no significant differences in plasma Ca, Na or K levels between SHR and WKY meal-fed animals. Plasma protein concentrations were also not significantly different (range 64–70 g/l) between SHR and WKY rats fed on the different diets. From the total plasma and protein contents ionized Ca was also calculated. Of particular interest was the finding that both total and ionized Ca levels were significantly ($P < 0{\cdot}05$) lower in SHR and WKY rats fed on the low-Ca–casein diet compared with counterparts fed on the soya-bean protein diet.

Table 2. *Expt 1. Body-weight gain and food intake of spontaneously hypertensive (SHR) and normotensive (WKY) rats fed on diets containing casein or soya-bean protein and 20, 5 or 0·5 g calcium/kg*†

(Mean values with their standard errors)

| Dietary treatment | Body-wt gain (g/d) | | | | Food intake‡ (g/d) | | | |
|---|---|---|---|---|---|---|---|---|
| | SHR | | WKY | | SHR | | WKY | |
| | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 20 g Ca/kg | | | | | | | | |
| Casein | 3·09 | 0·09 | 3·38 | 0·14 | 13·7 | 0·6 | 13·3 | 0·6 |
| Soya-bean protein | 2·79* | 0·07 | 2·61* | 0·10 | 12·6* | 0·5 | 11·9* | 0·6 |
| 5 g Ca/kg | | | | | | | | |
| Casein | 3·46 | 0·24 | 3·16 | 0·07 | 15·0 | 0·8 | 13·4 | 0·2 |
| Soya-bean protein | 3·16 | 0·11 | 3·13 | 0·21 | 14·1 | 0·2 | 13·5 | 0·4 |
| 0·5 g Ca/kg | | | | | | | | |
| Casein | 2·25 | 0·12 | 2·33 | 0·11 | 11·6 | 0·6 | 11·0 | 0·3 |
| Soya-bean protein | 2·59 | 0·13 | 2·47 | 0·12 | 12·7 | 0·5 | 11·4 | 0·4 |

Mean values were significantly different from those of rats fed on casein diets (Student's *t* test): * $P < 0·05$.
† For details of diets and procedures, see Table 1 and pp. 766–769.
‡ Food intake was calculated from total food consumption during final 40 d.

The effects of dietary protein source and level of Ca intake on ileal Ca absorption variables are presented in Table 4. No difference in the amount of Ca present in the ligated loop or the extent of Ca absorbed from the ileum between SHR and WKY rats fed on identical diets was observed. The amount of $^{40}Ca$ present in the intestinal loop was directly related to the level of Ca fed to individual animals ($r$ 0·85; $P < 0·01$). Although attempts were made to ensure similar $^{45}Ca$ specific activities in the small intestine of animals fed on diets containing different levels of Ca, by adjusting accordingly the dose of $^{45}Ca$ injected into the ligated ileal loop, this goal was not achieved and, therefore, a direct comparison of ileal Ca absorption in rats fed on different dietary levels of Ca was not made. Rather, Ca absorption variables in animals fed on diets that varied only in protein source were tested for statistical significance (Table 4). At comparable daily food intakes and, thus, oral Ca consumption levels a significantly ($P < 0·05$) higher percentage of $^{45}Ca$ was absorbed in both SHR and WKY rats fed on medium- and low-Ca–casein diets, compared with soya-bean protein-fed counterparts. Animals fed on the low-Ca diets exhibited a greater absorption efficiency of the $^{45}Ca$ dose when compared with animals fed on the medium- and high-Ca diets.

Fig. 1 shows the relative proportion of total intestinal $^{45}Ca$ present in a soluble form in animals fed on the different experimental diets. Common to both SHR and WKY rats was the observation that casein-fed animals had a significantly ($P < 0·05$) greater proportion of soluble $^{45}Ca$ than soya-bean protein-fed animals. It is noteworthy that the difference in soluble $^{45}Ca$ between casein and soya-bean protein-fed animals was considerably less in the high Ca-fed animals.

Temporal patterns of SBP between 8 and 13 weeks of age from SHR and WKY control rats fed on different experimental diets are shown in Fig. 2 (*a–c*). In all treatments SHR

Table 3. *Expt 1. Effect of dietary protein source on plasma minerals (mM) in spontaneously hypertensive (SHR) and normotensive (WKY) rats fed on diets containing casein or soya-bean protein and 20, 5 or 0·5 g calcium/kg*†

(Mean values with their standard errors)

| Dietary treatment | SHR | | | | | | | | WKY | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ca | | $Ca^{2+}$‡ | | Sodium | | Potassium | | Ca | | $Ca^{2+}$‡ | | Sodium | | Potassium | |
| | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 20 g Ca/kg | | | | | | | | | | | | | | | | |
| Casein | 2·83 | 0·08 | 1·32 | 0·02 | 136·2 | 4·78 | 5·56 | 0·51 | 2·60 | 0·35 | 1·17 | 0·15 | 137·1 | 11·3 | 5·12 | 0·97 |
| Soya-bean protein | 2·53 | 0·03 | 1·13 | 0·08 | 122·7 | 2·60 | 5·88 | 0·92 | 2·58 | 0·13 | 1·25 | 0·05 | 123·2 | 2·68 | 4·98 | 0·23 |
| 5 g Ca/kg | | | | | | | | | | | | | | | | |
| Casein | 2·43 | 0·10 | 1·08 | 0·03 | 135·3 | 2·50 | 4·64 | 0·18 | 2·35 | 0·18 | 1·03 | 0·05 | 126·1 | 3·90 | 4·78 | 0·26 |
| Soya-bean protein | 2·65 | 0·20 | 1·10 | 0·02 | 136·2 | 2·75 | 5·98 | 0·87 | 2·48 | 0·10 | 1·08 | 0·03 | 143·2 | 5·60 | 5·83 | 1·41 |
| 0·5 g Ca/kg | | | | | | | | | | | | | | | | |
| Casein | 1·48** | 0·18 | 0·55** | 0·10 | 115·3 | 9·57 | 4·96 | 1·10 | 1·65** | 0·15 | 0·75** | 0·10 | 125·8 | 1·30 | 4·22 | 0·51 |
| Soya-bean protein | 2·57 | 0·13 | 1·20 | 0·05 | 125·3 | 3·92 | 4·59 | 0·30 | 2·45 | 0·14 | 1·13 | 0·10 | 129·7 | 1·30 | 4·34 | 0·28 |

Mean values were significantly different from those of rats fed on soya-bean protein diets (Student's *t* test): ** $P < 0·01$.

† For details of diets and procedures, see Table 1 and pp. 766–769.

‡ Calculated values: $(Ca^{2+}(mM) = (6Ca - P/3)/(P + 6)$, where P is protein (g/dl)).

Table 4. *Expt 1. Effect of dietary protein source on calcium content and absorption from the ligated ileal loop of spontaneously hypertensive (SHR) and normotensive (WKY) rats fed on diets containing casein or soya-bean protein and 20, 5, or 0·5 g Ca/kg*†

(Mean values with their standard errors)

| Dietary treatment | $^{40}$Ca in loop (mg/loop) | | | | $^{45}$Ca specific activity (dpm/mg) | | | | $^{45}$Ca absorbed‡ (% dose) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SHR | | WKY | | SHR | | WKY | | SHR | | WKY | |
| | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 20 g Ca/kg | | | | | | | | | | | | |
| Casein | 10·3 | 3·1 | 13·2 | 4·0 | 2·4 | 0·2 | 2·1 | 0·3 | 36·6 | 4·4 | 37·9 | 1·8 |
| Soya-bean protein | 8·8 | 1·3 | 11·1 | 1·8 | 3·3 | 0·3 | 2·8 | 0·3 | 40·0 | 2·6 | 29·1 | 4·0 |
| 5 g Ca/kg | | | | | | | | | | | | |
| Casein | 2·3 | 0·6 | 2·5 | 1·2 | 3·9 | 0·6 | 4·7 | 0·9 | 35·8 | 3·8 | 31·9 | 0·9 |
| Soya-bean protein | 2·7 | 0·4 | 2·7 | 0·7 | 3·5 | 0·5 | 4·0 | 0·7 | 20·8* | 5·3 | 20·3* | 3·4 |
| 0·5 g Ca/kg | | | | | | | | | | | | |
| Casein | 0·08 | 0·02 | 0·07 | 0·03 | 1·4 | 0·3 | 1·5 | 0·3 | 87·8 | 2·3 | 80·6 | 4·6 |
| Soya-bean protein | 0·14 | 0·06 | 0·11 | 0·03 | 2·1 | 0·5 | 1·9 | 0·2 | 71·0* | 5·0 | 70·4* | 1·6 |

dpm, disintegrations/min.
Mean values were significantly different from those of rats fed on casein diets (Student's *t* test): * $P < 0·05$.
† For details of diets and procedures, see Table 1 and pp. 766–769.
‡ (1 − $^{45}$Ca (dpm) at 1·0 h/dose $^{45}$Ca (dpm) administered) × 100.

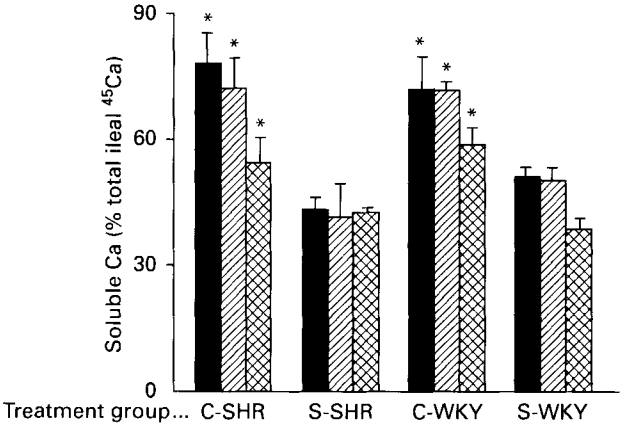


Fig. 1. Expt 1. The percentage of total radioactivity remaining in the ligated ileal loop as soluble $^{45}$Ca in spontaneously hypertensive (SHR) and normotensive (WKY) rats fed on casein (C) or soya-bean protein (S) diets containing 0·5 (■), 5 (▨) or 20 (⊠) g Ca/kg. Values are means with their standard errors represented by vertical bars. Means values for rats fed on C diets were significantly different from those for rats fed on S diets: * $P < 0·05$. For details of diets and procedures, see Table 1 and pp. 766–769.

exhibited significantly ($P < 0·001$) higher SBP (range 200–270 mmHg) at 10 weeks of age than WKY (140–190 mmHg) controls. Thus, the actual temporal development of hypertension was not influenced by the level of Ca intake. At 13 weeks, the SBP of SHR fed on high-Ca–casein and soya-bean protein diets (range 210–215 mmHg) was lower than,

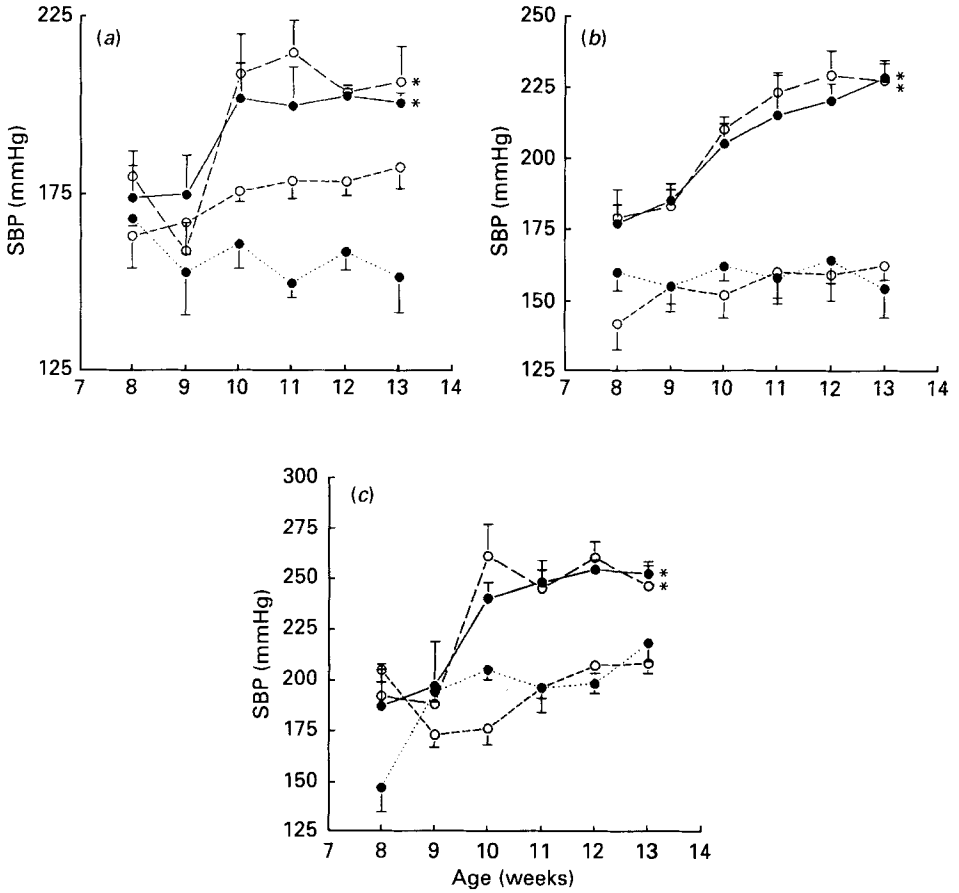


Fig. 2. Expt. 1. Temporal changes in systolic blood pressure (SBP) in spontaneously hypertensive (SHR) and normotensive (WKY) rats fed on casein (●) or soya-bean protein (○) diets containing (*a*) 20, (*b*) 5 and (*c*) 0·5 g calcium/kg. Values are means with their standard errors represented by vertical bars. Mean values for SHR were significantly different from those for WKY rats fed on similar diets, $^{*}P < 0{\cdot}001$. (●——●), SHR-C; (●...●), WKY-C; (○----○), SHR-S; (○---○), WKY-S. For details of diets and procedures, see Table 1 and pp. 766–769.

but not significantly different from, counterparts fed on medium-Ca–casein and soya-bean protein diets (225–230 mmHg). In contrast, significantly ($P < 0{\cdot}05$) higher SBP was observed in SHR fed on low-Ca–casein and soya-bean protein diets (range 240–260 mmHg) compared with medium-Ca–casein or soya-bean protein-fed animals. In all treatments no difference in SBP was observed between casein and soya-bean protein-fed animals, thus indicating that the apparent increase in Ca bioavailability in casein-fed animals did not result in a reduction in SBP in SHR and WKY animals.

*Expt 2*

In this experiment casein and soya-bean-protein diets were supplemented with CPP obtained from a tryptic digest of whole casein. The CPP isolate consisted of (g/kg) moisture 40, crude protein (nitrogen × 6·25) 820, crude ash 110, or CPP 920. The fortification of casein and soya-bean protein diets with CPP had no apparent effect on animal body-weight gain or daily food intake (body-weight gain g/d (means with SEM): casein 2·68 (SEM 0·1);

Table 5. *Expt 2. Food intake, polyethylene glycol (PEG) and $^{40}Ca$ content, $^{45}Ca$ specific activity, soluble $^{45}Ca$ and percentage $^{45}Ca$ absorbed in the ileum of spontaneously hypertensive rats at 1·5 h after ingestion of diets containing casein or soya-bean protein with or without caseinophosphopeptides (CPP)*†

(Mean values with their standard errors)

| | Dietary treatment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Casein | | Casein + CPP | | Soya-bean protein | | Soya-bean protein + CPP | |
| | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| Food intake (g/kg body-wt) | 35·5 | 4·2 | 39·6 | 5·3 | 31·1 | 3·7 | 36·8 | 3·0 |
| PEG (mg/loop) | 18·4 | 1·3 | 17·8 | 0·8 | 18·7 | 1·1 | 17·8 | 1·3 |
| Peptide (mg/loop) | 15·9 | 0·4 | 18·3 | 0·3 | 14·6 | 0·9 | 15·7 | 0·4 |
| $^{40}Ca$ (mg/loop) | 2·3 | 0·2 | 2·0 | 0·1 | 2·2 | 0·2 | 2·5 | 0·2 |
| $^{45}Ca$ SA (dpm/mg Ca) | 4·3 | 0·7 | 3·7 | 0·4 | 4·6 | 0·3 | 3·1 | 0·4 |
| $^{45}Ca$ absorbed‡ (% dose) | 43·2 | 2·0 | 49·3 | 3·4 | 29·2* | 2·2 | 41·9 | 2·8 |
| $^{45}Ca$ soluble§ (%) | 57·8 | 8·9 | 69·7 | 15·2 | 35·9* | 2·1 | 49·3 | 3·8 |

SA, specific activity; dpm, disintegrations/min.
Mean values were significantly different from those of other dietary treatment groups (ANOVA): * $P < 0·05$.
† For details of diets and procedures, see Table 1 and pp. 766–769.
‡ (1 − $^{45}Ca$ (dpm) at 1·0 h/dose administered) × 100.
§ Percentage of total ileal radioactivity remaining as soluble calcium.

casein + CPP 2·53 (SEM 0·06); soya-bean protein 2·17 (SEM 0·08); soya-bean protein + CPP 2·34 (SEM 0·06): food intake (g/d; (means with SEM): casein 14 (SEM 0·4); casein + CPP 13·9 (SEM 0·3); soya-bean protein 13·8 (SEM 0·2), soya-bean protein + CPP 13·7 (SEM 0·4). Similarly, no significant differences in plasma minerals (values not shown) were observed in SHR fed on the CPP-supplemented diets. The recovery of PEG in ileal contents of experimental animals was identical for all dietary treatments (Table 5), thus excluding intestinal transit time as a significant factor for *in situ* $^{45}Ca$ absorption variables in both experiments. The percentage of $^{45}Ca$ absorbed in animals fed on CPP-supplemented soya-bean protein diets was significantly ($P < 0·05$) higher than that of the soya-bean protein-fed controls and corresponded to similar values observed in casein-fed animals. These results were closely associated with concomitant increases in soluble $^{45}Ca$ in these animals (Table 5). The greater proportion of soluble $^{45}Ca$ in animals fed on casein and soya-bean protein diets supplemented with CPP were found to be directly associated with the recovery of a phosphopeptide fraction from ileal contents by gel-filtration chromatography, which co-eluted with $^{45}Ca$ radioactivity (Fig 3 (*a–d*)). This was particularly evident in the soya-bean protein-based diets (Fig. 3 (*c* and *d*)).

Despite the enhanced ileal absorption of $^{45}Ca$ in rats fed on the CPP-supplemented casein and soya-bean-protein diets, no detectable differences in SBP were observed over the experimental period (Fig. 4). The development of hypertension in SHR in the present experiment was confirmed to occur at about 8–10 weeks of age.

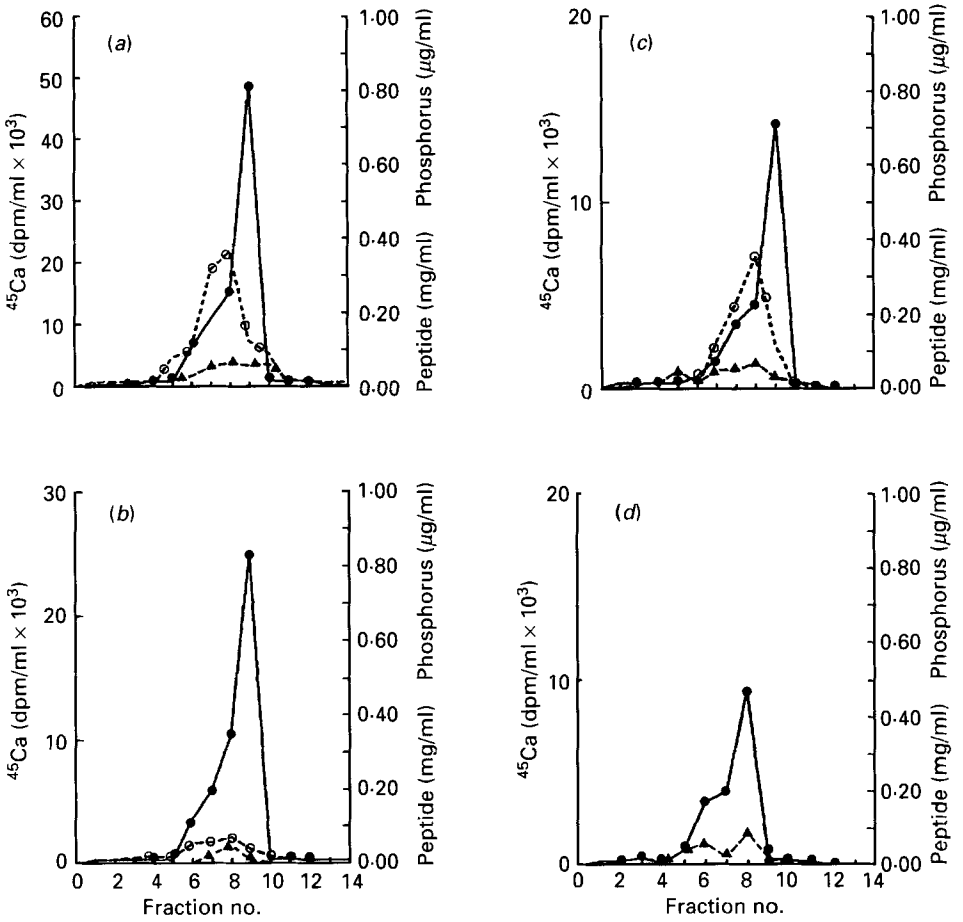


Fig. 3. Expt. 2. Gel filtration elution profiles for $^{45}$Ca (●), peptide (▲) and organic phosphorus (○) in the contents of ligated ileal loop of spontaneously hypertensive rats fed on diets containing (*a*) casein + caseinophosphopeptides (CPP), (*b*) casein, (*c*) soya-bean protein + CPP and (*d*) soya-bean protein. For details of diets and procedures, see Table 1 and pp. 766–769 dpm, Disintegrations/min.

## DISCUSSION

The present experiments were conducted to evaluate the nutritional aspects of Ca absorption in the distal small intestine. To evaluate further the physiological significance of changes in paracellular Ca absorption attributed to nutritional factors regulating Ca intake and bioavailability, SBP measurements from a genetically hypertensive animal model were taken.

Greater variations in body-weight gains and daily food intakes in both SHR and WKY rats were attributed to an interaction between level of dietary Ca intake and protein source. High dietary Ca intakes can lower the digestibility of dietary fat and, thus, the digestible energy of the diet by interfering with absorption of fatty acids released during digestion of lipids, or by reducing intestinal reabsorption of bile acids (Cheng *et al.* 1949; Yacowitz *et al.* 1967). The exacerbation of this effect in soya-bean protein-fed animals receiving a high dietary intake of Ca was consistent with the previously noted increased steroid excretion of bile acids in animals fed on soya-bean protein (Tanaka *et al.* 1984), as well as a marked

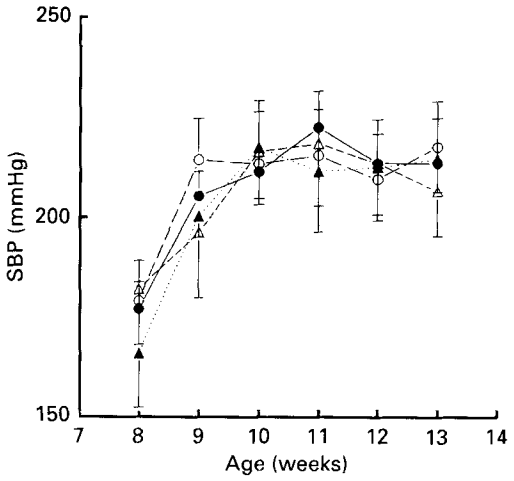


Fig. 4. Expt. 2. Temporal changes in systolic blood pressure (SBP) of spontaneously hypertensive rats fed on diets containing casein + caseinophosphopeptides (CPP) (△), casein (●), soya-bean protein + CPP (▲) and soya-bean protein (○). Values are means with their standard errors represented by vertical bars. For details of diets and procedures, see Table 1 and pp. 766–769.

inhibition in food intake caused by feeding fat or products of fat digestion (Gonzalez & Deutsch, 1985).

The absence of significant animal-strain differences in plasma electrolyte levels observed in the current study supports earlier findings of Vincent *et al.* (1977). In previous studies (Reeves & O'Dell, 1988; Hatton *et al.* 1989) blood Ca levels have been correlated with the level of dietary intake but, similar to the findings reported herein, an anti-hypertensive response in SHR fed on high-Ca diets was not observed. In contrast, other workers have reported a significant attenuation in the time-course of hypertension in SHR fed on high-Ca diets, but in the absence of significant changes in serum total Ca (Ayachi, 1979; Schleiffer *et al.* 1984). McCarron *et al.* (1981) noted that the development of hypertension in the SHR was associated with comparatively lower serum ionic Ca concentrations, albeit similar serum total Ca levels were reported for SHR and WKY strains. We were puzzled by our findings which showed that animals fed on low-Ca–soya-bean protein diets did not exhibit a similar hypocalcaemia to that observed in casein-fed counterparts. The relative hypocalcaemia was observed in both SHR and WKY animals indicating that Ca metabolism in Ca-depleted animals can be affected by the dietary protein source. Low dietary Ca intake increases parathyroid hormone (PTH) mRNA (Yamamoto *et al.* 1989) and can elevate circulating PTH (Grubenmann *et al.* 1978) and the mobilization of Ca from bone to maintain plasma Ca levels (Norman, 1985). The complex effect of a low dietary Ca intake on Ca homeostasis is emphasized by the noted increase in serum 1,25-dihydroxycholecalciferol (1,25-$(OH)_2$ vitamin D) observed in Ca-deficient rats (Trechsel *et al.* 1980), which in turn lowers PTH secretion (Au, 1984) by inhibiting cellular proliferation of the PTH gland (Szabo *et al.* 1989). In the present study we did not measure PTH or cholecalciferol and cannot speculate on the relative set-point of Ca-induced PTH secretion in animals fed on different protein sources. This finding, however, merits further investigation.

Previous reports have associated the development of hypertension in SHR with age-dependent changes in active transport of Ca (Toraason & Wright, 1981) or lower mean serum concentrations of 1,25-$(OH)_2$ vitamin D (Kurtz *et al.* 1986). Evidence of intestinal

Ca hyperabsorption observed in Ca balance studies with SHR (Jones *et al.* 1988) are contrary to in vitro Ca duodenal ($J_{m \rightarrow s}$) flux studies, which indicated a reduced duodenal Ca transport in SHR (McCarron *et al.* 1985). Recently, Blakeborough *et al.* (1990) reported impaired alkaline phosphatase (*EC* 3.1.3.1) and $Na^+$, $K^+$ and $Ca^{2+}$-ATPase (*EC* 3.6.1.3) (involved in regulating transcellular Ca transport) activities in the intestinal membranes of SHR. Our results extend these findings to show that no differences existed between SHR and WKY paracellular intestinal Ca absorption, which is of primary importance to overall Ca balance when there is a normal level of dietary Ca made available (Cramer & Copp, 1959; Marcus & Lengemann, 1962). The inverse relationship between dietary Ca intake and the proportion of absorbed Ca noted from ileal loops in both SHR and WKY rats agrees with the greater *in vitro* Ca mucosal–serosal ($J_{m \rightarrow s}$) and net transepithelial fluxes reported by others with ileal tissues collected from rats fed on low-Ca diets (Nellans & Kimberg, 1979).

It has recently been shown that low-Ca diets fed to SHR enhanced active Ca transport by stimulating intestinal brush-border membrane ATPase and alkaline phosphatase activity in the proximal intestine; however, the development of hypertension was not affected (Blakeborough *et al.* 1990). A similar finding was made in the present study with the noted increased efficiencies in intestinal Ca paracellular transport, although heightened hypertension still occurred in animals fed on the low-Ca diets. Thus, it is apparent that enhanced Ca absorption efficiency, whether it occurs by transcellular or paracellular transfer in Ca-deficient animals, is not sufficient to attenuate the development of hypertension. In contrast, there is some controversy as to whether dietary Ca supplementation alone will lower blood pressure in hypertensive subjects. The observation that SBP in both SHR and WKY rats fed on intermediate- and high-Ca diets was not significantly different is consistent with recent reports (Hatton *et al.* 1989), but is contrary to the findings from other laboratories (Ayachi, 1979; McCarron *et al.* 1981; McCarron, 1982). Considering the increased lumen concentrations of Ca in ileal contents of animals fed on high-Ca diets, as well as the higher proportion of soluble ileal Ca in animals fed on medium-Ca diets supplemented with CPP, it is apparent that dietary factors responsible for increasing the efficiency of passive diffusion of Ca from the ileum alone were neither sufficient nor the only contributing factor for reducing hypertension in the SHR. Moreover, it may be argued that the enhanced Ca bioavailability attributed to CPP-supplemented diets was too small to elicit a blood pressure-lowering effect in genetically hypertensive animals. It is believed that an increase in extracellular Ca with high dietary Ca intake has a direct effect on lowering blood pressure through the reduction of vascular smooth muscle contraction and promotion of relaxation of already contracted vasculature (Heagerty, 1990). However, an indirect effect of Ca in lowering blood pressure has also been shown to include the activity of renal tubules (McCarron *et al.* 1981; Resnick, 1987) and the sympathetic nervous system (Hatton *et al.* 1989; Wyss *et al.* 1989). Thus, it is equally probable that the increased paracellular Ca absorption derived from high Ca intake or enhanced Ca bioavailability in the present study did not induce other humoral factors that comprise the putative mechanisms underlying reduction of hypertension in the SHR.

The presence of phytates in soya-bean protein isolates has been well documented to reduce mineral absorption by complexing Ca and forming highly insoluble chelates (McCance & Widdowson, 1942). Alternatively, characteristic differences in paracellular Ca absorption noted between casein- and soya-bean protein-fed animals is partly explained by the presence of bioactive peptides, notably CPP in ileal contents of casein-fed animals. Numerous *in vitro* studies have previously demonstrated an inhibitory action of CPP derived from $\alpha_s$- and $\beta$-casein on calcium phosphate precipitation, resulting in increased Ca solubility (Mellander, 1963; Gerber & Jost, 1986). Our results confirm earlier in vivo studies

which attributed enhanced absorption of Ca in the small intestine to a CPP-mediated increase in soluble Ca content in ileal lumen contents not observed in animals fed on egg albumin, soya-bean protein or an amino acid mixture of casein (Lee *et al.* 1980, 1983). Furthermore, we extend these findings to animals with varying dietary Ca intakes. The greater ileal absorption of Ca observed in animals fed on casein diets with medium and low Ca content was not seen in animals fed on the high-Ca diet. This finding corresponded to the relatively small proportion of soluble Ca in ileal contents of casein-fed animals with a high dietary Ca intake, possibly reflecting a saturation of phosphoseryl groups on the CPP molecule (Mykkanen & Wasserman, 1980). This theory was examined further in Expt 2 where estimates of intestinal CPP content were made. The organic P content of ileal lumen contents of casein-fed animals after a 1·5 h feeding of a 200 g casein/kg diet agrees with previous studies (Lee *et al.* 1983). Performing a similar calculation made by Lee *et al.* (1980), it can be estimated from a PEG:casein ratio of 0·1 and a PEG ileal content of 18·4 mg/loop that approximately 1·52 mg P was derived from casein. This represents approximately 5·97 mg CPP (given that there is 60 mg P/g CPP) in the intestinal contents of animals 1·5 h after ingestion of the casein diet. Although the amount of CPP recovered from animals 1·5 h after ingestion of casein diets agrees with estimates calculated by Lee *et al.* (1980) in animals sampled 2·5 h after feeding, it was insufficient to solubilize the amount of ileal $^{40}$Ca present in animals fed on the high-Ca diets according to calculations of Naito & Suzuki (1974). Another possible explanation for these findings is that the amounts of soluble Ca present in the ligated ileal loops of casein- and soya-bean-protein-fed animals with high Ca intake were equally in excess of the amount required for passive diffusion to the blood to occur (Wasserman & Taylor, 1976). The presence of CPP from casein, therefore, would not have been the limiting factor in enhancing paracellular Ca absorption from the high-Ca diets. In contrast, animals fed on the medium-Ca diets had a relatively lower soluble lumen Ca content to interact with the CPP generated from a 200 g casein/kg diet thus explaining the relative difference in percentage of $^{45}$Ca absorbed in animals fed on casein and soya-bean protein diets respectively. Conversely, animals fed on the low-Ca diets had ileal Ca concentrations below the critical amount required for passive absorption, and although casein-fed animals generated sufficient CPP to solubilize ileal Ca in these animals the physiological significance of this effect was probably too small to make a difference to overall Ca balance. These findings stress the importance of dietary Ca intake on the potential CPP-mediated effects on ileal Ca absorption.

The co-elution of P-containing peptides with $^{45}$Ca in ileal contents represents a qualitative estimate of Ca bound to CPP within the intestine. Although sensitive to alkaline phosphatase activity, CPP are resistant to intestinal proteolytic enzymes (Mellander, 1963) and, furthermore, macropeptide fragments have been both qualitatively (Naito & Suzuki, 1974) and quantitatively isolated (Meisel & Frister, 1989) from ileal contents of casein-fed animals. In Expt 2 the supplementation of soya-bean-protein diets with CPP was found to have a more pronounced effect on increasing both the proportion of soluble $^{45}$Ca as well as disappearance of $^{45}$Ca from the intestinal loop than with CPP-supplemented casein diets. In vitro studies have indicated that unidentified constituents of milk reduce phytate-induced precipitation of Ca, theoretically enhancing Ca bioavailability (Platt *et al.* 1987). At the level of intraluminal Ca studied herein it is apparent that the intestinal milieu, reflecting the lumen concentration of Ca and presence of phytates or similar $Ca^{2+}$ ion chelators, will greatly influence the magnitude of effect exerted by CPP. It is important to note, however, that a substantial amount of $^{45}$Ca radioactivity shown to co-elute with peptide fractions derived from soya-bean protein digestion products was not associated with the presence of P or an equivalent amount of soluble Ca recovered from the intestinal contents. Carbonyl amino acid residues of soya-bean macropeptide digests forming acidic amino acid–Ca

complexes (Wasserman *et al.* 1956), which are not known to exhibit high Ca solubility (Naito *et al.* 1972), were possibly involved.

The extrapolation of our findings to human consumption of dairy products is difficult; however, a direct correlation between milk consumption and a protective effect against hypertension (Ackley *et al.* 1983; Garcia-Palmieri *et al.* 1984) has been suggested. The reduction in blood pressure in these studies was related to the intake of Ca from consumption of dairy products. In addition, protein-rich diets containing casein have been shown to attenuate the development of hypertension in stroke-prone hypertensive rats (Ikeda *et al.* 1987). The presence of bioactive peptides derived from casein hydrolysis products has also received attention concerning possible anti-hypertensive activity (Maruyama & Suzuki, 1982). We conclude that the apparent enhanced paracellular Ca bioavailability measured in animals fed on casein-containing diets in general, and CPP, in particular, was not a factor in lowering blood pressure in SHR. Our results with hypertensive rats raise the question as to the significance of increased paracellular Ca absorption to overall Ca utilization as influenced by a Ca homeostatic control and the physiological status of the individual.

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