Title

Acute oral calcium suppresses food intake through enhanced peptide-YY secretion mediated by the calcium-sensing receptor in rats

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Conflict of interest

The authors declare no conflict of interest.

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Calcium suppresses food intake via CaSR and PYY

Abbreviations

AIN-93G, American Institute of Nutrition-93G; BIIE, BIIE0246; $CaCO_3$, calcium carbonate; $CaCl_2$, calcium chloride; Ca lactate, calcium lactate; CaSR, calcium-sensing receptor; CCK, cholecystokinin; CMC, calboxymethyl cellulose; DMSO, dimethyl sulfoxide; DPP-4, dipeptidyl peptidase-4; DVZ, Devazepide; Ex9, exendin 9-39; GLP-1, glucagon like peptide-1; PYY, peptide-YY;

Abstract

Background: Dietary calcium has been proposed to reduce appetite in human studies. Postprandial satiety is mainly controlled by gut hormones. However, the effect of calcium on appetite and the role of gut hormones remain unclear.

Objective: We examined whether oral administration of calcium reduces food intake in rats and investigated the underlying mechanism.

Methods: Male Sprague-Dawley rats (8–12 wk) old were used after an overnight fasting. In a series of 2 trials with one-week interval between challenges, food intake was measured 0.5–24 h after oral gavage of a vehicle (saline containing 1.5% carboxymethyl cellulose) as the control treatment, or the vehicle containing various calcium compounds (calcium chloride ($CaCl_2$), calcium carbonate, calcium lactate, in a random order) at 150 mg calcium/kg dose. A conditional taste aversion test was conducted. In separate experiments, plasma calcium and gut hormone concentrations were measured 15 or 30 min after oral administration of the calcium compounds. In anesthetized rats, portal peptide-YY (PYY) concentrations were measured after intraluminal administration of a liquid meal with or without additional calcium.

Results: Oral $CaCl_2$ reduced food intake at the early period (30 min, ~20%, P <0.05) compared to control rats, without taste aversion. Plasma PYY concentration was higher (100%, P <0.05) in $CaCl_2$ preloaded rats than in control rats, 15 min after administration. In anesthetized rats, luminal meal+$CaCl_2$ induced a 4-fold higher increase in plasma PYY than the control treatment did. Oral administration of the calcium-sensing receptor (CaSR) agonist suppressed food intake (~30%, P <0.05), but $CaCl_2$ and CaSR agonist did not suppress food intake under treatment with a PYY receptor antagonist. Furthermore, CaSR antagonist attenuated the effect of $CaCl_2$ on food intake.

Conclusions: $CaCl_2$ suppresses food intake partly by increasing CaSR-mediated PYY secretion in rats. Our findings could at least partially explain the satiating effect of calcium.

Key Words

Calcium-sensing receptor, dietary calcium, meal, peptide-YY, satiety

Introduction

Overeating is a major cause of obesity, which remains one of the most serious global health problems. Appetite is regulated by complex systems, including the central nervous system, energy status, and gut endocrine systems. Research to understand appetite regulation and control by functional food ingredients or by pharmaceuticals is being widely undertaken.

Postprandial satiety is driven mostly by ingested macronutrients. In particular, proteins and fats are known to have potent satiating effects (1-6). Luminal digestive products such as peptides, amino acids, fatty acids, and monosaccharides (glucose and fructose) stimulate the secretion of various anorexic gut hormones from enteroendocrine cells. Cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), and peptide-YY (PYY) are known to be major players in inducing postprandial satiety (7). CCK is secreted by enteroendocrine “I cells”, which are primarily located in the proximal small intestine. GLP-1 and PYY are secreted by enteroendocrine “L cells”, which are abundantly present in the ileum and the large intestine.

Dietary calcium has been proposed to reduce appetite (or to enhance satiety) and to cause weight loss in human studies. Higher calcium and vitamin D intake at breakfast reduced energy intake in the following 24 h (8). Co-ingestion of calcium with meals in healthy adults temporarily suppressed appetite, which was accompanied by higher postprandial GLP-1 secretion (9-11). In overweight and obese adults with metabolic syndromes, a dietary pattern high in dairy products and calcium for 12 weeks had modestly lower appetite and higher postprandial PYY concentration (12). On the other hand, earlier studies do not support the suppressive effect of dietary calcium on appetite (13, 14). Weight loss induced by dietary calcium has been potentially explained not only by the satiating effect of calcium but also by enhanced fat excretion (15, 16).

92 Direct effects of calcium on gut hormone secretion in vivo are unclear, but the sensor for
93 extracellular calcium, namely, calcium-sensing receptor (CaSR) functions as a sensor for specific
94 amino acids and dietary peptides in enteroendocrine cells (17-23). A recent study demonstrated that
95 L-phenylalanine reduced food intake in rodent models, in a manner mediated by CaSR activation and
96 increased GLP-1 secretion (24).
97 Thus, the effect of calcium on appetite remains inconclusive, and involvement of gut hormones is
98 unclear in the possible satiating effect of orally administered calcium. It is also unclear whether the
99 effect of calcium depends on its chemical form. Accordingly, we hypothesized that oral
100 administration of calcium suppresses appetite through enhancing gut hormone secretion. To
101 investigate this hypothesis, we examined the effects of oral preload of various calcium compounds
102 on food intake, behaviors (duration before eating, single meal duration), and related parameters
103 (gastric emptying rate, intestinal transit, and plasm gut hormones) in rats, and examined the possible
104 involvement of gut hormones. Further experiments were conducted in anesthetized rats to elucidate
105 the underlying mechanisms of the effect of calcium on gut hormone secretion.
106
107 **Methods**
108 Animals, diets, and reagents
109 Male Sprague-Dawley rats (7 weeks old, 210-230 g) were purchased from Japan SLC, Inc.
110 (Hamamatsu, Japan), and fed an American Institute of Nutrition-93G diet (25). Experiments were
111 conducted after an acclimatization period at least for 1 week. Rats were housed individually in a
112 temperature- and humidity-controlled room (22 ± 2°C 55 ± 5%) with a standard light cycle (lights on
113 during 8:00 – 20:00). All rats were given access to diet and water ad libitum. All experimental
114 animal procedures were approved by the Hokkaido University Animal Committee (permission
115 numbers 14-0013 and 19-0064), and animals were maintained in accordance with the Hokkaido

University guidelines for the care and use of laboratory animals. Reagents were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) unless otherwise specified.

Food intake tests (Experiments 1-1, 1-2, 1-3, 3, 5)

Rats were acclimatized to oral administration before the experiment for 1 week. Rats were feed-deprived overnight and then received an oral gavage (10 mL/kg BW) of test liquids through a feeding tube (Fr 5, Atom Medical Corp. Tokyo, Japan) at around 10-11AM. Test materials were dissolved in a vehicle containing 1.5% calboxymethyl cellulose (CMC) in saline. The control group received the vehicle. Immediately after oral gavage of test liquids, rats had free access to the diet (AIN-93G) ad libitum, and food intake was measured at 0.5, 1, 2, 4, 8, and 24 h after the oral administration, by weighing the diet container manually and quickly.

Experiment 1: Effects of various calcium compounds on food intake, dose-response, and taste aversion test

In experiment 1-1, to compare the effects of various calcium sources, rats (n=8-9 /group) received calcium chloride ($CaCl_2$), calcium carbonate ($CaCO_3$), calcium DL-lactate pentahydrate (Ca lactate), at a dose of 150 mg/kg as calcium, or the vehicle. After one-week washout period, in experiment 1-2, rats (n=8-9 /group) received the vehicle, $CaCl_2$ (150 mg/kg as calcium) or L-Lysine (0.979 g/kg) as a positive control (26), to confirm the effect of $CaCl_2$. Rats were allocated to treatment groups not to receive the same treatment with previous round. In a separated experiment (experiment 1-3), rats (n=6 /group) received different doses (100, 150, 200 mg/kg as calcium) of $CaCl_2$. Food intake was measured as described above.

A conditional taste aversion test (experiment 1-4) was conducted based on previously reported protocol (27, 28). Rats (n=4-5 /group) were allowed access to two water bottles for 2 h (11:00-13:00) for ten days in order to acclimatize to a water-deprivation schedule. Water intake were measured

every day to confirm that the rats had taken the same amount of water from the two water bottles. On the day 11 (conditioning day), the rats were provided with two bottles of 0.3% saccharine solution for 30 min, and then received an oral gavage of various calcium solutions ($CaCl_2$, $CaCO_3$, or Ca lactate), vehicle (1.5% CMC), or an intraperitoneal injection of lithium chloride (127.2 mg/(mL · kg)) as a positive control. On the day 12 (rest day), rats were allowed to access to two water bottles for 2 h. On the day 13 (test day), a two-bottle preference test (one containing water, another containing saccharin solution) was performed for 30 min. Saccharine intake/total intake was determined as the saccharin preference ratio.

Experiment 2: Effects of oral calcium on short time (15 min and 30 min) food intake, time before initiating eating, first meal duration, and plasma calcium and gut hormone concentrations

Rats were feed-deprived overnight, and then received an oral gavage (10 mL/kg BW) of various calcium solutions ($CaCl_2$, $CaCO_3$, or Ca lactate) or its vehicle (1.5% CMC). In order to compare gastric emptying rate, acetaminophen (SIGMA, St. Louis, MO, USA) at a dose of 100 mg/kg was added to all of test solutions (29). The diet was given immediately after the administration, and rats had free access to the diet for 15 min (n=5 /group) or 30 min (n=6-8 /group). The time before initiating eating and the first meal duration were measured by using video recording in rats fed for 30 min. The first meal duration was defined as the duration from initiating eating to stop eating and take a rest for more than 5 minutes. Fifteen or 30 min after the administration, rats were anesthetized by isoflurane (MSD Animal Health, Inc., Tokyo, Japan), and a laparotomy was made immediately for blood sampling. Blood samples were taken from the portal vein and the vena cava under isoflurane anesthesia using a syringe filled with EDTA·2Na (final concentration 1.8 mg/mL, Dojindo, Kumamoto, Japan), Pefabloc SC (final concentration at 1 mg/mL, Roche, Basel. Switzerland), DPP-4 inhibitor (final concentration at 10 μL/mL, Millipore, Burlington, MA, U.S.A.), and Sigma's Protease inhibitor Cocktail (final concentration at 1 μL/mL, SIGMA). Then, rats were euthanized by

exsanguination. For measurement of calcium, blood samples were separately collected into a syringe containing heparin (final concentration at 50 IU/mL, Nacalai Tesque, Kyoto, Japan). Acetaminophen concentration was determined using an Acetaminophen Detection Kit (Kanto Chemical Co., Inc., Tokyo, Japan). Plasma total calcium concentration was determined using a Calcium E-Test Wako (Wako). Gut hormone concentrations were determined using the MILLIPLEX Multiplex Assay (MILLIPLEX MAP Rat Metabolic Hormone Magnetic Bead Panel - Metabolism Multiplex Assay, Millipore). To assess the effect of calcium preload on the intestinal transit of ingested food, we measured the maximum migration distance of food (chyme) remaining in the small intestine by visual observation immediately after blood collection (15 min postprandial) and euthanasia.

Experiment 3: Effects of gut hormone receptor antagonists on calcium-suppressed food intake

Food intake tests were conducted in rats treated with various gut hormone receptor antagonists. Rats (n=6-8 /group) received intraperitoneal injection of BIIE0246 solution (Tocris Bioscience, Bristol, UK dissolved in saline containing 7% DMSO, and dosed at 680 nmol/kg) as a PYY receptor (Y2R) antagonist, or its vehicle (7% DMSO in saline, 1 mL/kg), immediately after oral administration of $CaCl_2$ solution (at 150 mg calcium/kg in experiment 3-1, at 100 mg calcium/kg in experiment 3-2) or its vehicle (1.5% CMC in saline). Food intake was measured as described above. These experiments were separatory conducted.

In a separated experiment (experiment 3-3), rats (n=3-5 /group, Supplementary Figure 2) received intraperitoneal injection of Devazepide (DVZ, ML Laboratories, Liverpool, UK, dissolved in saline containing 5% dimethyl sulfoxide (DMSO) and 5% Tween 80, and dosed at 500 μg/kg) as a CCK receptor antagonist, exendin 9-39 (Ex9, Synthesized by Thermo Fisher Scientific Inc. Waltham, MA, USA, dissolved in saline, and dosed at 200 nmol/kg) as a GLP-1 receptor antagonist, BIIE0246 (340 nmol/kg) , or the vehicle (7% DMSO in saline, 1 mL/kg) immediately after oral administration of

190 $CaCl_2$ solution (150 mg calcium/kg) or its vehicle (1.5% CMC in saline). Food intake was measured
191 as described above.
192
193 Experiment 4: Effects of intestinal administration of a liquid meal with calcium on PYY secretion in
194 anesthetized rats (*in situ* experiment)
195 Rats (n=7-8 /group) were feed-deprived overnight, and a laparotomy was performed under
196 anesthesia with sodium pentobarbital (50 mg/kg, Somunopentyl; Kyoritsu Seiyaku Co., Tokyo,
197 Japan). A small tip of polyethylene catheter (SP28, Natsume Seisakusho Co.,Ltd., Tokyo, Japan)
198 connected to a silicone catheter (SILASCON No. 00, Kaneka Medics Kaneka Medix Corp., Osaka,
199 Japan) was inserted into the portal vein and fixed with an instant glue (30). Blood samples were
200 collected before (0 min as baseline) and after (7, 15, 30 min) duodenal administration (10 mL/kg) of
201 saline as control, $CaCl_2$ (93.6 mM, 37.5 mg calcium/kg ; a quarter dose of the oral administration), a
202 liquid meal (Ensure®H at 15 kcal/(10 mL · kg)), Abbott Japan, Tokyo, Japan, contains 8 mg
203 calcium/10 mL), or liquid meal (15 kcal/(10 mL · kg)), added with $CaCl_2$ (93.6 mM, 37.5 mg
204 calcium/kg). Blood samples were drawn into a syringe filled with heparin (final concentration at 50
205 IU/mL), aprotinin (from bovine lung, final concentration at 500 kIU/mL), and DPP-4 inhibitor (final
206 concentration, 10 μL/mL). PYY concentrations were determined using a PYY EIA kit (YK081;
207 Yanaihara Institute, Inc., Limited).
208
209 Experiment 5: Effects of gut hormone receptor antagonists on calcium-suppressed food intake
210 In experiment 5-1, a CaSR agonist (cinacalcet, CCT, purchased from SIGMA, dissolved in saline
211 containing 1% DMSO) was orally administered in feed-deprived rats (n=6-7 /group), at a dose of 50
212 mg/kg in combination with or without intraperitoneal BIIE0246 administration (680 nmol/kg). In a
213 separated experiment (experiment 5-2), a CaSR antagonist (NPS2143, purchased from SIGMA,
214 dissolved in saline containing 4% DMSO) was orally administered in feed-deprived rats (n=7-9

/group), at a dose of 40 mg/kg in combination with or without $CaCl_2$ (150 mg calcium/kg). Food intake was measured as described above.

Experiment 6: Effect of calcium on meal-induced PYY secretion in the presence or absence of CaSR antagonist in anesthetized rats (*in situ* experiment)

Another in situ experiment was performed in the presence of a CaSR antagonist. The portal catheter was implanted in anesthetized rats (n=7-8 /group) as described above, and NPS2143 (at 0.1 mg/(2 mL · kg)) in 1% DMSO) or its vehicle (1% DMSO at 2 mL/kg) was administered into the duodenum (– 5 min). Five minutes later, portal blood was collected, and then, the liquid meal (15 kcal/(10 mL · kg)) with or without $CaCl_2$ (93.6 mM, 37.5 mg calcium/kg) was administered into the duodenum (0 min). Portal blood samples were collected and plasma PYY concentrations were measured as described above.

Statistical analyses

The sample size was calculated based on experimental designs using G*Power software (version 3.1.9.2). In experiments 1-1, 1-2, 1-3 and 2, n=7 /group was found to be sufficient to provide >80% power to detect an effect size of 0.7 with an α level of 0.05 (one-way analysis of variance (ANOVA)). In experiments 1-4, (taste aversion test), n=4 /group was found to be sufficient to provide >80% power to detect an effect size of 1.0 with an α level of 0.05 (one-way ANOVA). In experiments 3 and 5, n=5 /group was found to be sufficient to provide >80% power to detect an effect size of 0.7 with an α level of 0.05 (two-way ANOVA). The number of rats varied group among treatments because some of rats unexpectedly refused oral administration on the day of experiment. In experiment 4 and 6, by using GLIMMPSE software (http://glimmpse.SampleSizeShop.org/) for repeated measures, the sample size (n=7 / group) was sufficient to provide >80% power with an α level of 0.05 to test the main effect (calcium) on PYY

240 secretory responses (approx. 40~100 pM difference between treatments observed in the present
241 study).
242 Data are expressed as means ± standard errors of the mean (SEM). Statistical analyses were
243 performed using JMP Pro version 14.3 software (SAS Institute, Inc., Cary, NC, USA). Normality of
244 data distribution was tested by the Shapiro-Wilk test, and homogeneity of variances was confirmed
245 by Bartlett's test. Data not normally distributed were log-transformed before ANOVA. Data at 0.5h
246 taken from food intake studies (experiment 1-1, 1-2, 1-3), and data taken from experiment 1-4 and 2
247 were analyzed by Dunnett's test to compare a set of means against the mean of a control group. Data
248 at 0.5h taken from food intake studies examining two factors (experiment 3-1, 3-2, 5-1, 5-2) were
249 analyzed by two-way ANOVA and Tukey–Kramer test to compare means one another. Time course
250 data from food intake studies (experiment 1-1, 1-2, 1-3, 3, 5) were analyzed by the mixed model with
251 unstructured covariance. For data on PYY responses during *in situ* experiments (experiments 4 and
252 6), the mixed model with unstructured covariance was performed, and the results (effects of time,
253 treatment (calcium, meal), and the interaction of factors) are presented in the figure. Student's t-test
254 was used for two group comparison (Supplementary Figure 2).-In all analyses, $P < 0.05$ was
255 considered statistically significant.
256
257 **Results**
258 Effects of oral preload of calcium on food intake and, behaviors (experiment 1 and 2)
259 Among the three calcium compounds tested at a 150 mg calcium/kg dose, $CaCl_2$ showed a
260 significant reduction in food intake measured after 30 min of refeeding, compared to the control
261 treatment (Fig. 1A). Total food intake for 24 h did not differ among treatments (control: 23.0 ± 0.7 g,
262 $CaCl_2$: 23.0 ± 0.9 g, $CaCO_3$: 23.1 ± 1.0 g, Ca lactate: 23.5 ± 0.9 g), indicating that rats treated with
263 $CaCl_2$ compensated for the initial decrease in food intake in the following period of time
264 (Supplementary Figure 1).

In a separate experiment (Fig. 1B), oral preload (approximately 1 g/kg) of lysine, indicating that our experimental method for testing the effects of certain compounds on rat food intake is compatible with the method previously reported.

Compared to 150 mg calcium/kg dose of $CaCl_2$, a lower dose (100 mg calcium/kg) or a higher dose (200 mg calcium/kg), had a smaller or larger effect on reducing food intake respectively, (Fig. 1C), which demonstrated the dose-responsive effect of oral preload of $CaCl_2$. No unfavorable effects, such as diarrhea, chronic anorexia, or overeating, were observed throughout the study.

Rats conditioned with calcium compounds at 150 mg calcium/kg showed preference ratios for saccharin solution similar to control rats, as examined by the conditional taste aversion test (Fig. 1D). LiCl-treated rats showed apparently lower preference ratios, indicating that conditional aversion was established by LiCl but not by calcium compounds. Although $CaCl_2$ at 200 mg calcium/kg had a potent effect on reducing food intake, rats conditioned with the dose had a lower saccharin preference ratio than control-treated rats (data not shown). Based on these results we employed a dose of 150 mg calcium/kg in further experiments.

Oral preload of calcium compounds did not affect 15 min food intake (Fig. 2A). Food intake for 30 min was significantly lower only in $CaCl_2$ treated rats than in control rats (Fig. 2A), consistent with the results shown in Fig. 1. Rats preloaded with $CaCl_2$ showed significantly shorter first meal duration, while the other groups had similar meal durations (15-20 min), as compared to the control rats (Fig. 2B). Most rats started to eat the meal immediately (~40 s) after oral administration of test solutions, but one rat in the $CaCl_2$-treated group started to eat after 90 s. The mean time before initiating eating did not significantly differ among treatments (Fig. 2C). There were no significant differences in acetaminophen and calcium concentrations in the portal vein, or calcium concentrations in the inferior vena cava, 15 and 30 min after the preload of various calcium compounds and re-feeding (Fig. 2D-F). Based on the above results, we selected $CaCl_2$ as the calcium compound to be used in subsequent experiments.

290

291 Involvement of gut hormones in calcium-reduced food intake (experiment 3)

292 Rats pretreated with calcium ($CaCl_2$ at 150 mg calcium/kg) and BIIE consumed similar amounts
293 of food as the control rats (Fig. 3A). In a separated experiment (Fig. 3B) oral $CaCl_2$ (100 mg
294 calcium/kg) significantly reduced food intake compared to the control group, but in rats pretreated
295 with calcium and BIIE, food intake was not significantly different compared to the control rats. The
296 effects of various gut hormone (CCK, GLP-1, and PYY) receptor antagonists were examined
297 (Supplementary Figure 3). Rats treated with calcium and BIIE had similar food intake to the control
298 group until 2 h, while other antagonist-treatments resulted in similar food intake to calcium treated
299 rats.

300 As shown in Fig. 2, food intake was significantly lower 30 min after oral preload with calcium
301 compared to preload with the control. No significant differences were observed in GLP-1 and GIP
302 concentrations (Supplementary Figure 2A and B). PYY concentration in calcium-treated rats was
303 significantly higher than that of the control rats at 15 min, but the difference had disappeared by 30
304 min (Supplementary Figure 2C). Chyme reached the proximal ileum (70% length of the whole small
305 intestine) 15 min after feeding the diet with or without calcium preload (Supplementary Figure 2D).

306

307 Effect of luminal calcium on PYY secretion (experiment 4)

308 Basal PYY concentrations were 152 ± 36 pM in the control, 172 ± 46 pM in the $CaCl_2$ group, 149
309 ± 16 pM in the Meal group, and 114 ± 30 pM in the Meal+ $CaCl_2$ group. Because basal PYY
310 concentrations varied as above, results are presented as changes from the basal concentration
311 (ΔPYY). Portal PYY concentrations were largely increased by luminal administration of a liquid
312 meal (Ensure H at 15 kcal/kg) in anesthetized rats, with significant differences compared to the
313 control group observed at 15 min (Fig. 4). In rats treated with a combination of liquid meal and

calcium (Meal+ $CaCl_2$), PYY levels were significantly higher than those in the control group both at 7 and 15 min.

Involvement of CaSR in the effect of calcium on food intake and PYY secretion (experiment 5 and 6)

Oral administration of the CaSR agonist cinacalcet significantly reduced food intake, and the suppressive effect of cinacalcet was abolished by treatment with the PYY receptor antagonist (BIIE0246) (Fig. 5A). As shown in Fig. 5B, oral administration with a CaSR antagonist (NPS2143) almost completely abolished the suppressive effect of calcium on food intake.

In rats without NPS2143 pretreatment, basal PYY concentrations were 137 ± 26 pM and 193 ± 27 pM in the Meal and Meal+ $CaCl_2$ groups, respectively. In rats pretreated with NPS2143, basal PYY concentrations were 130 ± 20 pM and 108 ± 7 pM in the Meal and Meal+ $CaCl_2$ groups, respectively. Because basal PYY concentrations varied as above, results are presented as changes from the basal concentration (ΔPYY). Portal PYY concentration increased 15 min after luminal infusion of the liquid meal (Fig. 6A, B). Delayed response compared that seen in the experiment above (Fig. 4) may be due to luminal preload of NPS2143 or its vehicle. In the absence of NPS2143 (Fig. 6A), PYY responses had a similar trend to the result in experiment 4 (Fig. 4), but in the presence of NPS2143(Fig. 6B), meal-induced PYY secretions almost completely matched with or without calcium.

Discussion

The aim of this study was to examine the effect of oral calcium on appetite in rats and elucidate the mechanism underlying its effect. We found that oral administration of $CaCl_2$ (100-150 mg calcium/kg) acutely reduced food intake in rats. Involvement of PYY in the suppressive effect of

339 calcium on food intake was suggested by using PYY receptor antagonist, and enhancement of PYY
340 secretion by calcium. The involvement of CaSR in the enhancement of PYY secretion by luminal
341 calcium was also suggested. Our findings could at least partially explain mechanisms for the
342 satiating of calcium observed in previous human studies.

343 Among calcium components tested, $CaCl_2$ at 150 mg calcium/kg showed significant and
344 reproducible reduction in food intake. In a separated experiment, Ca lactate at 200 mg calcium/kg
345 significantly reduced food intake only at 2 h (data not shown). These results demonstrate that dietary
346 calcium salts have a suppressive effect on food intake with various potencies, depending on their
347 molecular forms. Such differences might be due to the solubility or absorbability of the calcium
348 compounds (11), and interactions with other food components and luminal factors in the intestinal
349 lumen. Calcium is bound to dietary proteins and peptides (31, 32), which provides specific
350 bioactivity such as enhancing calcium absorption in the intestine. It is unclear whether such calcium
351 binding peptides promote or attenuate the satiating effect of orally given calcium. Studies using
352 food-derived calcium biding peptides would help to clarify this issue.

353 The dose (150 mg calcium/kg = 416 mg/kg of $CaCl_2$) is not supra-physiological based on various
354 points of view, as described below. Firstly, the LD50 value of $CaCl_2$ in rats (acute oral
355 administration) is reported as 3798-4179 mg/kg (33). Secondly, only a certain portion of orally given
356 liquid flows into the small intestine in a short time, and the liquid is diluted by stomach fluid and by
357 intestinal fluid. Accordingly, it is possible that the orally given calcium solution (150 mg calcium/10
358 mL = 375 mM) was diluted to the concentration comparable to luminal calcium concentration (~100
359 mM) (34, 35). Thirdly, adult rats usually consume 500 mg calcium/kg body weight in a single day
360 from the AIN-93G diet, based on the assumption below. The AIN-93G diet contains 5 mg calcium/g
361 (25), and young adult male rats daily consume 100 g of the diet per kg body weight. Thus, the dose
362 of calcium at 150 mg/kg is less than 1/3 of the daily intake of calcium (500 mg/kg) and far less than
363 LD50 (approx. 4000 mg/kg). Lastly, when considering nutritional supplements for humans,

manufacturers commonly recommend that consumers take calcium supplements more than half of the daily required amount of calcium (650-800 mg/day for adults in Japan).

A significant reduction in food intake was observed after 30 min but not within 15 min (Fig. 2A). This could be attributed to the first meal duration (Fig. 2B), but not to either the gastric emptying rate (Fig. 2F) or plasma calcium concentrations at 15 min (Fig. 2D, E). One possible speculation is that portal calcium concentrations transiently elevated before or just after 15 min. Previous studies demonstrated increment of calcium concentrations, at 1-5 h after calcium-rich meal ingestion (36) in human peripheral blood, and at 20 min after ileal infusion of calcium with casein phosphopeptide (37) in rat portal vein. Because gastrointestinal blood flow postprandially increases, and the magnitude, timing, and duration of increased blood flow differ among gastrointestinal arteries/veins (38), further examinations measuring blood calcium at multiple time points in multiple veins might detect an elevation of circulating calcium.

Almost all rats started to eat the diet within 30 sec after the oral administration regardless of treatment (Fig. 2C). These observations support the idea that the effect of calcium is not due to taste aversion. Because $CaCl_2$ preloaded rats quit eating within 15 min but others kept eating for a further 5 min (Fig. 2B), it is likely the preload of $CaCl_2$ accelerated meal-induced satiety.

Postprandial satiety is induced by increased secretion of gut hormones (7). Among the candidate gut hormones (Supplementary Figure 3), PYY appeared to be partly responsible for the initial reduction of food intake by calcium (Fig. 3A, B, Supplementary Figure 2C). Other unknown factors are possibly involved since PYY receptor antagonist did not completely abolish the effect of calcium on food intake. Our results (Supplementary Figure 2D) revealed that at least a portion of the ingested meal (as chyme) immediately (within 15 min) reaches the middle-distal small intestine where PYY-producing cells are abundant (39, 40), and there was no difference between treatments (± oral calcium). Thus, luminal digestive products such as peptide and fatty acids together with preloaded

388 calcium possibly stimulated PYY secretion in the middle and distal small intestine in the present
389 study.
390 Oral administration of calcium enhanced meal-induced PYY secretion during the first meal
391 (within 15 min, Supplementary Figure 2B), which likely provided satiation and caused rats to stop
392 eating. These results suggest that luminal calcium had a potent effect on enhancing meal-induced
393 PYY secretion, which is supported by the results of *in situ* experiments demonstrating an
394 enhancement of meal-induced PYY secretion by adding calcium (Fig. 4).
395 In this study, plasma PYY and GLP-1 showed different secretory responses (Supplementary
396 Figure 2). Failure of a GLP-1 receptor antagonist to attenuate the suppressive effect of calcium on
397 food intake (Fig. 3) is in line with the secretory response. Although it is well recognized that PYY
398 and GLP-1 are co-produced in enteroendocrine L cells, this is not always the case; several reports
399 have demonstrated that not all GLP-1- and PYY-producing cells co-localize in the intestine (40-42).
400 Studies demonstrating suppressive effect of oral administration calcium on food intake are
401 limited. Involvement of GLP-1 has been proposed in human studies (10, 11), while studies blocking
402 GLP-1 signal were not conducted. A previous human study (12) suggested involvement of PYY in
403 calcium-induced satiety, but the study demonstrated increase in postprandial PYY response after
404 chronic ingestion of calcium-enriched diet. That is adaptive enhancement of PYY response.
405 Although experimental models (humans vs rats) and designs (calcium-enriched meal vs calcium
406 solution, appetite score vs food intake) are largely different, these and our studies suggest
407 involvement of enteroendocrine L cells (GLP-1 or PYY) in the suppressive effect of calcium on
408 appetite. In the present study, rats treated with $CaCl_2$ compensatory consumed diet within 24 h to
409 have similar food intake to control rats. Accordingly, it is interesting to examine effects of chronic
410 administration of oral calcium in rat models on food intake, PYY and other gut hormones.
411 CaSR was primarily identified as an extracellular calcium sensor regulating calcium homeostasis
412 in the parathyroid gland. Subsequent studies revealed the role of CaSR as a sensor for luminal amino

413 acids and dietary peptides in CCK/GLP-1-releasing enteroendocrine cells (17, 20, 22, 43). Our
414 hypothesis is supported by the results using CaSR agonist and antagonist (Fig. 5, Fig. 6). If PYY-
415 producing cell-specific CaSR knock-down/knock-out animals and/or isolated PYY-producing
416 enteroendocrine cells are available, our hypothesis will be further investigated in the future.
417 Although CaSR reportedly functions in CCK/GLP-1-producing cells, it is unclear why these
418 hormones were not involved in the suppressive effect of calcium on food intake in the present study.
419 Calcium affects various functions in the gastrointestinal tract, such as digestion, nutrient absorption,
420 energy metabolism, immune responses, hormone secretion, motility and barrier function (44, 45).
421 Since CaSR reportedly functions in pancreatic β-cell to regulate insulin secretion (46), insulin may
422 be involved in calcium-reduced food intake. Because an elevation of plasma calcium concentration
423 was not observed, it is not possible to speculate the involvement of calcium absorbed into the
424 circulation.
425 The calcium compounds ($CaCl_2$, $CaCO_3$, and Ca lactate) tested in the present study are widely
426 used as food additives (pH adjuster, coagulant, stabilizer, etc.) and nutritional supplements. Although
427 our data provide a translational perspective that calcium supplements prevent not only calcium
428 deficiency (47) but also overeating, we need to be aware of the risk of hypercalcemia (48) when
429 applying this knowledge. The present study was conducted only in young adult male rats (8~12
430 weeks age), because male rats at such age stably and well consume the diet. However, gender (49),
431 aging, high energy diet, could affect appetite. Further studies are need in the future to compare the
432 effects of oral administration of calcium in female, aged, or obesity models.
433 In summary, we examined whether oral preload of calcium suppresses food intake in rats and
434 investigated the mechanisms underlying the effect of calcium on food intake. oral administration
435 (150 mg calcium/kg, approx. 1/3 of daily calcium intake) of calcium ($CaCl_2$) acutely (~4 h)
436 suppressed food intake without any adverse effects. The effect of calcium was not accompanied by
437 an elevation of plasma calcium, but involved PYY signal. Experiments employing pharmacological

agonist and antagonist suggested the involvement of CaSR in both suppressing food intake and enhancing PYY secretion. These results demonstrate that oral calcium suppresses food intake in rats partly through PYY secretion which is enhanced by calcium and mediated by CaSR. Our findings revealed a novel physiological interaction between dietary calcium and enteroendocrine systems that regulate satiety induction.

Author Contributions

A. I, S. O and T. H designed and performed the research; A. I, S. O, R. T, K. O and T. H analyzed the data; A. I, H. H and T. H wrote the manuscript; T. H had primary responsibility for the final content. All authors read and approved the final manuscript. The authors declare that they have no conflicts of interest.

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Figure captions

Fig. 1.

Effects of oral administration of various calcium compounds (A), L-lysine (B), and different doses of calcium chloride (C) on food intake, and on saccharin preference (D) in rats randomly assigned to receive various treatments after one-week washout period (experiment 1)

Food intake in control rats and rats after oral administration of $CaCl_2$, $CaCO_3$, and Ca lactate at 150 mg Ca/kg body weight (A), $CaCl_2$ at 150 mg Ca/kg and L-Lys at a dose of 0.979 mg/kg (B), or $CaCl_2$ at doses of 100, 150, and 200 mg Ca/kg (C), and the saccharine preference ratio in rats conditioned by oral administration of $CaCl_2$, $CaCO_3$, and Ca lactate at 150 mg calcium/kg or with an intraperitoneal injection of lithium chloride (LiCl). Values are mean ± SEM, n=8-9 (A, B), 6 (C), or 4-5 (D). *Different from control, $P < 0.05$.

Fig. 2.

Acute (15 or 30 min) effects of oral calcium on food intake (A), first meal duration (B), time before initiating eating (C), plasma calcium concentrations (D, E), and gastric emptying (F) in rats (experiment 2)

Food intake (A), first meal duration (B), and the duration before eating (C) monitored for 30 min. Plasma concentrations of calcium in the portal vein (D), calcium in vena cava (E), and acetaminophen in the portal vein (F) 15 min and 30 min after the oral administration of vehicle or various calcium compounds (150 mg calcium/kg). The results are expressed as the mean ± SEM, n=5-8. *Different from control, $P<0.05$.

Fig. 3.

Effects of a PYY receptor antagonists on calcium (150 mg calcium/kg (A), at 100 mg calcium/kg (B))-reduced food intake in rats randomly assigned to receive various treatments after one-week washout period (experiment 3)

Food intake in control rats and rats after oral administration of calcium chloride ($CaCl_2$) at 150 mg calcium/kg (A), at 100 mg calcium/kg (B), or its vehicle (1.5% CMC in saline) with an intraperitoneal administration of vehicle or a PYY receptor antagonist BIIE0246 (BIIE, 680 nmol/kg). Values shown are means ± SEM, n=6-8 (A), 8 (B). Statistical significance was assessed by two-way ANOVA followed by Tukey-Krammer' s test. Labeled means without a common letter differ, $P<0.05$.

Fig. 4.

Effects of calcium on meal-induced PYY secretion in anesthetized rats (experiment 4)

A liquid meal (Meal, 15 kcal/(10 mL · kg)), calcium chloride ($CaCl_2$, 37.5 mg calcium/(10 mL · kg)), their combination (Meal + $CaCl_2$), or saline (Control) was directly infused into the small intestinal lumen in anesthetized rats. Values shown are means ± SEM (n=7-8), and are presented as changes from the basal (0 min) concentration of plasma PYY. Statistical significance was assessed by the mixed model with unstructured covariance followed by Tukey-Krammer' s test. Labeled means at the same time point without a common letter differ, $P<0.05$.

Fig. 5.

Effects of CaSR agonist (A) and antagonist (B) on food intake in rats randomly assigned to receive various treatments after one-week washout period (experiment 5)

Food intake in control rats and rats after oral administration of a calcium sensing receptor antagonist cinacalcet (CCT, 50 mg/kg) with intraperitoneal administration of saline or a PYY receptor antagonist (BIIE0246, 680 nmol/kg) (A), calcium chloride ($CaCl_2$, 150 mg calcium/kg) or its vehicle (Control) with or without coadministration of a calcium sensing receptor antagonist NPS2143 (40 mg/kg) (B). The results are expressed as the mean ± SEM, n=6-7 (A), 7-9 (B). Statistical significance was assessed by two-way ANOVA followed by Tukey-Krammer' s test. Labeled means without a common letter differ, $P<0.05$.

Fig. 6.

Effects of calcium on meal-induced PYY secretion in the absence (A) or presence (B) of CaSR antagonist in anesthetized rats (experiment 6)

A liquid meal (Meal, 15 kcal/(10 mL · kg)) was directly infused into the small intestinal lumen with (Meal + $CaCl_2$) or without $CaCl_2$ (37.5 mg calcium/(10 mL · kg)), in the absence (A) or presence (B) of CaSR antagonist (NPS2143, 0.1 mg/kg), in anesthetized rats. Blood samples were drawn from the catheter inserted into the portal vein before and after administration. Values are means ± SEM, n=7-8, and are presented as changes from the basal (0 min) concentration of plasma PYY. Statistical significance was assessed by the mixed model with unstructured covariance.

Figure 1

Fig. 1

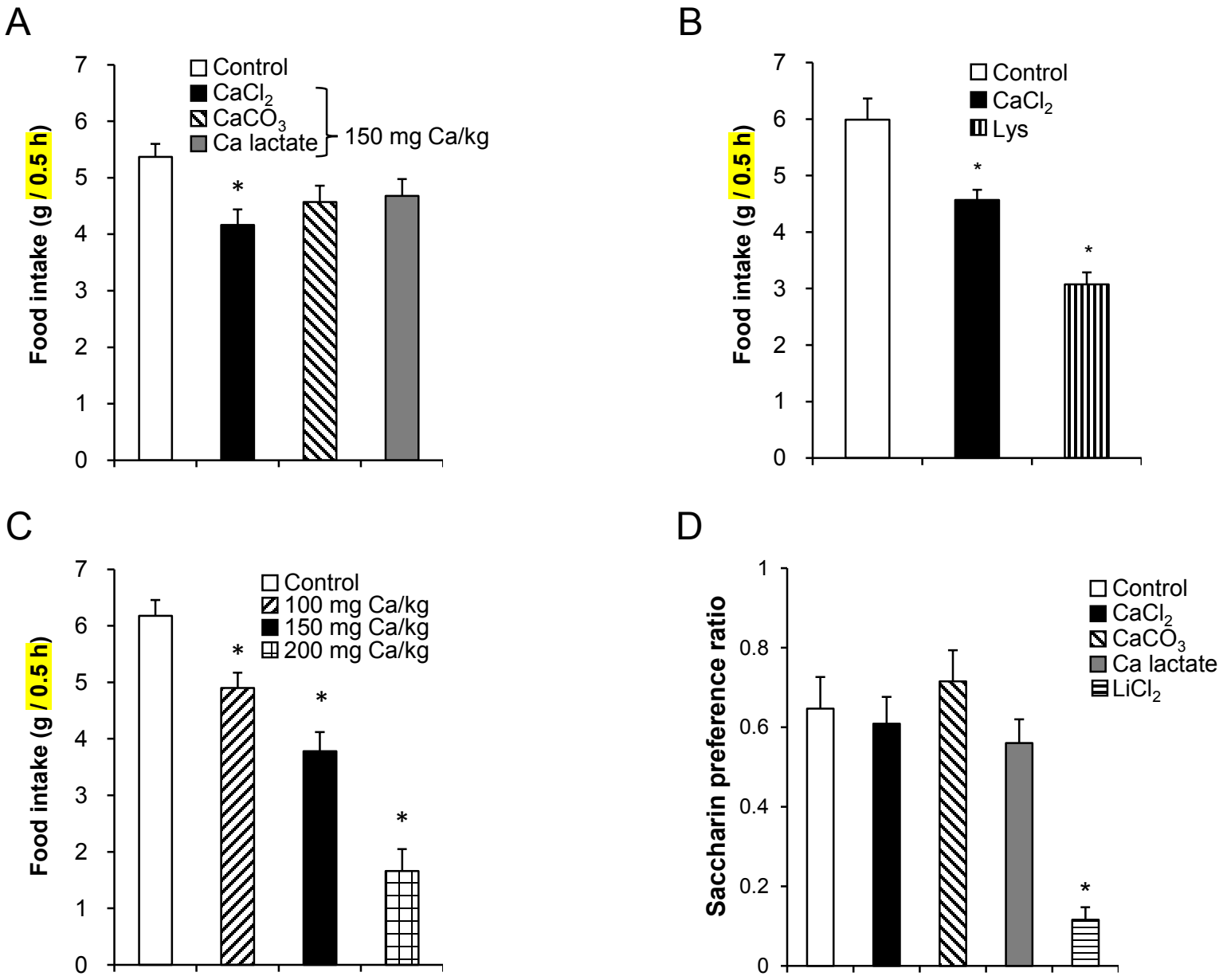

Figure 2

Fig. 2

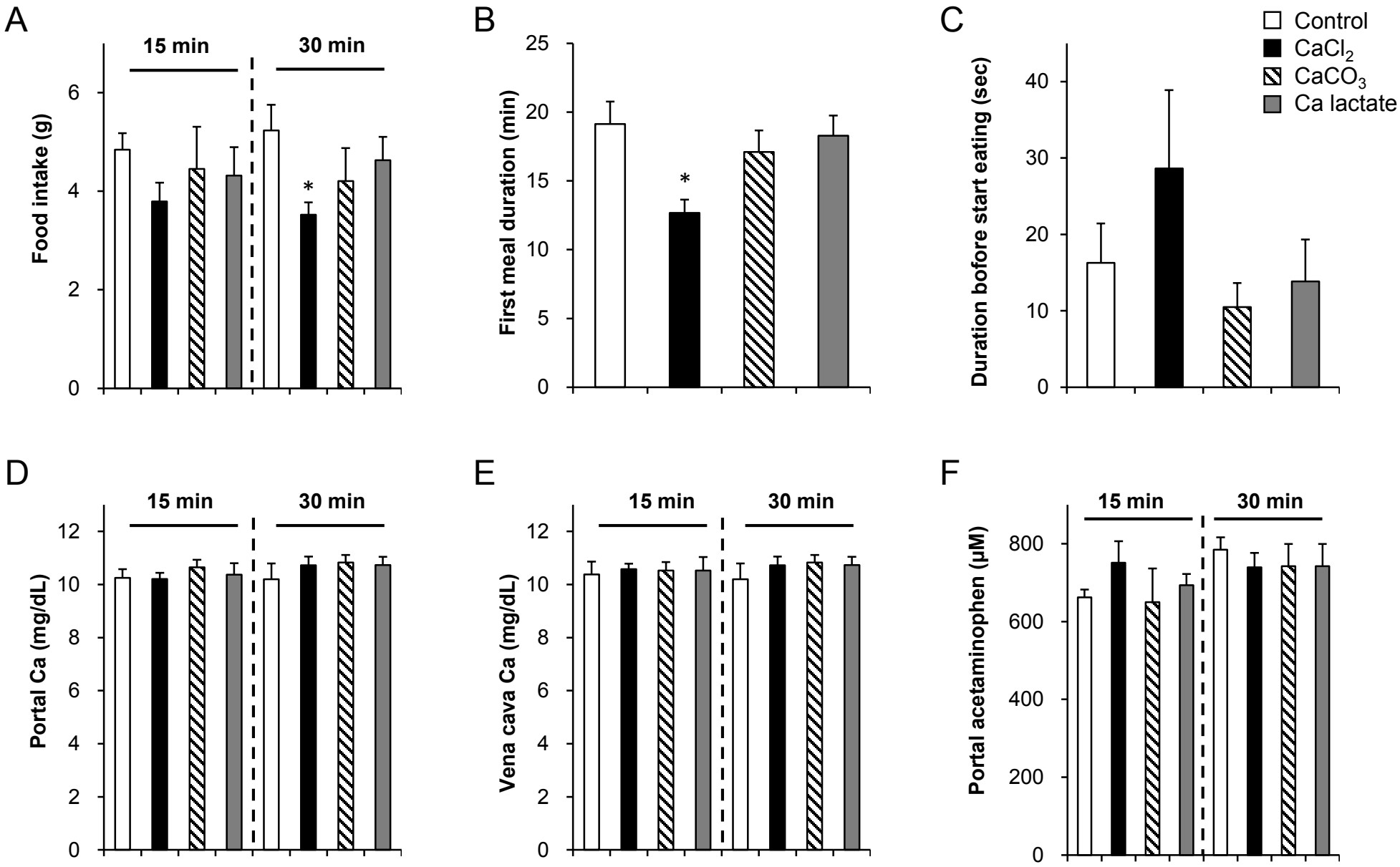

Figure 3

Fig. 3

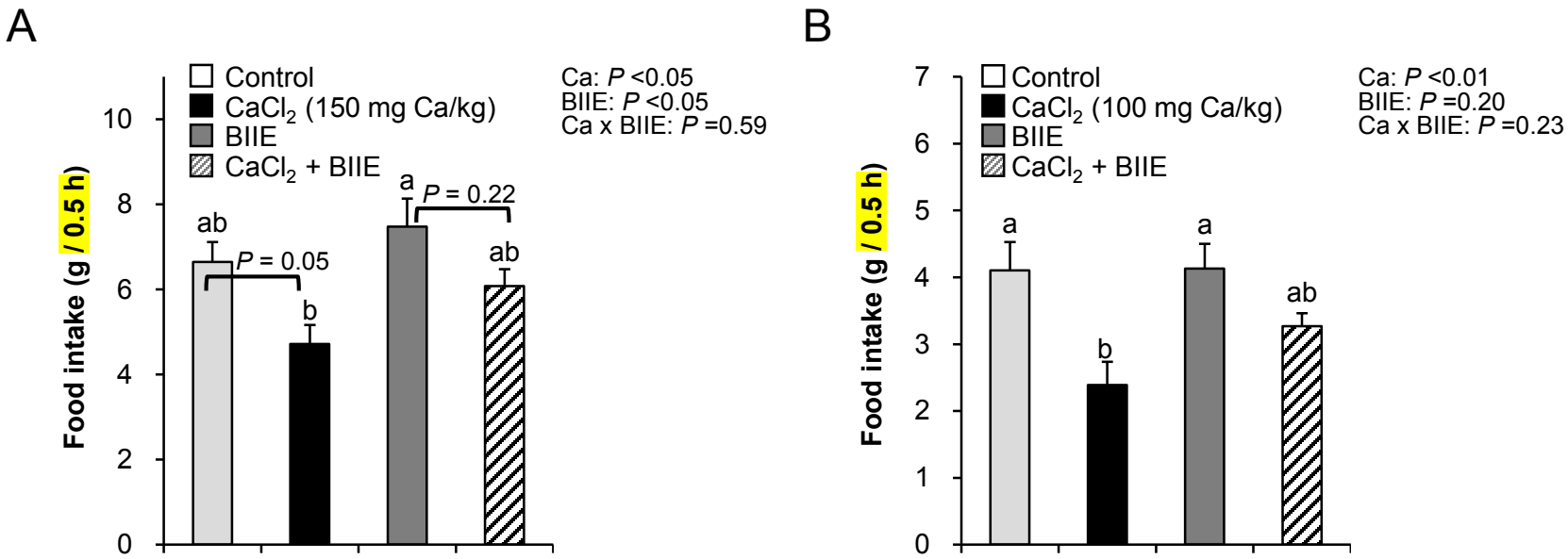

Figure 4

Fig. 4

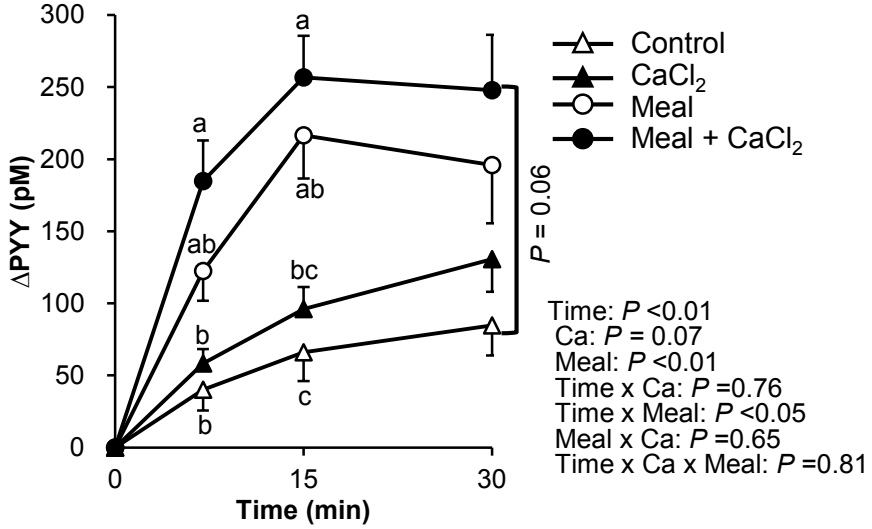

Figure 5

Fig. 5

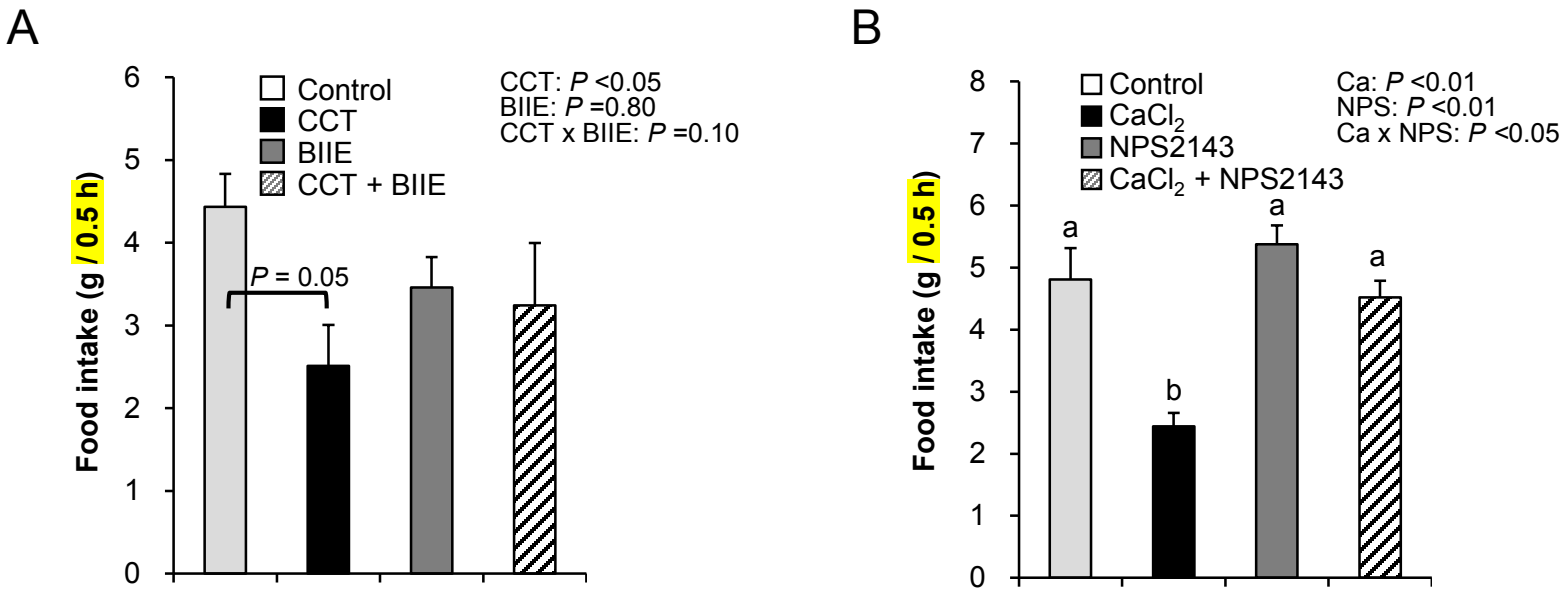

Figure 6

Fig. 6