



Title	Acute Oral Calcium Suppresses Food Intake Through Enhanced Peptide-YY Secretion Mediated by the Calcium-Sensing Receptor in Rats
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1 Title

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

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18 The authors declare no conflict of interest.

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33 Calcium suppresses food intake via CaSR and PYY

34 **Abbreviations**

35 AIN-93G, American Institute of Nutrition-93G; BIIE, BIIE0246; CaCO₃, calcium carbonate;

36 CaCl₂, calcium chloride; Ca lactate, calcium lactate; CaSR, calcium-sensing receptor; CCK,

37 cholecystokinin; CMC, calboxymethyl cellulose; DMSO, dimethyl sulfoxide; DPP-4, dipeptidyl

38 peptidase-4; DVZ, Devazepide; Ex9, exendin 9-39; GLP-1, glucagon like peptide-1; PYY,

39 peptide-YY;

40

41

42 **Abstract**

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

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

48 Methods: Male Sprague-Dawley rats (8–12 wk) old were used after an overnight fasting. In a series
49 of 2 trials with one-week interval between challenges, food intake was measured 0.5–24 h after oral
50 gavage of a vehicle (saline containing 1.5% carboxymethyl cellulose) as the control treatment, or the
51 vehicle containing various calcium compounds (calcium chloride (CaCl₂), calcium carbonate,
52 calcium lactate, in a random order) at 150 mg calcium/kg dose. A conditional taste aversion test was
53 conducted. In separate experiments, plasma calcium and gut hormone concentrations were measured
54 15 or 30 min after oral administration of the calcium compounds. In anesthetized rats, portal peptide-
55 YY (PYY) concentrations were measured after intraluminal administration of a liquid meal with or
56 without additional calcium.

57 Results: Oral CaCl₂ reduced food intake at the early period (30 min, ~20%, *P* <0.05) compared to
58 control rats, without taste aversion. Plasma PYY concentration was higher (100%, *P* <0.05) in CaCl₂
59 preloaded rats than in control rats, 15 min after administration. In anesthetized rats, luminal
60 meal+CaCl₂ induced a 4-fold higher increase in plasma PYY than the control treatment did. Oral
61 administration of the calcium-sensing receptor (CaSR) agonist suppressed food intake (~30%, *P*
62 <0.05), but CaCl₂ and CaSR agonist did not suppress food intake under treatment with a PYY
63 receptor antagonist. Furthermore, CaSR antagonist attenuated the effect of CaCl₂ on food intake.

64 Conclusions: CaCl₂ suppresses food intake partly by increasing CaSR-mediated PYY secretion in
65 rats. Our findings could at least partially explain the satiating effect of calcium.

66 **Key Words**

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

68

69

70 **Introduction**

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

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

83 Dietary calcium has been proposed to reduce appetite (or to enhance satiety) and to cause weight
84 loss in human studies. Higher calcium and vitamin D intake at breakfast reduced energy intake in the
85 following 24 h (8). Co-ingestion of calcium with meals in healthy adults temporarily suppressed
86 appetite, which was accompanied by higher postprandial GLP-1 secretion (9-11). In overweight and
87 obese adults with metabolic syndromes, a dietary pattern high in dairy products and calcium for 12
88 weeks had modestly lower appetite and higher postprandial PYY concentration (12). On the other
89 hand, earlier studies do not support the suppressive effect of dietary calcium on appetite (13, 14).
90 Weight loss induced by dietary calcium has been potentially explained not only by the satiating
91 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 plasma 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 ($22 \pm 2^\circ\text{C}$ $55 \pm 5\%$) 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

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

118

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

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

127

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

130 In experiment 1-1, to compare the effects of various calcium sources, rats (n=8-9 /group) received
131 calcium chloride (CaCl₂), calcium carbonate (CaCO₃), calcium DL-lactate pentahydrate (Ca lactate),
132 at a dose of 150 mg/kg as calcium, or the vehicle. After one-week washout period, in experiment 1-2,
133 rats (n=8-9 /group) received the vehicle, CaCl₂ (150 mg/kg as calcium) or L-Lysine (0.979 g/kg) as a
134 positive control (26), to confirm the effect of CaCl₂. Rats were allocated to treatment groups not to
135 receive the same treatment with previous round. In a separated experiment (experiment 1-3), rats
136 (n=6 /group) received different doses (100, 150, 200 mg/kg as calcium) of CaCl₂. Food intake was
137 measured as described above.

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

141 every day to confirm that the rats had taken the same amount of water from the two water bottles. On
142 the day 11 (conditioning day), the rats were provided with two bottles of 0.3% saccharine solution
143 for 30 min, and then received an oral gavage of various calcium solutions (CaCl₂, CaCO₃, or Ca
144 lactate), vehicle (1.5% CMC), or an intraperitoneal injection of lithium chloride (127.2 mg/(mL ·
145 kg)) as a positive control. On the day 12 (rest day), rats were allowed to access to two water bottles
146 for 2 h. On the day 13 (test day), a two-bottle preference test (one containing water, another
147 containing saccharin solution) was performed for 30 min. Saccharine intake/total intake was
148 determined as the saccharin preference ratio.

149

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

152 Rats were feed-deprived overnight, and then received an oral gavage (10 mL/kg BW) of various
153 calcium solutions (CaCl₂, CaCO₃, or Ca lactate) or its vehicle (1.5% CMC). In order to compare
154 gastric emptying rate, acetaminophen (SIGMA, St. Louis, MO, USA) at a dose of 100 mg/kg was
155 added to all of test solutions (29). The diet was given immediately after the administration, and rats
156 had free access to the diet for 15 min (n=5 /group) or 30 min (n=6-8 /group). The time before
157 initiating eating and the first meal duration were measured by using video recording in rats fed for 30
158 min. The first meal duration was defined as the duration from initiating eating to stop eating and take
159 a rest for more than 5 minutes. Fifteen or 30 min after the administration, rats were anesthetized by
160 isoflurane (MSD Animal Health, Inc., Tokyo, Japan), and a laparotomy was made immediately for
161 blood sampling. Blood samples were taken from the portal vein and the vena cava under isoflurane
162 anesthesia using a syringe filled with EDTA · 2Na (final concentration 1.8 mg/mL, Dojindo,
163 Kumamoto, Japan), Pefabloc SC (final concentration at 1 mg/mL, Roche, Basel, Switzerland), DPP-
164 4 inhibitor (final concentration at 10 μL/mL, Millipore, Burlington, MA, U.S.A.), and Sigma's
165 Protease inhibitor Cocktail (final concentration at 1 μL/mL, SIGMA). Then, rats were euthanized by

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

175

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

177 Food intake tests were conducted in rats treated with various gut hormone receptor antagonists.
178 Rats (n=6-8 /group) received intraperitoneal injection of BIIE0246 solution (Tocris Bioscience,
179 Bristol, UK dissolved in saline containing 7% DMSO, and dosed at 680 nmol/kg) as a PYY receptor
180 (Y2R) antagonist, or its vehicle (7% DMSO in saline, 1 mL/kg), immediately after oral
181 administration of CaCl₂ solution (at 150 mg calcium/kg in experiment 3-1, at 100 mg calcium/kg in
182 experiment 3-2) or its vehicle (1.5% CMC in saline). Food intake was measured as described above.
183 These experiments were separatory conducted.

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

190 CaCl₂ 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, CaCl₂ (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 CaCl₂ (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

215 /group), at a dose of 40 mg/kg in combination with or without CaCl₂ (150 mg calcium/kg). Food
216 intake was measured as described above.

217

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

220 Another *in situ* experiment was performed in the presence of a CaSR antagonist. The portal
221 catheter was implanted in anesthetized rats (n=7-8 /group) as described above, and NPS2143 (at 0.1
222 mg/(2 mL · kg)) in 1% DMSO) or its vehicle (1% DMSO at 2 mL/kg) was administered into the
223 duodenum (– 5 min). Five minutes later, portal blood was collected, and then, the liquid meal (15
224 kcal/(10 mL · kg)) with or without CaCl₂ (93.6 mM, 37.5 mg calcium/kg) was administered into the
225 duodenum (0 min). Portal blood samples were collected and plasma PYY concentrations were
226 measured as described above.

227

228 Statistical analyses

229 The sample size was calculated based on experimental designs using G*Power software (version
230 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%
231 power to detect an effect size of 0.7 with an α level of 0.05 (one-way analysis of variance
232 (ANOVA)). In experiments 1-4, (taste aversion test), n=4 /group was found to be sufficient to
233 provide >80% power to detect an effect size of 1.0 with an α level of 0.05 (one-way ANOVA). In
234 experiments 3 and 5, n=5 /group was found to be sufficient to provide >80% power to detect an
235 effect size of 0.7 with an α level of 0.05 (two-way ANOVA). The number of rats varied group
236 among treatments because some of rats unexpectedly refused oral administration on the day of
237 experiment. In experiment 4 and 6, by using GLIMMPSE software
238 (<http://glimmpse.SampleSizeShop.org/>) for repeated measures, the sample size (n=7 / group) was
239 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 \pm 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, CaCl₂ 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 \pm 0.7 g,
262 CaCl₂: 23.0 \pm 0.9 g, CaCO₃: 23.1 \pm 1.0 g, Ca lactate: 23.5 \pm 0.9 g), indicating that rats treated with
263 CaCl₂ compensated for the initial decrease in food intake in the following period of time
264 (Supplementary Figure 1).

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

268 Compared to 150 mg calcium/kg dose of CaCl₂, a lower dose (100 mg calcium/kg) or a higher
269 dose (200 mg calcium/kg), had a smaller or larger effect on reducing food intake respectively, (Fig.
270 1C), which demonstrated the dose-responsive effect of oral preload of CaCl₂. No unfavorable effects,
271 such as diarrhea, chronic anorexia, or overeating, were observed throughout the study.

272 Rats conditioned with calcium compounds at 150 mg calcium/kg showed preference ratios for
273 saccharin solution similar to control rats, as examined by the conditional taste aversion test (Fig.
274 1D). LiCl-treated rats showed apparently lower preference ratios, indicating that conditional aversion
275 was established by LiCl but not by calcium compounds. Although CaCl₂ at 200 mg calcium/kg had a
276 potent effect on reducing food intake, rats conditioned with the dose had a lower saccharin
277 preference ratio than control-treated rats (data not shown). Based on these results we employed a
278 dose of 150 mg calcium/kg in further experiments.

279 Oral preload of calcium compounds did not affect 15 min food intake (Fig. 2A). Food intake for
280 30 min was significantly lower only in CaCl₂ treated rats than in control rats (Fig. 2A), consistent
281 with the results shown in Fig. 1. Rats preloaded with CaCl₂ showed significantly shorter first meal
282 duration, while the other groups had similar meal durations (15-20 min), as compared to the control
283 rats (Fig. 2B). Most rats started to eat the meal immediately (~40 s) after oral administration of test
284 solutions, but one rat in the CaCl₂-treated group started to eat after 90 s. The mean time before
285 initiating eating did not significantly differ among treatments (Fig. 2C). There were no significant
286 differences in acetaminophen and calcium concentrations in the portal vein, or calcium
287 concentrations in the inferior vena cava, 15 and 30 min after the preload of various calcium
288 compounds and re-feeding (Fig. 2D-F). Based on the above results, we selected CaCl₂ as the calcium
289 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 (CaCl₂ 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 CaCl₂ (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 CaCl₂ group, 149
309 ± 16 pM in the Meal group, and 114 ± 30 pM in the Meal+ CaCl₂ group. Because basal PYY
310 concentrations varied as above, results are presented as changes from the basal concentration
311 (Δ PYY). 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

314 calcium (Meal+ CaCl₂), PYY levels were significantly higher than those in the control group both at
315 7 and 15 min.

316

317 Involvement of CaSR in the effect of calcium on food intake and PYY secretion (experiment 5 and
318 6)

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

323 In rats without NPS2143 pretreatment, basal PYY concentrations were 137 ± 26 pM and 193 ± 27
324 pM in the Meal and Meal+ CaCl₂ groups, respectively. In rats pretreated with NPS2143, basal PYY
325 concentrations were 130 ± 20 pM and 108 ± 7 pM in the Meal and Meal+ CaCl₂ groups,
326 respectively. Because basal PYY concentrations varied as above, results are presented as changes
327 from the basal concentration (Δ PYY). Portal PYY concentration increased 15 min after luminal
328 infusion of the liquid meal (Fig. 6A, B). Delayed response compared that seen in the experiment
329 above (Fig. 4) may be due to luminal preload of NPS2143 or its vehicle. In the absence of NPS2143
330 (Fig. 6A), PYY responses had a similar trend to the result in experiment 4 (Fig. 4), but in the
331 presence of NPS2143 (Fig. 6B), meal-induced PYY secretions almost completely matched with or
332 without calcium.

333

334

335 **Discussion**

336 The aim of this study was to examine the effect of oral calcium on appetite in rats and elucidate
337 the mechanism underlying its effect. We found that oral administration of CaCl₂ (100-150 mg
338 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, CaCl₂ 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 binding peptides would help to clarify this issue.

353 The dose (150 mg calcium/kg = 416 mg/kg of CaCl₂) is not supra-physiological based on various
354 points of view, as described below. Firstly, the LD50 value of CaCl₂ 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,

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

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

376 Almost all rats started to eat the diet within 30 sec after the oral administration regardless of
377 treatment (Fig. 2C). These observations support the idea that the effect of calcium is not due to taste
378 aversion. Because CaCl₂ preloaded rats quit eating within 15 min but others kept eating for a further
379 5 min (Fig. 2B), it is likely the preload of CaCl₂ accelerated meal-induced satiety.

380 Postprandial satiety is induced by increased secretion of gut hormones (7). Among the candidate
381 gut hormones (Supplementary Figure 3), PYY appeared to be partly responsible for the initial
382 reduction of food intake by calcium (Fig. 3A, B, Supplementary Figure 2C). Other unknown factors
383 are possibly involved since PYY receptor antagonist did not completely abolish the effect of calcium
384 on food intake. Our results (Supplementary Figure 2D) revealed that at least a portion of the ingested
385 meal (as chyme) immediately (within 15 min) reaches the middle-distal small intestine where PYY-
386 producing cells are abundant (39, 40), and there was no difference between treatments (\pm oral
387 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 CaCl₂ 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

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

443

444 **Author Contributions**

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

449

450 **References**

- 451 [1] Bensaïd A, Tomé D, Gietzen D, Even P, Morens C, Gausseres N, Fromentin G. Protein is more
452 potent than carbohydrate for reducing appetite in rats. *Physiol Behav* 2002; 75: 577-582.
- 453 [2] Bensaïd A, Tomé D, L'Heureux-Bourdon D, Even P, Gietzen D, Morens C, Gaudichon C,
454 Larue-Achagiotis C, Fromentin G. A high-protein diet enhances satiety without conditioned
455 taste aversion in the rat. *Physiol Behav* 2003; 78: 311-320.
- 456 [3] Westerterp-Plantenga MS. The significance of protein in food intake and body weight
457 regulation. *Curr Opin Clin Nutr Metab Care* 2003; 6: 635-638.
- 458 [4] French SJ, Conlon CA, Mutuma ST, Arnold M, Read NW, Meijer G, Francis J. The effects of
459 intestinal infusion of long-chain fatty acids on food intake in humans. *Gastroenterology* 2000;
460 119: 943-948.
- 461 [5] Kamphuis MM, Mela DJ, Westerterp-Plantenga MS. Diacylglycerols affect substrate oxidation
462 and appetite in humans. *Am J Clin Nutr* 2003; 77: 1133-1139.

- 463 [6] Zapata RC, Singh A, Chelikani PK. Peptide YY mediates the satiety effects of diets enriched
464 with whey protein fractions in male rats. *FASEB J* 2018; 32: 850-861.
- 465 [7] Camilleri M. Peripheral mechanisms in appetite regulation. *Gastroenterology* 2015; 148: 1219-
466 1233.
- 467 [8] Rebello CJ, O'Neil CE, Greenway FL. Dietary fiber and satiety: the effects of oats on satiety.
468 *Nutr Rev* 2016; 74: 131-147.
- 469 [9] Gonzalez JT, Stevenson EJ. Calcium co-ingestion augments postprandial glucose-dependent
470 insulinotropic peptide(1-42), glucagon-like peptide-1 and insulin concentrations in humans.
471 *Eur J Nutr* 2014; 53: 375-385.
- 472 [10] Gonzalez JT, Green BP, Brown MA, Rumbold PL, Turner LA, Stevenson EJ. Calcium
473 ingestion suppresses appetite and produces acute overcompensation of energy intake
474 independent of protein in healthy adults. *J Nutr* 2015; 145: 476-482.
- 475 [11] Chen YC, Smith HA, Hengist A, Chrzanowski-Smith OJ, Mikkelsen UR, Carroll HA, Betts
476 JA, Thompson D, Saunders J, Gonzalez JT. Co-ingestion of whey protein hydrolysate with
477 milk minerals rich in calcium potently stimulates glucagon-like peptide-1 secretion: an RCT in
478 healthy adults. *Eur J Nutr* 2019; 59: 2449-2462.
- 479 [12] Jones KW, Eller LK, Parnell JA, Doyle-Baker PK, Edwards AL, Reimer RA. Effect of a dairy-
480 and calcium-rich diet on weight loss and appetite during energy restriction in overweight and
481 obese adults: a randomized trial. *Eur J Clin Nutr* 2013; 67: 371-376.
- 482 [13] Jacobsen R, Lorenzen JK, Toubro S, Krog-Mikkelsen I, Astrup A. Effect of short-term high
483 dietary calcium intake on 24-h energy expenditure, fat oxidation, and fecal fat excretion. *Int J*
484 *Obes (Lond)* 2005; 29: 292-301.
- 485 [14] Lorenzen JK, Nielsen S, Holst JJ, Tetens I, Rehfeld JF, Astrup A. Effect of dairy calcium or
486 supplementary calcium intake on postprandial fat metabolism, appetite, and subsequent energy
487 intake. *Am J Clin Nutr* 2007; 85: 678-687.

- 488 [15] Christensen R, Lorenzen JK, Svith CR, Bartels EM, Melanson EL, Saris WH, Tremblay A,
489 Astrup A. Effect of calcium from dairy and dietary supplements on faecal fat excretion: a
490 meta-analysis of randomized controlled trials. *Obes Rev* 2009; 10: 475-486.
- 491 [16] Soerensen KV, Thorning TK, Astrup A, Kristensen M, Lorenzen JK. Effect of dairy calcium
492 from cheese and milk on fecal fat excretion, blood lipids, and appetite in young men. *Am J Clin*
493 *Nutr* 2014; 99: 984-991.
- 494 [17] Hira T, Nakajima S, Eto Y, Hara H. Calcium-sensing receptor mediates phenylalanine-induced
495 cholecystokinin secretion in enteroendocrine STC-1 cells. *FEBS J* 2008; 275: 4620-4626.
- 496 [18] Wang Y, Chandra R, Samsa LA, Gooch B, Fee BE, Cook JM, Vigna SR, Grant AO, Liddle
497 RA. Amino acids stimulate cholecystokinin release through the Ca²⁺-sensing receptor. *Am J*
498 *Physiol Gastrointest Liver Physiol* 2011; 300: G528-537.
- 499 [19] Liou AP, Sei Y, Zhao X, Feng J, Lu X, Thomas C, Pechhold S, Raybould HE, Wank SA. The
500 extracellular calcium-sensing receptor is required for cholecystokinin secretion in response to
501 L-phenylalanine in acutely isolated intestinal I cells. *Am J Physiol Gastrointest Liver Physiol*
502 2011; 300: G538-546.
- 503 [20] Nakajima S, Hira T, Hara, H. Calcium-sensing receptor mediates dietary peptide-induced CCK
504 secretion in enteroendocrine STC-1 cells. *Mol Nutr Food Res* 2012; 56: 753-760.
- 505 [21] Diakogiannaki E, Pais R, Tolhurst G, Parker HE, Horscroft J, Rauscher B, Zietek T, Daniel H,
506 Gribble FM, Reimann, F. Oligopeptides stimulate glucagon-like peptide-1 secretion in mice
507 through proton-coupled uptake and the calcium-sensing receptor. *Diabetologia* 2013; 56:
508 2688-2696.
- 509 [22] Pais R, Gribble FM, Reimann F. Signalling pathways involved in the detection of peptones by
510 murine small intestinal enteroendocrine L-cells. *Peptides* 2016; 77: 9-15.
- 511 [23] Muramatsu M, Hira T, Mitsunaga A, Sato E, Nakajima S, Kitahara Y, Eto Y, Hara, H.
512 Activation of the gut calcium-sensing receptor by peptide agonists reduces rapid elevation of

513 plasma glucose in response to oral glucose load in rats. *Am J Physiol Gastrointest Liver*
514 *Physiol* 2014; 306: G1099-1107.

515 [24] Alamshah A, Spreckley E, Norton M, Kinsey-Jones JS, Amin A, Ramgulam A, Cao Y,
516 Johnson R, Saleh K, Akalestou E, Malik Z, Gonzalez-Abuin N, Jomard A, Amarsi R, Moolla
517 A, Sargent PR, Gray GW, Bloom SR, Murphy KG. l-phenylalanine modulates gut hormone
518 release and glucose tolerance, and suppresses food intake through the calcium-sensing receptor
519 in rodents. *Int J Obes (Lond)* 2017; 41: 1693-1701.

520 [25] Reeves PG. Components of the AIN-93 diets as improvements in the AIN-76A diet. *J Nutr*
521 1997; 127: 838S-841S.

522 [26] Jordi J, Herzog B, Camargo SM, Boyle CN, Lutz TA, Verrey, F. Specific amino acids inhibit
523 food intake via the area postrema or vagal afferents. *J Physiol* 2013; 591: 5611-5621.

524 [27] Sasaki T, Kinoshita Y, Matsui S, Kakuta S, Yokota-Hashimoto H, Kinoshita K, Iwasaki Y,
525 Kinoshita T, Yada T, Amano N, Kitamura T. N-methyl-d-aspartate receptor coagonist d-serine
526 suppresses intake of high-preference food. *Am J Physiol Regul Integr Comp Physiol* 2015; 309:
527 R561-575.

528 [28] Iwasaki Y, Maejima Y, Suyama S, Yoshida M, Arai T, Katsurada K, Kumari P, Nakabayashi
529 H, Kakei M, Yada T. Peripheral oxytocin activates vagal afferent neurons to suppress feeding
530 in normal and leptin-resistant mice: a route for ameliorating hyperphagia and obesity. *Am J*
531 *Physiol Regul Integr Comp Physiol* 2015; 308: R360-369.

532 [29] Hira T, Yahagi A, Nishimura S, Sakaino M, Yamashita T, Hara H. Diunsaturated aldehyde,
533 trans,trans-2,4-decadienal in the intestinal lumen suppresses gastric emptying through
534 serotonin signaling in rats. *J Agric Food Chem* 2015; 63: 8177-8181.

535 [30] Hira T, Mochida T, Miyashita K, Hara H. GLP-1 secretion is enhanced directly in the ileum
536 but indirectly in the duodenum by a newly identified potent stimulator, zein hydrolysate, in
537 rats. *Am J Physiol Gastrointest Liver Physiol* 2009; 297: G663-671.

- 538 [31] Liu FR, Wang L, Wang R, Chen ZX. Calcium-binding capacity of wheat germ protein
539 hydrolysate and characterization of Peptide-calcium complex. *J Agric Food Chem*; 2013; 61:
540 7537-44.
- 541 [32] Walters ME, Esfandi R, Tsopmo A. Potential of Food Hydrolyzed Proteins and Peptides to
542 Chelate Iron or Calcium and Enhance their Absorption. *Foods* 2018; 7: 172.
- 543 [33] Organisation for Economic Co-operation and Development, Calcium chloride, in OECD SIDS
544 Initial Assessment Report for SIAM 15, 2002, Screening Information Data Set 10043-52-4.
- 545 [34] Brun LR, Brance ML, Rigalli A. Luminal calcium concentration controls intestinal calcium
546 absorption by modification of intestinal alkaline phosphatase activity. *Br J Nutr* 2012; 108:
547 229-233.
- 548 [35] Kiela PR, Ghishan FK. Molecular Mechanisms of Intestinal Transport of Calcium,
549 Phosphate, and Magnesium. *Physiology of the Gastrointestinal Tract: Sixth Edition*, (Ed: H.
550 M. Said), *Elsevier*, Amsterdam, Netherlands 2018; Ch. 59.
- 551 [36] Kruger MC, von Hurst PR, Booth CL, Kuhn-Sherlock B, Todd JM, Schollum LM.
552 Postprandial metabolic responses of serum calcium, parathyroid hormone and C-telopeptide of
553 type I collagen to three doses of calcium delivered in milk. *J Nutr Sci* 2014; 3:e6.
- 554 [37] Kumagai H, Koizumi A, Suda A, Sato N, Sakurai H, Kumagai H. Enhanced Calcium
555 Absorption in the Small Intestine by a Phytate-removed Deamidated Soybean Globulin
556 Preparation. *Biosci Biotechnol Biochem* 2004; 68: 1598–1600.
- 557 [38] Kvietys PR. *The Gastrointestinal Circulation*. San Rafael (CA): Morgan & Claypool Life
558 Sciences; 2010.
- 559 [39] Batterham RL, Cowley MA, Small CJ, Herzog H, Cohen MA, Dakin CL, Wren AM, Brynes
560 AE, Low MJ, Ghatei MA, Cone RD, Bloom SR. Gut hormone PYY(3-36) physiologically
561 inhibits food intake. *Nature* 2002; 418: 650-654.

- 562 [40] Svendsen B, Holst JJ. Regulation of gut hormone secretion. Studies using isolated perfused
563 intestines. *Peptides* 2016; 77: 47-53.
- 564 [41] Svendsen B, Pedersen J, Albrechtsen NJ, Hartmann B, Toräng S, Rehfeld JF, Poulsen SS,
565 Holst JJ. An analysis of cosecretion and coexpression of gut hormones from male rat proximal
566 and distal small intestine. *Endocrinology* 2015; 156: 847-857.
- 567 [42] Cho HJ, Kosari S, Hunne B, Callaghan B, Rivera LR, Bravo DM, Furness JB. Differences in
568 hormone localisation patterns of K and L type enteroendocrine cells in the mouse and pig small
569 intestine and colon. *Cell Tissue Res* 2015; 359: 693-698.
- 570 [43] Acar I, Cetinkaya A, Lay I, Ileri-Gurel E. The role of calcium sensing receptors in GLP-1 and
571 PYY secretion after acute intraduodenal administration of L-Tryptophan in rats. *Nutr Neurosci*
572 2018; 1-9.
- 573 [44] Liu G, Cao W, Jia G, Zhao H, Chen X, Wang J. Calcium-sensing receptor in nutrient sensing:
574 an insight into the modulation of intestinal homeostasis. *Br J Nutr* 2018; 120: 881-890.
- 575 [45] Tang L, Cheng CY, Sun X, Pedicone AJ, Mohamadzadeh M, Cheng SX. The Extracellular
576 Calcium-Sensing Receptor in the Intestine: Evidence for Regulation of Colonic Absorption,
577 Secretion, Motility, and Immunity. *Front Physiol* 2016; 7: 245.
- 578 [46] Squires PE, Jones PM, Younis MY, Hills CE. The calcium-sensing receptor and β -cell
579 function. *Vitam Horm* 2014; 95: 249-67.
- 580 [47] Balk EM, Adam GP, Langberg VN, Earley A, Clark P, Ebeling PR, Mithal A, Rizzoli R,
581 Zerbini CAF, Pierroz DD, Dawson-Hughes B, Committee, I. O. F. C. S. Global dietary
582 calcium intake among adults: a systematic review. *Osteoporos Int* 2017; 28: 3315-3324.
- 583 [48] Žofková I. Hypercalcemia. Pathophysiological aspects. *Physiol Res* 2016; 65: 1-10.
- 584 [49] Asarian L, Geary N. Sex differences in the physiology of eating. *Am J Physiol Regul Integr*
585 *Comp Physiol* 2013; 305: R1215–R1267.
- 586

587 **Figure captions**

588 **Fig. 1.**

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

592 Food intake in control rats and rats after oral administration of CaCl₂, CaCO₃, and Ca lactate at 150
593 mg Ca/kg body weight (A), CaCl₂ at 150 mg Ca/kg and L-Lys at a dose of 0.979 mg/kg (B), or
594 CaCl₂ at doses of 100, 150, and 200 mg Ca/kg (C), and the saccharine preference ratio in rats
595 conditioned by oral administration of CaCl₂, CaCO₃, and Ca lactate at 150 mg calcium/kg or with an
596 intraperitoneal injection of lithium chloride (LiCl). Values are mean ± SEM, n=8-9 (A, B), 6 (C), or
597 4-5 (D). *Different from control, P < 0.05.

598

599 **Fig. 2.**

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

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

608

609

610 **Fig. 3.**

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

614 Food intake in control rats and rats after oral administration of calcium chloride (CaCl₂) at 150 mg
615 calcium/kg (A), at 100 mg calcium/kg (B), or its vehicle (1.5% CMC in saline) with an intraperitoneal
616 administration of vehicle or a PYY receptor antagonist BIIE0246 (BIIE, 680 nmol/kg). Values shown
617 are means ± SEM, n=6-8 (A), 8 (B). Statistical significance was assessed by two-way ANOVA
618 followed by Tukey-Kramer' s test. Labeled means without a common letter differ, *P*<0.05.

619

620

621 **Fig. 4.**

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

623 A liquid meal (Meal, 15 kcal/(10 mL · kg)), calcium chloride (CaCl₂, 37.5 mg calcium/(10 mL · kg)),
624 their combination (Meal + CaCl₂), or saline (Control) was directly infused into the small intestinal
625 lumen in anesthetized rats. Values shown are means ± SEM (n=7-8), and are presented as changes
626 from the basal (0 min) concentration of plasma PYY. Statistical significance was assessed by the mixed
627 model with unstructured covariance followed by Tukey-Kramer' s test. Labeled means at the same
628 time point without a common letter differ, *P*<0.05.

629

630

631 **Fig. 5.**

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

634 Food intake in control rats and rats after oral administration of a calcium sensing receptor antagonist
635 cinacalcet (CCT, 50 mg/kg) with intraperitoneal administration of saline or a PYY receptor
636 antagonist (BIIE0246, 680 nmol/kg) (A), calcium chloride (CaCl₂, 150 mg calcium/kg) or its vehicle
637 (Control) with or without coadministration of a calcium sensing receptor antagonist NPS2143 (40
638 mg/kg) (B). The results are expressed as the mean ± SEM, n=6-7 (A), 7-9 (B). Statistical
639 significance was assessed by two-way ANOVA followed by Tukey-Kramer's test. Labeled means
640 without a common letter differ, *P*<0.05.

641

642 **Fig. 6.**

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

645 A liquid meal (Meal, 15 kcal/(10 mL · kg)) was directly infused into the small intestinal lumen with
646 (Meal + CaCl₂) or without CaCl₂ (37.5 mg calcium/(10 mL · kg)), in the absence (A) or presence (B)
647 of CaSR antagonist (NPS2143, 0.1 mg/kg), in anesthetized rats. Blood samples were drawn from the
648 catheter inserted into the portal vein before and after administration. Values are means ± SEM, n=7-
649 8, and are presented as changes from the basal (0 min) concentration of plasma PYY. Statistical
650 significance was assessed by the mixed model with unstructured covariance.

651

652

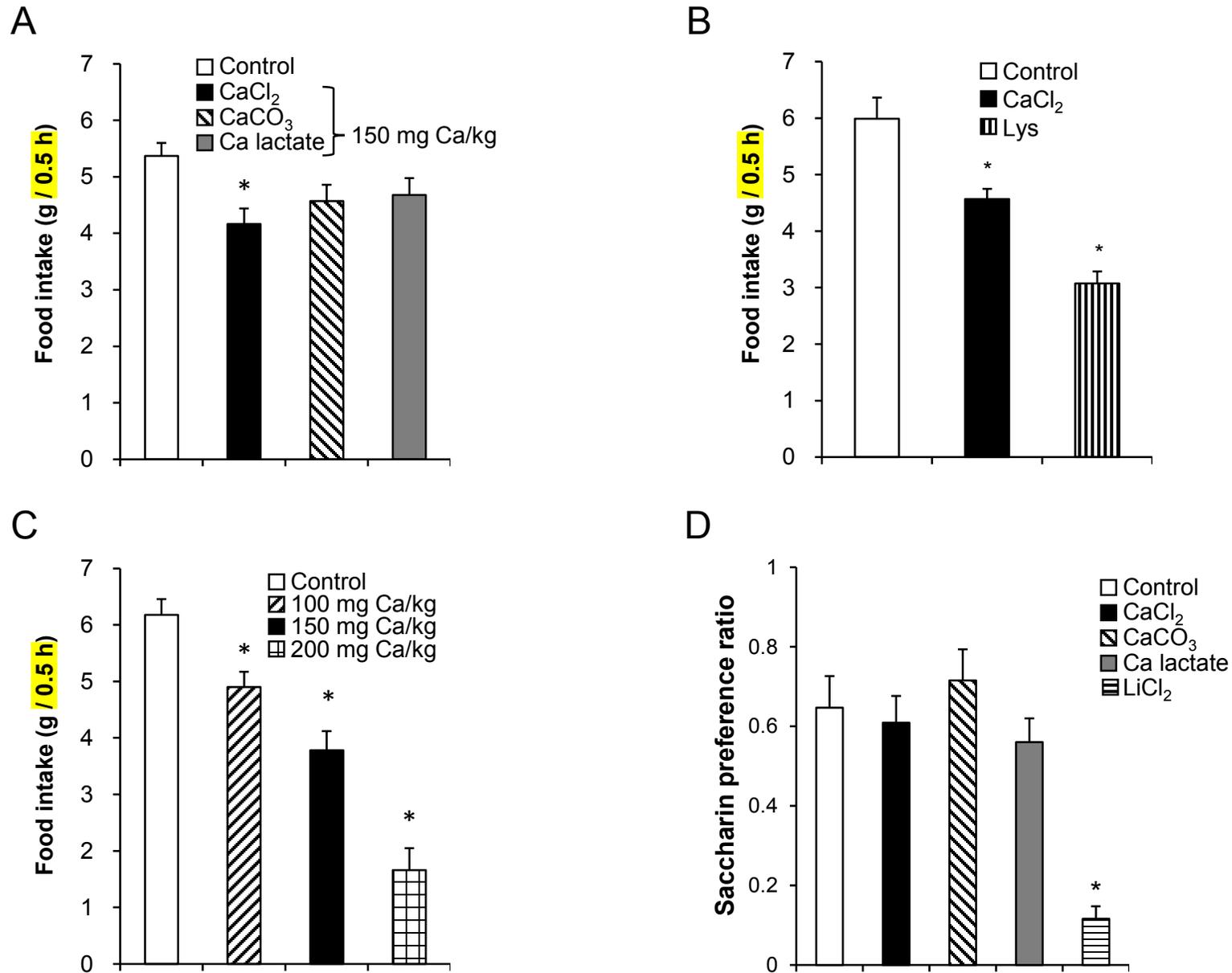
Fig. 1

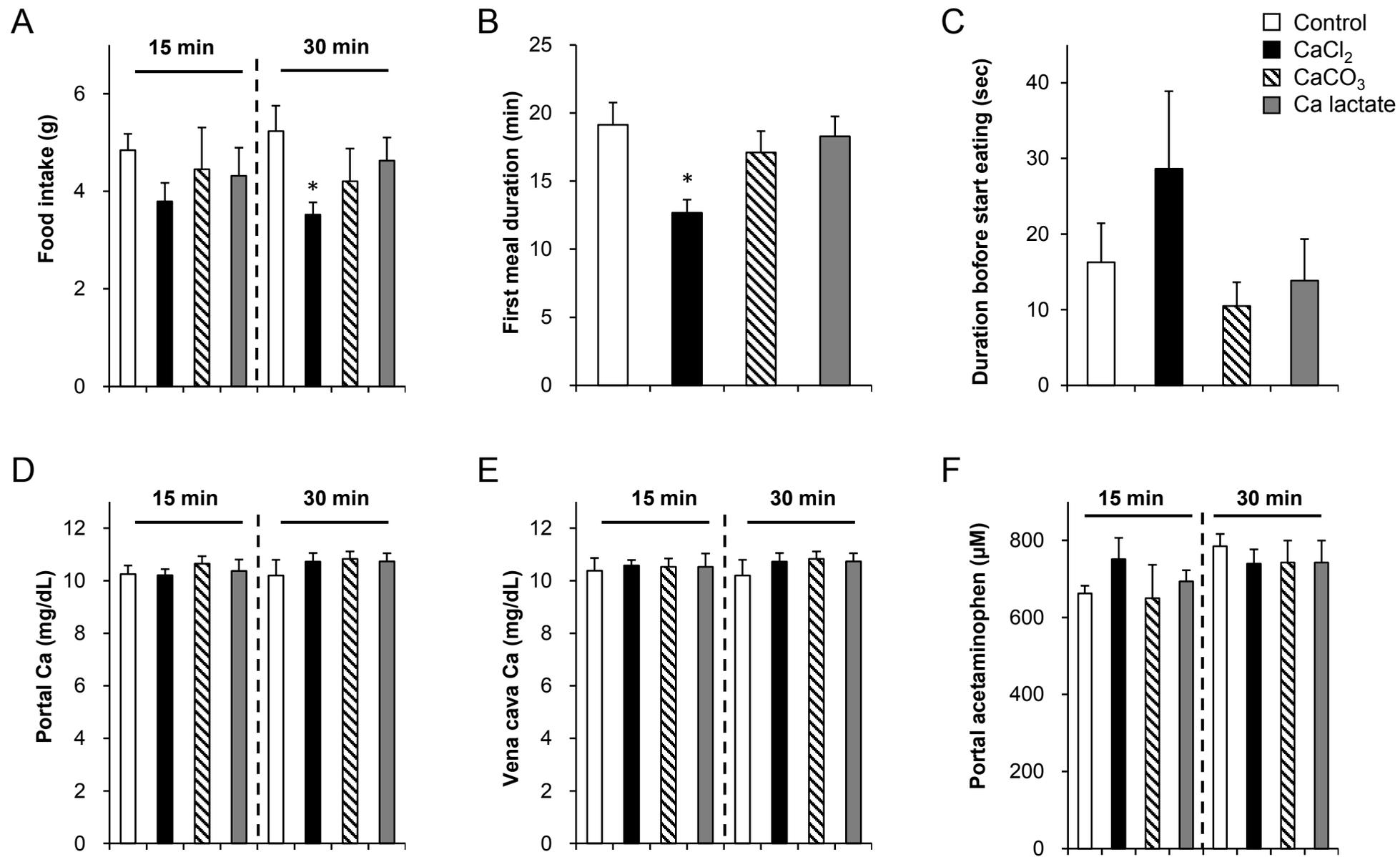
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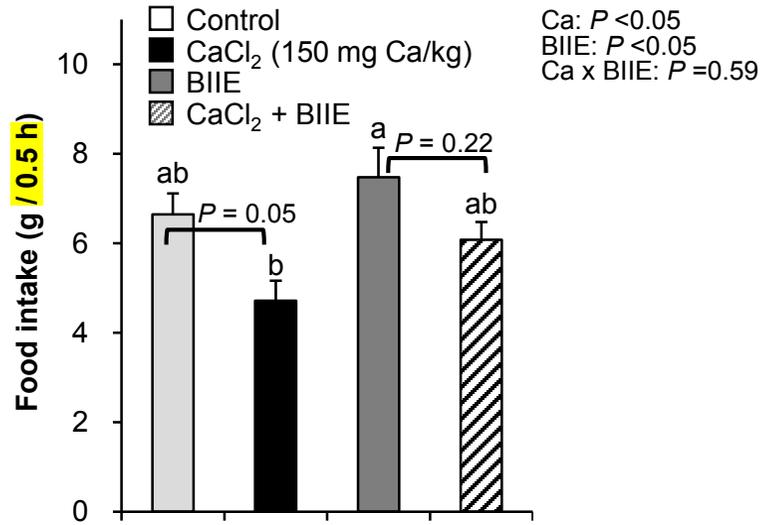
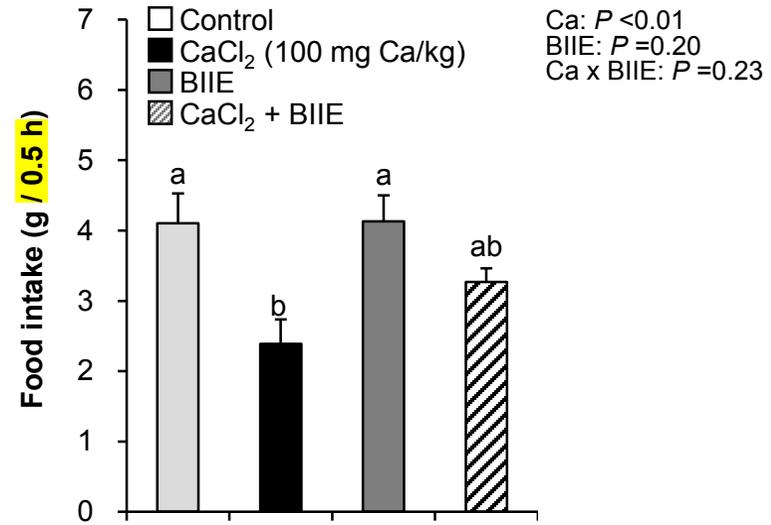
Fig. 3**A****B**

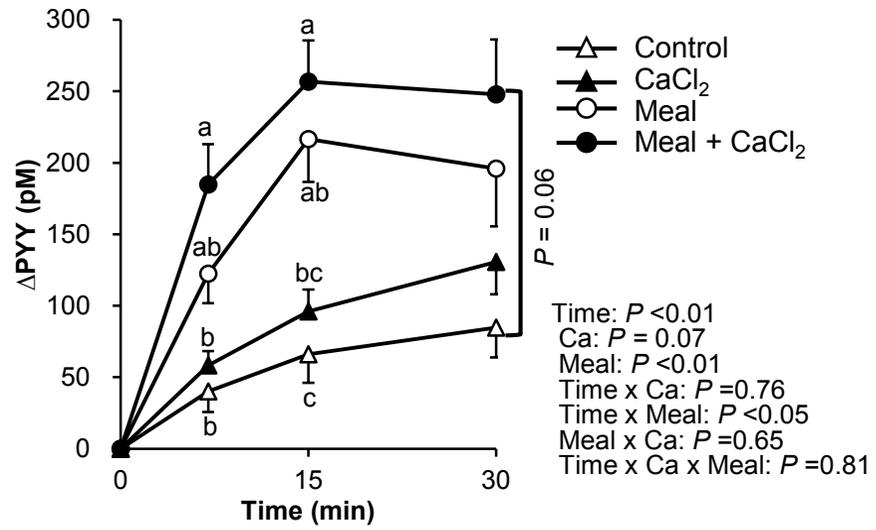
Fig. 4

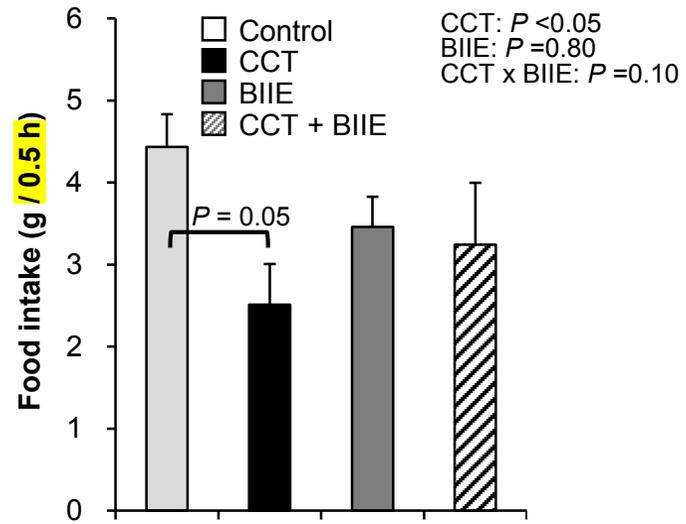
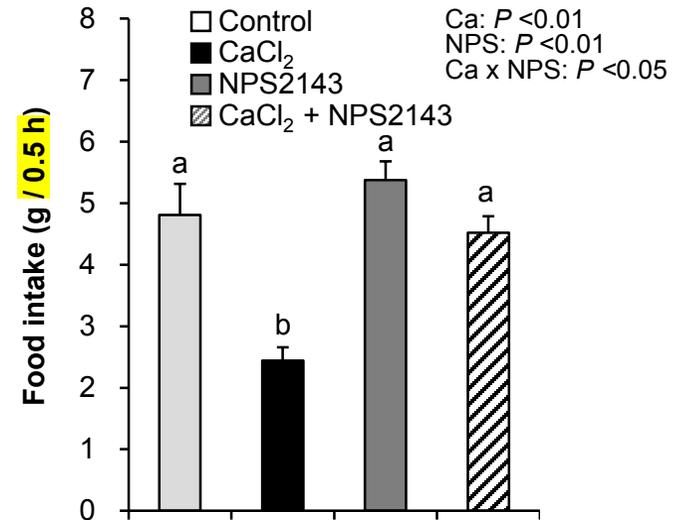
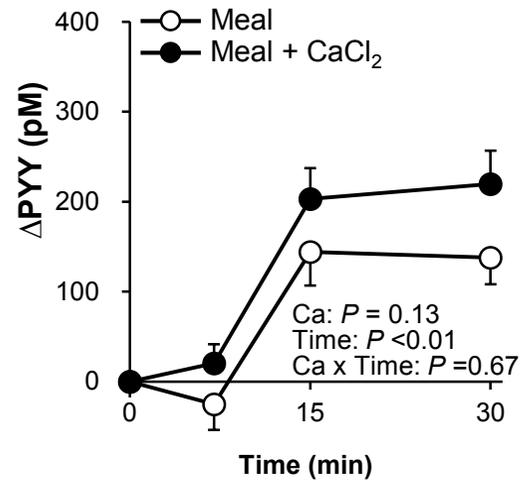
Fig. 5**A****B**

Fig. 6

A



B

