



Title	Calcium-sensing receptor mediates phenylalanine-induced cholecystokinin secretion in enteroendocrine STC-1 cells
Author(s)	Hira, Tohru; Nakajima, Shingo; Eto, Yuzuru; Hara, Hiroshi
Citation	FEBS Journal, 275(18), 4620-4626 https://doi.org/10.1111/j.1742-4658.2008.06604.x
Issue Date	2008-09
Doc URL	http://hdl.handle.net/2115/85371
Rights	This is the peer reviewed version of the following article: Hira, T., Nakajima, S., Eto, Y. and Hara, H. (2008), Calcium-sensing receptor mediates phenylalanine-induced cholecystokinin secretion in enteroendocrine STC-1 cells. The FEBS Journal, 275: 4620-4626, which has been published in final form at https://doi.org/10.1111/j.1742-4658.2008.06604.x . This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions. This article may not be enhanced, enriched or otherwise transformed into a derivative work, without express permission from Wiley or by statutory rights under applicable legislation. Copyright notices must not be removed, obscured or modified. The article must be linked to Wiley 's version of record on Wiley Online Library and any embedding, framing or otherwise making available the article or pages thereof by third parties from platforms, services and websites other than Wiley Online Library must be prohibited.
Type	article (author version)
File Information	FEBS J_275_4620.pdf



[Instructions for use](#)

1 *Title.*

2 Calcium-sensing receptor mediates phenylalanine-induced cholecystinin secretion in
3 enteroendocrine STC-1 cells

4 *Authors' names.*

5 Tohru Hira¹, Shingo Nakajima², Yuzuru Eto³ and Hiroshi Hara¹

6 *Addresses.*

7 ¹Division of Applied Biosciences, Research Faculty of Agriculture, Hokkaido University
8 Kita-9, Nishi-9, Kita-ku, Sapporo 060-8589, Japan

9 ²Division of Bio-systems and Sustainability, Graduate School of Agriculture, Hokkaido
10 University, Kita-9, Nishi-9, Kita-ku, Sapporo 060-8589, Japan

11 ³Institute of Life Sciences, Ajinomoto Co., Inc., 1-1, Suzuki-Cho, Kawasaki-ku,
12 Kawasaki-shi, 210-8681, Japan

13 *Correspondence author.*

14 Tohru Hira

15 Division of Applied Biosciences, Research Faculty of Agriculture, Hokkaido University
16 Kita-9, Nishi-9, Kita-ku, Sapporo 060-8589, Japan

17 Tel: +81-11-706-2811, Fax: +81-11-706-2504

18 E-mail: hira@chem.agr.hokudai.ac.jp

19 *Running title.*

20 Amino acid sensing in enteroendocrine cells

21 *Abbreviations.*

22 Phenylalanine; Phe, Cholecystinin; CCK, Calcium-sensing receptor; CaR

23 *Key words.*

24 Enteroendocrine cells, Calcium-sensing receptor, Cholecystinin, Phenylalanine

1 **SUMMARY**

2 Intraluminal L-phenylalanine (Phe) stimulates cholecystokinin (CCK) secretion *in vivo*
3 and *in vitro*. However, the cellular mechanism by which CCK-producing enteroendocrine
4 cells sense Phe is unknown. Calcium-sensing receptor (CaR), which can sense amino acids,
5 is expressed in the gastrointestinal tract. In the present study, we examined whether CaR
6 functions as a receptor for Phe in CCK-producing enteroendocrine cells. CCK secretion and
7 intracellular Ca^{2+} concentration in response to Phe were measured in the murine
8 CCK-producing enteroendocrine cell line STC-1 at various extracellular Ca^{2+} concentrations
9 or after treatment with a CaR antagonist.

10 At more than 20 mM, Phe induced dose-dependent CCK secretion and intracellular Ca^{2+}
11 mobilization in STC-1 cells. In the presence of 3.0 mM extracellular Ca^{2+} , 10 and 20 mM Phe
12 induced significantly higher CCK secretion than under normal conditions (1.2 mM
13 extracellular Ca^{2+}). Intracellular Ca^{2+} mobilization, induced by 10 or 20 mM Phe, was also
14 enhanced by increasing extracellular Ca^{2+} concentrations. In addition, intracellular Ca^{2+}
15 mobilization induced by addition of extracellular Ca^{2+} was augmented by the presence of
16 Phe. These results closely match the known CaR properties. Treatment with a specific CaR
17 antagonist (NPS2143) completely inhibited Phe-induced CCK secretion and the latter phase
18 of intracellular Ca^{2+} mobilization. CaR mRNA expression was demonstrated by RT-PCR in
19 STC-1 cells as well as in other mouse tissues including the kidney, thyroid, stomach and
20 intestine. In conclusion, CaR functions as a receptor for Phe to stimulate CCK secretion in
21 enteroendocrine STC-1 cells.

22

23 **INTRODUCTION**

24 Cholecystokinin (CCK) is secreted from enteroendocrine I cells in the upper small

1 intestine in response to food ingestion. Fatty acids, dietary protein, and peptides are potent
2 stimulants [1]. Aromatic amino acids (phenylalanine and tryptophan) are also known to
3 stimulate CCK and pancreatic secretion in humans and dogs [2-5]. The cellular mechanism
4 by which these amino acids stimulate CCK secretion in enteroendocrine cells is still unclear.

5 A previous paper reported that phenylalanine (Phe) stimulates CCK secretion via a
6 Ca^{2+} -dependent system in enteroendocrine STC-1 cells derived from the murine duodenum,
7 and speculated the involvement of a transporter or a plasma membrane receptor [6]. The
8 D-isomer of Phe does not cause any significant increase in CCK secretion In STC-1 cells,
9 which is consistent with the results of a previous study using isolated canine epithelial cells
10 [7]. Another paper demonstrated tryptophan (Trp)-induced CCK secretion in STC-1 cells [8].
11 However, the amino acid-sensing mechanism in STC-1 cells is still to be clarified.

12 The calcium-sensing receptor (CaR) was identified as an L-amino acid receptor [9].
13 Among several amino acids, aromatic amino acids (Phe, Trp) are potent ligands known to
14 activate CaR. The widespread expression of CaR in the gastrointestinal tract including
15 enteroendocrine cells suggests that CaR has roles in both gut function and the
16 enteroendocrine system [10-12]. Recent papers show that CaR senses amino acids in
17 gastric mucosal cells to stimulate gastric acid secretion [13,14], and that CaR is also
18 involved in colonic fluid secretion [15]. As speculated in some reviews [10,11], it is likely that
19 CaR is expressed and functions in intestinal enteroendocrine cells.

20 In the present study, we tested whether the extracellular Ca^{2+} concentration modulates
21 Phe-induced cellular responses (CCK secretion and intracellular Ca^{2+} mobilization) in
22 CCK-producing STC-1 cells. CaR mRNA expression and the effect of a CaR antagonist on
23 the Phe-induced responses were also investigated to determine the involvement of CaR.

24

1 RESULTS

2 *Phe induces CCK secretion in STC-1 cells*

3 At a normal extracellular Ca^{2+} concentration (1.2 mM), exposure to Phe (≥ 20 mM)
4 induced CCK secretion in a dose-dependent manner in STC-1 cells after 60 min (Fig. 1B).
5 Phe at 10 mM did not induce any significant increase in CCK secretion (Fig. 1A). In the
6 presence of 3 mM extracellular Ca^{2+} , 10-50 mM Phe induced an increase in CCK secretion.
7 The secretion induced by 10 and 20 mM Phe was significantly higher in the presence of 3
8 mM Ca^{2+} than that in the presence of 1.2 mM Ca^{2+} (Fig. 1A, B).

9 *Phe induces increases in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in STC-1 cells*

10 Exposure to 10 mM Phe induced a small elevation in $[\text{Ca}^{2+}]_i$ (~ 40 nM), but 20 mM and 50
11 mM Phe both induced much larger increases in $[\text{Ca}^{2+}]_i$ (80~90 nM) than did 10 mM Phe. The
12 increases in $[\text{Ca}^{2+}]_i$ induced by 20 and 50 mM Phe were sustained for more than 10 min.

13 *Extracellular Ca^{2+} enhances $[\text{Ca}^{2+}]_i$ response to Phe*

14 STC-1 cells were equilibrated with the Hepes buffer in the absence of extracellular Ca^{2+} ,
15 and then exposed to the buffer with various Ca^{2+} concentrations (0-3.0 mM) for 10 min. After
16 the $[\text{Ca}^{2+}]_i$ was stabilized, cells were exposed to 10 or 20 mM Phe (Fig. 3A, 10 mM Phe after
17 treatment with 3.0 mM Ca^{2+}). Exposure to 3 mM Ca^{2+} after equilibration to 0 mM Ca^{2+}
18 induced a rapid and sustained increase in $[\text{Ca}^{2+}]_i$ (Fig. 3A). In the presence of 3 mM
19 extracellular Ca^{2+} , 10 mM Phe induced an increase in $[\text{Ca}^{2+}]_i$ that was much higher than the
20 signal in the presence of 1.2 mM Ca^{2+} (Fig. 3B). Changes in $[\text{Ca}^{2+}]_i$ in response to 20 mM
21 Phe also showed an extracellular Ca^{2+} concentration-dependent increase (Fig. 3C). In the
22 absence of extracellular Ca^{2+} , 20 mM Phe failed to induce intracellular Ca mobilization (Fig.
23 3C).

24 *Pretreatment with Phe enhances $[\text{Ca}^{2+}]_i$ response to extracellular Ca^{2+}*

1 STC-1 cells were equilibrated with the Hepes buffer in the absence of extracellular Ca^{2+} ,
2 and subsequently exposed to the Hepes buffer with or without 20 mM Phe for 10 min.
3 Exposure to 20 mM Phe in the absence of extracellular Ca^{2+} did not affect $[\text{Ca}^{2+}]_i$ (data not
4 shown). Cells were then exposed to 1.2 or 3.0 mM Ca^{2+} . Exposure to 1.2 mM extracellular
5 Ca^{2+} in the presence of 20 mM Phe induced a higher increase in $[\text{Ca}^{2+}]_i$ than in the absence
6 of Phe (Fig. 4), which was similar to the response induced by 3.0 mM Ca^{2+} without Phe
7 pretreatment.

8 *Involvement of extracellular sodium*

9 Extracellular Na^+ was replaced with NMDG to examine whether Na^+ influx is responsible
10 for Phe-induced intracellular Ca^{2+} mobilization. Substitution of extracellular Na^+ by NMDG
11 did not affect the Phe-induced response.

12 *Involvement of the calcium-sensing receptor*

13 Treatment with a specific CaR antagonist, NPS2143 (25 μM), appeared to reduced the
14 prolonged Ca^{2+} responses induced by 50 mM Phe (Fig. 6A), but the initial increase remained
15 at a similar level as that for vehicle treatment (0.1% DMSO). Dose-dependent increases in
16 CCK secretion induced by Phe (20 and 50 mM) were completely abolished by the presence
17 of 50 μM NPS2143 (Fig. 6B). CCK secretion induced by 20 mM Phe was also abolished in
18 the presence of 25 μM NPS2143 (Fig. 6C).

19 *Expression of CaR mRNA in STC-1 cells and mouse tissues*

20 To examine the expression of CaR in STC-1 cells, RNA was extracted and subjected to
21 RT-PCR. We detected single bands of the expected DNA size (300 bp) for CaR (Fig. 7). CaR
22 mRNA was detected in each mouse tissue tested in the present study, though the kidney
23 and thyroid showed higher levels of expression than did the other tissues. CaR mRNA
24 expression tended to be higher in STC-1 cells than in the upper small intestine.

1

2 **DISCUSSION**

3 It is well known that Phe and Trp are potent stimulants of CCK secretion in humans and
4 dogs [2-5]. However, the underlying mechanism by which these amino acids trigger CCK
5 secretion remains unclear. Using a CCK-producing cell line, STC-1, Mangel et al. [6]
6 suggested the involvement of extracellular Ca^{2+} in Phe-induced CCK secretion in 1995,
7 though no subsequent reports on amino acid-induced CCK secretion appeared. The
8 discovery that CaR functions as an amino acid sensor by Conigreve et al. [9] led us to
9 investigate the CaR function in enteroendocrine cells.

10 Phe, at a concentration of more than 20 mM, induced an increase in CCK secretion (Fig.
11 1) under the normal extracellular Ca^{2+} concentration (1.2 mM). The effective concentration
12 of Phe (20 mM) in these cells, therefore, corresponds to the Phe concentrations (8-50 mM)
13 found to stimulate CCK or pancreatic secretion in human and dog studies [2-5]. CCK
14 secretion induced by 10 and 20 mM Phe was significantly enhanced by increasing the
15 extracellular Ca^{2+} concentration from 1.2 to 3.0 mM. These findings indicate that the
16 extracellular Ca^{2+} modulates Phe-induced CCK secretion, which suggests that CaR plays a
17 role in Phe-induced CCK secretion in STC-1 cells.

18 Exposure to Phe induced dose-dependent and rapid intracellular Ca^{2+} mobilization,
19 which was sustained for more than 10 min (Fig. 2). The lack of any CCK-releasing activity at
20 10 mM Phe under 1.2 mM extracellular Ca^{2+} condition indicates that the increase in $[\text{Ca}^{2+}]_i$
21 induced by 10 mM Phe is not sufficient to trigger significant CCK secretion in that condition.
22 Further, the absence of any intracellular Ca^{2+} mobilization by exposure to 20 mM Phe in the
23 absence of extracellular Ca^{2+} (Fig. 3C) indicates the involvement of an extracellular
24 Ca^{2+} -dependent mechanism. Pretreatment with higher concentrations of extracellular Ca^{2+}

1 enhanced the intracellular Ca^{2+} mobilization induced by subsequent exposure to Phe (Fig.
2 3A-C). This effect on the intracellular Ca^{2+} mobilization may be responsible for enhanced
3 CCK secretion in the presence of higher extracellular Ca^{2+} (Fig. 1). In the experiment with
4 the reverse exposure sequence (Ca^{2+} after Phe), pretreatment with Phe also enhanced
5 extracellular Ca^{2+} -induced intracellular Ca^{2+} mobilization (Fig 4). Such modulation of the
6 cellular response (receptor activity) by both ligands (Ca^{2+} and Phe) reflects the typical
7 properties of CaR [9]. Therefore, together with the above-mentioned results of CCK
8 secretion, these results strongly support the involvement of CaR in the sensing of Phe in
9 STC-1 cells.

10 It has been reported that glutamine induces intracellular Ca^{2+} mobilization to trigger
11 GLP-1 secretion from enteroendocrine cells via a Na^+ -dependent amino acid transport
12 system [16]. In the present study, removal of extracellular Na^+ did not affect the $[\text{Ca}^{2+}]_i$
13 response to Phe (Fig. 5). Therefore, it is unlikely that Phe induces the responses via an
14 amino acid transport system such as that reported for glutamine-induced GLP-1 secretion.

15 Both Phe-induced intracellular Ca^{2+} mobilization and CCK secretion in STC-1 cells were
16 reduced by treatment with a specific CaR antagonist, NPS2143 (Fig. 6). These data suggest
17 the involvement of CaR in Phe-sensing in STC-1 cells. The initial spike in $[\text{Ca}^{2+}]_i$, which
18 remained in the presence of NPS, might be induced by unknown pathways independent of
19 CaR. However, the activation of unknown pathways seems not to be sufficient to trigger
20 CCK secretion.

21 RT-PCR analysis confirmed the expression of CaR mRNA in STC-1 cells (Fig. 7).
22 Relatively lower CaR expression in the STC-1 cells and the intestinal tissue than in the
23 thyroid and kidney, which control systemic Ca^{2+} homeostasis, may reflect the susceptibility
24 to extracellular Ca^{2+} of those tissues. Changes in extracellular Ca^{2+} concentrations in the

1 intestinal mucosa are probably much higher than the systemic changes.

2 Several reviews have speculated on the role of CaR in nutrient sensing in
3 enteroendocrine cells [10,11]; however, reports are limited on the role in gastrin and acid
4 secretion in the stomach [13,14]. CaR is widely expressed throughout the body including the
5 gastrointestinal tract [17]. Only one paper [12] has reported that enteroendocrine cells
6 express CaR in the human colon tissue, but the function of CaR was not shown. In the
7 present study, we, for the first time, demonstrated that CaR is expressed and functions in
8 CCK-producing STC-1 cells. A recent paper demonstrated no significant increase in plasma
9 CCK by treatment with a CaR agonist (cinacalcet) in patients with secondary
10 hyperparathyroidism [18]. However, the plasma CCK response to a test meal tended to
11 increase in the cinacalcet treatment group, which supports the notion that CaR functions in
12 CCK secretion in humans. Further study is still needed to demonstrate the involvement of
13 CaR in nutrient sensing in enteroendocrine cells *in vivo*.

14 In summary, phenylalanine induced CCK secretion and intracellular Ca²⁺ mobilization in
15 enteroendocrine CCK-producing STC-1 cells. STC-1 cells expressed CaR mRNA and the
16 characteristic of CaR, that is, Phe-induced CCK secretion and Ca²⁺ mobilization were
17 enhanced by increasing extracellular Ca²⁺ concentration. A specific CaR antagonist blocked
18 the Phe-induced responses. Taken together, these results reveal that CaR functions as a
19 Phe receptor to induce intracellular Ca²⁺ mobilization and subsequent CCK secretion in
20 enteroendocrine cells.

21

22 **MATERIALS AND METHODS**

23 *Materials*

24 Cell culture consumables (Dulbecco's modified Eagle's medium, fetal bovine serum,

1 penicillin/streptomycin, trypsin-EDTA solution) were purchased from Invitrogen (Carlsbad,
2 CA). Fura-2-AM and Pluronic F-127 were obtained from Molecular Probes (Leiden,
3 Netherlands). NPS2143 was synthesized by Ajinomoto (Tokyo, Japan) and antagonist
4 activity was confirmed in HEK-293 cells transiently transfected with human CaR [19].
5 Poly-L-lysine solution (0.1%) and Hepes were purchased from Sigma (St. Louis, MO). Other
6 drugs are purchased from Wako (Osaka, Japan) unless specified.

7 *Cell culture*

8 STC-1 cells (a gift from Dr. D. Hanahan, University of California, San Francisco, CA)
9 were grown in Dulbecco's modified Eagle's medium (Invitrogen, Cat. No. 12100-038)
10 supplemented with 10% fetal bovine serum, 50 IU/ml penicillin, and 500 µg/ml streptomycin
11 in a humidified 5% CO₂ atmosphere at 37°C. Cells were routinely subcultured by
12 trypsinization upon reaching 80–90% confluency.

13 *CCK secretion study*

14 STC-1 cells were grown in 48-well culture plates at a density of 1.25×10^5 cells/well for
15 2-3 days until they reached 80-90% confluency. Cells were washed twice with Hepes buffer
16 to remove the culture media, and exposed to test agents dissolved in the same buffer for 60
17 min at 37°C. The Hepes buffer had the following composition: 140 mM NaCl, 4.5 mM KCl, 20
18 mM Hepes, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 10 mM D-glucose, and 0.1% BSA.

19 Supernatants were collected and centrifuged at 800 x g for 5 min at 4°C to remove
20 remaining cells, and then stored at -50°C until CCK concentration measurement with a
21 commercial enzyme immuno assay kit (Phoenix Pharmaceuticals Inc., Belmont CA). The
22 primary antiserum cross-reacts 100 % with sulphated- and non-sulphated CCK (26-33),
23 CCK-33 (porcine), caelurein, gastrin-1 (human), big gastrin-1 (human), 12.6% with CCK
24 (30-33), 0% with pancreatic polypeptide (human) and VIP (human, porcine, rat). Since

1 STC-1 cells do not express detectable levels of gastrin [20], we used an enzyme
2 immunoassay kit in which the antibody cross-reacts with gastrin.

3 *Intracellular Ca²⁺ measurement*

4 For measurement of intracellular Ca²⁺ concentrations ([Ca²⁺]_i), cells were grown on
5 0.025% poly-L-lysine-coated coverslips (1.3 cm²) at a density of 3-5 x 10⁵ cells/well in
6 24-well plates, and used 24–48 h after seeding, between passages 40 and 60. The
7 cytoplasmic [Ca²⁺] in cells grown on the coverslips was determined using a dual-excitation
8 spectrofluorophotometer (CAF-110; JASCO, Tokyo, Japan) with the calcium
9 sensitive-ratiometric dye, fura-2-AM. Cells cultured on a coverslip were loaded with 2 μM
10 fura-2-AM dissolved in Hepes buffer, containing 0.005% Pluronic F-127, at 37 °C for 20 min.
11 CaCl₂ was omitted from the Ca²⁺-free Hepes buffer, and NaCl was substituted with
12 *N*-methyl-D-glucamine (NMDG) in the Na⁺-free Hepes buffer. The pH of all buffers was
13 adjusted to 7.4. After loading the fura-2-AM, the coverslip was mounted into the folder and
14 washed with Hepes buffer. The folder was then inserted into the cuvette for the
15 spectrofluorophotometer, and the experimental liquid was continuously stirred in the cuvette
16 at 1000 rpm at 25°C. Fluorescent intensities were measured at an emission wavelength of
17 510 nm, and excitation wavelengths of 340 and 380 nm. After stabilization of basal
18 fluorescence, cells were exposed to the test agents. In order to estimate cytoplasmic
19 calcium concentration, cells were lysed with 0.2% Triton X-100 to obtain the maximum
20 fluorescent ratio (340 nm/380 nm) and then exposed to 10 mM EGTA to obtain the minimum
21 fluorescent ratio (340 nm/380 nm). The cytoplasmic calcium concentration was calculated
22 as described by Grynkiewicz et al. [21] according to the fluorescent ratio (340 nm/380 nm).
23 Data are expressed as changes in [Ca²⁺]_i from basal levels before exposure to the test
24 agents (0 min). Cell viability was assessed by exposing cells to 40 mM KCl after a challenge

1 with the test agents.

2

3 *RT-PCR*

4 RNA was isolated from STC-1 cells cultured in 75-cm² tissue culture flasks and from
5 BALB/c mouse (Japan SLC, Hamamatsu, Japan) tissues (gastric mucosa, upper small
6 intestinal mucosa, thyroid, kidney) using Trizol (Invitrogen). The study was approved by the
7 Hokkaido University Animal Committee, and the animals were maintained in accordance
8 with the guidelines for the care and use of laboratory animals of Hokkaido University. cDNA
9 was prepared from 1 µg RNA using Reversetranscript I reverse transcriptase (Wako), and
10 subjected to PCR using primers based on the mouse CaR mRNA sequence (GenBank
11 Accession No. NM013803, forward primer: 5'-gagcacatcccttcaaccatc-3', reverse primer:
12 5'-aagtcaatgcagatgtccctctc-3') and mouse glyceraldehyde-3-phosphate dehydrogenase
13 (GAPDH) mRNA sequence (GenBank Accession No. NM008084, forward primer:
14 5'-aaccttggcattgtggaagg-3', reverse primer: 5'-ccctgttgctgtagccgtat-3'). PCR conditions were
15 95°C for 5 min, then 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. PCR
16 products were separated by 1.5% agarose gel electrophoresis and visualized by ethidium
17 bromide staining.

18 *Statistical analysis*

19 Results are expressed as means ± SEM. Statistical significance was assessed using
20 one-way ANOVA and significant differences among mean values were determined by the
21 Tukey's HSD post hoc test ($P < 0.05$).

22

23 **REFERENCES**

24 1. Liddle RA (1997) Cholecystokinin cells. *Annu Rev Physiol* **59**, 221-242.

- 1 2. Go VL, Hofmann AF & Summerskill WH (1970) Pancreozymin bioassay in man based on
2 pancreatic enzyme secretion: potency of specific amino acids and other digestive
3 products. *J Clin Invest* **49**(8), 1558-1564.
- 4 3. Colombel JF, Sutton A, Chayvialle JA & Modigliani R (1988) Cholecystokinin release and
5 biliopancreatic secretion in response to selective perfusion of the duodenal loop with
6 aminoacids in man. *Gut* **29**(9), 1158-1166.
- 7 4. Meyer JH, Kelly GA & Jones RS (1976) Canine pancreatic response to intestinally
8 perfused oligopeptides. *Am J Physiol* **231**(3), 678-681.
- 9 5. Meyer JH, Kelly GA, Spingola LJ & Jones RS (1976) Canine gut receptors mediating
10 pancreatic responses to luminal L-amino acids. *Am J Physiol* **231**(3), 669-677.
- 11 6. Mangel AW, Prpic V, Wong H, Basavappa S, Hurst LJ, Scott L, Garman RL, Hayes JS,
12 Sharara AI, Snow ND, Walsh JH & Liddle RA (1995) Phenylalanine-stimulated secretion
13 of cholecystokinin is calcium dependent. *Am J Physiol* **268**(1 Pt 1): G90-94.
- 14 7. Koop I & Buchan AM (1992) Cholecystokinin release from isolated canine epithelial cells
15 in short-term culture. *Gastroenterology* **102**(1), 28-34.
- 16 8. Chang CH, Chey WY, Sun Q, Leiter A & Chang TM (1994) Characterization of the release
17 of cholecystokinin from a murine neuroendocrine tumor cell line, STC-1. *Biochim*
18 *Biophys Acta* **1221**(3), 339-47.
- 19 9. Conigrave AD, Quinn SJ & Brown EM (2000) L-amino acid sensing by the extracellular
20 Ca²⁺-sensing receptor. *Proc Natl Acad Sci U S A* **97**(9), 4814-4819.
- 21 10. Conigrave AD & Brown EM (2006) Taste receptors in the gastrointestinal tract. II.
22 L-amino acid sensing by calcium-sensing receptors: implications for GI physiology. *Am J*
23 *Physiol Gastrointest Liver Physiol* **291**(5), G753-761.
- 24 11. Kirchhoff P & Geibel JP (2006) Role of calcium and other trace elements in the

- 1 gastrointestinal physiology. *World J Gastroenterol* **12**(20), 3229-3236.
- 2 12. Sheinin Y, Kállay E, Wrba F, Kriwanek S, Peterlik M, Cross HS (2000)
- 3 Immunocytochemical localization of the extracellular calcium-sensing receptor in normal
- 4 and malignant human large intestinal mucosa. *J Histochem Cytochem* **48**(5), 595-602.
- 5 13. Dufner MM, Kirchhoff P, Remy C, Hafner P, Müller MK, Cheng SX, Tang LQ, Hebert SC,
- 6 Geibel JP & Wagner CA (2005) The calcium-sensing receptor acts as a modulator of
- 7 gastric acid secretion in freshly isolated human gastric glands. *Am J Physiol Gastrointest*
- 8 *Liver Physiol* **289**(6), G1084-1090.
- 9 14. Busque SM, Kerstetter JE, Geibel JP & Insogna K (2005) L-type amino acids stimulate
- 10 gastric acid secretion by activation of the calcium-sensing receptor in parietal cells. *Am J*
- 11 *Physiol Gastrointest Liver Physiol* **289**(4), G664-669.
- 12 15. Cheng SX, Geibel JP & Hebert SC (2004) Extracellular polyamines regulate fluid
- 13 secretion in rat colonic crypts via the extracellular calcium-sensing receptor.
- 14 *Gastroenterology* **126**(1), 148-158.
- 15 16. Reimann F, Williams L, da Silva Xavier G, Rutter GA & Gribble FM (2004) Glutamine
- 16 potently stimulates glucagon-like peptide-1 secretion from GLUTag cells. *Diabetologia*
- 17 **47**, 1592-1601.
- 18 17. Chattopadhyay N, Cheng I, Rogers K, Riccardi D, Hall A, Diaz R, Hebert SC, Soybel DI
- 19 & Brown EM (1998) Identification and localization of extracellular Ca(2+)-sensing
- 20 receptor in rat intestine. *Am J Physiol Gastrointest Liver Physiol* **274**, G122-130.
- 21 18. Díez JJ, Miguel JL, Codoceo R, Iglesias P, Bajo MA, Sánchez C, Del Peso G, Gil F,
- 22 Martínez-Ara J, Gancedo PG & Selgas R (2008) Effects of cinacalcet on gastrointestinal
- 23 hormone release in patients with secondary hyperparathyroidism undergoing dialysis.
- 24 *Nephrol Dial Transplant* **23**(4), 1387-1395.

- 1 19. Nemeth EF, Delmar EG, Heaton WL, Miller MA, Lambert LD, Conklin RL, Gowen M,
2 Gleason JG, Bhatnagar PK & Fox J (2001) Calcilytic compounds: potent and selective
3 Ca^{2+} receptor antagonists that stimulate secretion of parathyroid hormone. *J Pharmacol*
4 *Exp Ther* **299**(1), 323-31.
- 5 20. McLaughlin JT, Lomax RB, Hall L, Dockray GJ, Thompson DG & Warhurst G (1998)
6 Fatty acids stimulate cholecystokinin secretion via an acyl chain length-specific,
7 Ca^{2+} -dependent mechanism in the enteroendocrine cell line STC-1. *J Physiol* **513**(1),
8 11-18.
- 9 21. Grynkiewicz G, Poenie M & Tsien RY (1985) A new generation of Ca^{2+} indicators with
10 greatly improved fluorescence properties. *J Biol Chem* **260**, 3440-3450.

11

12 **FIGUER REGENDS**

13 Fig. 1. Effects of Phe and extracellular Ca^{2+} on CCK secretion in STC-1 cells.

14 STC-1 cells were exposed to 10 mM (A) or 20-50 mM (B) Phe in the presence of 1.2 mM
15 and 3.0 mM extracellular Ca^{2+} concentrations for 60 min. CCK concentrations in the
16 supernatant were measured by enzyme immunoassay kit. Values are means \pm SEM of 3-4
17 experiments. Values not sharing a common letter are significantly different ($P < 0.05$,
18 Tukey's test).

19

20 Fig. 2. Changes in intracellular Ca^{2+} concentration ($\Delta[\text{Ca}^{2+}]_i$) in STC-1 cells exposed to
21 10-50 mM Phe.

22 STC-1 cells were loaded with fura-2 and intracellular Ca^{2+} concentration was measured
23 ratiometrically using a spectrofluorophotometer. Cells were exposed to various
24 concentrations (10-50 mM) of Phe at 0 min (arrow). Data shows changes in intracellular

1 Ca^{2+} concentration ($\Delta[\text{Ca}^{2+}]_i$) from the basal intracellular Ca^{2+} concentrations. Values are
2 the means of 4-6 experiments.

3

4 Fig. 3. Effects of pretreatment with various extracellular Ca^{2+} concentrations on Phe-induced
5 intracellular Ca^{2+} mobilization.

6 STC-1 cells were equilibrated with the buffer in the absence of Ca^{2+} , and exposed to
7 various extracellular Ca^{2+} concentrations (0-3.0 mM) for 10 min. Cells were then exposed to
8 10 or 20 mM Phe after pretreatment with various concentrations of Ca^{2+} . A: A representative
9 data for the intracellular Ca^{2+} response to 10 mM Phe after pretreatment with 3.0 mM
10 extracellular Ca^{2+} . The responses in the boxed area are shown in Fig. 3B and C. B and C:
11 Changes in intracellular Ca^{2+} concentration ($\Delta[\text{Ca}^{2+}]_i$) in STC-1 cells exposed to 10 mM (B)
12 or 20 mM (C) Phe after pretreatment with various extracellular Ca^{2+} concentrations (0-3.0
13 mM). Values are means of 3-4 experiments.

14

15 Fig. 4. Effect of pretreatment with Phe on extracellular Ca^{2+} -induced intracellular Ca^{2+}
16 mobilization.

17 Changes in intracellular Ca^{2+} concentration ($\Delta[\text{Ca}^{2+}]_i$) in STC-1 cells exposed to
18 extracellular Ca^{2+} (1.2 mM or 3.0 mM) after pretreatment with 20 mM Phe. STC-1 cells were
19 equilibrated with the buffer in the absence of Ca^{2+} , and exposed to 0 or 20 mM Phe for 10
20 min. Data before (-1 min) and after exposure to extracellular Ca^{2+} were extracted. Values are
21 the means of 3-4 experiments.

22

23 Fig. 5. Effect of the removal of extracellular sodium on Phe-induced intracellular Ca^{2+}
24 mobilization.

1 STC-1 cells were equilibrated with a buffer in which the extracellular Na⁺ was replaced
2 with NMDG, and then exposed to 50 mM Phe under sodium-free conditions. Values are the
3 means of 3-4 experiments.

4

5 Fig. 6. Effects of CaR antagonist on Phe-induced intracellular Ca²⁺ mobilization and CCK
6 secretion.

7 A: Changes in intracellular Ca²⁺ concentration ($\Delta[Ca^{2+}]_i$) in STC-1 cells exposed to 50
8 mM Phe in the presence of 25 μ M NPS2143 (NPS) or 0.1% DMSO (Vehicle). Values are the
9 means of 6 experiments. B and C: CCK secretion from STC-1 cells exposed to 20-50 mM
10 Phe in the presence of 50 μ M (B) or 25 μ M NPS (C). Vehicle contained 0.1% DMSO. Values
11 are the means \pm SEM of 3-4 experiments. Values not sharing a common letter are
12 significantly different ($P < 0.05$, Tukey's test).

13

14 Fig. 7. CaR expression in STC-1 cells and mouse tissues.

15 RNA was extracted from STC-1 cells and mouse tissues (gastric mucosa, upper small
16 intestinal mucosa, thyroid, kidney), and then subjected to RT-PCR with CaR or GAPDH
17 primers. PCR products were loaded onto 1.5% agarose gel and stained with ethidium
18 bromide.

Fig. 1

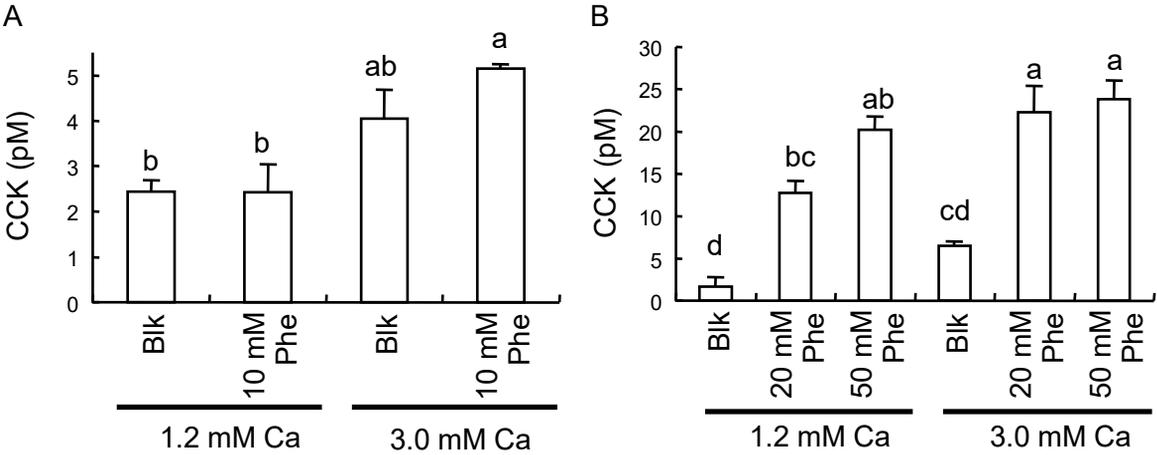


Fig. 2

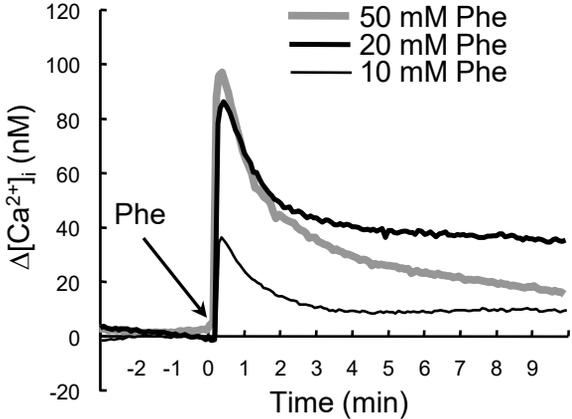


Fig. 3

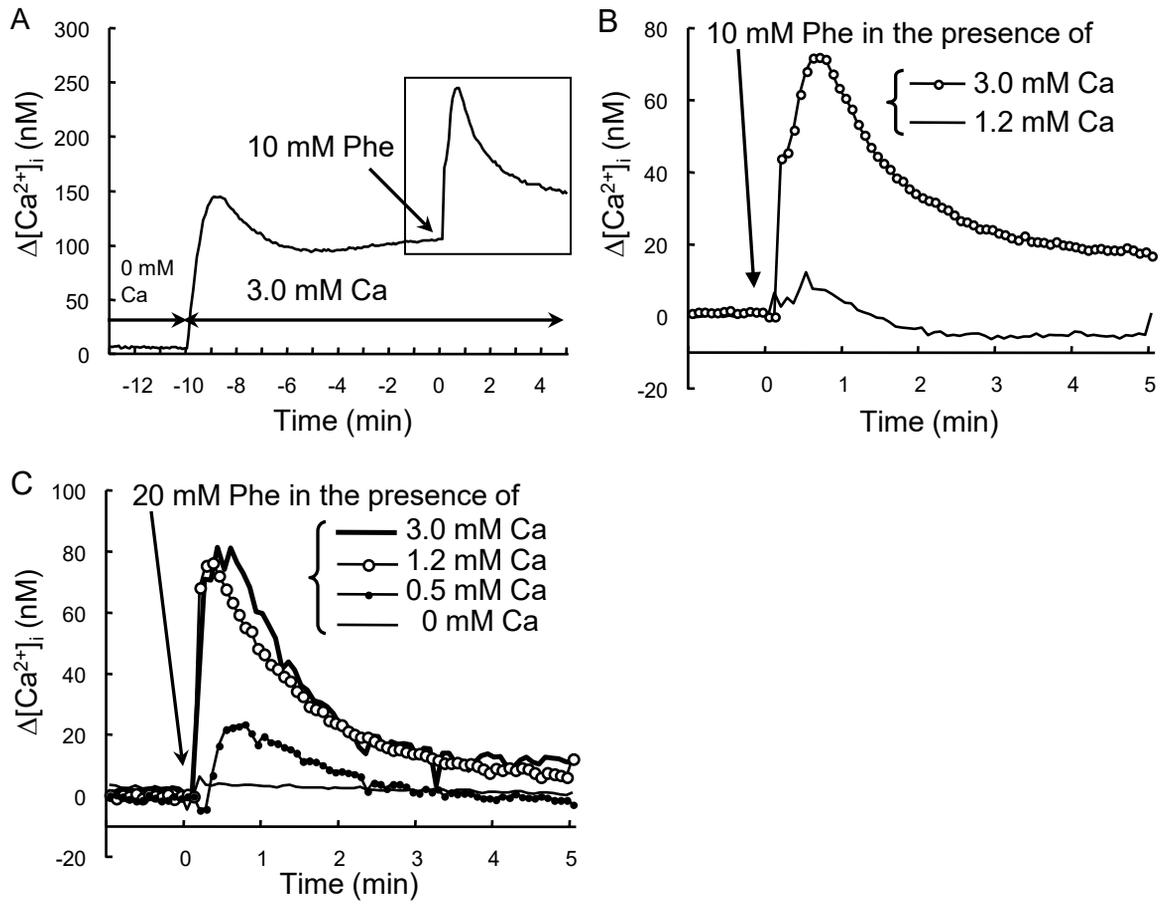


Fig. 4

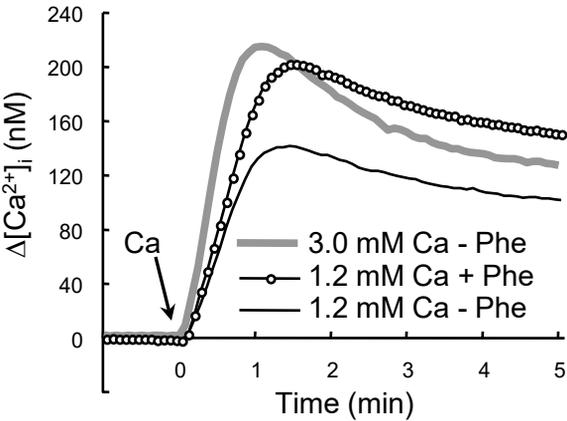


Fig. 5

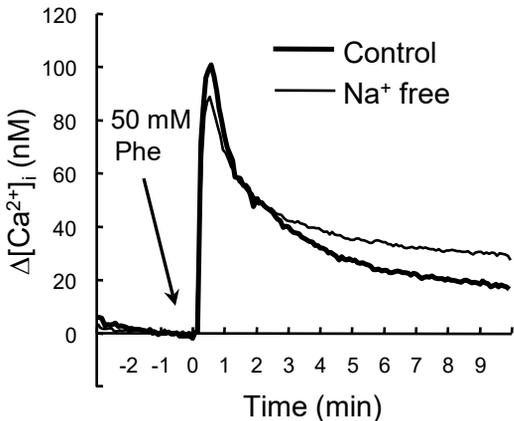


Fig. 6

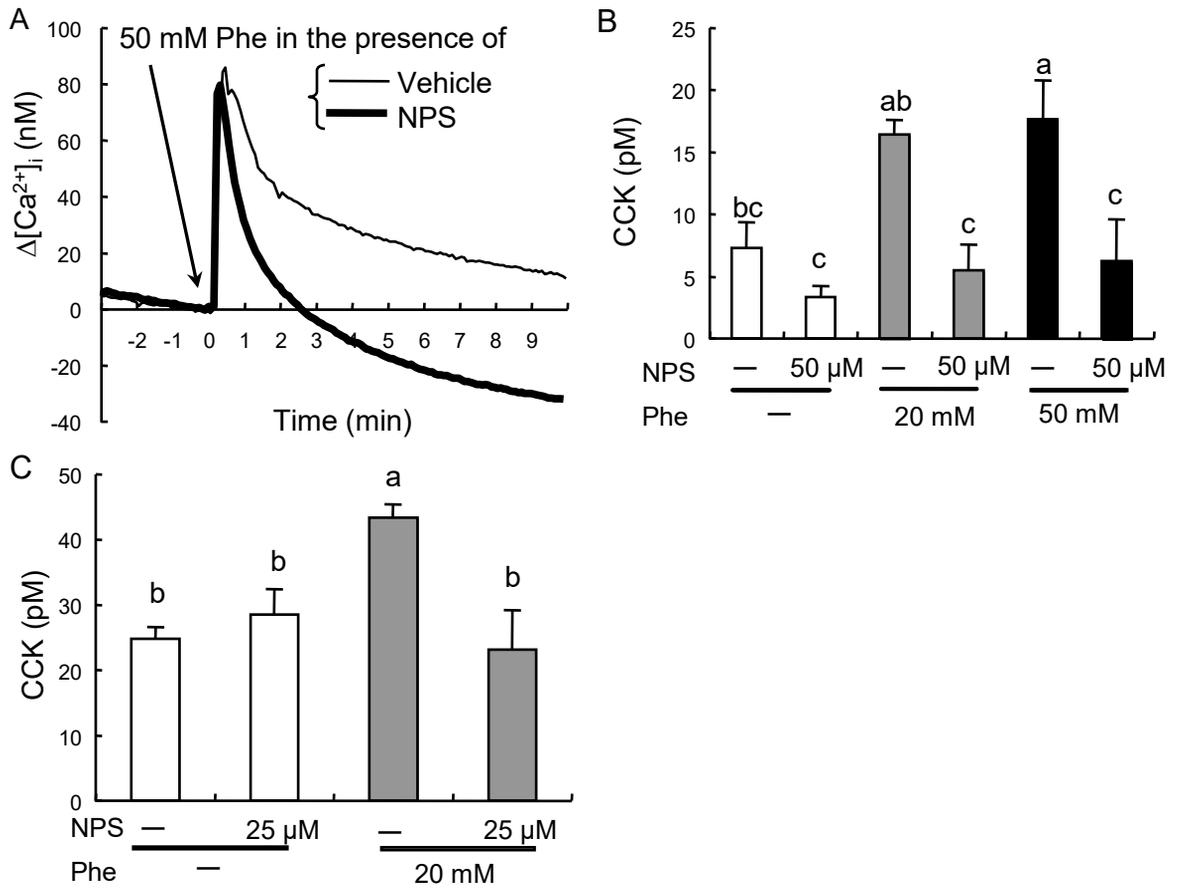


Fig. 7

