*Title.*

Calcium-sensing receptor mediates phenylalanine-induced cholecystokinin secretion in enteroendocrine STC-1 cells

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*Running title.*

Amino acid sensing in enteroendocrine cells

*Abbreviations.*

Phenylalanine; Phe, Cholecystokinin; CCK, Calcium-sensing receptor; CaR

Key words.

Enteroendocrine cells, Calcium-sensing receptor, Cholecystokinin, Phenylalanine

## SUMMARY

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

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


## INTRODUCTION

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

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

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

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

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

## RESULTS

*Phe induces CCK secretion in STC-1 cells*

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

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

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

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

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

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

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

*Involvement of extracellular sodium*

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

*Involvement of the calcium-sensing receptor*

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

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

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

## DISCUSSION

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

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

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

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

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

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

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

intestinal mucosa are probably much higher than the systemic changes.

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

In summary, phenylalanine induced CCK secretion and intracellular $Ca^{2+}$ mobilization in enteroendocrine CCK-producing STC-1 cells. STC-1 cells expressed CaR mRNA and the characteristic of CaR, that is, Phe-induced CCK secretion and $Ca^{2+}$ mobilization were enhanced by increasing extracellular $Ca^{2+}$ concentration. A specific CaR antagonist blocked the Phe-induced responses. Taken together, these results reveal that CaR functions as a Phe receptor to induce intracellular $Ca^{2+}$ mobilization and subsequent CCK secretion in enteroendocrine cells.

## MATERIALS AND METHODS

### *Materials*

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

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

*Cell culture*

STC-1 cells (a gift from Dr. D. Hanahan, University of California, San Francisco, CA) were grown in Dulbecco's modified Eagle's medium (Invitrogen, Cat. No. 12100-038) supplemented with 10% fetal bovine serum, 50 IU/ml penicillin, and 500 µg/ml streptomycin in a humidified 5% $CO_2$ atmosphere at 37°C. Cells were routinely subcultured by trypsinization upon reaching 80–90% confluency.

*CCK secretion study*

STC-1 cells were grown in 48-well culture plates at a density of 1.25 x $10^5$ cells/well for 2-3 days until they reached 80-90% confluency. Cells were washed twice with Hepes buffer to remove the culture media, and exposed to test agents dissolved in the same buffer for 60 min at 37°C. The Hepes buffer had the following composition: 140 mM NaCl, 4.5 mM KCl, 20 mM Hepes, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM D-glucose, and 0.1% BSA.

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

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

*Intracellular $Ca^{2+}$ measurement*

For measurement of intracellular $Ca^{2+}$ concentrations ($[Ca^{2+}]_i$), cells were grown on 0.025% poly-L-lysine-coated coverslips (1.3 $cm^2$) at a density of 3-5 x $10^5$ cells/well in 24-well plates, and used 24–48 h after seeding, between passages 40 and 60. The cytoplasmic $[Ca^{2+}]$ in cells grown on the coverslips was determined using a dual-excitation spectrafluorophotometer (CAF-110; JASCO, Tokyo, Japan) with the calcium sensitive-ratiometric dye, fura-2-AM. Cells cultured on a coverslip were loaded with 2 μM fura-2-AM dissolved in Hepes buffer, containing 0.005% Pluronic F-127, at 37 °C for 20 min. $CaCl_2$ was omitted from the $Ca^{2+}$-free Hepes buffer, and NaCl was substituted with *N*-methyl-D-glucamine (NMDG) in the $Na^+$-free Hepes buffer. The pH of all buffers was adjusted to 7.4. After loading the fura-2-AM, the coverslip was mounted into the folder and washed with Hepes buffer. The folder was then inserted into the cuvette for the spectrafluorophotometer, and the experimental liquid was continuously stirred in the cuvette at 1000 rpm at 25°C. Fluorescent intensities were measured at an emission wavelength of 510 nm, and excitation wavelengths of 340 and 380 nm. After stabilization of basal fluorescence, cells were exposed to the test agents. In order to estimate cytoplasmic calcium concentration, cells were lysed with 0.2% Triton X-100 to obtain the maximum fluorescent ratio (340 nm/380 nm) and then exposed to 10 mM EGTA to obtain the minimum fluorescent ratio (340 nm/380 nm). The cytoplasmic calcium concentration was calculated as described by Grynkiewies et al. [21] according to the fluorescent ratio (340 nm/380 nm). Data are expressed as changes in $[Ca^{2+}]_i$ from basal levels before exposure to the test agents (0 min). Cell viability was assessed by exposing cells to 40 mM KCl after a challenge

with the test agents.

*RT-PCR*

RNA was isolated from STC-1 cells cultured in 75-$cm^2$ tissue culture flasks and from BALB/c mouse (Japan SLC, Hamamatsu, Japan) tissues (gastric mucosa, upper small intestinal mucosa, thyroid, kidney) using Trizol (Invitrogen). The study was approved by the Hokkaido University Animal Committee, and the animals were maintained in accordance with the guidelines for the care and use of laboratory animals of Hokkaido University. cDNA was prepared from 1 μg RNA using Reversetranscript I reverse transcriptase (Wako), and subjected to PCR using primers based on the mouse CaR mRNA sequence (GenBank Accession No. NM013803, forward primer: 5'-gagcacatcccttcaaccatc-3', reverse primer: 5'-aagtcaatgcagatgtccctctc-3') and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA sequence (GenBank Accession No. NM008084, forward primer: 5'-aactttggcattgtggaagg-3', reverse primer: 5'-ccctgttgctgtagccgtat-3'). PCR conditions were 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 products were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

*Statistical analysis*

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

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**FIGUER REGENDS**

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

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

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

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

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

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

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

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

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

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

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

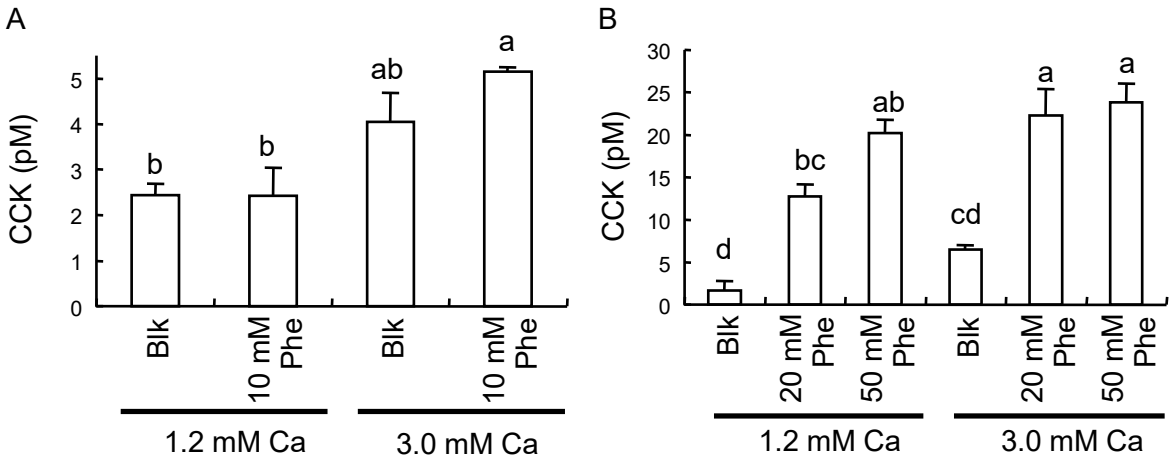
Fig. 6. Effects of CaR antagonist on Phe-induced intracellular $Ca^{2+}$ mobilization and CCK secretion.

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

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

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

Fig. 1


A
CCK (pM)
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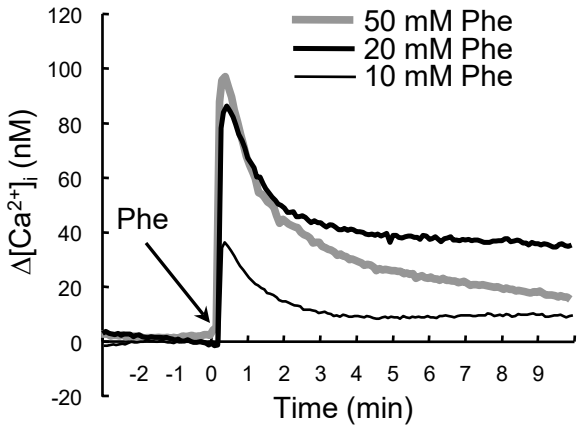
1.2 mM Ca
3.0 mM Ca

Fig. 2


50 mM Phe
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Phe
Δ[Ca2+]i (nM)
120
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40
20
0
-20
-2 -1 0 1 2 3 4 5 6 7 8 9
Time (min)

Fig. 3

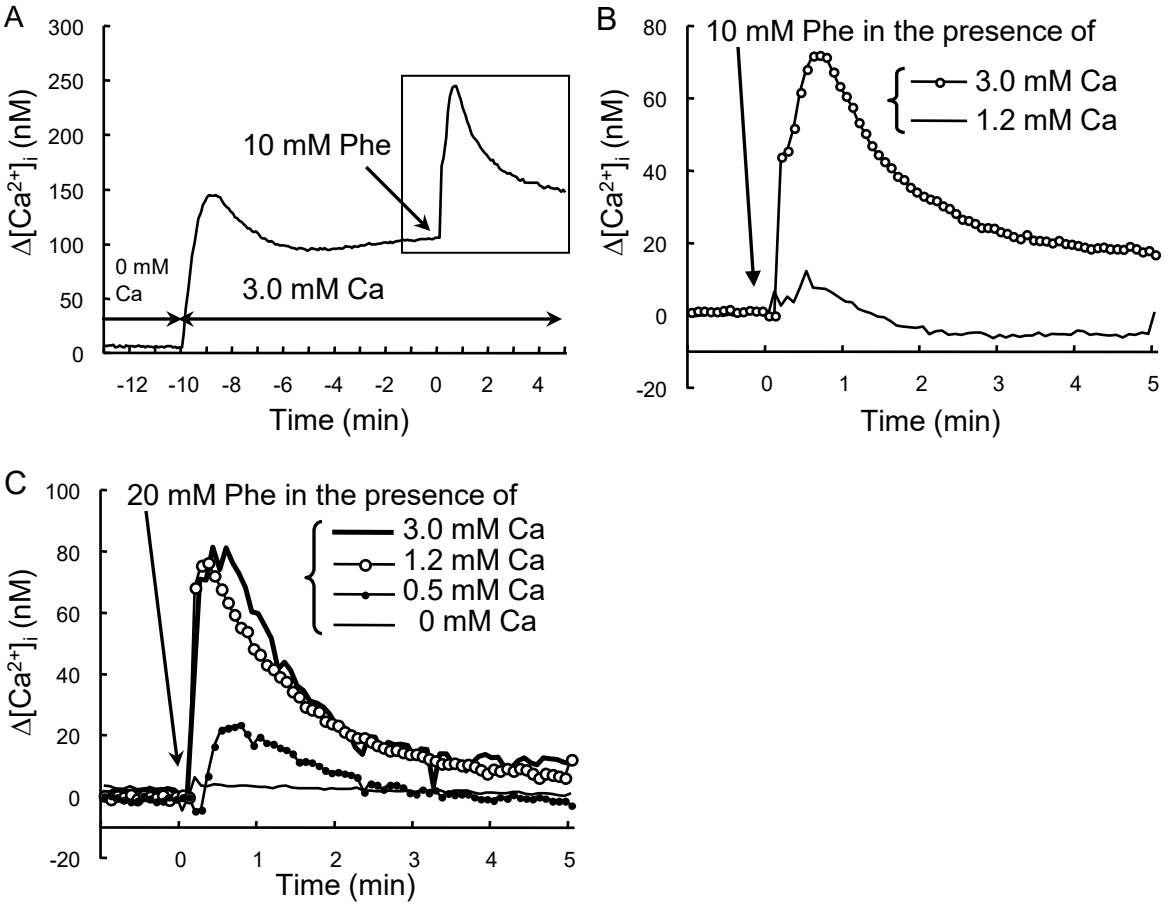

A
300
250
200
150
100
50
0
Δ[Ca2+]i (nM)
10 mM Phe
0 mM
Ca
3.0 mM Ca
-12 -10 -8 -6 -4 -2 0 2 4
Time (min)
B
80
60
40
20
0
-20
10 mM Phe in the presence of
3.0 mM Ca
1.2 mM Ca
Δ[Ca2+]i (nM)
0 1 2 3 4 5
Time (min)
C
100
80
60
40
20
0
-20
20 mM Phe in the presence of
3.0 mM Ca
1.2 mM Ca
0.5 mM Ca
0 mM Ca
Δ[Ca2+]i (nM)
0 1 2 3 4 5
Time (min)

Fig. 4

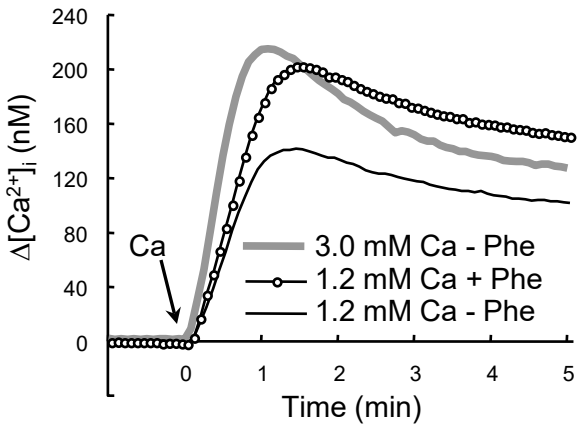

240
200
160
120
80
40
0
Δ[Ca2+]i (nM)
Ca
3.0 mM Ca - Phe
1.2 mM Ca + Phe
1.2 mM Ca - Phe
0
1
2
3
4
5
Time (min)

Fig. 5

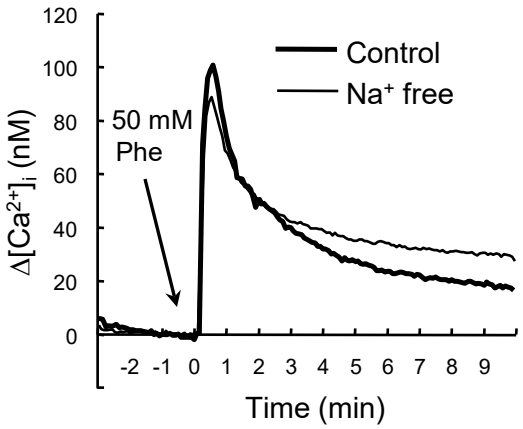

120
100
80
60
40
20
0
Δ[Ca2+]i (nM)
50 mM
Phe
Control
Na+ free
-2 -1 0 1 2 3 4 5 6 7 8 9
Time (min)

Fig. 6

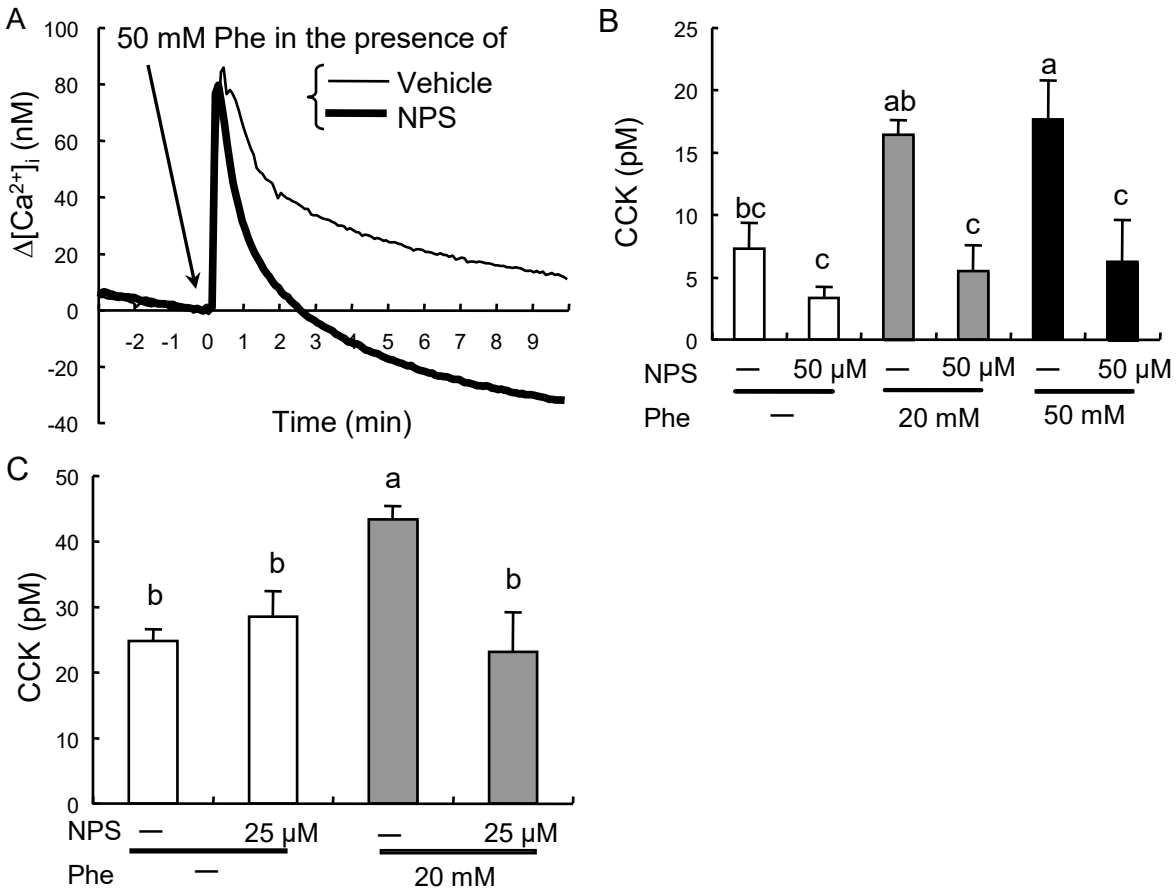
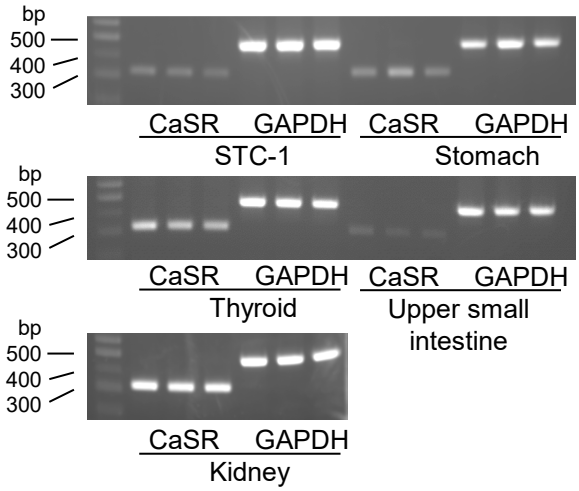

A
50 mM Phe in the presence of
Vehicle
NPS
Δ[Ca2+]i (nM)
Time (min)
B
CCK (pM)
bc
c
ab
c
a
c
NPS
50 μM
Phe
20 mM
50 mM
C
CCK (pM)
b
b
a
b
NPS
25 μM
Phe
20 mM

Fig. 7


bp
500
400
300
CaSR
GAPDH
CaSR
GAPDH
STC-1
Stomach
bp
500
400
300
CaSR
GAPDH
CaSR
GAPDH
Thyroid
Upper small intestine
bp
500
400
300
CaSR
GAPDH
Kidney