



Title	Soybean $\beta$ 51-63 peptide stimulates cholecystokinin secretion via a calcium-sensing receptor in enteroendocrine STC-1 cells
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1 **Title.**

2 Soybean  $\beta$ 51-63 peptide stimulates cholecystokinin secretion via a calcium-sensing  
3 receptor in enteroendocrine STC-1 cells

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19 **Summary**

20 We previously demonstrated that intraduodenal administration of an arginine-rich  
21  $\beta$ 51-63 peptide in soybean  $\beta$ -conglycinin suppresses food intake via cholecystokinin (CCK)  
22 secretion in rats. However, the cellular mechanisms by which the  $\beta$ 51-63 peptide induces  
23 CCK secretion remain to be clarified. In the present study, we examined whether the  
24 extracellular calcium-sensing receptor (CaR) mediates  $\beta$ 51-63-induced CCK secretion in

1 **murine** CCK-producing enteroendocrine cell-line **STC-1**. CCK secretion and changes in  
2 intracellular  $\text{Ca}^{2+}$  concentration in response to  $\beta$ 51-63 peptide were measured in STC-1  
3 cells under various extracellular  $\text{Ca}^{2+}$  concentrations and after treatment with a CaR  
4 antagonist. Intracellular  $\text{Ca}^{2+}$  concentrations in response to  $\beta$ 51-63 peptide and  
5 extracellular  $\text{Ca}^{2+}$  were also measured in CaR-expressing human embryonic kidney  
6 (HEK293) cells. The  $\beta$ 51-63 peptide induced CCK secretion and intracellular  $\text{Ca}^{2+}$   
7 mobilization in STC-1 cells under normal (1.2 mM) extracellular  $\text{Ca}^{2+}$  conditions in a  
8 dose-dependent manner. These responses to  $\beta$ 51-63 peptide were reduced by the removal  
9 of intra- or extra-cellular  $\text{Ca}^{2+}$  but enhanced by increasing extracellular  $\text{Ca}^{2+}$  concentrations.  
10 Intracellular  $\text{Ca}^{2+}$  mobilization induced by extracellular  $\text{Ca}^{2+}$  was also increased by the  
11 pretreatment with  $\beta$ 51-63 peptide. Treatment with a specific CaR antagonist (NPS2143)  
12 inhibited  $\beta$ 51-63-induced CCK secretion and intracellular  $\text{Ca}^{2+}$  mobilization. In addition,  
13 HEK293 cells transfected with CaR acquired sensitivity to the  $\beta$ 51-63 peptide. From these  
14 results, we conclude that CaR is the  $\beta$ 51-63 peptide sensor responsible for the stimulation  
15 of CCK secretion in enteroendocrine STC-1 cells.

16

17 *Abbreviations.*

18 Cholecystokinin; CCK, Calcium-sensing receptor; CaR

19 Key words.

20  $\beta$ 51-63 peptide, Enteroendocrine cells, Calcium-sensing receptor, Cholecystokinin

## 1 **1. Introduction**

2 Cholecystokinin (CCK) is a gut hormone produced by enteroendocrine 'I cells' located in  
3 the upper small intestine, which is known to regulate gallbladder contraction, pancreatic  
4 secretion, gastric emptying, and food intake. In our previous study, duodenal infusion of a  
5 peptone prepared from soybean  $\beta$ -conglycinin reduced food intake via CCK secretion in  
6 rats [1]. Furthermore, an arginine-rich fragment,  $\beta$ 51-63, in the  $\beta$ -subunit of  $\beta$ -conglycinin  
7 was identified as one of the active peptides [2]. However, whether the  $\beta$ 51-63 peptide acts  
8 directly on CCK-producing cells is not known. In addition, the mechanism by which the  
9  $\beta$ 51-63 peptide stimulates CCK secretion from CCK-producing cells remains unclear.

10 Dietary peptides and fatty acids are well known stimulants of CCK secretion [3]. The  
11 cellular mechanisms by which these nutrients stimulate CCK secretion have been studied  
12 using an enteroendocrine cell line, STC-1, derived from the murine duodenum [4]. The  
13 signal transduction pathway for dietary-peptide-induced CCK secretion in enteroendocrine  
14 cells involves an increase in intracellular  $\text{Ca}^{2+}$  mobilization and cyclic AMP [5, 6].

15 The extracellular calcium-sensing receptor (CaR) was first cloned from human  
16 parathyroid cells and is known to regulate  $\text{Ca}^{2+}$  homeostasis via parathyroid hormone  
17 secretion [7]. CaR is expressed in various tissues, including the gastrointestinal tract, and  
18 has several physiological roles; for example, CaR mediates gastrin and gastric acid  
19 secretion in human G-cells [8, 9] and also functions as a negative modulator of intestinal  
20 fluid transport [10]. CaR is activated not only by extracellular  $\text{Ca}^{2+}$ , but also by multiple  
21 ligands, such as amino acids [11-13], polyamines [14, 15], and basic polypeptides  
22 (protamine, poly-lysine, poly-arginine) [16-18]. Recently, glutathione ( $\gamma$ Glu-Cys-Gly) has  
23 been reported to have ligand activity for CaR in HEK293 cells expressing CaR [19],  
24 indicating that some short peptides can also activate CaR. In a very recent study, we

1 demonstrated that CaR is expressed in CCK-producing STC-1 cells and mediates  
2 phenylalanine-induced CCK secretion [11]. These studies led us to investigate the  
3 possibility that CaR has a role as a dietary peptide receptor to regulate CCK secretion in  
4 enteroendocrine cells.

5 In the present study, we examined whether  $\beta$ 51-63 peptide could stimulate CCK  
6 secretion in STC-1 cells and then investigated the intracellular signaling pathways involved  
7 in  $\beta$ 51-63-induced CCK secretion. The involvement of extracellular  $\text{Ca}^{2+}$  and CaR in the  
8  $\beta$ 51-63-induced cellular responses (CCK secretion and intracellular  $\text{Ca}^{2+}$  mobilization) was  
9 also investigated in STC-1 cells and CaR-expressing HEK-293 cells to determine the role of  
10 CaR in the peptide-sensing mechanism.

11

## 12 **2. Materials and Methods**

### 13 *2.1. Materials*

14 Cell culture consumables (Dulbecco's modified Eagle's medium, fetal bovine serum,  
15 penicillin/streptomycin, and trypsin-EDTA solution) were purchased from Invitrogen  
16 (Carlsbad, CA). The  $\beta$ 51-63 peptide (VRIRLLQRFNKRS) was synthesized by Bachem  
17 (Bubendorf, Switzerland). Fura-2-AM and Pluronic F-127 were obtained from Molecular  
18 Probes (Leiden, Netherlands). NPS2143 was synthesized by Ajinomoto (Tokyo, Japan) and  
19 antagonist activity was confirmed in HEK-293 cells transiently transfected with human CaR  
20 [20]. YM-254890 was provided by Astellas Pharma Inc (Tokyo, Japan). Poly-L-lysine  
21 solution (0.1%), HEPES, thapsigargin, pertussis toxin (PTX) and denatonium benzoate were  
22 purchased from Sigma (St. Louis, MO). *O, O'*-Bis (2-aminophenyl) ethylenglycol-*N, N, N'*,  
23 *N'*-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM) was purchased from Dojinndo  
24 (Kumamoto, Japan). Other reagents were purchased from Wako (Osaka, Japan) unless

1 specified.

## 2 2.2. Cell culture

3 STC-1 cells (a gift from Dr. D. Hanahan, University of California, San Francisco, CA)  
4 were grown in Dulbecco's modified Eagle's medium (Invitrogen, Cat. No. 12100-038)  
5 supplemented with 10% fetal bovine serum, 50 IU/ml penicillin, and 500 µg/ml streptomycin  
6 in a humidified 5% CO<sub>2</sub> atmosphere at 37°C. Cells were routinely subcultured by  
7 trypsinization upon reaching 80–90% confluency. HEK293 cells (JCRB9068) were  
8 purchased from Human Science Research Resources Bank (Osaka, Japan), and cells  
9 were grown in DMEM/F12 medium containing 10% FCS.

## 10 2.3. CCK secretion study

11 STC-1 cells were grown in 48-well culture plates at a density of  $1.25 \times 10^5$  cells/well for  
12 2-3 days until they reached 80-90% confluency. Cells were washed twice with HEPES buffer  
13 to remove the culture media, and exposed to test agents dissolved in the same buffer for 60  
14 min at 37°C. The HEPES buffer had the following composition: 140 mM NaCl, 4.5 mM KCl,  
15 20 mM HEPES, 1.2 mM CaCl<sub>2</sub>, 1.2 mM MgCl<sub>2</sub>, 10 mM D-glucose, and 0.1% BSA. After  
16 incubation for 60 min, supernatants from the 48-well culture plates were collected and  
17 centrifuged at 800 x g for 5 min at 4°C to remove the remaining cells, and then stored at  
18 -50°C until CCK concentration measurement with a commercial enzyme immunoassay  
19 (EIA) kit (Phoenix Pharmaceuticals Inc., Belmont CA). The primary antiserum cross-reacts  
20 100% with sulphated- and non-sulphated CCK (26-33), CCK-33 (porcine), caelurein,  
21 gastrin-1 (human), and big gastrin-1 (human); 12.6% with CCK (30-33); and 0% with  
22 pancreatic polypeptide (human) and VIP (human, porcine, rat). Since STC-1 cells do not  
23 express detectable levels of gastrin [21], we used an EIA kit in which the antibody  
24 cross-reacts with gastrin. The coefficients of intra-assay and inter-assay variation are < 5%

1 and < 14%, respectively.

#### 2 *2.4. Intracellular Ca<sup>2+</sup> measurement in STC-1 cells*

3 For measurement of intracellular Ca<sup>2+</sup> concentrations ([Ca<sup>2+</sup>]<sub>i</sub>), cells were grown on  
4 0.025% poly-L-lysine-coated coverslips (1.3 cm<sup>2</sup>) at a density of 3-5 x 10<sup>5</sup> cells/well in  
5 24-well plates, and used 24–48 h after seeding, between passages 30 and 50. The  
6 cytoplasmic [Ca<sup>2+</sup>] in cells grown on the coverslips was determined using a dual-excitation  
7 spectrofluorophotometer (CAF-110; JASCO, Tokyo, Japan) with the Ca<sup>2+</sup>  
8 sensitive-ratiometric dye, fura-2-AM. Cells cultured on a coverslip were loaded with 2 μM  
9 fura-2-AM dissolved in Hepes buffer containing 0.005% Pluronic F-127 at 37 °C for 20 min.  
10 CaCl<sub>2</sub> was omitted from the Ca<sup>2+</sup>-free Hepes buffer. The pH of all buffers was adjusted to  
11 7.4. After loading the fura-2-AM, the coverslip was mounted into the folder and washed with  
12 Hepes buffer. The folder was then inserted into the cuvette for the spectrofluorophotometer,  
13 and the experimental liquid was continuously stirred in the cuvette at 1000 rpm at 25°C.  
14 Fluorescent intensities were measured at an emission wavelength of 510 nm, and  
15 excitation wavelengths of 340 and 380 nm. After stabilization of basal fluorescence, cells  
16 were exposed to the test agents. In order to estimate cytoplasmic calcium concentration,  
17 cells were lysed with 0.2% Triton X-100 to obtain the maximum fluorescent ratio (340  
18 nm/380 nm) and then exposed to 10 mM O, O'-Bis(2-aminoethyl)ethyleneglycol-N, N, N',  
19 N'-tetraacetic acid (EGTA) to obtain the minimum fluorescent ratio (340 nm/380 nm). The  
20 cytoplasmic calcium concentration was calculated as described by Grynkiewicz et al. [22],  
21 according to the fluorescent ratio (340 nm/380 nm). The data are expressed as changes in  
22 [Ca<sup>2+</sup>]<sub>i</sub> from basal levels before exposure to the test agents (0 min). Cell viability was  
23 assessed by exposing cells to 40 mM KCl after a challenge with the test agents.

#### 24 *2.5. Intracellular Ca<sup>2+</sup> assay in HEK293 cells expressing mouse CaR.*

1 HEK293 cells were transiently transfected with an expression vector (pcDNA3.1,  
2 Invitrogen) containing a mouse CaR cDNA fragment or an empty vector (mock) using  
3 FuGENE6 transfection reagent (Roche, Basel, Switzerland) for 5-6 hours. Cells were then  
4 trypsinized and seeded in 96-well plates at a density of  $7 \times 10^4$  cells / well for 24 hr. It was  
5 estimated that approximately 60% of the cells were successfully transfected by using  
6 GFP-expressing vector in the experimental condition. Intracellular  $Ca^{2+}$  concentrations  
7 were measured using the FlexStation system and a FLIPR Calcium 3 assay kit (Molecular  
8 devices, Sunnyvale, CA, U.S.A.)

## 9 *2.6. Statistical analysis*

10 Results are expressed as means  $\pm$  SEM. Statistical significance was assessed using  
11 one-way ANOVA and significant differences among mean values were determined by  
12 Tukey's HSD post-hoc test ( $P < 0.05$ ). t-test?

13

## 14 **3. Results**

### 15 *3.1. $\beta$ 51-63 peptide induces CCK secretion in STC-1 cells.*

16 At a normal extracellular  $Ca^{2+}$  concentration (1.2 mM), exposure to the  $\beta$ 51-63 peptide  
17 for 60 min induced dose-dependent CCK secretion in STC-1 cells (Fig. 1). This  
18 demonstrates that  $\beta$ 51-63 peptide can directly act on CCK-producing cells to induce CCK  
19 secretion.  $\beta$ 51-63 peptide at 1.2 mM was the minimum concentration needed to induce a  
20 statistically significant increase in CCK level compared to the control (no  $\beta$ 51-63 peptide).  
21 Therefore, we used  $\beta$ 51-63 peptide mainly at 1.2 mM in the following studies.

### 22 *3.2. Involvement of intracellular calcium mobilization ( $[Ca^{2+}]_i$ ) in $\beta$ 51-63-induced CCK 23 secretion in STC-1 cells.*

24 Exposure to  $\beta$ 51-63 peptide induced dose-dependent elevations in  $[Ca^{2+}]_i$  under normal

1 extracellular  $\text{Ca}^{2+}$  conditions, and the elevation of  $[\text{Ca}^{2+}]_i$  was sustained for more than 30  
2 min (Fig. 2A).  $\beta 51-63$  peptide at 1.2 mM induced a biphasic increase in  $[\text{Ca}^{2+}]_i$  that had an  
3 initial peak at around 1 min and a large elevation peaking at around 25 min after the  
4 exposure. After pre-treatment with an intracellular calcium chelator, BAPTA-AM for 30 min,  
5 exposure to  $\beta 51-63$  peptide did not induce any significant increase in CCK secretion (Fig.  
6 2B).

### 7 *3.3. Extracellular $\text{Ca}^{2+}$ modulates $\beta 51-63$ -induced CCK secretion and intracellular $\text{Ca}^{2+}$* 8 *mobilization*

9 To investigate whether extracellular  $\text{Ca}^{2+}$  affects the  $\beta 51-63$ -induced responses,  
10 intracellular  $\text{Ca}^{2+}$  mobilization and CCK secretion were examined under various  
11 extracellular  $\text{Ca}^{2+}$  concentrations. In the absence of extracellular  $\text{Ca}^{2+}$ ,  $\beta 51-63$  failed to  
12 induce statistically significant increase in CCK secretion (Fig. 3A).  $\beta 51-63$  peptide did not  
13 induce sustained increases in intracellular  $\text{Ca}^{2+}$  concentration under extracellular  $\text{Ca}^{2+}$ -free  
14 conditions, but the initial peak around 1 min after exposure to  $\beta 51-63$  peptide partially  
15 remained (Fig. 3C).  $\beta 51-63$  peptide induced an insignificant increase in CCK secretion in  
16 the presence of 1 mM  $\text{Ca}^{2+}$ , but it induced a significant increase in CCK secretion in the  
17 presence of 2 mM  $\text{Ca}^{2+}$  (Fig. 3B). In order to examine the effect of conditioning with various  
18 extracellular  $\text{Ca}^{2+}$  concentrations on intracellular  $\text{Ca}^{2+}$  responses to  $\beta 51-63$  peptide, STC-1  
19 cells equilibrated with a  $\text{Ca}^{2+}$ -free buffer were pretreated with various extracellular  $\text{Ca}^{2+}$   
20 concentrations (0.1-2.0 mM) for 10 min, and then cells were exposed to 1.2 mM  $\beta 51-63$   
21 peptide. In the presence of 0.1-2.0 mM extracellular  $\text{Ca}^{2+}$ , 1.2 mM  $\beta 51-63$  peptide induced  
22 biphasic intracellular  $\text{Ca}^{2+}$  mobilizations as well as the response shown in Fig. 3C (with 1.2  
23 mM  $\text{Ca}^{2+}$ ). However, peak responses to  $\beta 51-63$  peptide were enhanced dependent on by  
24 increasing extracellular  $\text{Ca}^{2+}$  concentrations (Fig. 3D).

1 *3.4.  $\beta$ 51-63 peptide modulates extracellular  $\text{Ca}^{2+}$ -induced intracellular  $\text{Ca}^{2+}$  mobilization.*

2 STC-1 cells were exposed to  $\beta$ 51-63 peptide and extracellular  $\text{Ca}^{2+}$  in the reverse order  
3 to that employed in the experiments shown in Fig. 3 to examine the effect of  $\beta$ 51-63 peptide  
4 on extracellular  $\text{Ca}^{2+}$ -sensing activity. Cells were exposed to various concentrations of  
5 extracellular  $\text{Ca}^{2+}$  (0.1-2.0 mM) after pretreatment for 20 min with a  $\text{Ca}^{2+}$ -free buffer that  
6 either did or did not contain 1.2 mM  $\beta$ 51-63 peptide. With or without  $\beta$ 51-63 pretreatment,  
7 the addition of extracellular  $\text{Ca}^{2+}$  induced dose-dependent transient increases (peaking  
8 around 1-2 min after exposure) in  $[\text{Ca}^{2+}]_i$ . In the presence of  $\beta$ 51-63 peptide, extracellular  
9  $\text{Ca}^{2+}$  induced a greater increase in intracellular  $\text{Ca}^{2+}$  concentrations (maximum response at  
10  $\sim$ 1000 nM) than those observed in the absence of  $\beta$ 51-63 peptide (maximum response at  
11  $\sim$ 300 nM) (Fig. 4). The data shown in Fig. 3 and 4 demonstrates that  $\beta$ 51-63 peptide and  
12 extracellular  $\text{Ca}^{2+}$  positively modulate cellular responses (CCK secretion and intracellular  
13  $\text{Ca}^{2+}$  mobilization) each other in STC-1 cells. Such modulation ~~modification~~ by extracellular  
14  $\text{Ca}^{2+}$  of responses to the  $\beta$ 51-63 peptide and the basic properties of the  $\beta$ 51-63 peptide  
15 suggest the involvement of a calcium-sensing receptor (CaR), which is activated by  
16 extracellular  $\text{Ca}^{2+}$ , amino acids [11-13], and basic polypeptides [18].

17 *3.5. Calcium-sensing receptor (CaR) mediates  $\beta$ 51-63-induced  $\text{Ca}^{2+}$  mobilization and CCK*  
18 *secretion.*

19 A specific CaR antagonist, NPS2143, was used to determine the involvement of CaR in  
20 the  $\beta$ 51-63-induced intracellular  $\text{Ca}^{2+}$  mobilization and CCK secretion in STC-1 cells. In the  
21 presence of NPS2143 (25  $\mu\text{M}$ ), intracellular  $\text{Ca}^{2+}$  mobilization in response to  $\beta$ 51-63  
22 peptide was strongly suppressed (Fig. 5A), but the initial response remained at a level  
23 similar to that for the vehicle treatment (0.1% DMSO). CCK secretion induced by  $\beta$ 51-63  
24 peptide was significantly decreased but not completely eliminated by the presence of 25

1  $\mu\text{M}$  NPS2143. A bitter taste receptor (T2Rs) agonist, denatonium [23] induced increases in  
2 CCK secretion (not statistically significant by Turkey's test, but significantly different from  
3 blank control by Student's t-test). This increment was not suppressed by NPS2143  
4 treatment (Fig. 5B).

### 5 *3.6. CaR, expressed transiently in HEK293 cells, responds to $\beta$ 51-63 peptide*

6 HEK-293 cells transfected with either a CaR-expressing or an empty vector (mock)  
7 were exposed to  $\beta$ 51-63 peptide or  $\text{Ca}^{2+}$  in the presence of 1 mM extracellular  $\text{Ca}^{2+}$ .  
8 Addition of extracellular  $\text{Ca}^{2+}$  at more than 200  $\mu\text{M}$  (Fig. 6A) and  $\beta$ 51-63 peptide at more  
9 than 20  $\mu\text{M}$  (Fig. 6B) induced increase in intracellular  $\text{Ca}^{2+}$  mobilization in the  
10 CaR-expressing cells in a dose-dependent manner. Intracellular  $\text{Ca}^{2+}$  responses to both  
11 extracellular  $\text{Ca}^{2+}$  and  $\beta$ 51-63 peptide peaked at 5-10 seconds after exposure.  
12 Mock-transfected cells did not respond to either  $\beta$ 51-63 peptide or extracellular  $\text{Ca}^{2+}$  at the  
13 concentrations tested. Intracellular  $\text{Ca}^{2+}$  mobilizations induced by  $\beta$ 51-63 and extracellular  
14  $\text{Ca}^{2+}$  were abolished in the presence of 25  $\mu\text{M}$  NPS2143 (Fig. 6C and D).

### 15 *3.7 Involvement of the $G\alpha_q$ and $G\alpha_i/o$ pathway*

16 Initial peak in  $\beta$ 51-63-induced intracellular  $\text{Ca}^{2+}$  mobilization was decreased by  
17 sarco/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPases (SERCA) blocker, thapsigargin (Fig. 7A).  
18 Treatments with a  $G\alpha_q$  inhibitor, YM-254890 [24], also abolished the initial peak in  $\text{Ca}^{2+}$   
19 mobilization induced by  $\beta$ 51-63 peptide (Fig. 7B), but the latter increment remained and  
20 was tended to be rather higher than that for the vehicle treatment (0.1 % DMSO). CCK  
21 secretion induced by  $\beta$ 51-63 peptide was only slightly decreased by treatment with  
22 YM-254890 (Fig. 7C). PTX treatment for blocking  $G\alpha_i/o$  pathway [5] also did not reduce  
23  $\beta$ 51-63-induced CCK secretion (Fig. 7D).

24

#### 1 4. Discussion

2 It is well known that dietary proteins and peptides in the small intestinal ~~gastrointestinal~~  
3 lumen are physiological stimulants of CCK secretion. The presence of luminal dietary  
4 peptides is thought to be sensed by intestinal CCK-producing I cells. However, the  
5 peptide-sensing mechanism that leads to induction of CCK release is still not well  
6 understood. A recent paper demonstrated that a G protein-coupled receptor (GPCR),  
7 GPR93, functions as a receptor for a peptone [6]. However, another GPCR, CaR, is also  
8 known to sense several basic peptides (protamine, poly-l-lysine, and poly-l-arginine)  
9 [16-18] and a tri-peptide (glutathione) [19]. Very recently, we reported that CaR is  
10 expressed in enteroendocrine STC-1 cells and mediates phenylalanine-induced CCK  
11 secretion [11]. The  $\beta$ 51-63 peptide consists of 13 amino acids (VRIRLLQRFNKRS) and is a  
12 basic peptide containing the arginine cluster. We hypothesized that CaR in STC-1 cells is  
13 also responsive to a basic peptide,  $\beta$ 51-63. In the present study, we demonstrated a novel  
14 function of CaR as a receptor for a dietary peptide,  $\beta$ 51-63, that induces CCK secretion in  
15 enteroendocrine STC-1 cells.

16 Exposure to  $\beta$ 51-63 peptide induced CCK secretion in a dose-dependent manner, and  
17  $\beta$ 51-63 peptide concentrations greater than 1.2 mM induced statistically significant  
18 increments (Fig. 1).  $\beta$ 51-63 peptide also induced biphasic and sustained increase in  
19 intracellular  $\text{Ca}^{2+}$  concentration (Fig. 2A). These results indicate that the  $\beta$ 51-63 peptide  
20 acts directly on CCK-producing cells to trigger intracellular  $\text{Ca}^{2+}$  mobilization and  
21 subsequent CCK secretion, as speculated in our previous experiment in rats [2]. Treatment  
22 with an intracellular  $\text{Ca}^{2+}$  chelator (BAPTA-AM) abolished  $\beta$ 51-63-induced CCK secretion  
23 (Fig. 2B), indicating that intracellular  $\text{Ca}^{2+}$  mobilization is responsible for the CCK secretion.

24 CCK secretion and intracellular  $\text{Ca}^{2+}$  mobilization induced by  $\beta$ 51-63 peptide were

1 reduced by removal of extracellular  $\text{Ca}^{2+}$  (Fig. 3A, C). In the presence of 2 mM  $\text{Ca}^{2+}$ ,  
2  $\beta$ 51-63-induced CCK secretion and intracellular  $\text{Ca}^{2+}$  mobilization appeared to be  
3 increased compared to that in the presence of 1 mM  $\text{Ca}^{2+}$  (Fig. 3B, D). In addition,  $\beta$ 51-63  
4 pretreatment markedly enhanced the sensitivity to extracellular  $\text{Ca}^{2+}$  in STC-1 cells (Fig. 4).  
5 The reason for insignificant difference between intracellular  $\text{Ca}^{2+}$  responses to 1 mM and 2  
6 mM extracellular  $\text{Ca}^{2+}$  may be due to saturation of intracellular  $\text{Ca}^{2+}$  responses in STC-1, as  
7 the maximum response to depolarization stimuli (40 mM KCl) is usually around 1000 nM in  
8 our experiments. These results demonstrate the dependence of  $\beta$ 51-63-induced cellular  
9 responses on extracellular  $\text{Ca}^{2+}$ . Further, these results closely match the CaR  
10 characteristics; that is, receptor activity depends on the presence of extracellular  $\text{Ca}^{2+}$  or  
11 another CaR agonist, as shown in parathyroid cells and HEK 293 cells expressing CaR [12,  
12 13, 18]. Intracellular  $\text{Ca}^{2+}$  responses to extracellular  $\text{Ca}^{2+}$  after pretreatment with  $\beta$ 51-63  
13 peptide (Fig. 4) were also higher than the responses to  $\beta$ 51-63 peptide in the presence of  
14 various extracellular  $\text{Ca}^{2+}$  concentrations (Fig. 3D). Modulation of the receptor activity may  
15 be different between  $\text{Ca}^{2+}$  and  $\beta$ 51-63 peptide, and it also may depend on which ligand is  
16 given first.

17 Treatment with the specific CaR antagonist NPS2143 suppressed  $\beta$ 51-63-induced CCK  
18 secretion and intracellular  $\text{Ca}^{2+}$  mobilization (Fig. 5), indicating that CaR is responsible for  
19  $\beta$ 51-63-induced CCK secretion and intracellular  $\text{Ca}^{2+}$  mobilization in STC-1 cells. In  
20 addition, the transfection of a CaR expression vector allowed HEK293 cells to respond to  
21 the  $\beta$ 51-63 peptide as well as to extracellular  $\text{Ca}^{2+}$  (Fig. 6A and B). Responses to both  
22 extracellular  $\text{Ca}^{2+}$  and  $\beta$ 51-63 peptide in HEK293 cells were effectively blocked by  
23 NPS2143 (Fig. 6C and D). These results strongly support the notion that CaR functions as  
24 a  $\beta$ 51-63 peptide receptor.

1 CCK secretion induced by  $\beta$ 51-63 peptide was not completely inhibited by removal of  
2 extracellular  $\text{Ca}^{2+}$  (Fig. 3A), nor by treatment with NPS2143 (Fig. 5B). In addition to the  
3 result that  $\beta$ 51-63-induced CCK secretion was not saturated (Fig. 1), these results suggest  
4 an involvement of other receptor(s) for  $\beta$ 51-63 detection and CCK secretion. It is possible  
5 that the initial peak in the  $\beta$ 51-63-induced intracellular  $\text{Ca}^{2+}$  mobilization (Fig. 3B and 5A) is  
6 responsible for the CCK secretion observed in the absence of extracellular  $\text{Ca}^{2+}$  (Fig. 3A)  
7 and in the presence of NPS2143 (Fig. 5B). This peak was abolished by treatments with  
8 specific  $G\alpha_q$  inhibitor YM-254890 or sarco/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPases (SERCA)  
9 blocker thapsigargin. But the sustained increase in intracellular  $\text{Ca}^{2+}$  concentrations and  
10 CCK secretion induced by  $\beta$ 51-63 peptide were not reduced (Fig.7A-C). These results  
11 suggest that the  $G\alpha_q$ -IP<sub>3</sub> pathway is in some way related to  $\beta$ 51-63-induced CCK secretion  
12 but is not the main pathway.  $\beta$ 51-63-induced CCK secretion was not attenuated by PTX  
13 (Fig. 7D), suggesting the  $G\alpha_i/o$  pathway is not involved. Several papers reported that CaR  
14 mediates increases in  $[\text{Ca}^{2+}]_i$  and diacyl glycerol production through coupling to  $G\alpha_q$  or  
15  $G\alpha_i/o$  [25, 26]. However, the downstream pathways of CaR may differ among cell types. It  
16 is possible that CaR in STC-1 cells couples to another G-protein ( $G\alpha_{\text{gust}}$  and/or  $G\alpha_{12/13}$ )  
17 such as taste receptors [27]. ~~or interacts to other? signal transducer (IP3R, PLCg1, PKC)~~  
18 ~~directly as well as keratinocyte and griptite 293 MSR cells (ref).~~

19 A recent paper demonstrated an insignificant increase in plasma CCK resulting from  
20 treatment with a CaR agonist (cinacalcet) in patients with secondary hyperparathyroidism  
21 [28]. However, the plasma CCK response to a test meal tended to increase in the  
22 cinacalcet treatment group. This supports the notion that CaR mediates CCK secretion in  
23 the human intestine. Only one paper has shown that enteroendocrine cells in the human  
24 colon express CaR [29], but the function and specific gut hormone in those cells are

1 unknown. Further studies on the role of CaR in enteroendocrine cells *in vivo* (in rats, mice,  
2 and humans) are necessary.

3 In summary, soybean  $\beta$ 51-63 peptide induced CCK secretion and intracellular  $\text{Ca}^{2+}$   
4 mobilization in enteroendocrine CCK-producing STC-1 cells.  $\beta$ 51-63-induced CCK  
5 secretion and  $\text{Ca}^{2+}$  mobilization were modulated by increases in extracellular  $\text{Ca}^{2+}$   
6 concentration, and a specific CaR antagonist blocked the  $\beta$ 51-63-induced responses. In  
7 addition, CaR-expressing HEK-293 cells acquired sensitivity to both extracellular  $\text{Ca}^{2+}$  and  
8  $\beta$ 51-63 peptide. These results reveal that CaR functions as a peptide  $\beta$ 51-63 receptor that  
9 induces intracellular  $\text{Ca}^{2+}$  mobilization and subsequent CCK secretion in enteroendocrine  
10 cells.

11

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15 YM-254890, respectively.

16

17

18

- 1 **Reference** ~追加すると本文中の番号も変えることになるので、間違いの無いように。
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12  
13

## 1 **Figure legends**

2 Fig. 1. CCK secretion in response to  $\beta$ 51-63 peptide in STC-1 cells.

3 STC-1 cells cultured in 48-well plates were exposed to various concentrations of  $\beta$ 51-63  
4 peptide (0.3 – 2.4 mM) in HEPES buffer containing 1.2 mM  $\text{Ca}^{2+}$  for 60 min. CCK  
5 concentrations in the supernatant were measured by EIA. Values are means  $\pm$  SEM of 3-4  
6 wells. Bars not sharing the same letters differ significantly ( $P < 0.05$  by Tukey's post-hoc  
7 test).

8

9 Fig. 2. Involvement of intracellular  $\text{Ca}^{2+}$  mobilization in  $\beta$ 51-63-induced CCK secretion in  
10 STC-1 cells.

11 A: STC-1 cells cultured on coverslips were loaded with fura-2-AM. Intracellular  $\text{Ca}^{2+}$   
12 concentrations were measured ratiometrically using a spectrofluorometer. Cells were  
13 exposed to 0.012-1.2 mM  $\beta$ 51-63 peptide in HEPES buffer containing 1.2 mM  $\text{Ca}^{2+}$  at 0 min.  
14 Data are expressed as changes from basal state (0 min) ( $\Delta[\text{Ca}^{2+}]_i$ ). Values are means of 4  
15 experiments. B: STC-1 cells cultured in 48-well plates were exposed to 1.2 mM  $\beta$ 51-63  
16 peptide for 60 min after pretreatment with intracellular  $\text{Ca}^{2+}$  chelator BAPTA-AM (50  $\mu\text{M}$ ) or  
17 vehicle (0.1% DMSO, dimethyl sulfoxide) for 30 min. CCK concentrations in the  
18 supernatant were measured by EIA. Values are means  $\pm$  SEM of 4 wells. Bars not sharing  
19 the same letters differ significantly ( $P < 0.05$  by Tukey's post-hoc test).

20

21 Fig. 3. Effect of various extracellular  $\text{Ca}^{2+}$  concentrations on  $\beta$ 51-63-induced CCK secretion  
22 and intracellular  $\text{Ca}^{2+}$  mobilization.

23 STC-1 cells cultured in 48-well plates were equilibrated with a  $\text{Ca}^{2+}$ -free buffer. Cells were  
24 then exposed to the  $\beta$ 51-63 peptide in buffer containing 0 or 1.2 mM  $\text{Ca}^{2+}$  (A) and 1.0 or 2.0

1 mM Ca<sup>2+</sup> (B) for 60 min. Values are means ± SEM of 4 wells. Bars not sharing the same  
2 letters differ significantly ( $P < 0.05$  by Tukey's post-hoc test). Fura-2-AM-loaded STC-1 cells  
3 were equilibrated with the buffer in the absence of Ca<sup>2+</sup> and were exposed to 1.2 mM  
4 β51-63 peptide for 30 min in Hepes buffer with 0-1.2 mM Ca<sup>2+</sup> (C). Data are expressed as  
5 changes from basal state (0 min) ( $\Delta[\text{Ca}^{2+}]_i$ ). STC-1 cells were pretreated with 0.1-2 mM  
6 extracellular Ca<sup>2+</sup> for 10 min after equilibration with Ca<sup>2+</sup>-free condition. Then, cells were  
7 exposed to 1.2 mM β51-63 peptide for 30 min (D). The data are expressed as maximum  
8 changes induced by β51-63 peptide from basal state (0 min) ( $\Delta[\text{Ca}^{2+}]_i$ ). Values are means  
9 of 3-4 experiments. Plots not sharing the same letters differ significantly ( $P < 0.05$  by  
10 Tukey's post-hoc test).

11

12 Fig. 4. Effect of β51-63 peptide on extracellular Ca<sup>2+</sup>-sensing in STC-1 cells.

13 STC-1 cells were pretreated with a Ca<sup>2+</sup>-free buffer containing or not containing 1.2 mM  
14 β51-63 peptide for 20 min. Cells were then exposed to various concentrations of  
15 extracellular Ca<sup>2+</sup> (0.1-2 mM) for 10 min. The data are expressed as maximum changes in  
16 intracellular Ca<sup>2+</sup> concentration induced by extracellular Ca<sup>2+</sup> from basal state (0 min)  
17 ( $\Delta[\text{Ca}^{2+}]_i$ ). Values are means of 4 experiments. Plots not sharing the same letters differ  
18 significantly ( $P < 0.05$  by Tukey's post-hoc test).

19

20 Fig. 5. Involvement of CaR in β51-63-induced intracellular Ca<sup>2+</sup> mobilization and CCK  
21 secretion.

22 A: Cells were exposed to 1.2 mM β51-63 peptide in the presence of 25 μM NPS2143 or  
23 vehicle (0.1% DMSO). Data are expressed as changes from basal state (0 min) ( $\Delta[\text{Ca}^{2+}]_i$ ).

24 Values are means of 4 experiments. B: STC-1 cells were exposed to 1.2 mM β51-63

1 peptide and 5 mM denatonium in the presence of CaR antagonist NPS2143 (25  $\mu$ M) or  
2 vehicle (0.1% DMSO). Values are means  $\pm$  SEM of 4-8 wells. Bars not sharing the same  
3 letters differ significantly ( $P < 0.05$  by Tukey's post-hoc test).

4

5 Fig. 6. Effects of extracellular  $\text{Ca}^{2+}$  and  $\beta$ 51-63 peptide on intracellular  $\text{Ca}^{2+}$  mobilization in  
6 HEK-293 cells transfected with CaR.

7 HEK-293 cells were transfected with mouse CaR expression plasmid. Mock-transfection  
8 was carried out using an empty plasmid. Cells were loaded with fluo-3-AM in 96-well culture  
9 plate, and intracellular  $\text{Ca}^{2+}$  concentrations were measured using the FlexStation system  
10 and a FLIPR Calcium 3 assay kit. Cells were equilibrated with 1 mM extracellular  $\text{Ca}^{2+}$ , and  
11 then exposed to additional  $\text{Ca}^{2+}$  or  $\beta$ 51-63 peptide (10-1000  $\mu$ M). HEK293 transfected with  
12 CaR were exposed to extracellular  $\text{Ca}^{2+}$  (C) and  $\beta$ 51-63 peptide (D) in the presence of  
13 NPS2143. Values are the peak fluorescence observed in response to addition of  
14 extracellular  $\text{Ca}^{2+}$  (A, C) or  $\beta$ 51-63 peptide (B, D), and are means of 3 wells.

15

16 Fig. 7. Effects of thapsigargin and G protein inhibitors on  $\beta$ 51-63-induced intracellular  $\text{Ca}^{2+}$   
17 mobilization or CCK secretion.

18 STC-1 cells were exposed to 1.2 mM  $\beta$ 51-63 in the presence of 5  $\mu$ M YM-254980 (A) or 10  
19  $\mu$ M Thapsigargin (B). Data are expressed as changes from basal state (0 min) ( $\Delta[\text{Ca}^{2+}]_i$ ),  
20 and as means of 3 experiments. STC-1 cells were exposed to 1.2 mM  $\beta$ 51-63 in the  
21 presence of 5  $\mu$ M YM-254980 (C) or after pretreatment with 200 ng/ml PTX for 5 hours (D).  
22 CCK concentrations in the supernatants were measured by EIA. Values are means  $\pm$  SEM  
23 of 3-4 wells. Bars not sharing the same letters differ significantly ( $P < 0.05$  by Tukey's  
24 post-hoc test).

Fig.1

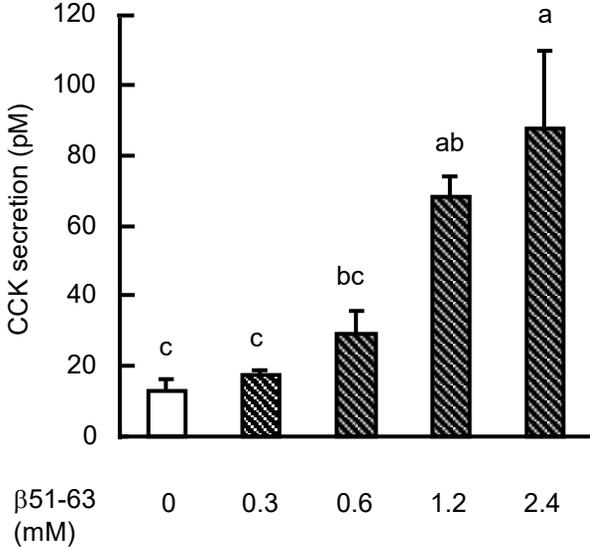


Fig.2

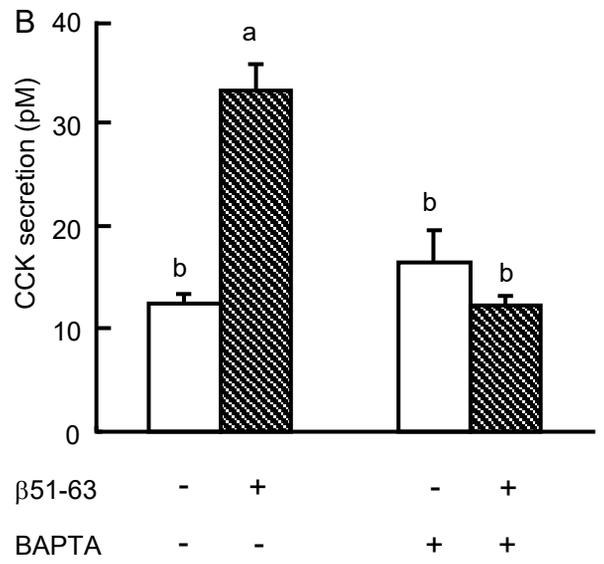
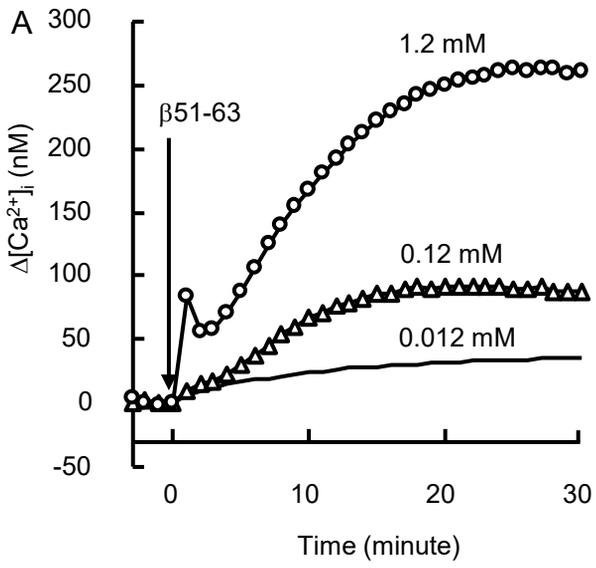


Fig. 3

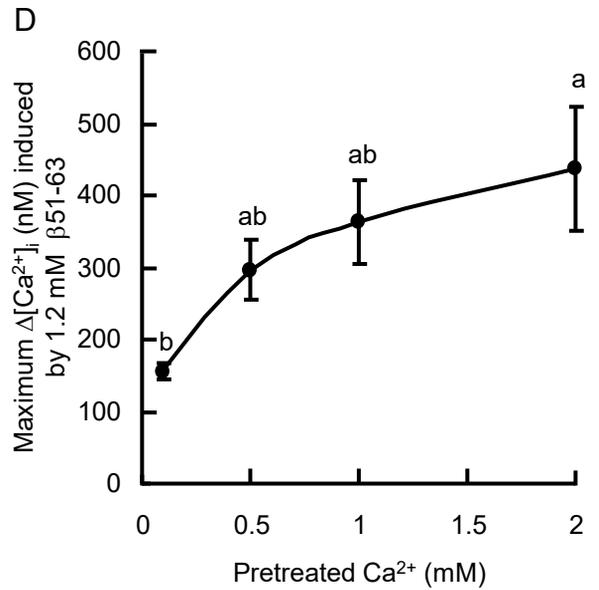
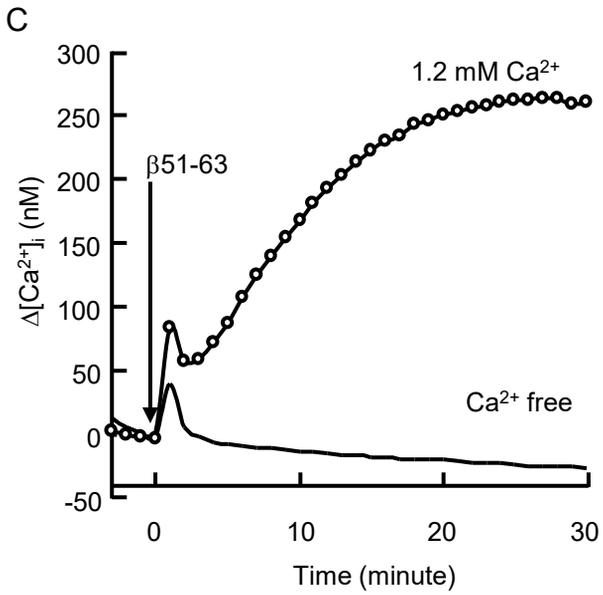
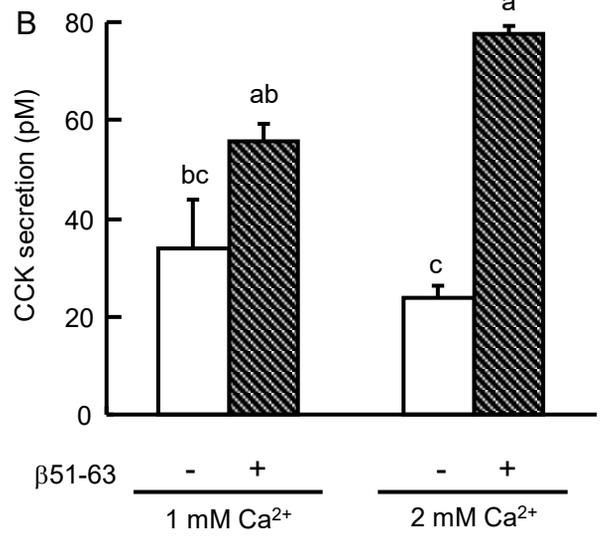
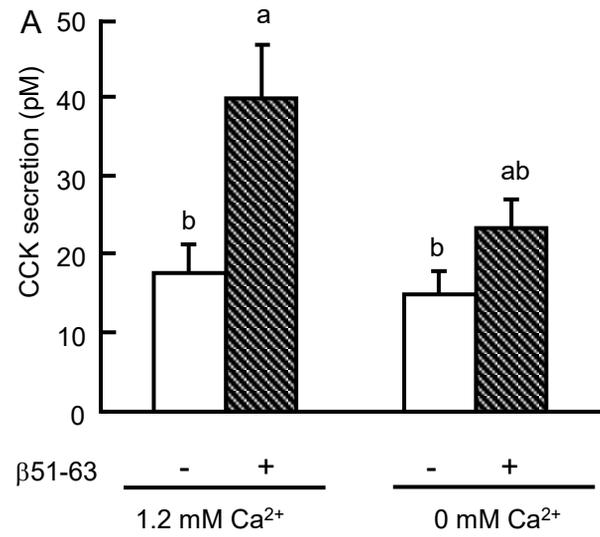


Fig. 4

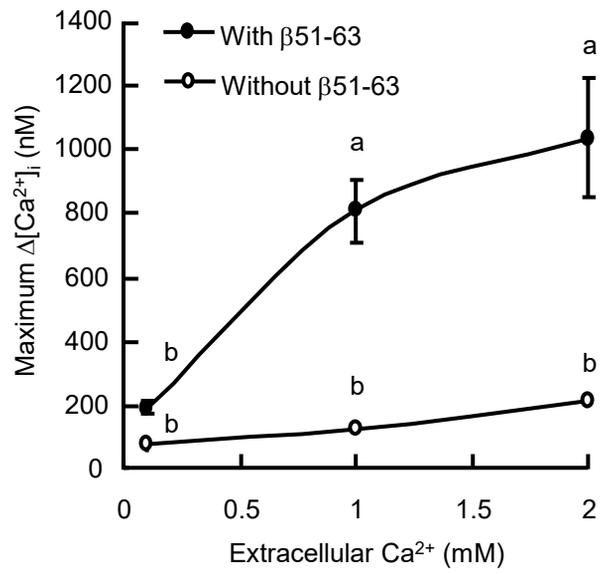


Fig. 5

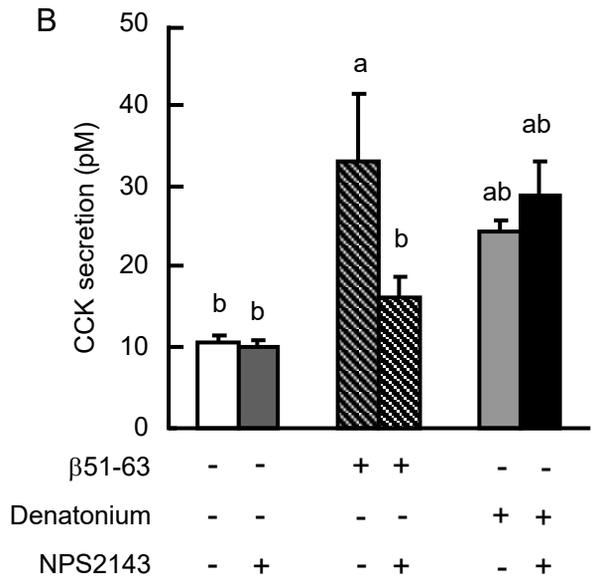
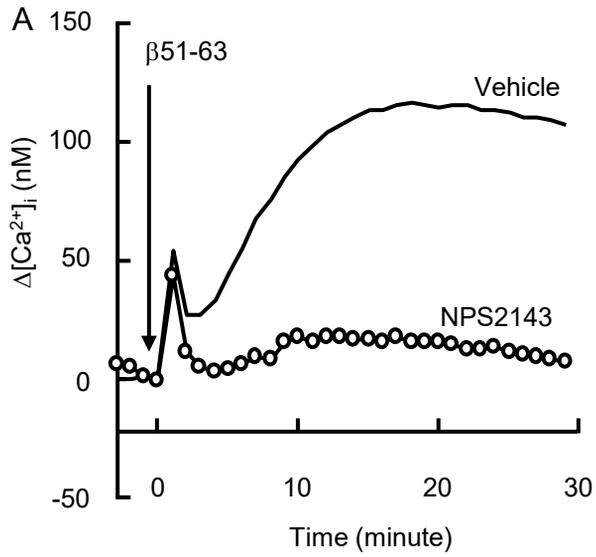


Fig. 6

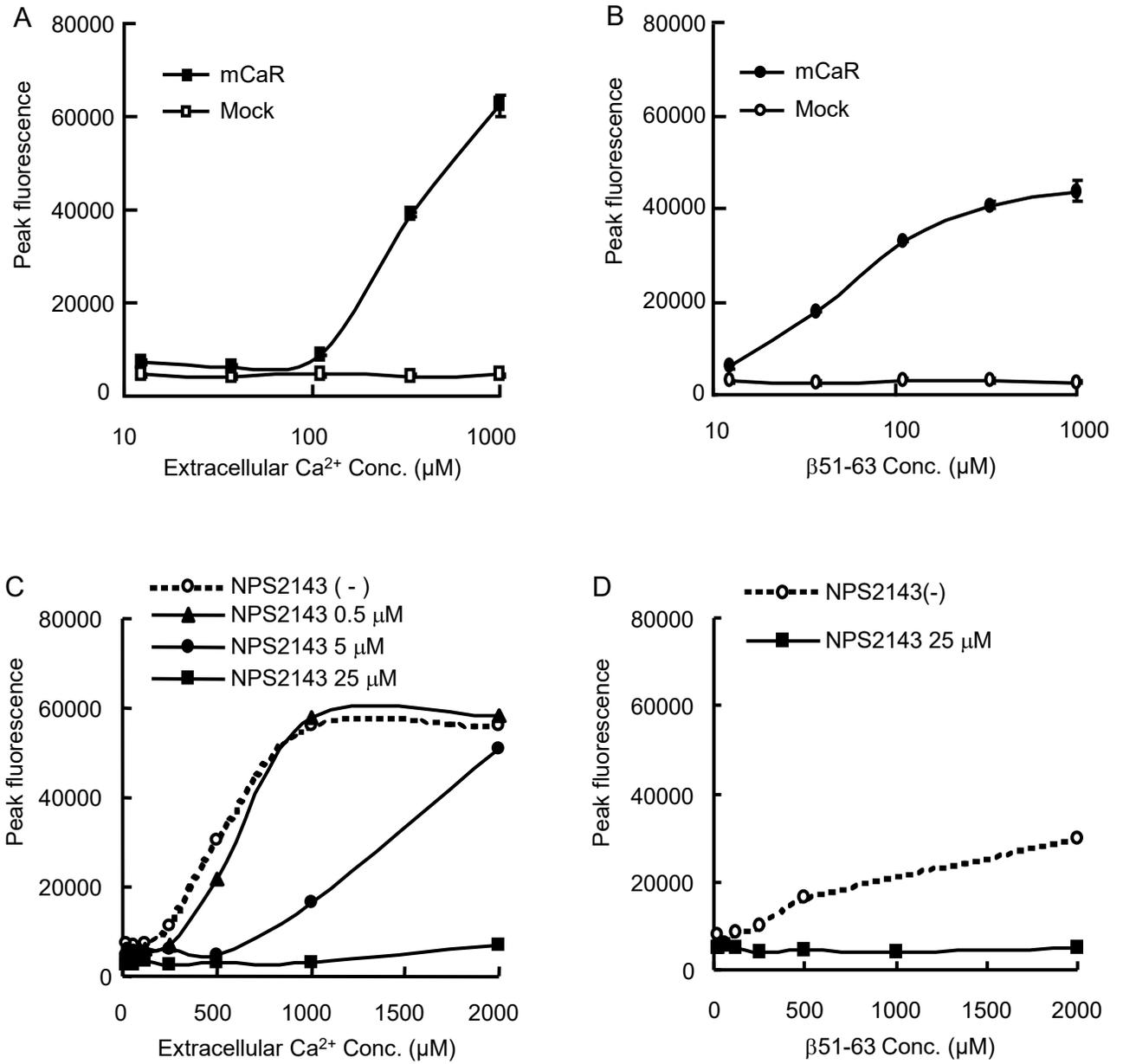


Fig. 7

