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Soybean β51-63 peptide stimulates cholecystokinin secretion via a calcium-sensing receptor in enteroendocrine STC-1 cells

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Summary

We previously demonstrated that intraduodenal administration of an arginine-rich β51-63 peptide in soybean β-conglycinin suppresses food intake via cholecystokinin (CCK) secretion in rats. However, the cellular mechanisms by which the β51-63 peptide induces CCK secretion remain to be clarified. In the present study, we examined whether the extracellular calcium-sensing receptor (CaR) mediates β51-63-induced CCK secretion in

murine CCK-producing enteroendocrine cell–line STC-1. CCK secretion and changes in intracellular Ca^{2+} concentration in response to β51-63 peptide were measured in STC-1 cells under various extracellular Ca^{2+} concentrations and after treatment with a CaR antagonist. Intracellular Ca^{2+} concentrations in response to β51-63 peptide and extracellular Ca^{2+} were also measured in CaR-expressing human embryonic kidney (HEK293) cells. The β51-63 peptide induced CCK secretion and intracellular Ca^{2+} mobilization in STC-1 cells under normal (1.2 mM) extracellular Ca^{2+} conditions in a dose-dependent manner. These responses to β51-63 peptide were reduced by the removal of intra- or extra-cellular Ca^{2+} but enhanced by increasing extracellular Ca^{2+} concentrations. Intracellular Ca^{2+} mobilization induced by extracellular Ca^{2+} was also increased by the pretreatment with β51-63 peptide. Treatment with a specific CaR antagonist (NPS2143) inhibited β51-63-induced CCK secretion and intracellular Ca^{2+} mobilization. In addition, HEK293 cells transfected with CaR acquired sensitivity to the β51-63 peptide. From these results, we conclude that CaR is the β51-63 peptide sensor responsible for the stimulation of CCK secretion in enteroendocrine STC-1 cells.

Abbreviations.

Cholecystokinin; CCK, Calcium-sensing receptor; CaR

Key words.

β51-63 peptide, Enteroendocrine cells, Calcium-sensing receptor, Cholecystokinin

1. Introduction

Cholecystokinin (CCK) is a gut hormone produced by enteroendocrine 'I cells' located in the upper small intestine, which is known to regulate gallbladder contraction, pancreatic secretion, gastric emptying, and food intake. In our previous study, duodenal infusion of a peptone prepared from soybean β-conglycinin reduced food intake via CCK secretion in rats [1]. Furthermore, an arginine-rich fragment, β51-63, in the β-subunit of β-conglycinin was identified as one of the active peptides [2]. However, whether the β51-63 peptide acts directly on CCK-producing cells is not known. In addition, the mechanism by which the β51-63 peptide stimulates CCK secretion from CCK-producing cells remains unclear.

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

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

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

In the present study, we examined whether β51-63 peptide could stimulate CCK secretion in STC-1 cells and then investigated the intracellular signaling pathways involved in β51-63-induced CCK secretion. The involvement of extracellular Ca^{2+} and CaR in the β51-63-induced cellular responses (CCK secretion and intracellular Ca^{2+} mobilization) was also investigated in STC-1 cells and CaR-expressing HEK-293 cells to determine the role of CaR in the peptide-sensing mechanism.

2. Materials and Methods

2.1. Materials

Cell culture consumables (Dulbecco's modified Eagle's medium, fetal bovine serum, penicillin/streptomycin, and trypsin-EDTA solution) were purchased from Invitrogen (Carlsbad, CA). The β51-63 peptide (VRIRLLQRFNKRS) was synthesized by Bachem (Bubendolf, Switzerland). 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 [20]. YM-254890 was provided by Astellas Pharma Inc (Tokyo, Japan). Poly-L-lysine solution (0.1%), Hepes, thapsigargin, pertussis toxin (PTX) and denatonium benzoate were purchased from Sigma (St. Louis, MO). *O*, *O*'-Bis (2-aminophenyl) ethylenglycol-*N, N, N', N'*-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM) was purchased from Dojinndo (Kumamoto, Japan). Other reagents were purchased from Wako (Osaka, Japan) unless

specified.

2.2. 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. HEK293 cells (JCRB9068) were purchased from Human Science Research Resources Bank (Osaka, Japan), and cells were grown in DMEM/F12 medium containing 10% FCS.

2.3. 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. After incubation for 60 min, supernatants from the 48-well culture plates were collected and centrifuged at 800 x g for 5 min at 4°C to remove the remaining cells, and then stored at -50°C until CCK concentration measurement with a commercial enzyme immunoassay (EIA) 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), and big gastrin-1 (human); 12.6% with CCK (30-33); and 0% with pancreatic polypeptide (human) and VIP (human, porcine, rat). Since STC-1 cells do not express detectable levels of gastrin [21], we used an EIA kit in which the antibody cross-reacts with gastrin. The coefficients of intra-assay and inter-assay variation are < 5%

and < 14%, respectively.

2.4. Intracellular Ca^{2+} measurement in STC-1 cells

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 30 and 50. The cytoplasmic $[Ca^{2+}]$ in cells grown on the coverslips was determined using a dual-excitation spectrofluorophotometer (CAF-110; JASCO, Tokyo, Japan) with the Ca^{2+} 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. 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 spectrofluorophotometer, 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 *O, O*'-Bis(2-aminoethyl)ethyleneglycol-*N, N, N′, N′*-tetraacetic acid (EGTA) to obtain the minimum fluorescent ratio (340 nm/380 nm). The cytoplasmic calcium concentration was calculated as described by Grynkiewies et al. [22], according to the fluorescent ratio (340 nm/380 nm). The 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.

2.5. Intracellular Ca^{2+} assay in HEK293 cells expressing mouse CaR.

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

2.6. 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 Tukey's HSD post-hoc test ($P < 0.05$). t-test?

3. Results

3.1. β51-63 peptide induces CCK secretion in STC-1 cells.

At a normal extracellular Ca^{2+} concentration (1.2 mM), exposure to the β51-63 peptide for 60 min induced dose-dependent CCK secretion in STC-1 cells (Fig. 1). This demonstrates that β51-63 peptide can directly act on CCK-producing cells to induce CCK secretion. β51-63 peptide at 1.2 mM was the minimum concentration needed to induce a statistically significant increase in CCK level compared to the control (no β51-63 peptide). Therefore, we used β51-63 peptide mainly at 1.2 mM in the following studies.

3.2. Involvement of intracellular calcium mobilization ($[Ca^{2+}]_i$) in β51-63-induced CCK secretion in STC-1 cells.

Exposure to β51-63 peptide induced dose-dependent elevations in $[Ca^{2+}]_i$ under normal

extracellular Ca^{2+} conditions, and the elevation of $[Ca^{2+}]_i$ was sustained for more than 30 min (Fig. 2A). β51-63 peptide at 1.2 mM induced a biphasic increase in $[Ca^{2+}]_i$ that had an initial peak at around 1 min and a large elevation peaking at around 25 min after the exposure. After pre-treatment with an intracellular calcium chelator, BAPTA-AM for 30 min, exposure to β51-63 peptide did not induce any significant increase in CCK secretion (Fig. 2B).

3.3. Extracellular Ca^{2+} modulates β51-63-induced CCK secretion and intracellular Ca^{2+} mobilization

To investigate whether extracellular Ca^{2+} affects the β51-63-induced responses, intracellular Ca^{2+} mobilization and CCK secretion were examined under various extracellular Ca^{2+} concentrations. In the absence of extracellular Ca^{2+}, β51-63 failed to induce statistically significant increase in CCK secretion (Fig. 3A). β51-63 peptide did not induce sustained increases in intracellular Ca^{2+} concentration under extracellular Ca^{2+}-free conditions, but the initial peak around 1 min after exposure to β51-63 peptide partially remained (Fig. 3C). β51-63 peptide induced an insignificant increase in CCK secretion in the presence of 1 mM Ca^{2+}, but it induced a significant increase in CCK secretion in the presence of 2 mM Ca^{2+} (Fig. 3B). In order to examine the effect of conditioning with various extracellular Ca^{2+} concentrations on intracellular Ca^{2+} responses to β51-63 peptide, STC-1 cells equilibrated with a Ca^{2+}-free buffer were pretreated with various extracellular Ca^{2+} concentrations (0.1-2.0 mM) for 10 min, and then cells were exposed to 1.2 mM β51-63 peptide. In the presence of 0.1-2.0 mM extracellular Ca^{2+}, 1.2 mM β51-63 peptide induced biphasic intracellular Ca^{2+} mobilizations as well as the response shown in Fig. 3C (with 1.2 mM Ca^{2+}). However, peak responses to β51-63 peptide were enhanced dependent on ~~by increasing~~ extracellular Ca^{2+} concentrations (Fig. 3D).

3.4. β51-63 peptide modulates extracellular Ca^{2+}-induced intracellular Ca^{2+} mobilization.

STC-1 cells were exposed to β51-63 peptide and extracellular Ca^{2+} in the reverse order to that employed in the experiments shown in Fig. 3 to examine the effect of β51-63 peptide on extracellular Ca^{2+}-sensing activity. Cells were exposed to various concentrations of extracellular Ca^{2+} (0.1-2.0 mM) after pretreatment for 20 min with a Ca^{2+}-free buffer that either did or did not contain 1.2 mM β51-63 peptide. With or without β51-63 pretreatment, the addition of extracellular Ca^{2+} induced dose-dependent transient increases (peaking around 1-2 min after exposure) in $[Ca^{2+}]_i$. In the presence of β51-63 peptide, extracellular Ca^{2+} induced a greater increase in intracellular Ca^{2+} concentrations (maximum response at ~1000 nM) than those observed in the absence of β51-63 peptide (maximum response at ~300 nM) (Fig. 4). The data shown in Fig. 3 and 4 demonstrates that β51-63 peptide and extracellular Ca^{2+} positively modulate cellular responses (CCK secretion and intracellular Ca^{2+} mobilization) each other in STC-1 cells. Such modulation ~~modification~~ by extracellular Ca^{2+} of responses to the β51-63 peptide and the basic properties of the β51-63 peptide suggest the involvement of a calcium-sensing receptor (CaR), which is activated by extracellular Ca^{2+}, amino acids [11-13], and basic polypeptides [18].

3.5. Calcium-sensing receptor (CaR) mediates β51-63-induced Ca^{2+} mobilization and CCK secretion.

A specific CaR antagonist, NPS2143, was used to determine the involvement of CaR in the β51-63-induced intracellular Ca^{2+} mobilization and CCK secretion in STC-1 cells. In the presence of NPS2143 (25 μM), intracellular Ca^{2+} mobilization in response to β51-63 peptide was strongly suppressed (Fig. 5A), but the initial response remained at a level similar to that for the vehicle treatment (0.1% DMSO). CCK secretion induced by β51-63 peptide was significantly decreased but not completely eliminated by the presence of 25

μM NPS2143. A bitter taste receptor (T2Rs) agonist, denatonium [23] induced increases in CCK secretion (not statistically significant by Turkey's test, but significantly different from blank control by Student's t-test). This increment was not suppressed by NPS2143 treatment (Fig. 5B).

3.6. CaR, expressed transiently in HEK293 cells, responds to β51-63 peptide

HEK-293 cells transfected with either a CaR-expressing or an empty vector (mock) were exposed to β51-63 peptide or Ca^{2+} in the presence of 1 mM extracellular Ca^{2+}. Addition of extracellular Ca^{2+} at more than 200 μM (Fig. 6A) and β51-63 peptide at more than 20 μM (Fig. 6B) induced increase in intracellular Ca^{2+} mobilization in the CaR-expressing cells in a dose-dependent manner. Intracellular Ca^{2+} responses to both extracellular Ca^{2+} and β51-63 peptide peaked at 5-10 seconds after exposure. Mock-transfected cells did not respond to either β51-63 peptide or extracellular Ca^{2+} at the concentrations tested. Intracellular Ca^{2+} mobilizations induced by β51-63 and extracellular Ca^{2+} were abolished in the presence of 25 μM NPS2143 (Fig. 6C and D).

3.7 Involvement of the Gαq and Gαi/o pathway

Initial peak in β51-63-induced intracellular Ca^{2+} mobilization was decreased by sarco/endoplasmic reticulum Ca^{2+}-ATPases (SERCA) blocker, thapsigargin (Fig. 7A). Treatments with a Gαq inhibitor, YM-254890 [24], also abolished the initial peak in Ca^{2+} mobilization induced by β51-63 peptide (Fig. 7B), but the latter increment remained and was tended to be rather higher than that for the vehicle treatment (0.1 % DMSO). CCK secretion induced by β51-63 peptide was only slightly decreased by treatment with YM-254890 (Fig. 7C). PTX treatment for blocking Gαi/o pathway [5] also did not reduce β51-63-induced CCK secretion (Fig. 7D).

4. Discussion

It is well known that dietary proteins and peptides in the small intestinal ~~gastrointestinal~~ lumen are physiological stimulants of CCK secretion. The presence of luminal dietary peptides is thought to be sensed by intestinal CCK-producing I cells. However, the peptide-sensing mechanism that leads to induction of CCK release is still not well understood. A recent paper demonstrated that a G protein-coupled receptor (GPCR), GPR93, functions as a receptor for a peptone [6]. However, another GPCR, CaR, is also known to sense several basic peptides (protamine, poly-l-lysine, and poly-l-arginine) [16-18] and a tri-peptide (glutathione) [19]. Very recently, we reported that CaR is expressed in enteroendocrine STC-1 cells and mediates phenylalanine-induced CCK secretion [11]. The β51-63 peptide consists of 13 amino acids (VRIRLLQRFNKRS) and is a basic peptide containing the arginine cluster. We hypothesized that CaR in STC-1 cells is also responsive to a basic peptide, β51-63. In the present study, we demonstrated a novel function of CaR as a receptor for a dietary peptide, β51-63, that induces CCK secretion in enteroendocrine STC-1 cells.

Exposure to β51-63 peptide induced CCK secretion in a dose-dependent manner, and β51-63 peptide concentrations greater than 1.2 mM induced statistically significant increments (Fig. 1). β51-63 peptide also induced biphasic and sustained increase in intracellular Ca^{2+} concentration (Fig. 2A). These results indicate that the β51-63 peptide acts directly on CCK-producing cells to trigger intracellular Ca^{2+} mobilization and subsequent CCK secretion, as speculated in our previous experiment in rats [2]. Treatment with an intracellular Ca^{2+} chelator (BAPTA-AM) abolished β51-63-induced CCK secretion (Fig. 2B), indicating that intracellular Ca^{2+} mobilization is responsible for the CCK secretion.

CCK secretion and intracellular Ca^{2+} mobilization induced by β51-63 peptide were

reduced by removal of extracellular Ca^{2+} (Fig. 3A, C). In the presence of 2 mM Ca^{2+}, β51-63-induced CCK secretion and intracellular Ca^{2+} mobilization appeared to be increased compared to that in the presence of 1 mM Ca^{2+} (Fig. 3B, D). In addition, β51-63 pretreatment markedly enhanced the sensitivity to extracellular Ca^{2+} in STC-1 cells (Fig. 4). The reason for insignificant difference between intracellular Ca^{2+} responses to 1 mM and 2 mM extracellular Ca^{2+} may be due to saturation of intracellular Ca^{2+} responses in STC-1, as the maximum response to depolarization stimuli (40 mM KCl) is usually around 1000 nM in our experiments. These results demonstrate the dependence of β51-63-induced cellular responses on extracellular Ca^{2+}. Further, these results closely match the CaR characteristics; that is, receptor activity depends on the presence of extracellular Ca^{2+} or another CaR agonist, as shown in parathyroid cells and HEK 293 cells expressing CaR [12, 13, 18]. Intracellular Ca^{2+} responses to extracellular Ca^{2+} after pretreatment with β51-63 peptide (Fig. 4) were also higher than the responses to β51-63 peptide in the presence of various extracellular Ca^{2+} concentrations (Fig. 3D). Modulation of the receptor activity may be different between Ca^{2+} and β51-63 peptide, and it also may depend on which ligand is given first.

Treatment with the specific CaR antagonist NPS2143 suppressed β51-63-induced CCK secretion and intracellular Ca^{2+} mobilization (Fig. 5), indicating that CaR is responsible for β51-63-induced CCK secretion and intracellular Ca^{2+} mobilization in STC-1 cells. In addition, the transfection of a CaR expression vector allowed HEK293 cells to respond to the β51-63 peptide as well as to extracellular Ca^{2+} (Fig. 6A and B). Responses to both extracellular Ca^{2+} and β51-63 peptide in HEK293 cells were effectively blocked by NPS2143 (Fig. 6C and D). These results strongly support the notion that CaR functions as a β51-63 peptide receptor.

CCK secretion induced by β51-63 peptide was not completely inhibited by removal of extracellular Ca^{2+} (Fig. 3A), nor by treatment with NPS2143 (Fig. 5B). In addition to the result that β51-63-induced CCK secretion was not saturated (Fig. 1), these results suggest an involvement of other receptor(s) for β51-63 detection and CCK secretion. It is possible that the initial peak in the β51-63-induced intracellular Ca^{2+} mobilization (Fig. 3B and 5A) is responsible for the CCK secretion observed in the absence of extracellular Ca^{2+} (Fig. 3A) and in the presence of NPS2143 (Fig. 5B). This peak was abolished by treatments with specific Gαq inhibitor YM-254890 or sarco/endoplasmic reticulum Ca^{2+}-ATPases (SERCA) blocker thapsigargin. But the sustained increase in intracellular Ca^{2+} concentrations and CCK secretion induced by β51-63 peptide were not reduced (Fig.7A-C). These results suggest that the Gαq-IP_3 pathway is in some way related to β51-63-induced CCK secretion but is not the main pathway. β51-63-induced CCK secretion was not attenuated by PTX (Fig. 7D), suggesting the Gαi/o pathway is not involved. Several papers reported that CaR mediates increases in $[Ca^{2+}]_i$ and diacyl glycerol production through coupling to Gαq or Gαi/o [25, 26]. However, the downstream pathways of CaR may differ among cell types. It is possible that CaR in STC-1 cells couples to another G-protein (Gαgust and/or Gα12/13) such as taste receptors [27]. ~~or interacts to other? signal transducer (IP3R, PLCg1, PKC) directly as well as keratinocyte and griptite 293 MSR cells (ref).~~

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

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

In summary, soybean β51-63 peptide induced CCK secretion and intracellular Ca^{2+} mobilization in enteroendocrine CCK-producing STC-1 cells. β51-63-induced CCK secretion and Ca^{2+} mobilization were modulated by increases in extracellular Ca^{2+} concentration, and a specific CaR antagonist blocked the β51-63-induced responses. In addition, CaR-expressing HEK-293 cells acquired sensitivity to both extracellular Ca^{2+} and β51-63 peptide. These results reveal that CaR functions as a peptide β51-63 receptor that induces intracellular Ca^{2+} mobilization and subsequent CCK secretion in enteroendocrine cells.

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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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Figure legends

Fig. 1. CCK secretion in response to β51-63 peptide in STC-1 cells.

STC-1 cells cultured in 48-well plates were exposed to various concentrations of β51-63 peptide (0.3 – 2.4 mM) in Hepes buffer containing 1.2 mM Ca^{2+} for 60 min. CCK concentrations in the supernatant were measured by EIA. Values are means ± SEM of 3-4 wells. Bars not sharing the same letters differ significantly ($P < 0.05$ by Tukey's post-hoc test).

Fig. 2. Involvement of intracellular Ca^{2+} mobilization in β51-63-induced CCK secretion in STC-1 cells.

A: STC-1 cells cultured on coverslips were loaded with fura-2-AM. Intracellular Ca^{2+} concentrations were measured ratiometrically using a spectrofluorometer. Cells were exposed to 0.012-1.2 mM β51-63 peptide in Hepes buffer containing 1.2 mM Ca^{2+} at 0 min. Data are expressed as changes from basal state (0 min) ($\Delta[Ca^{2+}]_i$). Values are means of 4 experiments. B: STC-1 cells cultured in 48-well plates were exposed to 1.2 mM β51-63 peptide for 60 min after pretreatment with intracellular Ca^{2+} chelator BAPTA-AM (50 μM) or vehicle (0.1% DMSO, dimethyl sulfoxide) for 30 min. CCK concentrations in the supernatant were measured by EIA. Values are means ± SEM of 4 wells. Bars not sharing the same letters differ significantly ($P < 0.05$ by Tukey's post-hoc test).

Fig. 3. Effect of various extracellular Ca^{2+} concentrations on β51-63-induced CCK secretion and intracellular Ca^{2+} mobilization.

STC-1 cells cultured in 48-well plates were equilibrated with a Ca^{2+}-free buffer. Cells were then exposed to the β51-63 peptide in buffer containing 0 or 1.2 mM Ca^{2+} (A) and 1.0 or 2.0

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

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

Fig. 5. Involvement of CaR in β51-63-induced intracellular Ca^{2+} mobilization and CCK secretion.
A: Cells were exposed to 1.2 mM β51-63 peptide in the presence of 25 μM NPS2143 or vehicle (0.1% DMSO). Data are expressed as changes from basal state (0 min) ($\Delta[Ca^{2+}]_i$). Values are means of 4 experiments. B: STC-1 cells were exposed to 1.2 mM β51-63

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

Fig. 6. Effects of extracellular Ca^{2+} and β51-63 peptide on intracellular Ca^{2+} mobilization in HEK-293 cells transfected with CaR.

HEK-293 cells were transfected with mouse CaR expression plasmid. Mock-transfection was carried out using an empty plasmid. Cells were loaded with fluo-3-AM in 96-well culture plate, and intracellular Ca^{2+} concentrations were measured using the FlexStation system and a FLIPR Calcium 3 assay kit. Cells were equilibrated with 1 mM extracellular Ca^{2+}, and then exposed to additional Ca^{2+} or β51-63 peptide (10-1000 μM). HEK293 transfected with CaR were exposed to extracellular Ca^{2+} (C) and β51-63 peptide (D) in the presence of NPS2143.,Values are the peak fluorescence observed in response to addition of extracellular Ca^{2+} (A, C) or β51-63 peptide (B, D), and are means of 3 wells.

Fig. 7. Effects of thapsigargin and G protein inhibitors on β51-63-induced intracellular Ca^{2+} mobilization or CCK secretion.

STC-1 cells were exposed to 1.2 mM β51-63 in the presence of 5 μM YM-254980 (A) or 10 μM Thapsigargin (B). Data are expressed as changes from basal state (0 min) ($\Delta[Ca^{2+}]_i$), and as means of 3 experiments. STC-1 cells were exposed to 1.2 mM β51-63 in the presence of 5 μM YM-254980 (C) or after pretreatment with 200 ng/ml PTX for 5 hours (D). CCK concentrations in the supernatants were measured by EIA. Values are means ± SEM of 3-4 wells. Bars not sharing the same letters differ significantly ($P < 0.05$ by Tukey's post-hoc test).

Fig.1

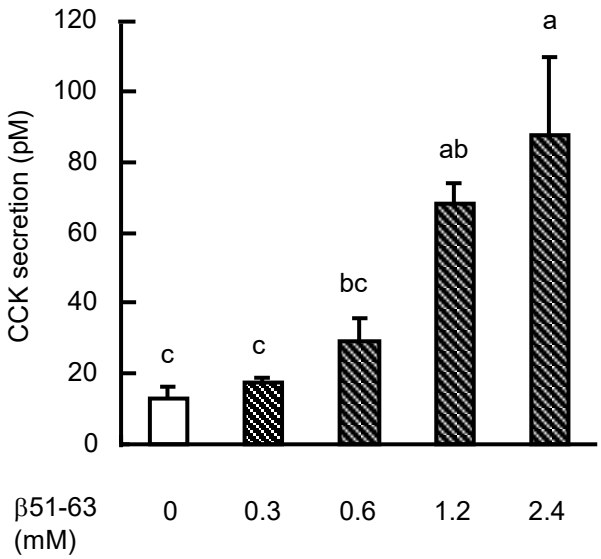

120
100
80
60
40
20
0
CCK secretion (pM)
c
c
bc
ab
a
β51-63 (mM)
0
0.3
0.6
1.2
2.4

Fig.2

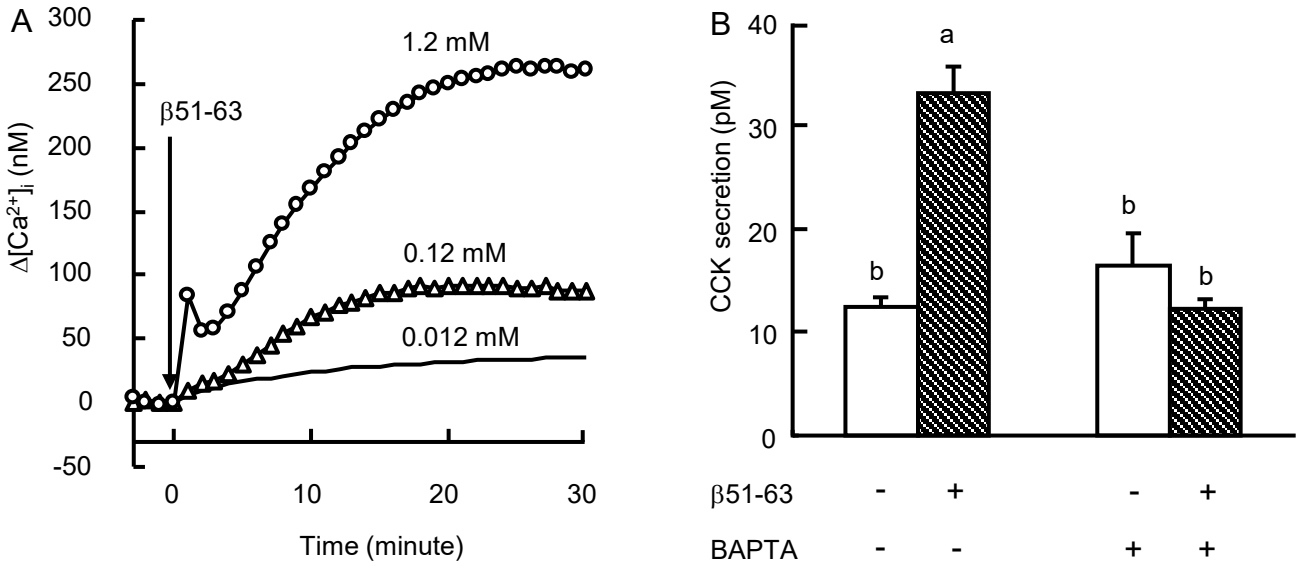

A
300
250
200
150
100
50
0
-50
Δ[Ca2+]i (nM)
β51-63
1.2 mM
0.12 mM
0.012 mM
0
10
20
30
Time (minute)
B
40
30
20
10
0
CCK secretion (pM)
a
b
b
b
β51-63 - + - +
BAPTA - - + +

Fig. 3

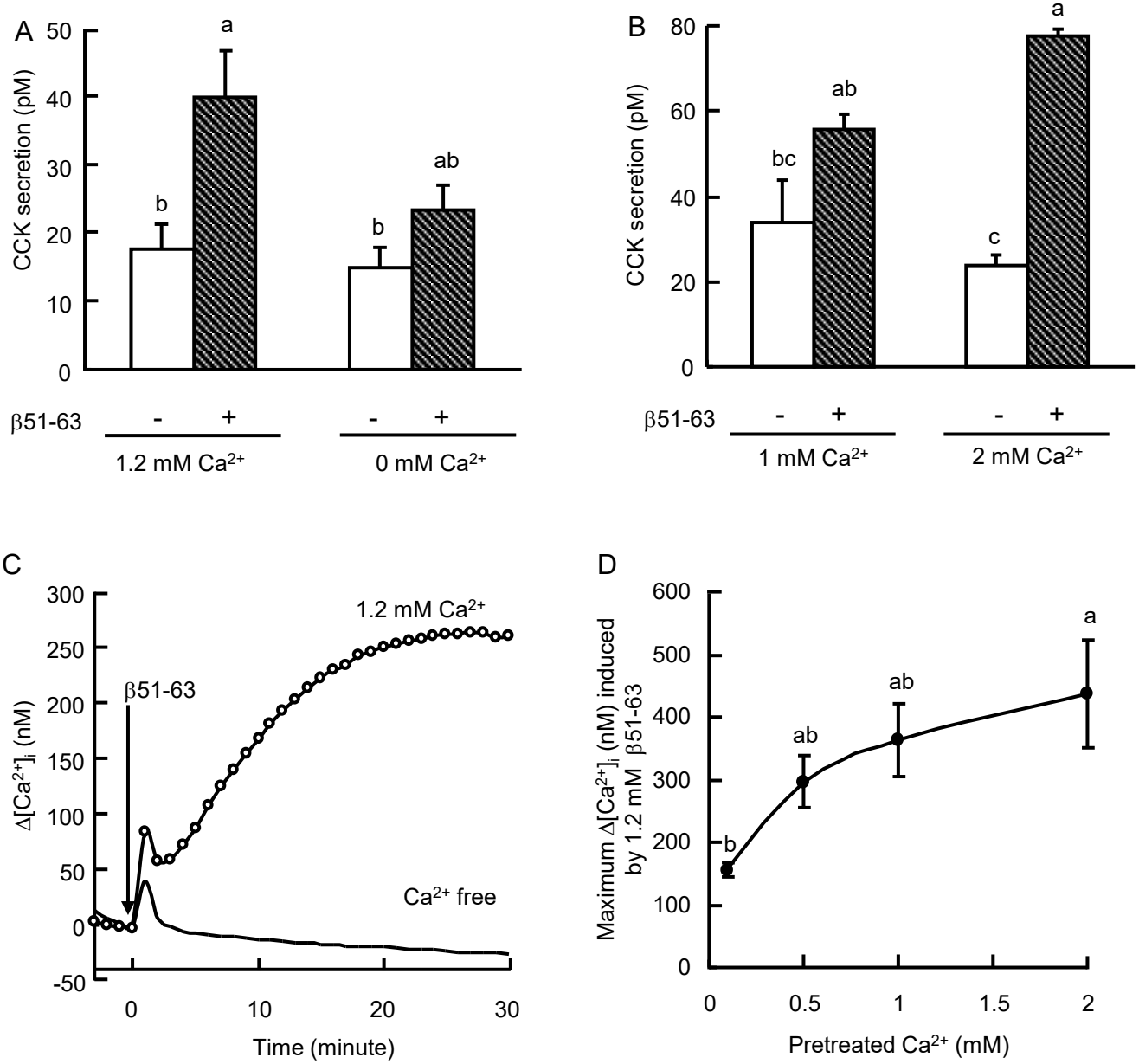

A
CCK secretion (pM)
50
40
30
20
10
0
a
b
ab
b
β51-63
-
+
-
+
1.2 mM Ca2+
0 mM Ca2+
B
CCK secretion (pM)
80
60
40
20
0
a
ab
bc
c
β51-63
-
+
-
+
1 mM Ca2+
2 mM Ca2+
C
Δ[Ca2+]i (nM)
300
250
200
150
100
50
0
-50
1.2 mM Ca2+
β51-63
Ca2+ free
0
10
20
30
Time (minute)
D
Maximum Δ[Ca2+]i (nM) induced by 1.2 mM β51-63
600
500
400
300
200
100
0
a
ab
ab
b
0
0.5
1
1.5
2
Pretreated Ca2+ (mM)

Fig. 4

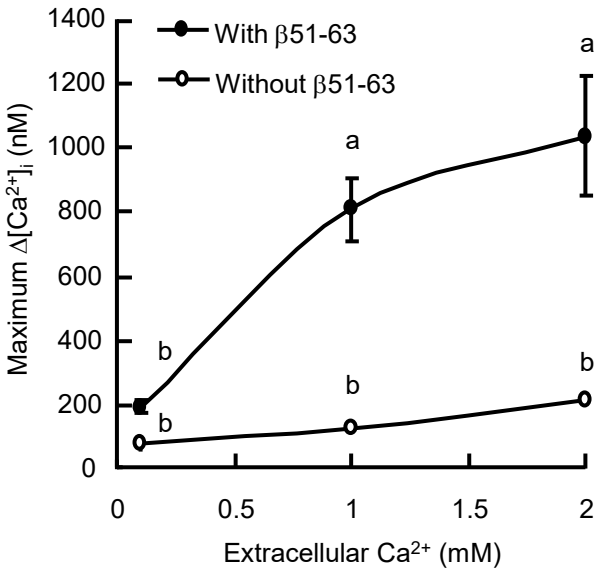

With β51-63
Without β51-63
Maximum Δ[Ca2+]i (nM)
Extracellular Ca2+ (mM)
1400
1200
1000
800
600
400
200
0
0
0.5
1
1.5
2
a
a
b
b
b
b

Fig. 5

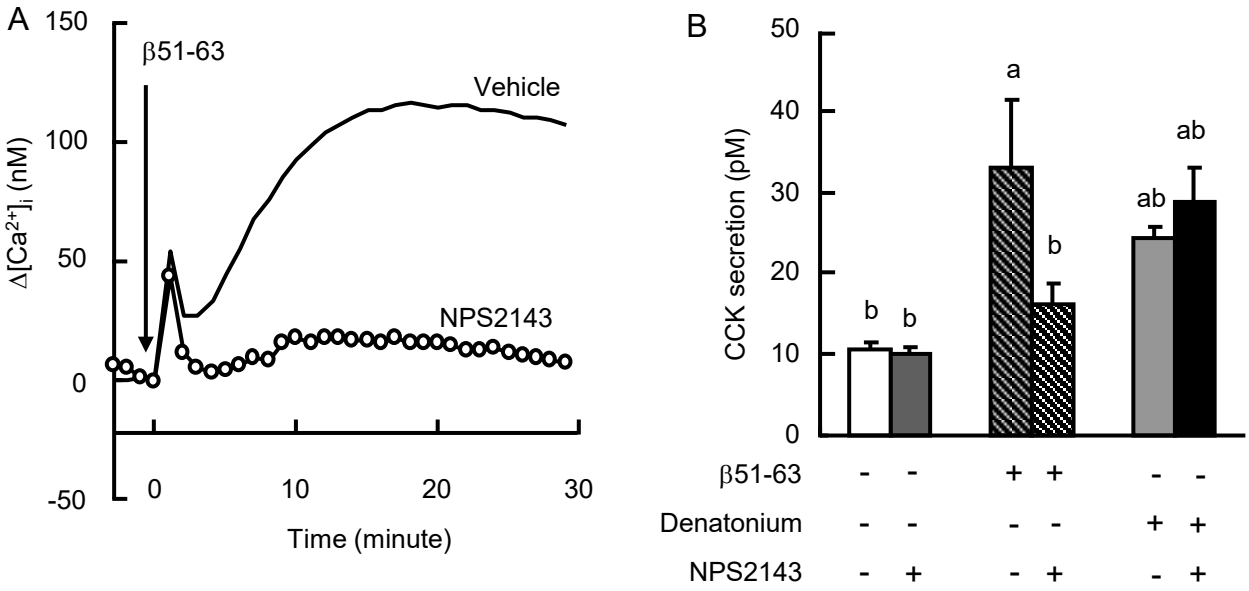

A
150
100
50
0
-50
β51-63
Vehicle
NPS2143
Δ[Ca2+]i (nM)
0
10
20
30
Time (minute)
B
50
40
30
20
10
0
CCK secretion (pM)
a
b
b
ab
ab
b
β51-63 - - + + - -
Denatonium - - - - + +
NPS2143 - + - + - +

Fig. 6

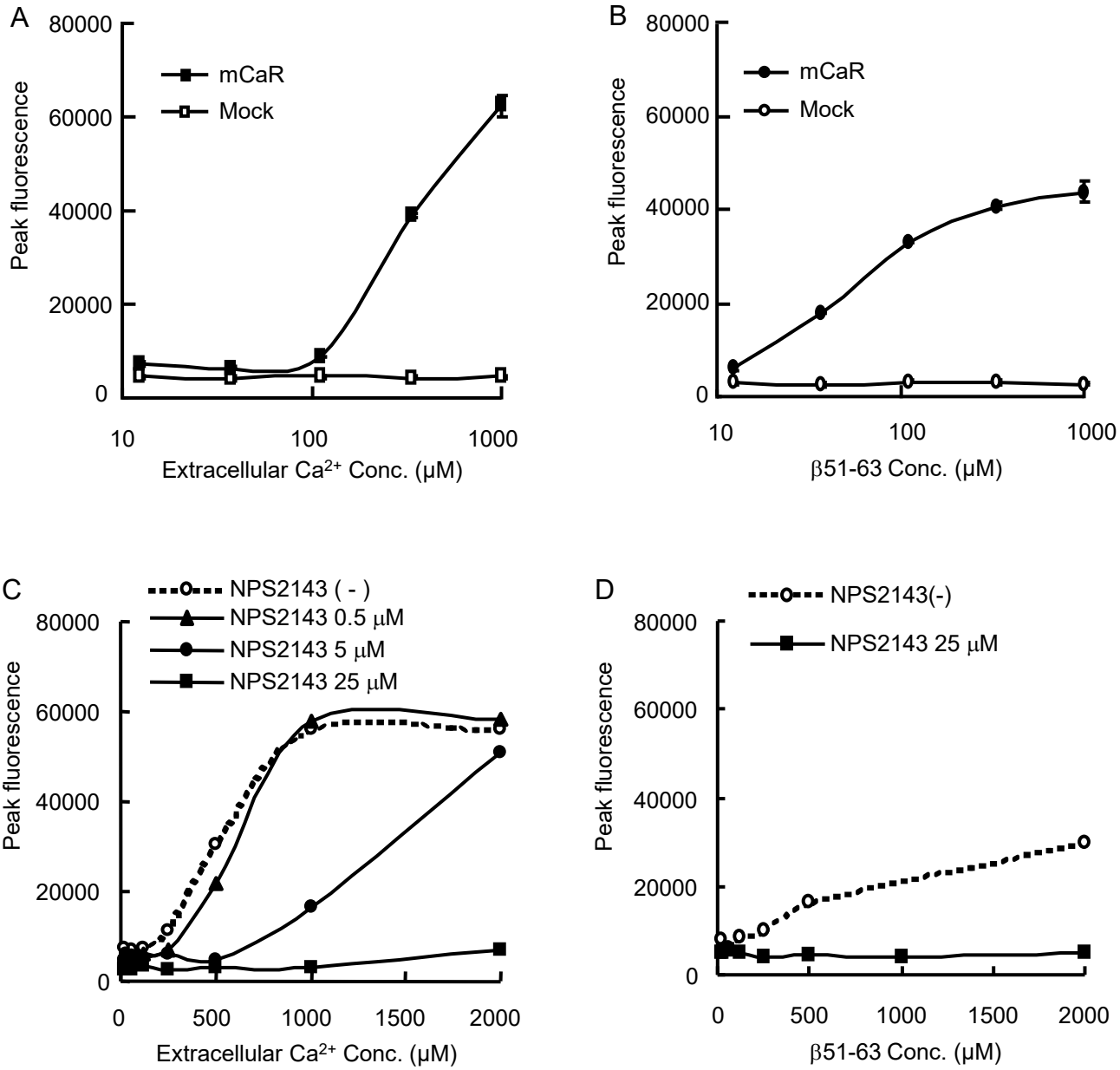
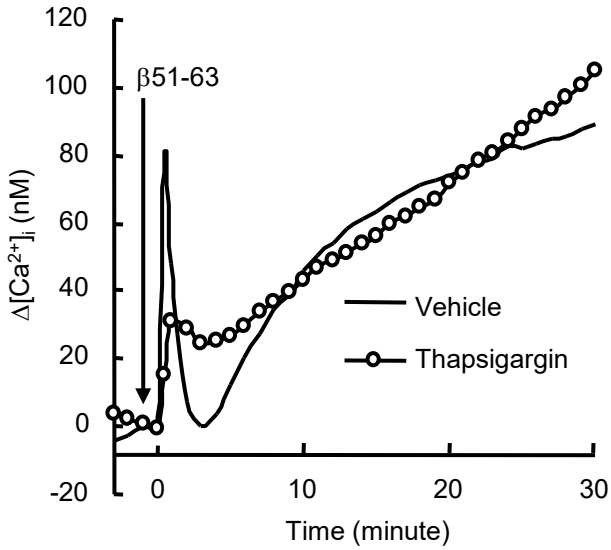
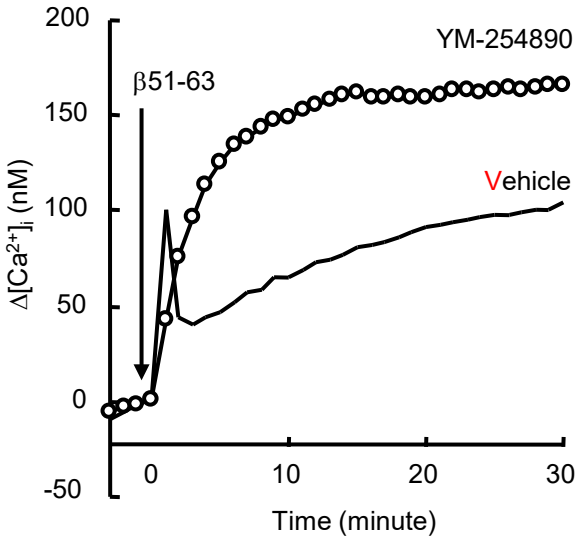
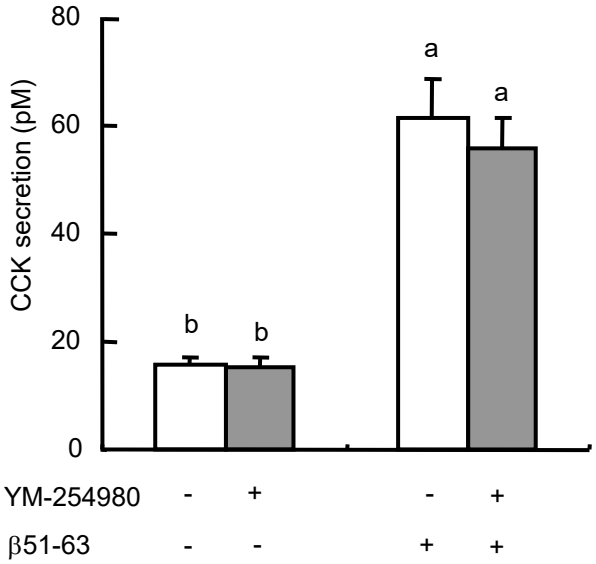
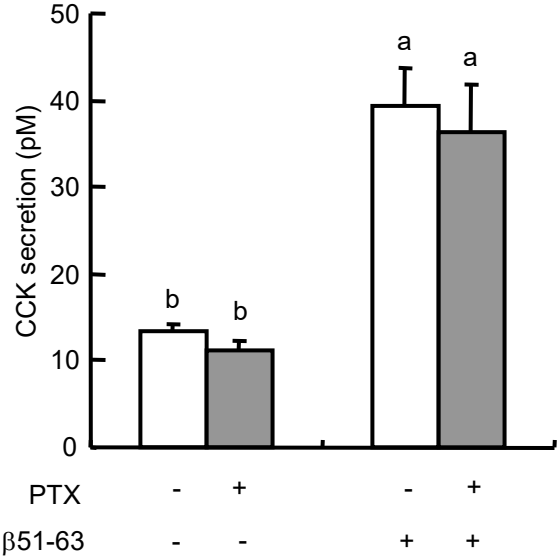

A
80000
60000
40000
20000
0
Peak fluorescence
mCaR
Mock
10
100
1000
Extracellular Ca2+ Conc. (μM)
B
80000
60000
40000
20000
0
Peak fluorescence
mCaR
Mock
10
100
1000
β51-63 Conc. (μM)
C
NPS2143 (-)
NPS2143 0.5 μM
NPS2143 5 μM
NPS2143 25 μM
80000
60000
40000
20000
0
Peak fluorescence
0
500
1000
1500
2000
Extracellular Ca2+ Conc. (μM)
D
NPS2143(-)
NPS2143 25 μM
80000
60000
40000
20000
0
Peak fluorescence
0
500
1000
1500
2000
β51-63 Conc. (μM)

Fig. 7


120
100
80
60
40
20
0
-20
β51-63
Δ[Ca2+]i (nM)
Vehicle
Thapsigargin
0
10
20
30
Time (minute)


200
150
100
50
0
-50
YM-254890
β51-63
Vehicle
Δ[Ca2+]i (nM)
0
10
20
30
Time (minute)


80
60
40
20
0
CCK secretion (pM)
a
a
b
b
YM-254980 - + - +
β51-63 - - + +


50
40
30
20
10
0
CCK secretion (pM)
a
a
b
b
PTX - + - +
β51-63 - - + +