Substance P-Induced Intracellular Ca^{2+} Signaling in Epithelial Cells of Isolated Crypt from Guinea Pig Distal Colon

Ken-ichi SHIOKAWA, Yutaka HOSODA, Yukiko SHIMODA, Mutsuaki SUZUKI, Shin-ichiro KARAKI, Michal CEREGRZYN and Atsukazu KUWAHARA

Laboratory of Physiology, Graduate School of Nutritional and Environmental Sciences, and Institute for Environmental Sciences, University of Shizuoka, 52-1 Yada, Shizuoka, 422-8526, Japan

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ABSTRACT

Substance P-induced $[\text{Ca}^{2+}]_i$ signaling in a single epithelial cell of isolated crypt from guinea pig distal colon was investigated by Ca^{2+} imaging technique. Substance P (SP), neurokinin A (NKA), and neurokinin B (NKB) induced a concentration-dependent transient increase in $[\text{Ca}^{2+}]_i$ with a rank order of potency of $\text{SP} > \text{NKA} > \text{NKB}$. A NK_1 receptor antagonist, FK888 ($>10^{-8}$ M) significantly reduced the response of SP (10^{-7} M). A phosphatidylinositol-specific phospholipase C (PI-PLC) inhibitor, 1-*O*-octadecyl-2-methyl-*rac*-glycero-3-phosphorylcholine (10^{-5} M) inhibited the SP-induced response. Ca^{2+} -free solution did not affect the SP-induced peak response, but the area under the curve (AUC) was decreased to 52% of control. Thapsigargin (10^{-5} M) also decreased the AUC of the SP-induced response to 52% of control. The combination of Ca^{2+} -free solution and thapsigargin abolished the SP-induced response. Ca^{2+} channel blockers, verapamil (10^{-4} M), nifedipine (10^{-5} M) and Ni^{2+} (10^{-3} M) did not affect the SP-induced responses, while flunarizine (10^{-4} M) and octanol (6×10^{-5} M) reduced the AUC, but not peak responses. These results suggest that SP stimulates NK_1 receptors on crypt epithelial cells in guinea pig distal colon. Furthermore, SP activates PI-PLC, and increases in $[\text{Ca}^{2+}]_i$ by releasing Ca^{2+} from a thapsigargin-sensitive store and by an influx of extracellular Ca^{2+} *via* flunarizine and octanol-sensitive, but verapamil, nifedipine and Ni^{2+} -insensitive Ca^{2+} channels.

Substance P (SP) is a neuropeptide of the tachykinin family. The family also includes neurokinin A (NKA) and neurokinin B (NKB) (29). In the gastrointestinal tract, SP and NKA are widely distributed within the enteric nervous system. The actions of tachykinins in the gastrointestinal tract are mediated by NK_1 , NK_2 and NK_3 receptors activated by SP, NKA and NKB, respectively (14, 15). One of the effects of SP is the stimulation of intestinal secretion,

which has been investigated both *in vivo* and *in vitro* experiments (6, 19, 20, 22, 32). Kachur *et al.* have reported that SP evokes anion secretion in muscle-stripped segments of guinea-pig ileum by acting directly on epithelial cells (19). On the other hand, it has been reported that SP-induced anion secretion is regulated in part by neural mediation in the jejunum and ileum of guinea pig (20, 32). Kuwahara and Cooke have reported that SP evokes Cl^- secretion by activating cholinergic and noncholinergic neurons and by releasing histamine from mast cells in the guinea pig distal colon (22). Furthermore, Wang *et al.* have reported that SP-evoked ion transport in mouse small intestine is mediated by enteric nerves and mast cells (42). In the guinea pig distal colon, the nonneural components are almost absent or

Correspondence to: Dr. Atsukazu Kuwahara
Laboratory of Physiology, Institute for Environmental Sciences, University of Shizuoka 52-1 Yada, Shizuoka, 422-8526, Japan
TEL/FAX: +81-54-264-5707
E-mail: kuwahara@sea.u-shizuoka-ken.ac.jp

much weaker than the neural component (6, 16, 22). However in our recent study, we have reported that SP induces a massive Cl^- secretion by the direct stimulation of epithelial cells in interaction with prostaglandin E_2 (PGE_2) (16). From this evidence, we have hypothesized that SP-induced Cl^- secretion via the direct stimulation of epithelia may occur in inflammatory conditions.

SP has been reported to activate the intracellular Ca^{2+} signal transduction system in various cells (31, 35). Changes in the concentration of free intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) are widely recognized to control a variety of functions in many cell types. For example, in colonic smooth muscle, SP binds NK_1 receptor and increases in $[\text{Ca}^{2+}]_i$ that is elicited by an influx of Ca^{2+} through Ca^{2+} channels and by releasing from intracellular Ca^{2+} stores (28). In our previous report, we showed that SP induces a transient increase in $[\text{Ca}^{2+}]_i$ in guinea pig distal colonic epithelia (16). However, the signaling mechanism of SP in intact crypt epithelial cells has not been well characterized yet. Therefore, the aim of this study is to characterize the SP-induced $[\text{Ca}^{2+}]_i$ mobilization of epithelial cells using intact crypts isolated from guinea pig distal colon.

METHODS

Isolation of colonic crypts. Male Hartley guinea pigs (250–800 g) from Japan SLC Inc. (Shizuoka, Japan) were allowed food and water *ad libitum* before the experiments. The guinea pigs were stunned and exsanguinated according to the Guide for Animal Experimentation of the National Institute for Physiological Sciences in Japan. Segments of distal colon 5–10 cm proximal to the anus were removed, flushed with cold Krebs-Ringer solution and stored in Krebs-Ringer solution on ice. The Krebs-Ringer solution contained the following (in mM): 117.0 NaCl, 4.7 KCl, 1.2 MgCl_2 , 1.2 NaH_2PO_4 , 25.0 NaHCO_3 , 2.5 CaCl_2 and 11.0 glucose. The solutions were gassed with 95% O_2 –5% CO_2 and buffered at pH 7.2–7.4. For Ca^{2+} -free solutions, CaCl_2 was removed from the Krebs-Ringer solution and 3 mM EDTA was added. The distal colonic segments (about 4 cm) were rinsed with cold Krebs-Ringer solution containing 3 mM dithiothreitol (DTT) (solution I), and filled PBS(–) containing 25 mM EDTA and 3 mM DTT (solution II) until a moderate tension was achieved by clamping both ends. Then, the tissues were incubated in Krebs-Ringer solution for 3 min at 37°C. After discarding the luminal contents, the segments were filled with solution I again until

achieving a moderate tension, and palpated gently for 3 min. Then, the isolated crypts in the luminal solution were collected by centrifugation (4°C, 1000 rpm for 1 min). A portion of the supernatant was then removed and tissue fragments containing crypts were rinsed twice with Krebs-Ringer solution.

Measurement of $[\text{Ca}^{2+}]_i$ in isolated crypt cells. The isolated crypts were suspended in a Krebs-Ringer solution containing 0.1% BSA, 5 μM Indo 1-acetoxymethyl ester (AM) and 0.05% Cremophore EL for 10 min in the dark at room temperature (21–23°C). To fix the crypts, the suspension of dye-loaded crypts was seeded for 20 min in a refrigerator on a glass perfusion vessel coated with a cell adhesive, Cell-Tak. Then, the vessel was placed in a 5% CO_2 incubator for 60–70 min at 37°C to allow the loading of Indo 1, which is hydrolyzed from Indo 1-AM by intracellular esterase. After the dye loading, the vessel was washed with Krebs-Ringer solution containing 0.1% BSA. The vessel was then placed on the stage of an inverted microscope. The vessel was continuously perfused at a flow rate of 2 mL/min with oxygenated Krebs-Ringer solution containing 0.1% BSA at 32°C. The measurements of $[\text{Ca}^{2+}]_i$ were performed after the temperature in the vessel became stable.

A laser-scanning confocal imaging system (ACAS Ultima 575 UVC; Meridian Instruments, Okemos, Michigan, USA) connected to a Zeiss Axiovert microscope (Axiovert 135, Zeiss, Germany; $\times 40$; water immersion objective) was used for $[\text{Ca}^{2+}]_i$ image acquisition and processing. Indo 1 was excited using 350–360 nm line. Twin photomultiplier channels detected bands of fluorescence centered on 405 and 485 nm. Ratiometric images were collected every 10 seconds during stimulation. The area of interest was encompassed and ratio images of individual cell were calculated on a pixel by pixel basis and recorded for later data analysis. Data were always expressed as ratios of emitted fluorescence (12). In the preliminary experiments, the cells that were the most responsive to tachykinins were located at the bottom region of the crypts (Fig. 1). Therefore, in the present study, single epithelial cells located at the bottom one-fifth of the isolated crypt were collected for quantitative analysis.

Experimental protocols. An isolated crypt was perfused with normal Krebs-Ringer solution containing 0.1% BSA before stimulation. Then, the perfusate was replaced by Krebs-Ringer solution containing SP, NKA, or NKB, and the changes in $[\text{Ca}^{2+}]_i$ were

examined. Non-cumulative concentration-response curves were determined for SP, NKA and NKB. EC₅₀ value of SP was obtained by the nonlinear-square procedure to fit Michaelis-Menten equation using KyPlot, a data analysis and graph-creating software (Kyence Inc., Tokyo, Japan). The peak values of responses were expressed as a normalized ratio indicating the peak of [Ca²⁺]_i. The area under the curve (AUC) of peak response was calculated using the integral of the normalized ratio for 3 min. AUC indicates the amount of increase in [Ca²⁺]_i induced by the response. For experiment using a NK₁ receptor blocker, FK888, the crypts were perfused with Krebs-Ringer solution containing a variety of concentrations of FK888 (10⁻¹⁰–10⁻⁶ M) from 10 min before SP stimulation. A phosphatidylinositol specific phospholipase C (PI-PLC) inhibitor, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphorylcholine (ET-18-OCH₃) (10⁻⁵ M) was also preperfused from 10 min before SP stimulation. A sarco-endoplasmic reticulum Ca²⁺-ATPase (SERCA) inhibitor, thapsigargin (10 M) was added from 10–30 min before SP stimulation. For extracellular Ca²⁺-free experiments, normal perfusing Krebs-Ringer solution was replaced by Ca²⁺-free solution from 15–20 min before SP stimulation. The experiment of combination of thapsigargin and Ca²⁺-free solution was also performed. A variety of Ca²⁺ channel blocker including verapamil (10⁻⁴ M), nifedipine (10⁻⁵ M), flunarizine (10⁻⁴ M), octanol (6 × 10⁻⁵ M), and Ni²⁺ (10⁻³ M) were preperfused from 10 min before addition of SP, respectively.

Chemicals. Substance P, neurokinin A and neurokinin B were from Peptide Institute Inc. (Osaka, Japan). NK₁ receptor antagonist, FK888 was a gift from Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan) (7) Indo 1-AM was from Dojindo Laboratories (Kumamoto, Japan). Thapsigargin was purchased from Research Biochemicals Inc. (MA, USA). Ethylenedioxybis(ethylamine)-N, N, N', N'-tetraacetic acid disodium salt (EDTA) was from Stratagene (CA, USA). 1, 4-Dithiothreitol (DTT), albumin bovine serum (BSA), verapamil, nifedipine, flunarizine, octanol, and dimethyl sulfoxide (DMSO) were from Sigma (MO, USA). NiCl₂ was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Cremophore EL was from Nakalai-tesque (Kyoto, Japan). Cell-Tak was from Becton Dickinson Labware (MA, USA).

Statistics. Values are presented as means ± SEM. Student's *t* test or ANOVA followed by Dunnett test

were used to determine the statistical significance of the difference between the control and experimental groups. To determine significant differences among each group, Tukey test was used. *P* < 0.05 was considered statistically significant.

RESULTS

Effects of tachykinins on [Ca²⁺]_i in colonic crypt cells

Under control conditions, [Ca²⁺]_i in epithelial cells located in isolated crypt was stable. The application of SP to the perfusate induced an increase in [Ca²⁺]_i in epithelial cells located in the isolated crypt (Fig. 1). When the crypt was stimulated with 10⁻⁷ M SP in the presence of extracellular Ca²⁺, the [Ca²⁺]_i level in epithelial cells in the crypt increased to a transient peak level in individual cells within 30–60 s. Thereafter, the [Ca²⁺]_i decreased, and returned to basal level within 3 min even in the presence of SP. There was considerable variability in the magnitude of [Ca²⁺]_i to SP among individual crypt cells as shown in Fig. 1, lower panel. The time course changes in individual cells of [Ca²⁺]_i are shown in Fig. 1, upper

Fig. 1 Effects of SP on [Ca²⁺]_i of isolated crypt epithelial cells. Upper graph shows time course of SP-induced changes in [Ca²⁺]_i of individual four crypt cells (No. 1–4 as shown in below). *Upper graph inset*: Micrograph of a crypt isolated from guinea pig distal colon obtained under resting condition by inverted microscope. Lower pictures show digital pseudo color images of SP-induced changes in [Ca²⁺]_i in isolated crypt. Numbers in the most left picture indicate cell numbers identical to the upper traces. Numbers under the each picture indicate the time after the starting experiment. Solid line indicates presence of 10⁻⁷ M SP in the perfusate.

panel. Jacobi *et al.* have reported that there is no cell-to-cell coupling in rat colonic crypts [17]. Thus, in further experiments, data were collected from the most responsive cells located in the bottom one-fifth of the crypt for further quantitative analysis. SP, NKA and NKB induced concentration-dependent increases in $[Ca^{2+}]_i$ as shown in Fig. 2 ($n=3-14$). The lowest concentration of tachykinins evoking an increase in $[Ca^{2+}]_i$ was higher than 1 nM. The maximal response to SP was achieved at 10^{-7} M (normalized ratio: 2.51 ± 0.18 , $n=14$, $P<0.05$ and $P<0.01$ vs. NKA and vs. NKB, respectively). The EC_{50} of the SP-induced response was 10.3 ± 18.9 nM. However, in the range of concentrations used, we did not achieve the maximum possible responses for NKA and NKB. The rank order of potency was $SP > NKA >> NKB$, as shown in Fig. 2. Tachykinins did not induce rhythmic oscillations of $[Ca^{2+}]_i$ in crypt epithelial cells.

Effect of a NK_1 receptor antagonist, FK888 on SP-induced response

To determine the tachykinin receptor subtypes present on the colonic crypt epithelial cells, we examined the effect of NK_1 receptor antagonist, FK888 on SP (10^{-7} M)-induced increase in $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} . The concentration of SP was chosen based on the concentration of the

maximum response mentioned above. FK888 concentration-dependently decreased the SP (10^{-7} M)-induced increase in $[Ca^{2+}]_i$ as shown in Fig. 3. FK888 (10^{-6} M) completely abolished the SP (10^{-7} M)-induced increase in $[Ca^{2+}]_i$ ($P<0.001$, $n=5-6$).

Effect of a PI-PLC inhibitor, ET-18-OCH₃ on SP-induced response

To test the participation of PI-PLC in the SP-induced increase in $[Ca^{2+}]_i$, a PI-PLC inhibitor, ET-18-OCH₃ was used. As a result, ET-18-OCH₃ (10^{-5} M) significantly inhibited the SP-induced peak response from 2.96 ± 0.07 to 2.26 ± 0.13 ($n=3$, $P<0.01$) (Fig. 4).

Effects of extracellular Ca^{2+} free solution and thapsigargin on SP-induced response

In the Ca^{2+} free conditions, SP (10^{-7} M) still induced an increase in $[Ca^{2+}]_i$ and the value of the peak of the normalized ratio did not change in comparison with Ca^{2+} -present conditions; 2.51 ± 0.11 ($n=7$) in control and 2.16 ± 0.05 ($n=3$) in Ca^{2+} -free conditions ($P>0.05$, Fig. 5A and C). However, the SP-induced increase in $[Ca^{2+}]_i$ in the Ca^{2+} -free conditions was more transient than the Ca^{2+} -present conditions (Fig. 5A). AUC for the SP-induced increase in total $[Ca^{2+}]_i$ decreased in the extracellular Ca^{2+} -free condition from the control value of 117.5 ± 15.2 ($n=7$) to

Fig. 2 Concentration-response curves for the peak values of $[Ca^{2+}]_i$ induced by SP, NKA and NKB. Crypts were exposed to various concentrations of tachykinins (10^{-10} – 10^{-6} M), and changes in $[Ca^{2+}]_i$ in single crypt epithelial cells were indicated as normalized ratio. Values are expressed as mean $\pm$ SEM, $n=3-14$. * $P<0.05$, ** $P<0.01$ vs. SP, † $P<0.05$ vs. NKA by Tukey test.

Fig. 3 Effect of NK_1 receptor antagonist, FK888 on SP-induced change in $[Ca^{2+}]_i$. Crypts were exposed to various concentrations of FK888 (10^{-10} – 10^{-6} M), and changes in $[Ca^{2+}]_i$ in single crypt epithelial cells stimulated by 10^{-7} M SP were measured. Data are expressed as means $\pm$ SEM, $n=5-6$, *** $P<0.001$ vs. 0 M FK888 by Dunnett test.

Fig. 4 Effect of a PI-PLC inhibitor, ET-18-OCH₃ on SP-induced increase in [Ca²⁺]_i. Crypts were exposed to ET-18-OCH₃ (10⁻⁵ M) from 10 min before SP stimulation, and the SP (10⁻⁷ M)-induced increase in [Ca²⁺]_i in single crypt epithelial cells were measured. Data are expressed as means $\pm$ SEM, $n=3$. ** $P<0.01$ vs. control by student's t test.

60.9 $\pm$ 9.3 (52% of control, $n=3$, $P<0.05$) (Fig. 5D).

To test whether or not the increase in [Ca²⁺]_i caused by SP is mediated by Ca²⁺ release from an intracellular store in addition to a Ca²⁺ influx, we used thapsigargin, a sarco-endoplasmic reticulum Ca²⁺-ATPase (SERCA) inhibitor, to exhaust the intracellular Ca²⁺ pool. First, we tested whether thapsigargin has an effect on [Ca²⁺]_i in epithelial cells of isolated crypt from guinea pig distal colon. Thapsigargin (10⁻⁵ M) itself gradually and progressively increased [Ca²⁺]_i in epithelial cells of isolated crypt in the presence of extracellular Ca²⁺ as shown in Fig. 5B (solid line). Thirty min after the addition of thapsigargin (10⁻⁵ M), [Ca²⁺]_i achieved 1.48 $\pm$ 0.11 ($n=4$) as expressed the normalized ratio. However, when the solution was changed to Ca²⁺ free 15 min after the addition of thapsigargin, [Ca²⁺]_i decreased to the basal level even in the presence of thapsigargin: 1.00 $\pm$ 0.03 ($n=5$, $P<0.01$ vs. Ca²⁺ present condition).

When SP (10⁻⁷ M) was added to the perfusate 30 min after the treatment with thapsigargin, SP caused a transient increase in [Ca²⁺]_i, which then returned to the level achieved by thapsigargin alone (Fig. 5B solid line). The peak value was 2.30 $\pm$ 0.14 ($n=4$), as much as the value in the absence of thapsigargin (Fig. 5C). However, the values of the peak ratio in-

Fig. 5 Effects of removal of extracellular Ca²⁺ on SP-induced increase in [Ca²⁺]_i in the presence and absence of thapsigargin. **A**: Representative traces of 10⁻⁷ M SP-induced change in [Ca²⁺]_i in the presence (solid line) or absence (dotted line) of extracellular Ca²⁺. **B**: Representative traces of change in [Ca²⁺]_i in the presence of 10⁻⁵ M thapsigargin and 10⁻⁷ M SP (solid line). From 15 to 20 min after the addition of thapsigargin, perfusate was changed to Ca²⁺ free solution (dotted line). **C**: Peak ratios of 10⁻⁷ M SP-induced increase in [Ca²⁺]_i in the presence and absence of 10⁻⁵ M thapsigargin and extracellular Ca²⁺. **D**: Area under the curves (AUCs) of 10⁻⁷ M SP-induced increases in ratio values integrated for 3 min in the presence and absence of 10⁻⁵ M thapsigargin and extracellular Ca²⁺. Data are expressed as means $\pm$ SEM, $n=3-7$. * $P<0.05$ and *** $P<0.001$ by Tuckey test.

duced by SP in the presence of thapsigargin were decreased because the baseline was increased by the treatment with thapsigargin. Thus, the AUC was decreased to 61.4 ± 10.5 (52% of control, $n=4$, $P<0.05$ vs. control, Fig. 5D). The combination of the removal of extracellular Ca^{2+} and thapsigargin pretreatment completely abolished the SP-induced increase in $[\text{Ca}^{2+}]_i$ (1.10 ± 0.06 as normalized ratio, $P<0.001$ vs. control, only Ca^{2+} free and only thapsigargin, Fig. 5C; 0.6 ± 2.2 as AUC, $P<0.001$ vs. control, $P<0.05$ vs. Ca^{2+} free and thapsigargin, Fig. 5D; $n=5$).

Effects of Ca^{2+} channel blockers on SP-induced response

To identify the type of Ca^{2+} channels involving in the SP-induced increase in $[\text{Ca}^{2+}]_i$, a variety of Ca^{2+} channel blockers, verapamil (10^{-4} M), nifedipine (10^{-5} M), flunarizine (10^{-4} M), octanol (6×10^{-5} M), and Ni^{2+} (10^{-3} M) were preperfused from 10 min before SP stimulation. As a result, the peak values of the SP-induced increase in $[\text{Ca}^{2+}]_i$ were not significantly different between 2.95 ± 0.10 ($n=20$) as control normalized ratio and each group of Ca^{2+} channel blockers (verapamil: 2.51 ± 0.08 , nifedipine: 2.96 ± 0.16 , flunarizine: 2.58 ± 0.21 , octanol: 2.46 ± 0.39 , and Ni^{2+} : 2.86 ± 0.31 ; $n=4-6$; Fig. 6B). Although there was no statistically significant difference among the peak values by ANOVA, returns of $[\text{Ca}^{2+}]_i$ to basal levels after the peak in the presence of flunarizine and octanol, but not verapamil, nifedipine, nor Ni^{2+} , were observed to be faster than that of control, respectively (Fig. 6A). Flunarizine and octanol significantly decreased the AUCs of normalized ratios by SP from 138.1 ± 6.9 ($n=20$) as control to 85.2 ± 6.8 and 85.4 ± 22.2 , respectively ($n=5-6$, $P<0.05$; Fig. 6C).

DISCUSSION

The present results show that SP induces an increase in $[\text{Ca}^{2+}]_i$ in epithelial cells of isolated crypt from guinea pig distal colon (Fig. 1). The elevation in $[\text{Ca}^{2+}]_i$ arose from a combination of Ca^{2+} release from thapsigargin-sensitive intracellular stores and from an activation of extracellular Ca^{2+} entry pathway.

The SP-induced increase in $[\text{Ca}^{2+}]_i$ was concentration-dependent in the 1 nM – $1 \mu\text{M}$ range (Fig. 2). In the present experiments, much higher concentrations of SP were needed to induce an increase in $[\text{Ca}^{2+}]_i$ than those usually found in blood. One reason is that tachykinins function as neurotransmitters and/or modulators in the gastrointestinal tract. In-

deed, neurotransmitter substances like SP need much higher concentration to induce their effects (1, 5, 23, 42). Magnitudes of $[\text{Ca}^{2+}]_i$ varied with individual crypt epithelial cells. This heterogeneity was consistently observed and is unlikely to result from cell damage since crypt consists of different cell types such as columnar cells, goblet cells, enteroendocrine cells, and caveolated cells. Among them, the columnar cells are the predominant cell type in the crypt. Furthermore, in guinea pig distal colon, Halm and Roger have reported that vacuolated columnar cells in the lower two-thirds of the crypt appear to be responsible for active Cl^- secretion (13). Leipziger

Fig. 6 Effects of Ca^{2+} blockers on SP-induced increase in $[\text{Ca}^{2+}]_i$. Crypts were exposed to various Ca^{2+} channel blockers, verapamil (10^{-4} M), nifedipine (10^{-5} M), flunarizine (10^{-4} M), octanol (6×10^{-5} M), Ni^{2+} (10^{-3} M) from 10 min before SP stimulation, and the SP (10^{-7} M)-induced increase in $[\text{Ca}^{2+}]_i$ were measured, respectively. A: Representative traces of the SP (10^{-7} M)-induced change in $[\text{Ca}^{2+}]_i$ in control (solid line) and in the presence of flunarizine (dashed line) and octanol (dotted line), respectively. B: Peak ratios of 10^{-7} M SP-induced increase in $[\text{Ca}^{2+}]_i$ in the presence of various Ca^{2+} channel blockers. C: AUC for 3 min of 10^{-7} M SP-induced increase in $[\text{Ca}^{2+}]_i$. Data are expressed as means $\pm$ SEM, $n=4-20$. * $P<0.05$ by Dunnett test.

et al. have also found that an ATP-induced increase in [Ca²⁺]_i is most pronounced in cells of the crypt base (25). Therefore, in the present study, we have carefully chosen crypt epithelial cells located at the base of isolated crypt to measure [Ca²⁺]_i as shown in Fig 1.

In many non-excitabile tissues, agonist-provoked inositol lipid hydrolysis has been shown to induce oscillation in [Ca²⁺]_i (2, 3, 26). For example, Vajana-phanich *et al.* have reported that carbachol has a biphasic effect on [Ca²⁺]_i in T84 cells; there is an initial Ca²⁺ transit followed by a more sustained response that frequently has an oscillatory characters (41). Furthermore, in rat duodenocytes, the initial transient peak in [Ca²⁺]_i stimulated by carbachol has been reported to be followed by a sustained plateau or rhythmic oscillation (3). However, in the present study, SP did not induce Ca²⁺ oscillation. Leipziger *et al.* have reported that ATP induces an initial peak and a secondary plateau increase in [Ca²⁺]_i (25). However, in the present study, the pattern of SP-induced increase in [Ca²⁺]_i in single epithelial cells from isolated crypt was transient, returning to basal or near basal values within 1–2 min after the initial peak response while SP was still present. The reason for these differences in the Ca²⁺ signaling pattern is presently unclear, but may be related to differences in species, region, stimulants, or [Ca²⁺]_i measurement systems for those which were used in the present study.

Three different receptor subtypes (NK₁, NK₂ and NK₃) are involved in mediating the biological actions of tachykinins. SP is thought to bind NK₁ receptor to induce various biological actions (14, 15, 29). NKA can recognize to some extent NK₁ receptor as well as SP since natural tachykinins can act as full agonists on each one of the three receptors, albeit at different concentrations (28). In the present experiments, all tested tachykinins induced increases in [Ca²⁺]_i in single epithelial cells from isolated crypt and the rank order of potency was SP>NKA>>NKB (Fig. 2). Furthermore, SP-induced increases in [Ca²⁺]_i were concentration-dependently inhibited by NK₁ receptor antagonist, FK888 (Fig. 3). Although we did not use NK₁ receptor-specific agonists, present pharmacological analysis suggests that SP-induced increases in [Ca²⁺]_i are mediated by NK₁ receptor located on mucosal epithelium of guinea pig distal colon. This is supported by observations that NK₁ immunoreactivity is located on the mucosal epithelium of guinea pig large intestine and NK₁ receptor mRNA and protein are expressed in isolated epithelial cells of guinea pig distal colon (5, 37).

Thus, the present results suggest that NK₁ receptor is linked to Ca²⁺-signaling pathways in single epithelial cells in the crypt of guinea pig distal colon. Among the mammalian tachykinins, SP and NKA have been found in the gastrointestinal tract (27). NK₂ receptor immunoreactivity at the bases of the crypts is intense in the proximal and faint in the distal colon of guinea pigs (33). Therefore, NK₂ receptor may partially contribute to tachykinin-induced Ca²⁺ mobilization in guinea pig distal colon. In contrast, immunoreactivity analysis of the gastrointestinal tract in a variety of species has shown little or no presence of NKB (39). In the present study, NKB also induced an increase in [Ca²⁺]_i but the response was the smallest in the tachykinin family. Goldhill and Angel have reported that NK₃ receptor antagonist has no effect on SP-induced Cl⁻ secretion and that NK₃ receptor activation was abolished by tetrodotoxin in guinea pig distal colon (11). These results indicate that NK₃ receptors are not located in the epithelial cells of distal colonic crypt. Taken together, the present results suggest that the contribution of NK₃ receptor to Ca²⁺ signaling in the epithelial cells of distal colonic crypt is small in the case of guinea pigs.

By the pretreatment of the tissues with a PI-PLC inhibitor, ET-18-OCH₃, the peak values of the SP-induced increase in [Ca²⁺]_i were reduced (Fig. 4). This result indicates that the SP-induced response is due to the activation of PI-PLC. Thus, it is suggested that SP stimulates NK₁ receptor on the cell-surface of crypt epithelial cells, the receptors activate PI-PLC *via* G protein, the PI-PLC produces inositol 1, 4, 5-triphosphate (IP₃), and the IP₃ releases Ca²⁺ from intracellular Ca²⁺ store. In the human colonic adenocarcinoma cell line, HT29, it has been reported that the type 3 IP₃ receptor, an intracellular Ca²⁺ channel on the endoplasmic reticulum, is expressed (30). Thus, a part of the SP-induced increase in [Ca²⁺]_i in crypt epithelial cells of guinea pig distal colon might be due to the type 3 IP₃ receptor.

In the present study, SP could induce a transient increase in [Ca²⁺]_i in the absence of extracellular Ca²⁺ (Fig. 5A). The peak ratio of SP-induced increase in [Ca²⁺]_i in the absence of extracellular Ca²⁺ was similar to that in the presence of extracellular Ca²⁺ (Fig. 5C). Thus, the results suggest that the SP-induced transient increase in [Ca²⁺]_i is caused by the release of Ca²⁺ from intracellular stores. However, the increase in [Ca²⁺]_i was not entirely due to the Ca²⁺ release from intracellular stores because the amount of intracellular Ca²⁺ present during SP-in-

duced stimulation in the extracellular Ca^{2+} free condition decrease to 52% of the control AUC (Fig. 5A and D). These results suggest that an influx of Ca^{2+} from extracellular space also contributes to the SP-induced increase in $[\text{Ca}^{2+}]_i$. Therefore, we have first examined the source of intracellular Ca^{2+} stores using thapsigargin, a sarco-endoplasmic reticulum Ca^{2+} -ATPase (SERCA) inhibitor, and next examined the extracellular Ca^{2+} influx using channel blockers.

In crypts of rat large intestine, it has been reported that mRNAs for SERCA2a, SERCA2b and SERCA3 are expressed, and highly expressed in the case of SERCA3 (44). Thapsigargin itself gradually increased $[\text{Ca}^{2+}]_i$ in crypt epithelial cells in the presence of extracellular Ca^{2+} (Fig. 5B). It is known that the release of Ca^{2+} from intracellular stores induced by thapsigargin is indirect, while the inhibition of SERCAs prevents the pump from counterbalancing the passive Ca^{2+} leak from the stores to the cytosol (40). However, when the extracellular Ca^{2+} removed, the thapsigargin-induced increase in $[\text{Ca}^{2+}]_i$ returned to the basal level within 10 min (Fig. 5B). These results suggest that thapsigargin can empty intracellular Ca^{2+} from a thapsigargin-sensitive store. In the present experiments, the amount of free Ca^{2+} released into cytosol by SP was decreased by pretreatment with thapsigargin to 52% (Fig. 5B and D). Moreover, the combination of thapsigargin pretreatment and an extracellular Ca^{2+} -free condition completely abolished the response of SP (Fig. 5B, C and D). Therefore, the results suggest that the sources of an SP-induced increase in $[\text{Ca}^{2+}]_i$ are thapsigargin-sensitive intracellular Ca^{2+} store and an influx of Ca^{2+} from extracellular space.

The mechanism of Ca^{2+} influx from extracellular space in intestinal epithelial cells is currently unclear. Previous immunochemical and pharmacological results have suggested that the L-type Ca^{2+} channels are expressed at intestinal epithelial cells (34, 43). Reinlib *et al.* reported that calbachol-induced increase in $[\text{Ca}^{2+}]_i$ was blocked by verapamil and nifedipine in T84 cell line (34). However, our results showed that the SP-induced increase in $[\text{Ca}^{2+}]_i$ was not blocked by verapamil nor nifedipine in crypt epithelial cells of guinea pig distal colon (Fig. 6B and C). Therefore, it is suggested that the SP-induced increase in $[\text{Ca}^{2+}]_i$ is not due to the L-type Ca^{2+} channel. The response is different from that of the calbachol-induced response. Moreover, in our results, the SP-induced response was insensitive to Ni^{2+} ion (Fig. 6B and C). The concentration of Ni^{2+} used in the present study (10^{-3} M) was enough to block three different T-type Ca^{2+} channels: α_{1G} ,

α_{1H} , and α_{1I} (24). Therefore, it is suggested that the T-type Ca^{2+} channel does not contribute the SP-induced increase in $[\text{Ca}^{2+}]_i$. On the other hand, flunarizine and octanol reduced the AUC of the SP-induced increase in $[\text{Ca}^{2+}]_i$ about both 60% of control, respectively (Fig. 6C). Since, in the present results, Ca^{2+} -free solution reduced the SP-induced increase in $[\text{Ca}^{2+}]_i$ about 50% (Fig. 5D), it is suggested that flunarizine and octanol inhibit the almost all SP-induced influx of Ca^{2+} , respectively. It is known that flunarizine and octanol inhibit T-type Ca^{2+} channels. However, we already suggested that T-type Ca^{2+} channels may hardly be involved in the SP-induced increase in $[\text{Ca}^{2+}]_i$ as mentioned above. One possibility to solve this discrepancy might be that flunarizine and octanol inhibit the other unknown Ca^{2+} channels.

Although the mechanisms of tachykinin-induced Cl^- secretion is a matter of some controversy (5, 6, 11, 22, 42), there are few reports about the direct action of tachykinins on isolated crypt cells (16, 36). What is the significance of the direct action of SP on crypt epithelial cells? SP is a neurotransmitter of intrinsic primary afferent neurons in the enteric nervous system (8). It is considered that the afferent neurons monitor the luminal condition of chemical changes, distension of the intestine, and mechanical distortion of the mucosa (9). In the normal state, there are tonic reflexes: axonal reflex and classical reflex (afferent-reflex center-efferent). If SP is continuously released at a neuro-epithelial site as a result of the axonal reflex, the released SP always affects epithelial functions: secretion and absorption. However, a previous study of ours has recently shown that an increase in $[\text{Ca}^{2+}]_i$ in crypt epithelial cell is not enough to induce Cl^- secretion, although SP can directly stimulate crypt epithelial cells and induce an increase in $[\text{Ca}^{2+}]_i$ in guinea pig distal colon (16). If an increase in $[\text{Ca}^{2+}]_i$ stimulated by SP in crypt epithelial cells promoted Cl^- secretion in the normal state, diarrhea would occur. Therefore, the reason why an increase in $[\text{Ca}^{2+}]_i$ alone can not induce massive Cl^- secretion may be a self-defense mechanism to reduce excessive water loss. What is the physiological role of the SP-induced increase in $[\text{Ca}^{2+}]_i$ in crypt epithelial cells? We have also reported that SP can induce a massive Cl^- secretion when intracellular cAMP increases, for example in the presence of PGE_2 (16). This suggests that SP can induce Cl^- secretion by direct action on crypt epithelial cells in inflammatory conditions, cooperating with PGE_2 to flush harmful contents out of the lumen. Therefore, the direct action of SP in intesti-

nal epithelia should be considered important in relation to intestinal inflammation.

In conclusion, the present study has provided basic information of the effect of tachykinins on Ca²⁺ mobilization in crypt epithelial cells in the distal colon; 1) tachykinins induces a transient increase in [Ca²⁺]_i in crypt epithelial cells in the guinea pig distal colon; 2) the response is mediated by the stimulation of NK₁ receptors; 3) the response is mediated by the activation of PI-PLC; 4) the sources of SP-induced first peak increase in [Ca²⁺]_i are thapsigargin-sensitive intracellular Ca²⁺ store; 5) and an influx of Ca²⁺ from extracellular space is *via* flunarizine and octanol sensitive Ca²⁺ channels, but it is probably not T-type.

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REFERENCES

- Baron S. A., Jaffe B. M. and Gintzler A. R. (1983) Release of substance P from the enteric nervous system: direct quantitation and characterization. *J. Pharmacol. Exp. Ther.* **227**, 365–368.
- Bird G. S., Takemura H., Thastrup O., Putney J. W. Jr and Menniti F. S. (1992) Mechanisms of activated Ca²⁺ entry in the rat pancreatoma cell line, AR4-2J. *Cell Calcium* **13**, 49–58.
- Chew C. S., Safsten B. and Flemstrom G. (1998) Calcium signaling in cultured human and rat duodenal enterocytes. *Am. J. Physiol.* **275**, G296–G304.
- Cooke H. J. (1998) "Enteric tears": chloride secretion and its neural regulation. *News Physiol. Sci.* **13**, 269–274.
- Cooke H. J., Sidhu M., Fox P., Wang Y. Z. and Zimmermann E. M. (1997) Substance P as a mediator of colonic secretory reflexes. *Am. J. Physiol.* **272**, G238–G245.
- Frieling T., Dobrev G., Weber E., Becker K., Rupprecht C., Neunlist M. and Schemann M. (1999) Different tachykinin receptors mediate chloride secretion in the distal colon through activation of submucosal neurones. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **359**, 71–79.
- Fujii T., Murai M., Morimoto H., Maeda Y., Yamaoka M., Hagiwara D., Miyake H., Ikari N. and Matsuo M. (1992) Pharmacological profile of a high affinity dipeptide NK₁ receptor antagonist, FK888. *Br. J. Pharmacol.* **107**, 785–789.
- Furness J. B. (2000) Types of neurons in the enteric nervous system. *J. Auton. Nerv. Syst.* **81**, 87–96.
- Furness J. B., Kunze W. A. and Clerc N. (1999) Nutrient tasting and signaling mechanisms in the gut II: The intestine as a sensory organ: neural, endocrine, and immune responses. *Am. J. Physiol.* **277**, G922–G928.
- Gallacher D. V., Hanley M. R., Petersen O. H., Roberts M. L., Squire-Pollard L. G. and Yule D. I. (1990) Substance P and bombesin elevate cytosolic Ca²⁺ by different molecular mechanisms in a rat pancreatic acinar cell line. *J. Physiol.* **426**, 193–207.
- Goldhill J. and Angel I. (1998) Mechanism of tachykinin NK₁ receptor-mediated colonic ion transport in the guinea pig. *Eur. J. Pharmacol.* **363**, 161–168.
- Gryniewicz G., Poenie M. and Tsien R. Y. (1985) A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**, 3440–3450.
- Halm D. R. and Rick R. (1992) Secretion of K and Cl across colonic epithelium: cellular localization using electron microprobe analysis. *Am. J. Physiol.* **262**, C1392–C1402.
- Holzer P. and Holzer-Petsche U. (1997) Tachykinins in the gut. Part I. Expression, release and motor function. *Pharmacol. Ther.* **73**, 173–217.
- Holzer P. and Holzer-Petsche U. (1997) Tachykinins in the gut. Part II. Roles in neural excitation, secretion and inflammation. *Pharmacol. Ther.* **73**, 219–263.
- Hosoda Y., Karaki S., Shimoda Y. and Kuwahara A. (2002) Substance P-evoked Cl[−] secretion in guinea pig distal colonic epithelia: interaction with PGE₂. *Am. J. Physiol.* **283**, G347–G356.
- Jacobi C., Leipziger J., Nitschke R., Ricken S. and Greger R. (1998) No evidence for cell-to-cell coupling in rat colonic crypts: studies with Lucifer Yellow and with photobleaching. *Pflügers Arch.* **436**, 83–89.
- Kachintorn U., Vajanaphanich M., Traynor-Kaplan A. E., Dharmasathaphorn K. and Barrett K. E. (1993) Activation by calcium alone of chloride secretion in T84 epithelial cells. *Br. J. Pharmacol.* **109**, 510–517.
- Kachur J. F., Miller R. J., Field M. and Rivier J. (1982) Neurohumoral control of ileal electrolyte transport II. Neurotensin and substance P. *J. Pharmacol. Exp. Ther.* **220**, 456–463.
- Keast J. R., Furness J. B. and Costa M. (1985) Different substance P receptors are found on mucosal epithelial cells and submucosal neurons of the guinea-pig small intestine. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **329**, 382–387.
- Krause J. E., Chirgwin J. M., Carter M. S., Xu Z. S. and Hershey A. D. (1987) Three rat preprotachykinin mRNAs encode the neuropeptides substance P and neurokinin A. *Proc. Natl. Acad. Sci. USA* **84**, 881–885.
- Kuwahara A. and Cooke H. J. (1990) Tachykinin-induced anion secretion in guinea pig distal colon: role of neural and inflammatory mediators. *J. Pharmacol. Exp. Ther.* **252**, 1–7.
- Kuwahara A. and Yanaihara N. (1987) Action of the newly discovered mammalian tachykinins, substance K and neurokinin K, on gastroduodenal motility of anesthetized dog. *Regul. Pept.* **17**, 221–228.
- Lee J. H., Gomora J. C., Cribbs L. L. and Perez-Reyes E. (1999) Nickel block of three cloned T-type calcium channels: low concentrations selectively block $\alpha 1H$. *Biophys. J.* **77**, 3034–3042.
- Leipziger J., Kerstan D., Nitschke R. and Greger R. (1997) ATP increases [Ca²⁺]_i and ion secretion via a basolateral P2Y₂ receptor in rat distal colonic mucosa. *Pflügers Arch.* **434**, 77–83.
- Ljungstrom M. and Chew C. S. (1991) Calcium oscillations and morphological transformations in single cultured gastric parietal cells. *Am. J. Physiol.* **260**, C67–C78.
- Maggi C. A. (1995) The mammalian tachykinin receptors. *Gen. Pharmacol.* **26**, 911–944.
- Maggi C. A., Holzer P. and Giuliani S. (1994) Effect of ω -conotoxin on cholinergic and tachykininergic excitatory neurotransmission to the circular muscle of the guinea-pig colon. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **350**, 529–536.

29. Maggi C. A., Patacchini R., Rovero P. and Giachetti A. (1993) Tachykinin receptors and tachykinin receptor antagonists. *J. Auton. Pharmacol.* **13**, 23–93.
30. Maranto A. R. (1994) Primary structure, ligand binding, and localization of the human type 3 inositol 1, 4, 5-triphosphate receptor expressed in intestinal epithelium. *J. Biol. Chem.* **269**, 1222–1230.
31. Mayer E. A., Sun X. P., Supplisson S., Kodner A., Regoli M. and Sachs G. (1991) Neurokinin receptor-mediated regulation of [Ca], and Ca-sensitive ion channels in mammalian colonic muscle. *Ann. N.Y. Acad. Sci.* **632**, 439–441.
32. Perdue M. H., Galbraith R. and Davison J. S. (1987) Evidence for substance P as a functional neurotransmitter in guinea pig small intestinal mucosa. *Regul. Pept.* **18**, 63–74.
33. Portbury A. L., Furness J. B., Southwell B. R., Wong H., Walsh J. H. and Bunnett N. W. (1996) Distribution of neurokinin-2 receptors in the guinea-pig gastrointestinal tract. *Cell Tissue Res.* **286**, 281–292.
34. Reinlib L., Mikkelsen R., Zahniser D., Dharmasathaphorn K. and Donowitz M. (1989) Carbachol-induced cytosolic free Ca^{2+} increases in T84 colonic cells seen by microfluorimetry. *Am. J. Physiol.* **257**, G950–G960.
35. Sarosi G. A. Jr, Kimball B. C., Barnhart D. C., Zhang W. and Mulholland M. W. (1998) Tachykinin neuropeptide-evoked intracellular calcium transients in cultured guinea pig myenteric neurons. *Peptides* **19**, 75–84.
36. Satoh Y., Williams M. R. and Habara Y. (1999) Effects of AIF4- and ATP on intracellular calcium dynamics of crypt epithelial cells in mouse small intestine. *Cell Tissue Res.* **298**, 295–305.
37. Southwell B. R. and Furness J. B. (2001) Immunohistochemical demonstration of the NK₁ tachykinin receptor on muscle and epithelia in guinea pig intestine. *Gastroenterology* **120**, 1140–1151.
38. Sternini C., Anderson K., Frantz G., Krause J. E. and Brecha N. (1989) Expression of substance P / neurokinin A-encoding preprotachykinin messenger ribonucleic acids in the rat enteric nervous system. *Gastroenterology* **97**, 348–356.
39. Tateishi K., Kishimoto S., Kobayashi H., Kobuke K. and Matsuoka Y. (1990) Distribution and localization of neurokinin A-like immunoreactivity and neurokinin B-like immunoreactivity in rat peripheral tissue. *Regul. Pept.* **30**, 193–200.
40. Treiman M., Caspersen C. and Christensen S. B. (1998) A tool coming of age: thapsigargin as an inhibitor of sarco-endoplasmic reticulum Ca^{2+} -ATPases. *Trends Pharmacol. Sci.* **19**, 131–135.
41. Vajanaphanich M., Schultz C., Tsien R. Y., Traynor-Kaplan A. E., Pandol S. J. and Barrett K. E. (1995) Cross-talk between calcium and cAMP-dependent intracellular signaling pathways: implications for synergistic secretion in T84 colonic epithelial cells and rat pancreatic acinar cells. *J. Clin. Invest.* **96**, 386–393.
42. Wang L., Stanis A. M., Wershil B. K., Galli S. J. and Perdue M. H. (1995) Substance P induces ion secretion in mouse small intestine through effects on enteric nerves and mast cells. *Am. J. Physiol.* **269**, G85–G92.
43. Wang X. T., Nagaba Y., Cross H. S., Wrba F., Zhang L. and Guggino S. E. (2000) The mRNA of L-type calcium channel elevated in colon cancer: protein distribution in normal and cancerous colon. *Am. J. Pathol.* **157**, 1549–1562.
44. Wu K. D., Lee W. S., Wey J., Bungard D. and Lytton J. (1995) Localization and quantification of endoplasmic reticulum Ca^{2+} -ATPase isoform transcripts. *Am. J. Physiol.* **269**, C775–C784.