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Substance P-evoked Cl^- secretion in guinea pig distal colonic epithelia: interaction with PGE_2

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Hosoda, Yutaka, Shin-Ichiro Karaki, Yukiko Shimoda, and Atsukazu Kuwahara. Substance P-evoked Cl^- secretion in guinea pig distal colonic epithelia: interaction with PGE_2. *Am J Physiol Gastrointest Liver Physiol* 283: G347–G356, 2002; 10.1152/ajpgi.00504.2001.—Interaction between substance P (SP) and PGE_2 on Cl^- secretion in the guinea pig distal colonic epithelia was investigated. A short-circuit current (I_{sc}) was measured as an index of ion transport. Mucosa preparations deprived of muscle and submucosa of distal colon were mounted in the Ussing flux chamber and treated with TTX and piroxicam to remove the influences of neuronal activity and endogenous PG synthesis, respectively. Although SP (10^{-7} M) itself evoked little increase in I_{sc}, exogenous PGE_2 concentration dependently enhanced the response of SP. The effect of PGE_2 on the SP-evoked response was mimicked by forskolin and 8-bromoadenosine cAMP. Depletion of Ca^{2+} from the bathing solution reduced the PGE_2-dependent response of SP. Effects of PGE_2, SP, and SP in the presence of PGE_2 on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in isolated crypt cells were measured by the confocal microscope fluorescence imaging system. SP, but not PGE_2, temporally evoked an increase in $[Ca^{2+}]_i$ but declined to the baseline within 3 min. A return of the SP-evoked increase in $[Ca^{2+}]_i$ was slower in the presence of PGE_2 than SP alone. These results suggest that PGE_2 synergistically enhances SP-evoked Cl^- secretion via an interaction between the intracellular cAMP and $[Ca^{2+}]_i$ in the epithelial cells. In conclusion, SP and PGE_2 could cooperatively induce massive Cl^- secretion in guinea pig distal colon at epithelial levels.

colonic crypt; adenosine 3′,5′-cyclic monophosphate; Ca^{2+}, crosstalk; inflammation

SUBSTANCE P (SP) is a member of the tachykinin family widely distributed in the enteric nervous system (ENS) of small and large intestines (15, 16, 24, 25). The tachykinin family includes neurokinin A (NKA) and B (NKB) in addition to SP. These tachykinins are involved in smooth muscle contractions, blood flow, local immune functions, and epithelial transport. The effects of tachykinins are mediated by at least three different receptor subtypes: neurokinin-1 (NK_1), NK_2, and NK_3. These receptors are coupled to G protein, and its stimulation induces an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) as a second messenger (25). In the intestine, SP and NKA, but not NKB, are mainly found in the intrinsic primary afferent neurons, interneurons, and motorneurons in the ENS (15). Grady et al. (17) reported the immunoreactivity of neurokinin receptors in the gastrointestinal tract of the rat and that NK_1 receptors are distributed in myenteric and submucosal neurons and in interstitial cells of Cajal, NK_2 receptors are localized to circular and longitudinal muscle cells and to nerve endings in the plexuses, and NK_3 receptors are detected in numerous myenteric and submucosal neurons. Recently, Southwell and Furness (44) reported that NK_1 receptor immunoreactivity was detected both on the muscle and on the mucosal epithelium of the guinea pig small and large intestines. It is known that endogenous SP and NKA in the intestine interact with other enteric transmitters, such as ACh, and regulate intestinal motility, and fluid and electrolyte transport (15).

Presence of SP in nerve fibers close to epithelial cells suggests a role for SP in the regulation of epithelial transport. In a previous study, Kuwahara and Cooke (23) reported that the exogenous addition of SP evokes Cl^- secretion in the guinea pig distal colon and that it is mediated by neurons and a nonneuronal pathway. Involvement of ENS on SP-evoked Cl^- secretion has been intensively studied, but little is known about the nonneuronal pathway of SP-induced Cl^- secretion in the colon (10, 14, 23).

It has been reported that SP-evoked Cl^- secretion is inhibited in the porcine jejunum (46), dog (35), guinea pig (23), and human colon (38) by pretreatment with cyclooxigenase (COX) inhibitor, and that SP induces PGE_2 synthesis (46). These effects are typically ascribed to agonist-stimulated release of PGs from the subepithelium. These reports also suggest that SP evokes Cl^- secretion via activation of enteric neurons and/or production of PG in mammalian colonic mucosa. PGs are well known as inflammatory mediators and secretagogues in the gastrointestinal tract (12, 13, 32, 41, 43). PGs are reported to act both on the epithelium

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and on the submucosal plexus to evoke ion transport (12, 13). Homaidan et al. (20) reported that PGE_2 binding sites are detected and cAMP levels are increased by PGE_2 in the crypt cells. An increasing body of evidence indicates that SP is involved in the pathophysiology of intestinal secretion and inflammation in animals and humans (6, 28, 29, 45). The administration of SP receptor antagonists reduces secretory and inflammatory changes in rat models of acute and chronic intestinal inflammation (7, 34). Furthermore, SP immunoreactivity and SP binding are increased in the colons of patients with inflammatory bowel disease (22, 27). Thus it is possible that SP and PGE_2 interact with each other in inflammatory conditions in the intestine. However, the interaction of SP and PGE_2 on epithelial cells and the cellular mechanism of SP-evoked Cl^- secretion have not been investigated in depth.

In the present study, we investigated the nonneuronal pathway of SP-evoked Cl^- secretion in the guinea pig distal colon. In particular, we focused on the interaction between SP and PGE_2 on Cl^- secretion. Results show that synergistic action of SP and PGE_2 on Cl^- secretion occurs at the epithelial cell level of guinea pig distal colon.

MATERIALS AND METHODS

Ussing flux chamber experiment. Male albino guinea pigs (Hartley-Hazleton; Nippon SLC, Hamamatsu, Japan) ranging in weight from 400 to 900 g were allowed food and water ad libitum before the experiments. The animals were stunned and exsanguinated according to the method approved by the *Guide for Animal Experimentation* of the National Institute for Physiological Sciences of Japan. In the present experiments, tissue was prepared according to a previous study (4) to remove the neural influence. Segments of distal colon were removed, flushed with Krebs-Ringer solution, and cut along the mesenteric border. Tissues were then laid flat on an acrylic board with the mucosal side up. Mucosal preparation was made by longitudinally peeling off mucosa using a pair of glass slides. This procedure removes submucosal ganglia, the submucosal layer, and external muscle layers, including myenteric plexus (4). Four sets of mucosal sheets were mounted between halves of Ussing flux chambers in which the total cross-sectional area was 0.64 cm^2. Mucosal and serosal surfaces of the tissues were bathed with 10 ml of Krebs-Ringer solution by recirculation from a reservoir maintained at 37°C during the experiment. Tissues were left in the solution for 0.5–1 h before the experiment. Krebs-Ringer solution contained (in mM) 120 NaCl, 6 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 14.4 $NaHCO_3$, 2.5 $CaCl_2$, and 11.5 glucose. The Cl^--free solution contained (in mM) 2.7 K_2SO_4, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 54.9 Na_2SO_4, 13 $NaHCO_3$, 1.7 $CaSO_4$, 60.4 mannitol, and 11.5 glucose. The solution was gassed with 95% O_2-5% CO_2 and buffered at pH 7.2. For a Ca-free solution, $CaCl_2$ was removed from the Krebs-Ringer solution and 3 mM EDTA was added. The potential difference (PD) across the tissue was measured by paired Ag-AgCl electrodes in Krebs-agar bridge and clamped to 0 mV by applying a short-circuit current (I_{sc}) by Ag-AgCl electrodes with a voltage-clamp apparatus (SS-1335; Nihon-Koden, Tokyo, Japan). Tissue conductance (G_t) was calculated by determining the current necessary to change PD by 10 mV. Responses were continuously recorded on a chart recorder (Recti-Horitz-8K; Nihon-Denki Sanei, Tokyo, Japan) and Mac/Lab8 system (ADInstruments; Cattle Hill, Australia). ΔI_{sc} was calculated on the basis of the value before and after stimulation.

To check the neuronal influence, tissues were electrically stimulated by passing a current parallel to the plane of the tissue via a pair of aluminum foil ribbon electrodes placed on the submucosal surface of the tissues. Rectangular stimulus pulses of 25 V, 10 Hz, and 0.5 ms duration were applied. To test the residual neuronal influence of the effect of SP on I_{sc} and endogenous PG synthesis, TTX (10^{-7} M), piroxicam (10^{-6} M), or both TTX and piroxicam were pretreated 10 min before the addition of SP (10^{-7} M). All following experiments were done in the presence of TTX (10^{-7} M) and piroxicam (10^{-6} M). Various concentrations of PGE_2 ($10^{-7} - 10^{-4}$ M) were added to serosal bathing solution 10 min before the addition of SP (10^{-7} M), and changes in I_{sc} were measured. A combination of ω-conotoxin GVIA (3×10^{-7} M) and ω-conotoxin MVIIC (3×10^{-7} M) was used to block the release of neurotransmitters from nerve terminals, as done in a previous study (19). Bumetanide (5×10^{-4} M) and Cl^--free solution were used to investigate whether the ion component of change in I_{sc} was Cl^- secretion. Forskolin and 8-bromoadenosine cAMP (8-br-cAMP) were used to stimulate adenylate cyclase and as exogenous cAMP, respectively.

Isolation of crypts. Distal colonic segments (~4 cm) were rinsed with cold Krebs-Ringer solution with 3 mM dithiothreitol and then filled with PBS (−), including 25 mM EDTA and 3 mM dithiothreitol, until a moderate tension was achieved by clamping both ends. Tissue was then incubated in Krebs-Ringer solution for 3 min at 37°C. Then, the luminal solution containing isolated crypts was collected by centrifugation (4°C; 1,000 rpm for 1 min). A portion of the supernatant was then removed, and the tissue fragments containing crypts were rinsed twice with Krebs-Ringer solution.

Measurement of $[Ca^{2+}]_i$ in isolated crypt cells. Isolated crypts were suspended with 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. Suspension of dye-loaded crypt was seeded on a specially designed glass perfusion vessel coated with cell adhesive Cell-Tak to fix the crypts for 20 min in a refrigerator. Then, the vessel was placed in a 5% CO_2 incubator for 60–90 min at 37°C to allow indo 1-AM loading. After dye loading, the vessel was washed with Krebs-Ringer solution containing 0.1% BSA. The vessel was then placed on the stage of a laser-scanning confocal imaging system (ACAS Ultima 575 UVC; Meridian Instruments, Okemos, MI) with an inverted microscope (Axiovert 135; Zeiss) magnification ×40 (water immersion objective). The vessel was continuously perfused at 2 ml/min of flow rate with the oxygenated Krebs-Ringer solution containing 0.1% BSA at 32°C. Indo 1-AM was excited using the 350- to 360-nm line. Twin photomultiplier channels detected bands of fluorescence centered on 405 and 485 nm. Ratiometric images were collected every 10 s in the stimulated and unstimulated conditions for analysis of temporal change in $[Ca^{2+}]_i$.

Isolated crypts were perfused before stimulation with normal Krebs-Ringer solution containing 0.1% BSA. The crypts were then continuously stimulated by replacing the normal perfused Krebs-Ringer solution with Krebs-Ringer solution containing either SP (10^{-7} M) or PGE_2 (10^{-5} M), and changes in $[Ca^{2+}]_i$ were measured. In other experiments, Krebs-Ringer solution containing PGE_2 (10^{-5} M) was perfused for 10 min, then the Krebs-Ringer solution containing SP (10^{-6} M) and PGE_2 was further perfused, and changes in $[Ca^{2+}]_i$ were measured.

Chemicals. Substance P was purchased from Peptide Institute (Osaka, Japan); bumetanide, DMSO, TTX, ω-conotoxins, and 8-br-cAMP were from Sigma (St. Louis, MO); piroxicam and forskolin were from Biomol Research Laboratories (Plymouth Meeting, PA); PGE_2 was from Cayman (Ann Arbor, MI). Bumetanide and piroxicam were dissolved in dimethyl sulphoxide. The other drugs were dissolved in distilled water. Volume of dissolved drugs in H_2O or dimethyl sulphoxide added to the bathing solutions did not exceed 100 and 10 μl, respectively.

Statistics. All data are expressed as means ± SE. ANOVA was followed by the Tukey test to determine significant differences between each experimental tissue. $P < 0.05$ was considered statistically significant. Concentration-response curves were fitted to Michaelis-Menten binding curves by the nonlinear-square procedure using KyPlot, a data analysis and graph-creating software (50). We considered the PGE_2-evoked sustained phase (see RESULTS) of I_{sc} consisted of two components of ion transport: K^+ secretion as negative I_{sc} and Cl^- secretion as positive I_{sc} (19). Therefore, the equation to fit the curve was calculated as the sum of the two following Michaelis-Menten equations: $I = I_K \cdot C/(C + EC_{50,K}) + I_{Cl} \cdot C/(C + EC_{50,Cl})$, where I, I_K, and I_{Cl} are net K^+ and Cl^- I_{sc}, respectively; C is PGE_2 concentration, and $EC_{50,K}$ and $EC_{50,Cl}$ are the half effective concentrations K^+ I_{sc} and Cl^- I_{sc}, respectively, constrained with $I_K \leq 0$ and I_{Cl}, $EC_{50,K}$, and $EC_{50,Cl} \geq 0$.

RESULTS

Effects of TTX and piroxicam on SP-evoked increase in I_{sc}. The present experiment was designed to examine the interaction between SP and PGE_2 on ion transport using a mucosal preparation of guinea pig distal colon. The average PD, basal I_{sc}, and G_t just before the addition of SP in the control, TTX-pretreated, piroxicam-pretreated, and both TTX- and piroxicam-pretreated groups were not significantly different, respectively. The PD, I_{sc}, and G_t of the control group were 4.2 ± 0.2 mV, −55.5 ± 6.8 μA/cm^2 and 12.0 ± 0.9 mS/cm^2, respectively ($n = 4$). In the mucosal preparations, SP (10^{-7} M) evoked a biphasic increase in I_{sc} (1st phase: 21.9 ± 6.1 μA/cm^2; 2nd phase: 105.9 ± 26.3 μA/cm^2; $n = 4$) (Fig. 1). TTX (10^{-7} M) pretreatment decreased the SP-evoked responses to 13.9 ± 5.4 μA/cm^2 (1st phase, $P = 0.56$) and 28.3 ± 26.3 μA/cm^2 (2nd phase, $P < 0.05$), respectively ($n = 4$) (Fig. 1*B*). Piroxicam (10^{-6} M) pretreatment also decreased the SP-evoked responses to 5.1 ± 1.6 μA/cm^2 (1st phase, $P = 0.07$) and 18.6 ± 14.7 μA/cm^2 (2nd phase, $P < 0.05$), respectively ($n = 4$). Moreover, combination of TTX and piroxicam pretreatment decreased the SP-evoked responses to 4.7 ± 1.4 μA/cm^2 (1st phase, $P = 0.06$) and 15.6 ± 10.6 μA/cm^2 (2nd phase, $P < 0.05$), respectively ($n = 4$). The SP response in the presence of piroxicam and TTX was not significantly different from the response with piroxicam or TTX alone. In the present experiments, the SP-evoked biphasic increase in I_{sc} was inhibited by TTX and piroxicam, as mentioned above. This result indicated that the effect of SP via neurons and endogenous PGs remained even in the mucosal preparations. Moreover, the electrical field stimulation (25 V, 10 Hz, and 0.5 ms duration) for 2 min increased basal I_{sc} in the absence of TTX (105.9 ± 8.0 μA/cm^2, $n = 4$), and the response was completely abolished by pretreatment with TTX (10^{-7} M). Thus for further experiments, all tissues were pretreated with TTX (10^{-7} M) and piroxicam (10^{-6} M) to completely remove the neuronal effect and production of endogenous PGs in tissues.

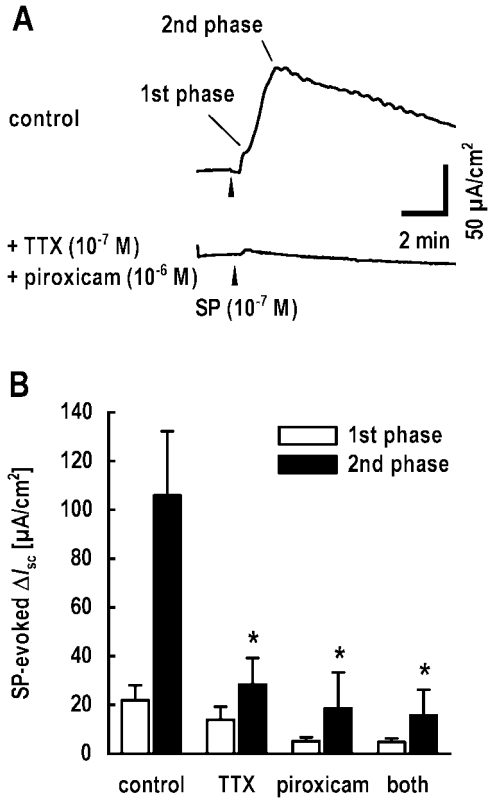

Fig. 1. Effects of TTX and piroxicam on substance P (SP)-evoked change in short-circuit current (I_{sc}) of aganglionated tissues from guinea pig distal colon. SP (10^{-7} M) was added to the serosal bathing solution in pretreatment with TTX (10^{-7} M), piroxicam (10^{-6} M), or both, and SP-evoked changes in I_{sc} were measured. *A*: representative traces to illustrate effects of SP on basal I_{sc} in the presence or absence of TTX and piroxicam on SP-evoked changes in I_{sc}. Vertical and horizontal bars are I_{sc} and time, respectively. *B*: effects of TTX, piroxicam, or TTX + piroxicam on SP-evoked responses. Peak values were expressed as means ± SE, $n = 4$, $^*P < 0.05$ vs. control.

Effect of PGE_2 on basal I_{sc} and SP-evoked increase in I_{sc}. To investigate the interaction between SP and PGE_2 on ion transport, tissues were pretreated with PGE_2 ($10^{-9} - 10^{-4}$ M) 10 min before the addition of SP (10^{-7} M). Figure 2*A* shows a representative trace of the SP-evoked response 10 min after the addition of PGE_2 (10^{-5} M) in the presence of TTX and piroxicam. The serosal addition of PGE_2 ($>10^{-7}$ M) concentration-dependently evoked biphasic changes in I_{sc} in both transient and sustained phases (Fig. 2*B*). The maximal increase in I_{sc} was observed at 10^{-5} M PGE_2. The transient phase was observed ~1 min after the addition of PGE_2, and the sustained phase lasted for >20 min (Fig. 2*A*). Values of PGE_2 (10^{-5} M)-evoked transient phase and sustained phase were 202.3 ± 24.2 and 61.7 ± 9.5 μA/cm^2, respectively (Fig. 2*B*). EC_{50} of the transient phase of PGE_2 was 2.46 × 10^{-6} M. On the

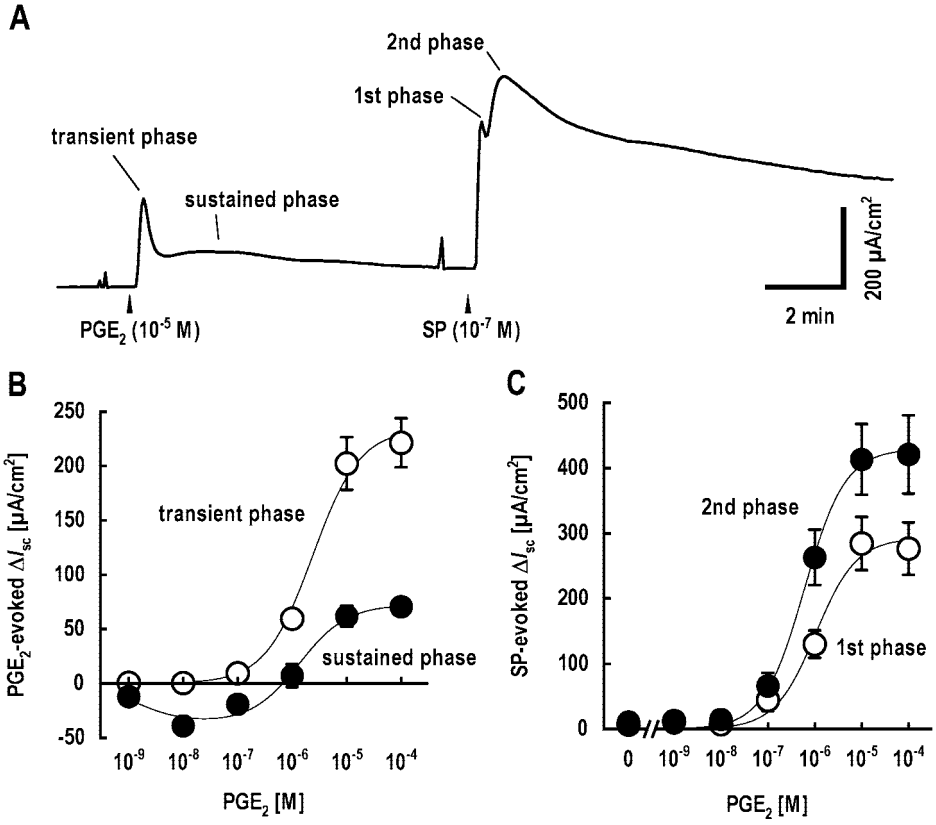

Fig. 2. Effects of PGE_2 and the further addition of SP on I_{sc} in the presence of TTX and piroxicam. Various concentrations (10^{-9} – 10^{-4} M) of PGE_2 were added to the serosal bathing solution, and SP (10^{-7} M) was added to the serosal bathing solution 10 min after the addition of PGE_2. PGE_2 and the further addition of SP-evoked changes in I_{sc} were measured. All tissues were pretreated with TTX (10^{-7} M) and piroxicam (10^{-6} M). *A*: representative trace of effects of PGE_2 (10^{-5} M) and the further addition of SP (10^{-7} M) on I_{sc}. *B* and *C*: concentration-response curves of PGE_2 (*B*) and the further addition of SP (*C*)-evoked changes in I_{sc}. Peak values were expressed as means ± SE, n = 4–15.

other hand, low concentrations of PGE_2 ($<10^{-7}$ M) evoked a decrease in I_{sc}, and the responses reached a plateau at 10 min or more. Maximal decrease was observed at 10^{-8} M PGE_2 (−39.0 ± 0.7 μA/cm², n = 4). Curve fitting of the sustained phase was calculated by the sum of the two divided components, including K^+ and Cl^- secretion based on a previous study (19). $EC_{50,A}$ and $EC_{50,B}$ were 1.42×10^{-9} M and 1.33×10^{-6} M, respectively.

Serosal addition of SP (10^{-7} M) in the presence of TTX and piroxicam evoked little biphasic increase in I_{sc}, as described above (Fig. 1). However, in the presence of PGE_2, SP (10^{-7} M) evoked an apparent increase in I_{sc}, and the responses were dependent on the concentration of PGE_2 in the 1st phase (peak: 15–20 s after the addition of SP) and 2nd phase (0.5 - 2 min) (Fig. 2, *A* and *C*). The maximum values of SP (10^{-7} M) evoked a biphasic increase in I_{sc}, achieved at 10^{-5} M of PGE_2 pretreatment (1st phase: 284.5 ± 40.8 μA/cm²; 2nd phase: 413 ± 54.3 μA/cm², n = 10). The EC_{50}s of SP-evoked 1st and 2nd phase responses were 1.05×10^{-6} M and 6.03×10^{-7} M, respectively.

In addition, to confirm no contribution of neurotransmitter release by PGE_2 or SP in the presence of TTX, ω-conotoxins were used to block neurotransmitter release by inhibition of presynaptic Ca^{2+} channels. As a result, the combination of ω-conotoxin GVIA (3×10^{-7} M) and ω-conotoxin MVIIC (3×10^{-7} M) did not affect both the responses of PGE_2 (10^{-5} M) and SP (10^{-7} M) in the presence of PGE_2.

Effects of Cl^--free solution and bumetanide on PGE_2- and PGE_2-dependent SP-evoked increase in I_{sc}. To determine the ionic basis for the increase in I_{sc} induced by PGE_2- and PGE_2-dependent SP-evoked responses, bumetanide, an inhibitor of the Na^+-K^+-$2Cl^-$ cotransporter and a Cl^--free solution were used.

Bumetanide (5×10^{-4} M) was added 10 min before the application of piroxicam and TTX. Concentration of bumetanide was chosen based on the previous experiment (21). In the bumetanide-treated condition, PD and basal I_{sc}, but not G_t, were significantly changed from 4.2 ± 0.7 to −0.6 ± 1.2 mV and from −58.7 ± 9.4 to 7.8 ± 18.0 μA/cm², respectively (n = 5). Pretreatment with bumetanide did not alter the response of PGE_2 (Fig. 3*A*). On the other hand, bumetanide significantly reduced the PGE_2-dependent SP-evoked increase in I_{sc} from the control value of the 1st phase (262.9 ± 25.2 μA/cm²) and the 2nd phase (422.3 ± 30.1 μA/cm²)(n = 12) to 64.8 ± 8.6 and 15.6 ± 1.6 μA/cm² (n = 6), respectively (Fig. 3*B*).

Bathing solution was replaced by a Cl^--free solution 10 min before the addition of TTX and piroxicam. Depletion of Cl^- from the bathing solution significantly changed in PD, basal I_{sc}, and G_t from 2.5 ± 0.8 to −2.6 ± 1.7 mV, −42.1 ± 15.1 to 17.6 ± 17.4 μA/cm², and 14.7 ± 0.8 to 9.9 ± 1.3 mS/cm², respectively (n = 5–9). In the Cl^--free solution, PGE_2-evoked transient increase in I_{sc} was significantly decreased from 162.2 ± 25.2 μA/cm² (n = 12) to 64.8 ± 8.6 μA/cm² (n = 10), whereas the sustained phase was not affected (Fig.

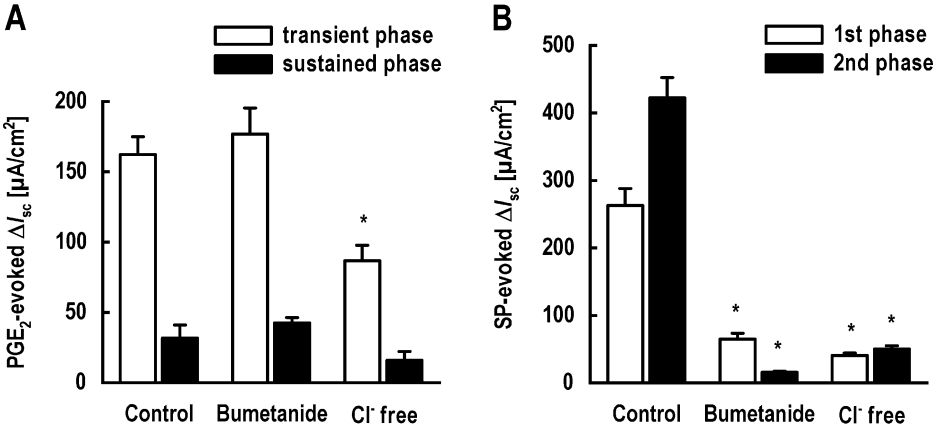

Fig. 3. Effects of bumetanide and Cl^--free solution on PGE_2 and PGE_2-dependent SP-evoked increase in I_{sc}. Bumetanide (5×10^{-4} M) was added to the serosal bathing solution or the bathing solution was changed to Cl^--free solution before the addition of TTX and piroxicam. Ten min after these procedures, PGE_2 (10^{-5} M) was added to the serosal bathing solution, and 10 min later, SP (10^{-7} M) was added to the serosal bathing solution. Effects of bumetanide and Cl^--free solution on PGE_2 (*A*) and the further addition of SP (*B*)-evoked increases in I_{sc} were measured. Peak values were expressed as means ± SE, $n = 5$–12, $^*P < 0.05$ vs. control.

3*A*). Both phases of the SP-evoked increases in I_{sc} significantly decreased by the depletion of Cl^- from the control value of the 1st phase (262.9 ± 25.2 μA/cm^2) and the 2nd phase (422.3 ± 30.1 μA/cm^2) ($n = 12$) to 40.8 ± 3.5 and 50.2 ± 5.0 μA/cm^2 ($n = 9$), respectively (Fig. 3*B*).

Effects of PGE_2, forskolin, or 8-br-cAMP on SP-evoked increase in I_{sc}. To determine whether the increase in intracellular cAMP mimics the effect of PGE_2 on the SP-evoked increase in I_{sc}, an adenylate cyclase activator forskolin or a membrane-permeable cAMP analog 8-br-cAMP were used. Serosal addition of forskolin (10^{-5} M) evoked an increase in basal I_{sc} (64.6 ± 13.2 μA/cm^2, $n = 3$). On the other hand, 8-br-cAMP (10^{-3} M) evoked a decrease in basal I_{sc} (−16.8 ± 11.9 μA/cm^2, $n = 4$), but the changes were not statistically significant. The addition of SP (10^{-7} M) 10 min after treatment with forskolin or 8-br-cAMP in the presence of TTX and piroxicam evoked the following biphasic increases in I_{sc}: forskolin pretreatment, 1st phase 327.6 ± 7.3 μA/cm^2, 2nd phase 361.5 ± 19.1 μA/cm^2, $n = 3$; 8-br-cAMP pretreatment, 1st phase 188.7 ± 33.4 μA/cm^2, 2nd phase 317.6 ± 64.9 μA/cm^2, $n = 4$ (Fig. 4).

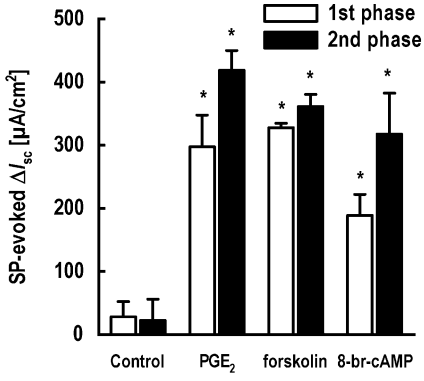

Fig. 4. Effects of PGE_2, forskolin and 8-bromoadenosine cAMP (8-br-cAMP) on the SP-evoked increase in I_{sc}. PGE_2 (10^{-5} M), forskolin (10^{-5} M) or 8-br-cAMP (10^{-3} M) were added to the serosal bathing solution 10 min before the addition of SP (10^{-7} M), and the SP-evoked increase in I_{sc} was measured. All tissues were pretreated with TTX (10^{-7} M) and piroxicam (10^{-6} M). Peak values were expressed as means ± SE, $n = 3$–4, $^*P < 0.05$ vs. control.

To confirm that the SP-evoked increase in I_{sc} in the presence of forskolin is due to Cl^- secretion, as with the PGE_2-dependent SP-evoked responses, bumetanide was used. Pretreatment of the tissues with bumetanide (5×10^{-4} M) did not affect the forskolin (10^{-5} M)-evoked response, but significantly inhibited SP-evoked responses from the control values of 522.3 ± 122.1 μA/cm^2 (1st phase) and 562.5 ± 40.6 μA/cm^2 (2nd phase) to 89.9 ± 5.9 μA/cm^2 (1st phase) and 19.7 ± 4.5 μA/cm^2 (2nd phase) ($n = 4$), respectively.

Effect of Ca^{2+} in bathing solution on PGE_2- and PGE_2-dependent SP-evoked increase in I_{sc}. To investigate the affect of extracellular Ca^{2+} on PGE_2- and PGE_2-dependent SP-evoked increase in I_{sc}, serosal, mucosal, or both sides bathing solution were replaced by a Ca^{2+}-free solution before the addition of TTX and piroxicam. Depletion of serosal and both sides Ca^{2+} significantly changed in G_t from 13.3 ± 1.4 and 10.9 ± 0.9 mS/cm^2 to 31.5 ± 3.1 and 34.8 ± 4.9 mS/cm^2 ($n = 4$), respectively.

Serosal and both sides, but not mucosal Ca^{2+}-free solution, significantly increased the PGE_2 (10^{-5} M)-evoked sustained phase of I_{sc} (control: 34.1 ± 8.6 μA/cm^2, $n = 10$; serosal Ca^{2+} free: 122.9 ± 27.5 μA/cm^2, $n = 4$; both sides Ca^{2+} free: 138.7 ± 11.6 μA/cm^2, $n = 4$) but not the transient phase (Fig. 5*A*).

Serosal and both sides, but not mucosal Ca^{2+}-free solution, also significantly decreased the PGE_2-dependent SP (10^{-7} M)-evoked increase in I_{sc} (control: 1st phase 340.9 ± 39.3 μA/cm^2, 2nd phase 478.8 ± 37.0 μA/cm^2, $n = 10$; serosal Ca^{2+} free: 1st phase 12.2 ± 5.1 μA/cm^2, 2nd phase 6.8 ± 3.1 μA/cm^2, $n = 3$; both sides Ca^{2+} free: 1st phase 87.3 ± 15.7 μA/cm^2, 2nd phase 12.1 ± 23.9 μA/cm^2, $n = 4$) (Fig. 5*B*).

Effects of PGE_2, SP, and SP in the presence of PGE_2 on $[Ca^{2+}]_i$ in isolated colonic crypt cells. Isolated crypt cells were used to investigate the involvement with Ca^{2+} signaling pathway in PGE_2-dependent SP-evoked responses. Perfusion with a solution containing PGE_2 (10^{-5} M) did not affect $[Ca^{2+}]_i$ in isolated crypt cells. On the other hand, SP (10^{-7} M) evoked a transient increase in $[Ca^{2+}]_i$ (peak value of the normalized ratio: 2.70 ± 0.19, $n = 7$) and returned to basal level within 3 min (Fig. 6). The presence of PGE_2 in the perfusate

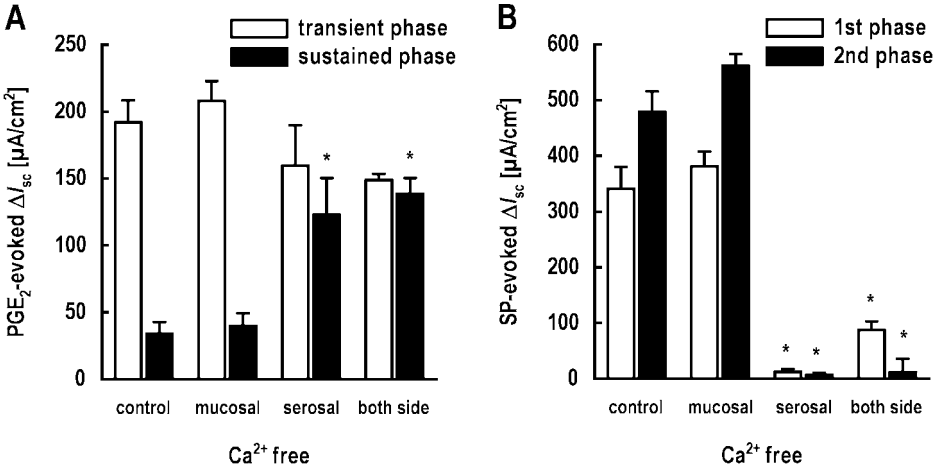

Fig. 5. Effects of removal of Ca^{2+} from bathing solution on PGE_2 and PGE_2-dependent SP-evoked increase in I_{sc}. The bathing solution was changed to Ca^{2+}-free solution before the addition of TTX (10^{-7} M) and piroxicam (10^{-6} M). Ten minutes after these procedures, PGE_2 (10^{-5} M) was added to the serosal bathing solution, and 10 min later, SP (10^{-7} M) was added to the serosal bathing solution. Effects of Ca^{2+}-free solution on PGE_2 (*A*) and the further addition of SP (*B*)-evoked increases in I_{sc} were measured. Peak values were expressed as means ± SE, $n = 3$–10, $^*P < 0.05$.

did not affect the peak values of the SP-evoked increase in $[Ca^{2+}]_i$ (2.89 ± 0.18, $n = 6$) (Fig. 6). However, the return of $[Ca^{2+}]_i$ to the basal level was slower in the presence of PGE_2 (Fig. 6*A*). The area under the curves (AUCs; change in normalized ratio × s) for 3 min were compared. Results show that the presence of PGE_2 significantly increased the AUC of normalized ratio for 3 min by SP from 97.5 ± 13.9 to 173.7 ± 9.6 ($n = 6$).

DISCUSSION

In the present study, we have shown the direct action of SP and the interaction with PGE_2 on Cl^- secretion in the guinea pig distal colonic epithelia. Previous studies have shown that the SP-evoked nonneurally mediated secretion is much weaker than neurally mediated secretion (10, 14, 23). We have also obtained a similar result (Fig. 1*A*). However, in the present study, we found that SP could induce massive Cl^- secretion at nonneural, perhaps epithelial cell levels, and in as large amounts as the neurally mediated responses when a high concentration of PGE_2 was present. Moreover, we have shown that SP evokes a direct and transient increase in $[Ca^{2+}]_i$, and SP-evoked nonneural massive Cl^- secretion is due to an increase

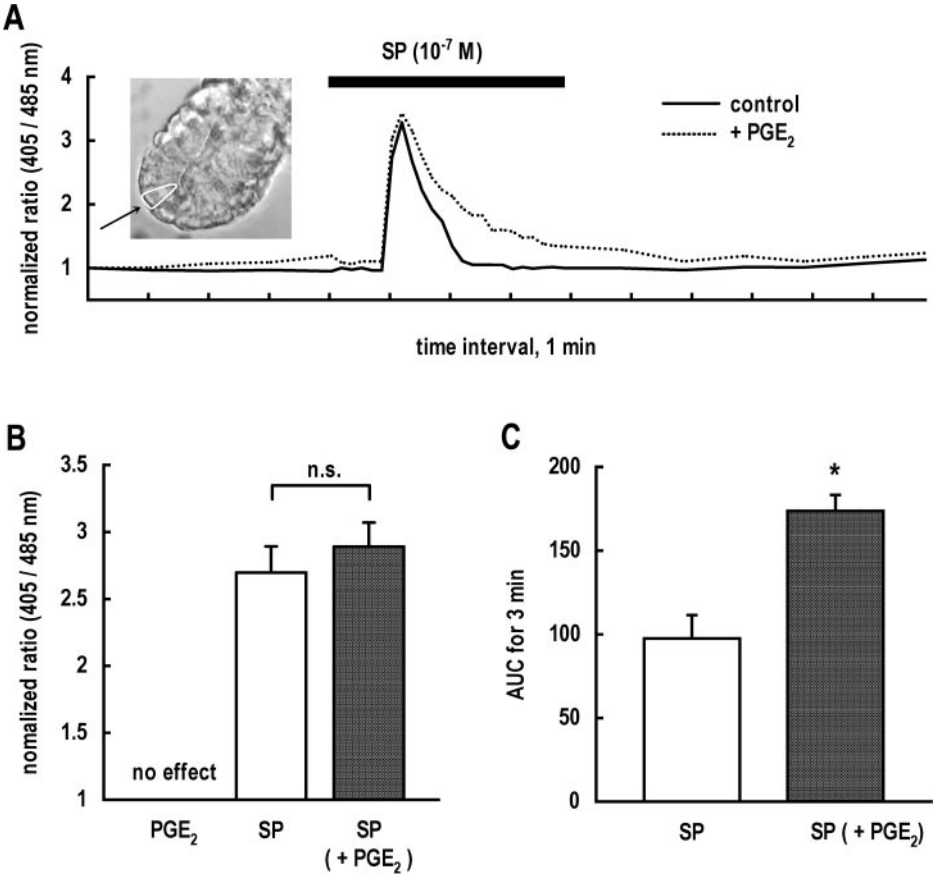

Fig. 6. Effects of PGE_2, SP, and SP in the presence of PGE_2 on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in isolated crypt cells. Normalized ratio of 405-to-485 nm fluorescence intensity were measured as an index of $[Ca^{2+}]_i$ when the isolated crypt cells were perfused with Krebs-Ringer solution containing PGE_2 (10^{-5} M) or SP (10^{-7} M). In addition, 10 min after perfusion with Krebs-Ringer solution containing PGE_2 (10^{-5} M), Krebs-Ringer solution containing PGE_2 (10^{-5} M) plus SP (10^{-6} M) was perfused, and changes in $[Ca^{2+}]_i$ were measured. *A*: time courses of changes in $[Ca^{2+}]_i$ induced by SP (10^{-7} M) and SP (10^{-7} M) in the presence of PGE_2 (10^{-5} M). *Inset*: example of isolated crypt. Arrow indicates a selected crypt cell that SP potently affected. *B*: normalized ratio induced by PGE_2, SP, and SP in the presence of PGE_2. *C*: area under the curve of response induced by SP or SP in the presence of PGE_2 for 3 min. Peak and area values were expressed as means ± SE, $n = 6$–7, $^*P < 0.05$. ns, Not significant.

in intracellular cAMP level. Previous studies (20) have indicated that PGE_2 increases the intracellular cAMP level of colonic crypt cells via EP_2 receptors. Therefore, it is suggested that SP may act in concert with PGE_2 to evoke Cl^- secretion as a crosstalk between Ca^{2+} and cAMP at the epithelial cell level and that this massive Cl^- secretion to flush out noxious agents from the intestinal lumen is not mediated via neurons in the inflammatory condition.

A previous study (23) has shown that the effect of SP on ion transport in the colon is mediated by neurons and mast cell-derived mediators including histamine and PGE_2. In the present study, we chose to make aganglionated mucosal preparations to investigate the direct action of SP and PGE_2 on the epithelium and found that the response of SP was comparatively smaller than those of previous studies (14, 23) using mucosa-submucosa preparations (Fig. 1). The present mucosal preparations were histologically checked and there was no evidence that submucosal ganglia remained (data not shown). However, the preparations proved insufficient to remove ganglia, including nerve fibers, because the SP-evoked response was still sensitive to TTX. It is reported that SP stimulates NK_1 and/or NK_3 receptors on cell somas and dendrites of secretomotor neurons (14). The result raises the possibility that ganglia are located in the mucosal plexus in the colon (30, 31). Moreover, Riegler et al. (38) have reported that NK_1 immunoreactivity on nerve cells is detected in mucosal lamina propria in the human colon. The SP-evoked response was also reduced by the inhibition of PG synthesis using the COX-inhibitor, piroxicam (Fig. 1). The result suggests that the SP-evoked response is due to endogenous PGs. Moreover, the SP-evoked response in the combination of TTX and piroxicam was not significantly different from TTX or piroxicam alone (Fig. 1). Therefore, the results suggest that a part of the SP-evoked response is dependent on both neuronal activity and PG synthesis. Frieling et al. (12) have reported that PGE_2-evoked Cl^- secretion in guinea pig distal colon is mediated by nerve-dependent and -independent mechanisms. Some part of the SP-evoked response might be due to the interaction between SP and endogenous PGE_2 at the submucosal neuron level. In the present study, we investigated the interaction between SP and PGE_2 on the epithelium. Therefore, the tissues were treated with TTX and piroxicam together to avoid any effect of neuronal activity and endogenous PG synthesis. Moreover, we considered that the release of any neurotransmitters from nerve terminals is not involved, because ω-conotoxins do not affect any responses in the presence of TTX.

In the present experiment, pretreatment of the tissues with PGE_2 concentration dependently enhanced the SP-evoked responses although SP itself also evoked a small increase in I_{sc} (Fig. 2, *A* and *C*). The SP-evoked response in the presence of PGE_2 was inhibited by bumetanide and a Cl^--free bathing solution (Fig. 3*B*). These results indicate that the SP-evoked increase in I_{sc} in the presence of PGE_2 is mainly due to Cl^- secretion. Homaidan et al. (20) reported that PGE_2 increases cAMP level in isolated rabbit colonic crypt cells via the EP_2 subtype of PGE receptors. Therefore, the results suggest that SP can evoke Cl^- secretion in the colonic epithelial cells when intracellular cAMP level increases. We have further tested whether activators of cAMP mimic the effect of PGE_2 on the SP-evoked response using an adenylate cyclase activator, forskolin, or a membrane-permeable cAMP analog, 8-br-cAMP. Data showed that pretreatment with forskolin and 8-br-cAMP could mimic the effect of PGE_2 pretreatment on the SP-evoked increase in I_{sc} (Fig. 4). The SP-evoked increase in I_{sc} in the presence of forskolin also resulted in Cl^- secretion, because the response was bumetanide sensitive. Thus the effect of PGE_2 on the SP-evoked Cl^- secretion is probably due to the increase in intracellular cAMP level in the guinea pig distal colonic epithelial cells. Yajima et al. (49) showed similar results, namely that bethanechol-evoked Cl^- secretion is enhanced when the tissue is pretreated with PGE_2, VIP, and 8-br-cAMP.

It has been suggested that all tachykinin receptors are coupled to $G_{q/11}$ protein, and tachykinins evoke an increase in $[Ca^{2+}]_i$ by the SP > NKA ≫ NKB potency order in isolated guinea pig colonic crypt cells (unpublished observations by K. Shiokawa, Y. Hosoda, Y. Shimoda, M. Suzuki, S. Karaki, M. Ceregrzyn, and A. Kuwahara). Cooke et al. (10) showed that the NK_1 receptor mRNA is expressed and binding of SP is inhibited by the NK_1 receptor antagonist GR-82334 on guinea pig colonic crypt cells. It has been reported that NK_1 receptor immunoreactivity is located on the mucosal epithelium of the guinea pig distal colon (44), but NK_2 receptors are rare (33). These reports suggest that SP stimulates NK_1 receptors and increases in $[Ca^{2+}]_i$ in the distal colonic crypt cells. Therefore, we investigated the role of Ca^{2+} on Cl^- secretion by SP in the presence of PGE_2 on the colonic epithelia. Removal of Ca^{2+} from serosal or both sides bathing solution significantly reduced the SP-evoked increase in I_{sc} in the presence of PGE_2 (Fig. 5*B*). The result indicates that the SP-evoked increase in I_{sc} in the presence of PGE_2 depends on serosal-side extracellular Ca^{2+}. It has been reported that an increase in $[Ca^{2+}]_i$ opens *1*) the calcium-activated chloride channel on the apical membrane and *2*) the basolateral K^+ channel. It also enhances the driving force for Cl^- secretion (2, 3, 18). In the present study, we measured the change in $[Ca^{2+}]_i$ in isolated guinea pig distal colonic crypt cells when the crypts were perfused with PGE_2, SP, or SP in the presence of PGE_2 using a confocal laser-microscope and the calcium imaging system. Results showed that SP evoked a transient increase in $[Ca^{2+}]_i$ both in the presence and absence of PGE_2. However, SP in the absence of PGE_2 evoked little electrogenic ion transport in the mucosal preparations (Fig. 2*C*), although $[Ca^{2+}]_i$ increased considerably (Fig. 6). Thus these results suggest that a transient increase in $[Ca^{2+}]_i$ itself in colonic crypt cells cannot evoke Cl^- secretion; however, SP can evoke Cl^- secretion through a transient increase in $[Ca^{2+}]_i$ with an increase in intracellular cAMP level in the epithelia. Mall et al. (26) reported similar results that carba-

chol-induced increase in $[Ca^{2+}]_i$ can induce Cl^- secretion only in the presence of cAMP. Carew and Thorn (5) have also reported that autocrine release of PGs from epithelial cells is sufficient to support the carbachol-induced Cl^- secretion and that carbachol-evoked Cl^- secretion is dependent on continuous basal production of cAMP in the epithelium. This evidence and our present results suggest that Ca^{2+}-dependent Cl^- secretion is also dependent on cAMP. The mechanism of cAMP-dependent Cl^- secretion is considered to be that an increase in cAMP in the epithelial cell opens the cAMP-dependent Cl^- channel and the K^+ channel on the apical and basolateral membranes, respectively (18). Therefore, it is suggested that the SP-induced increase in $[Ca^{2+}]_i$ may open the Ca^{2+}-dependent K^+ channel (mentioned above) and produce an electrical driving force for anion secretion. In addition, although there was no significant difference between peak values of the SP-induced increase in $[Ca^{2+}]_i$ in the presence and absence of PGE_2, the return to the basal level was significantly slower in the presence of SP and PGE_2 than with SP alone (Fig. 6). Therefore, it is suggested that the effect of PGE_2 on the SP-evoked increase in $[Ca^{2+}]_i$ may contribute to the massive Cl^- secretion. However, the SP-evoked increase in I_{sc} in the Ussing flux chamber experiments was comparatively long-lasting although the SP-evoked increase in $[Ca^{2+}]_i$ in isolated crypt cells was transient. Therefore, we have hypothesized that the transient increase in $[Ca^{2+}]_i$ in colonic epithelial cells might have a role as a trigger of change in intracellular pathways affecting the cAMP regulation and evoking massive Cl^- secretion although there is no firm evidence yet. Moreover, Carew and Thorn (5) showed that PGE_2 secretion in the nanomolar range (1 nM) is sufficient to support the carbachol-induced Cl^- secretion. In the case of SP, >100 times higher concentrations of PGE_2 ($>10^{-7}$ M) were necessary to evoke massive Cl^- secretion (Fig. 2*C*). It is hypothesized that the difference in sensitivity to the PGE_2 level between SP and carbachol might be due to the role of the respective chemical transmitters. Thus in the normal condition with a low concentration of PGE_2, acetylcholine affects the epithelium to evoke Cl^- secretion as a physiological function, whereas in an inflammatory condition with a high concentration of PGE_2, SP might affect the epithelium to evoke massive Cl^- secretion as a pathophysiological action.

In the present study, PGE_2 itself concentration dependently evoked a transient increase and a sustained response in I_{sc} (Fig. 2, *A* and *B*). Pretreatment of the tissues with bumetanide did not alter the PGE_2-evoked response, but a Cl^--free solution decreased the transient phase (Fig. 3*A*). Rechkemmer et al. (36) suggested that the PGE_2-evoked net I_{sc} is consistent with the sum of the electrogenic K^+ and Cl^- secretion and was bumetanide insensitive. Recently, Halm and Halm (19) reported more detailed experiments to describe prostanoids-evoked K^+ and Cl^- secretion in guinea pig distal colon. They suggested that at low concentrations (<30 nM) and high concentrations (>100 nM), PGE_2 stimulates K^+ secretion via EP_2 receptors and Cl^- secretion via DP receptors, respectively. In the present experiment, we had the same results of I_{sc} response, and bumetanide was also insensitive to the PGE_2-evoked response. It has been reported that bumetanide completely blocks PGE_2-evoked K^+ secretion but not Cl^- secretion (36). From our results, the ionic basis of the PGE_2-evoked increase in I_{sc} could not be defined, but some part of the transient phase may be due to Cl^- secretion. Furthermore, HCO_3^- might also contribute to an increase in I_{sc} evoked by PGE_2, especially in the Cl^--free condition (36).

Removal of Ca^{2+} from serosal or both sides bathing solution significantly enhanced the sustained phase of the PGE_2-evoked increase in I_{sc} but not the transient phase (Fig. 5*A*). Calderaro et al. (3) reported that a Ca^{2+}-free solution increases the intracellular cAMP concentration and PGE_2-evoked Cl^- secretion in rabbit distal colonic epithelia. They suggest that an increase in Cl^- secretion in a Ca^{2+}-free solution is due to lower cyclic nucleotide phosphodiesterase activity and higher adenylate cyclase activity than in a Ca^{2+} containing solution. Thus serosal Ca^{2+} may continuously inhibit the PGE_2-evoked sustained phase by decreasing the cAMP level in guinea pig colonic epithelia.

Although the cellular sources of PGs in the present study cannot be defined, it is well established that they can be released from lamina propria cells, including basophils, fibroblasts, macrophages, and mast cells (9). Sharon and Stenson (42) reported that levels of PGs markedly rise in inflammatory bowel disease. Singer et al. (43) also reported that COX-2 protein is not detected in normal human colonic epithelial cells but is detected in Crohn's disease and ulcerative colitis epithelial cells. In general, COX-1 is thought to be responsible for production of the PGs associated with the maintenance of gastrointestinal integrity, whereas COX-2 is believed to be responsible for the production of PGs associated with the mediation of inflammation (40). Furthermore, Mantyh et al. (27) reported that high concentrations of NK_1 receptor binding sites are expressed in the colon of inflammatory bowel disease. From their results, it is suggested that SP may also be involved in the pathophysiology of intestinal inflammation. Mast cells have been implicated in the pathophysiology of intestinal inflammation. Wang et al. (47) reported that mast cell-deficient mice exhibit a reduced ileal secretory response to SP. Thus mast cells may be responsible as one source of PGE_2 release. Taken together, the present results suggest that in pathophysiological states, an increased level of PGE_2 enhances SP-evoked Cl^- secretion to ensure the secretory responses induced by SP.

In the gastrointestinal tract, SP as a neurotransmitter is involved in the physiological control of several digestive functions, including blood flow, intestinal motility, and fluid and ion transport (15). In addition to these effects, many experimental results suggest that SP acts as a mediator for the regulation of intestinal inflammation, as mentioned above. Watanabe et al. (48) showed that SP-immunoreactive nerve fibers are increased in the colonic mucosa of ulcerative colitis

patients. A recent publication by Renzi et al. (37) showed that mRNA expression and immunoreactivity for the NK_1 receptor are dramatically increased in the crypt cells of both Crohn's disease and ulcerative colitis patients. These reports suggest that the SP and NK_1 receptor may be involved in inflammatory reactions in the human distal colon. Moreover, Stucchi et al. (45) suggested that the NK_1 receptor antagonist can have a therapeutic effect in the treatment of chronic ulcerative colitis. Our present findings provide evidence that SP can be a strong secretagogue when the intestinal PGE_2 level is increased. It has been reported that COX (PG synthesizing enzyme) activity level increases in the inflammatory condition (40, 43).

What is the functional role of the synergistic action between SP and PGE_2 observed in the present study? SP is well known as a neurotransmitter in ENS (11, 15). The neural pathways involved in secretory reflexes have not been clearly defined. Classical reflexes contribute to the control of ion transport. In addition to the classical reflexes, axonal reflexes must also be considered potential regulatory mechanisms of ion transport. SP is a key transmitter both in axonal and classical reflexes. In the present experiment, SP still evoked Cl^- secretion by direct action on the epithelium, although the response was smaller than that induced by the classical reflex (23). Therefore, in the physiological state, classical reflexes involving SP are probably important for flushing secretory IgA into the lumen continuously and for maintenance of mucous fluidity necessary to lubricate the luminal contents during their propulsion along the gastrointestinal tract. On the other hand, in the pathophysiological state, axonal reflexes where SP is directly released to the epithelium may be important for flushing out deleterious antigens or microorganisms. Inflammation is characterized by the presence of increased numbers of immune cells, including mast cells and macrophages, etc. Antigen challenge of sensitized tissue causes Cl^- secretion that is mediated, in part, by PGs (1, 39). The release of PGE_2 during anaphylaxis has also been reported (8). In the guinea pig distal colon, PGE_2 and PGD_2 evoke Cl^- secretion by both acting on the epithelial cells directly and through mediation by neurons (12, 13). Much experimental data suggest that SP also acts as a mediator in the regulation of intestinal inflammation, as mentioned above. Therefore, in the inflammatory condition, excessive secretion caused by the synergistic action between SP and PGE_2 may participate to protect mucosal lining by flushing the crypts of potentially deleterious antigens or microorganisms. In the present study, we have shown one example of the neuroimmune interaction on Cl^- secretion by SP and PGE_2. Although details of the mechanism of Cl^- secretion in interaction between neruotransmitters and immunomediators are not yet clear, this interaction may have an important role for host defense mechanisms.

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