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[1]*Division of Gastroenterology and* [4]*Department of Surgery, Beth Israel Deaconess Medical Center, Boston 02215;* [2]*Department of Mechanical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02141; and* [3]*University Clinic of Surgery, Vienna General Hospital, A-1090 Vienna, Austria*

Riegler, Martin, Ignazio Castagliuolo, Peter T. C. So, Margaret Lotz, Chi Wang, Michael Wlk, Tacettin Sogukoglu, Enrico Cosentini, Georg Bischof, Gerhard Hamilton, Bela Teleky, Etienne Wenzl, Jeffrey B. Matthews, and Charalabos Pothoulakis. Effects of substance P on human colonic mucosa in vitro. *Am. J. Physiol.* 276 (*Gastrointest. Liver Physiol.* 39): G1473–G1483, 1999.—Previous studies indicated that the peptide substance P (SP) causes Cl^--dependent secretion in animal colonic mucosa. We investigated the effects of SP in human colonic mucosa mounted in Ussing chamber. Drugs for pharmacological characterization of SP-induced responses were applied 30 min before SP. Serosal, but not luminal, administration of SP (10^{-8} to 10^{-6} M) induced a rapid, monophasic concentration and Cl^--dependent, bumetanide-sensitive short-circuit current (I_{sc}) increase, which was inhibited by the SP neurokinin 1 (NK_1)-receptor antagonist CP-96345, the neuronal blocker TTX, the mast cell stabilizer lodoxamide, the histamine 1-receptor antagonist pyrilamine, and the PG synthesis inhibitor indomethacin. SP caused TTX- and lodoxamide-sensitive histamine release from colonic mucosa. Two-photon microscopy revealed NK_1 (SP)-receptor immunoreactivity on nerve cells. The tyrosine kinase inhibitor genistein concentration dependently blocked SP-induced I_{sc} increase without impairing forskolin- and carbachol-mediated I_{sc} increase. We conclude that SP stimulates Cl^--dependent secretion in human colon by a pathway(s) involving mucosal nerves, mast cells, and the mast cell product histamine. Our results also indicate that tyrosine kinases may be involved in this SP-induced response.

genistein; histamine; short-circuit current; neurokinin 1 receptor

SUBSTANCE P (SP), an 11-amino acid neuropeptide (11), is present in enteric nerves and sensory neurons of the small and large intestine (13, 18, 58). An increasing body of evidence indicates that SP is involved in the pathophysiology of intestinal secretion and inflammation in animals and humans. Administration of SP-receptor antagonists in rats reduced secretory and inflammatory changes in animal models of acute and chronic intestinal inflammation (8, 9, 33, 36, 40). Furthermore, SP immunoreactivity and SP binding are increased in colon of patients with inflammatory bowel disease (25, 32). Interestingly, mice genetically deficient in the SP neurokinin 1 (NK_1) receptor are almost protected from the secretory and inflammatory changes mediated by *Clostridium difficile* toxin A (10), providing direct evidence for the importance of these receptors in intestinal secretion and inflammation.

Several electrophysiological studies demonstrate that SP induces secretion in animal intestine. For example, serosal application of SP causes a rapid increase of short-circuit current (I_{sc}) in pig (7, 38), guinea pig (21, 23), and mouse (53) small intestine, and guinea pig (29) and dog colon (42) mounted in Ussing chamber. These studies (7, 21, 23, 29, 38, 42, 53) also provide pharmacological evidence that enteric nerves and mast cells may be involved in these SP-mediated responses, in agreement with previous findings showing a SP-mucosal mast cell interaction in vivo and in vitro (47, 53, 57). Morphological studies also indicate that mast cells are in intimate contact with nerves in rat small intestine (50) and human colon (49). However, the effects of SP on human colonic mucosa have not been investigated.

We studied the effects of SP on human colonic mucosal electrophysiology in vitro using Ussing chambers. Participation of enteric nerves and mast cells and the involvement of the secretagogues histamine and PGs in SP-mediated changes in electrical parameters were also examined. Furthermore, using a specific antibody directed against the COOH terminus of the human SP receptor, we provide direct immunohistochemical evidence for the presence of SP receptors on human colonic mucosal nerves. Because tyrosine phosphorylation has been demonstrated to be involved in SP-mediated signal transduction in nonepithelial cells in vitro (30), the effect of the tyrosine kinase inhibitor genistein on SP-induced colonic responses was also investigated.

MATERIALS AND METHODS

Materials

All chemicals and drugs were obtained from Sigma (St. Louis, MO) unless otherwise indicated. The NK_1 (SP)-receptor antagonist CP-96345 and its inactive enantiomer CP-96344 were kindly provided by Pfizer Diagnostics (Groton, CT). Lodoxamide was obtained from Upjohn (Kalamazoo, MI).

Methods

Ussing chamber experiments. In this study a total of 104 individual specimens of tumor-free left-sided colon was used. After removal of the seromuscular layer by blunt dissection,

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two to six mucosal sheets from each specimen, measuring 5–10 cm^2, were vertically mounted in Ussing chambers (DCTSYS, Precision Instrument Design; 1.0 cm^2 surface area), as previously described (15, 19, 43). Luminal and serosal sides were bathed at 37°C in 8 ml of nutrient buffer containing (in mM) 122.0 NaCl, 2.0 $CaCl_2$, 1.3 $MgSO_4$, 5.0 KCl, 20.0 glucose, and 25.0 $NaHCO_3$ (pH 7.45 when gassed with 95% O_2-5% CO_2). When Cl^--free buffer was used, Cl^- was replaced by an equimolar concentration of isethionate (42, 53). Potential difference (PD) and I_{sc} were continuously measured and recorded every 1–10 min. Luminal and serosal solutions were connected via Ag-AgCl electrodes to a voltage-current clamp (model VCC600, Physiological Instruments). Resistance was calculated using Ohm's law from the open circuit PD and the I_{sc}. PD values were given in millivolts (lumen negative), I_{sc} values in microamperes per square centimeter, and resistance in ohms times square centimeter. PD and resistance values were corrected for the junctional potentials (<0.1 mV) between luminal and serosal solutions and the buffer resistance, respectively, as previously described (43). Drug-induced I_{sc} and resistance responses are presented as changes from values before drug administration and given as I_{sc} and resistance, respectively. Baseline values for I_{sc} and resistance were 82 ± 10 μA/cm^2 and 101 ± 16 Ω·cm^2, respectively (n = 72). The protocol for use of human tissues was approved by the Ethics Committee of Beth Israel Deaconess Medical Center and University Clinic of Vienna.

Measurements of epithelial permeability. Epithelial permeability to [^{3}H]mannitol was determined as previously described (43). After an equilibration period of 30 min, [^{3}H]mannitol (26.4 Ci/mmol; DuPont NEN, Boston, MA) was added to 8 ml of serosal buffer at a final concentration of 0.32 nM. Luminal aliquots of 200 μl were taken for scintillation counting using 5 ml of "Quicksave A" scintillation fluid (Zinser, Maidenhead, UK) and replaced with 200 μl of fresh buffer to eliminate a transepithelial solute gradient. The radioactivity of [^{3}H]mannitol in the luminal fluid was measured in counts per min (cpm/200 μl) and was determined for two subsequent 30-min periods before and after administration of serosal 10^{-6} M SP.

Determination of relative paracellular resistance. To determine the separate contribution of the paracellular and transcellular pathway to the epithelial resistance response during SP-induced I_{sc} increase, we used the approach of Yonath and Civan (60) and Parkos et al. (37). Conductance, the reciprocal of transepithelial resistance, was plotted against I_{sc} for each of the three phases of SP-induced I_{sc} changes: *phase 1*, period of increase of I_{sc} 0–15 min after administration of SP; *phase 2*, period of decrease of I_{sc} 15–30 min after; *phase 3*, period of further I_{sc} decrease 30–45 min after. By regression analysis, the value for conductance at the x-axis intercept (where I_{sc} would equal zero) was determined, and the reciprocal of this value was used to ascertain the relative paracellular resistance during each of the three phases [resistance (Ω·cm^2) = 1/conductance (mS/cm^2) × 1,000].

Experimental Design

After 30 min of baseline incubation, colonic tissues were incubated under serosal presence or absence of 10^{-9} to 10^{-6} M SP or luminal presence or absence of 10^{-6} M SP for 30 min. After the concentration response effects of SP were established (see RESULTS), a 10^{-6} M concentration of SP was used in all remaining experiments. This concentration was previously used to study SP-related changes in electrical parameters in mouse small intestine (53) and dog colon (42). In another set of experiments, 10^{-9} to 10^{-6} M CP-96345 (active NK_1-receptor antagonist) (40, 48) or 10^{-6} M CP-96344 (inactive enantiomer of the NK_1-receptor antagonist) was added to the serosal side 30 min before administration of 10^{-6} M SP. Tissues were also incubated with Cl^--free buffer 30 min before serosal application of 10^{-6} M SP, whereas a paired control was incubated with Cl^--containing buffer.

Pharmacological blockade of SP effects. Thirty minutes before serosal administration of 10^{-6} M SP, human colonic tissues were exposed serosally to either the muscarinic-receptor antagonist atropine (10^{-5} to 10^{-7} M) (38, 53) or to the nicotinic-receptor antagonist hexamethonium (10^{-4} to 10^{-7} M), the neurotoxin TTX (10^{-5} to 10^{-8} M) (3), the mast cell stabilizer lodoxamide (10^{-5} to 10^{-8} M) (28), the histamine 1 (H_1)-receptor antagonist pyrilamine (10^{-6} to 10^{-8} M) (53, 54), the H_2-receptor antagonist ranitidine (10^{-5} to 10^{-7} M) (53), the PG synthesis inhibitor indomethacin (10^{-5} to 10^{-8} M) (42, 54), the inhibitor of the Na^+-K^+-2Cl^- cotransporter bumetanide (10^{-5} to 10^{-7} M) (54), the Na^+-K^+-ATPase inhibitor ouabain (10^{-6} M, 10^{-7} M) (35), or the K^+-channel blocker charybdotoxin (10^{-6} M, 10^{-7} M) (44).

In another series, five human colonic explants from a single individual were mounted in Ussing chambers in parallel and incubated with buffer alone (control) or serosal buffer containing 10^{-6} to 10^{-9} M of the tyrosine kinase inhibitor genistein (52), 60 min before and during 30 min of serosal exposure to SP (n = 8). We also investigated the effect of genistein (10^{-6} M) on the I_{sc} increase induced by the secretagogues forskolin (2×10^{-6} M) and carbachol (2×10^{-5} M; n = 4, paired) (41). Preliminary experiments showed that a period of 60 min was required to obtain stabilization of genistein-induced changes in electrical parameters (see RESULTS).

Compounds used in our experiments were made up as follows: SP in distilled water; TTX in citrate buffer; atropine, hexamethonium, and indomethacin in sodium chloride; lodoxamide, pyrilamine, ranitidine, CP-96345, and CP-96344 in 95% ethanol; and bumetanide, ouabain, charybdotoxin, genistein, forskolin, and carbachol in DMSO. In all experiments, the volume of drug added to the bathing solution did not exceed 7 μl per 7 ml of half-chamber volume. Preliminary experiments indicated that none of the vehicles altered baseline electrophysiological parameters (data not shown).

Effect of drugs on basal human colonic electrophysiology. Incubation of tissues with 10^{-6} M of indomethacin for 30 min or genistein for 60 min caused a 25% ($P < 0.05$ vs. baseline, n = 6) and 30% ($P < 0.01$ vs. baseline, n = 8) decrease of human colonic I_{sc}, respectively. Serosal presence of ouabain for 30 min caused a statistically significant decrease of colonic I_{sc} (baseline vs. 30 min of ouabain: 78 ± 6.1 vs. 26.4 ± 4.8 μA/cm^2; $P < 0.01$, n = 6), without changing transepithelial resistance. None of the other drugs used in this study had an effect on basal electrophysiological values (data not shown). Human colonic mucosa displayed stable transepithelial resistance over the 2-h incubation, indicating excellent tissue viability (data not shown).

Histamine assay. Histamine release from human colonic mucosa was measured by a commercially available ELISA assay (enzyme immunoassay kit ref. 1153, Immunotech, Westbrook, ME). Four human colonic explants from a single individual were mounted in Ussing chambers in parallel and incubated with either serosal buffer alone or buffer containing 10^{-6} M of either TTX or lodoxamide 30 min before serosal administration of 10^{-6} M of SP. The fourth tissue was incubated with buffer alone and received vehicle instead of SP (n = 6, quadruplicate). Histamine (pg·ml^{-1}·cm^{-2}) was determined in serosal aliquots (50 μl) taken before and 5 and 10 min after administration of serosal SP. All samples were stored at −70°C for no more than 3 days before histamine measurements. Histamine concentration was also deter-

mined in luminal aliquots taken before and 10 min after SP administration ($n = 3$).

Histology. After Ussing chamber experiments colonic tissues were processed for light microscopy as previously described (15, 43). None of the colonic tissues used showed any signs of inflammation or malignancy. Furthermore, neither SP nor any of the drugs used caused morphological changes in human colon (data not shown).

SP-receptor antiserum. Antiserum generated against a peptide representing the last 15 amino acids of the human SP receptor COOH terminus was prepared by Immuno-Dynamics (La Jolla, CA) according to the *m*-maleim-idobenzoyl-*N*-hydroxysuccimide coupling method described by Kitigawa and Aikawa (27) and characterized by ELISA. This antiserum immunoprecipitated photoaffinity-labeled SP receptors expressed in Chinese hamster ovary cells transfected with the human SP receptor (31).

Immunofluorescent labeling. Freshly frozen mucosal preparations of human colon were cut (5 μm) and fixed in 4% paraformaldehyde. Sections were washed in Tris-buffered saline (TBS, pH 7.5) and incubated with blocking solution (TBS, pH 7.5, containing 50 mM ammonium chloride, 1% normal donkey serum, and 3% BSA) for 1 h at room temperature. Sections were next incubated for 1 h with 1:200 dilution of either the NK_1-receptor antiserum or with rabbit preimmune serum at room temperature. In some experiments the NK_1-receptor antiserum (1:200 dilution) was preincubated overnight at room temperature with the COOH-terminal 15-amino acid peptide used to generate the antiserum before addition to the sections. For immunofluorescent labeling of nerve cells, sections were incubated for 1 h with a mouse monoclonal IgG1 antibody directed against rat neurofilament polypeptide (1:50 dilution; NCL-NF200, Novocastra Laboratories, Newcastle upon Tyne, UK). All dilutions were made in blocking buffer. The sections were then washed in TBS and incubated for 1 h at room temperature with FITC-conjugated anti-rabbit antibody or tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG antibody (all Jackson ImmunoResearch Laboratories, West Grove, PA) (1:50 dilution). After being washed in TBS, sections were mounted in antibleach solution (10% PBS, 90% glycerol, containing 1 mg/ml *n*-propyl gallate), examined, and photographed using a laser confocal microscope (Zeiss, Thornwood, NY) or a two-photon scanning microscope.

Two-photon fluorescence microscopy. The two-photon scanning system was adapted to a Zeiss Axiovert 110 microscope as previously described (34, 59). The excitation source is a Ti-Sapphire laser (Mira 900, Coherent, Palo Alto, CA) tuned to 730 nm. The typical power incident on the sample is <5 mW. The samples were imaged with a Zeiss ×40 fluor (1.2 numerical aperture, oil) objective. For the colocalization experiments, the FITC staining was imaged with a filter combination consisting of a 35-nm wide band-pass filter center at 535 nm, a 500-nm long-pass filter, and a BG-39 infrared filter; the TRITC staining was imaged with a filter combination consisting of a 600-nm long-pass filter and two BG-39 Schott glass infrared filters. The filter combination is chosen to minimize interference between the two color channels (FITC, green; TRITC, red). We have further obtained an image of whole tissue fluorescence (see Fig. 6*D*) including FITC and TRITC fluorescence and tissue autofluorescence using a combination of two BG-39 Schott glass filters, as described previously (59).

Statistical Analysis

All data are expressed as means ± SE, and probabilities were regarded as significant when they reached a 95% level of confidence ($P < 0.05$) using Student's *t*-test for paired and unpaired observations.

RESULTS

SP Effects on Colonic Electrophysiology

The effects of different concentrations of SP on human colonic electrophysiology were compared using I_{sc}, transepithelial PD, and electrical resistance. Serosal administration of 10^{-6}, 10^{-7}, and 10^{-8} M SP to human colon induced a concentration-dependent I_{sc} increase (Fig. 1*B*) and resistance decrease (Fig. 1*D*), whereas 10^{-9} M had no effect. In all subsequent experiments a 10^{-6} M concentration of SP was used to obtain maximal responses.

Serosal SP exposure (10^{-6} M) caused a rapid, transient I_{sc} increase and resistance decrease, which peaked after 10 min (Fig. 1, *A* and *C*; $P < 0.001$ vs. controls) and returned toward baseline values after 40 min. Luminal addition of 10^{-6} M SP did not cause changes in electrical parameters ($n = 6$, data not shown).

As shown in Fig. 2, preincubation of tissues with 10^{-6}, 10^{-7}, and 10^{-8} M of the NK_1-receptor antagonist CP-96345 concentration dependently inhibited SP (10^{-6} M)-induced I_{sc} and resistance changes by 80, 60, and 20%, respectively, whereas administration of 10^{-9} M had no effect. In contrast, the inactive enantiomer of the NK_1-receptor antagonist CP-96344 (10^{-6} M) did not have an effect on SP-induced changes in electrical parameters. These results indicate that SP induces I_{sc} increase by acting on NK_1 receptors in human colon.

Effect of SP on Paracellular Epithelial Resistance

To determine whether the SP-induced resistance decrease is due to an increase in transcellular or paracellular epithelial conductance, we assessed the effect of serosal SP on transepithelial [^{3}H]mannitol flux and relative paracellular resistance of human colon. SP (10^{-6} M) did not alter serosal-to-luminal [^{3}H]mannitol flux and relative paracellular resistance, compared with paired control tissues ($P > 0.05$) (luminal [^{3}H]mannitol after 30 and 60 min in control vs. SP-exposed tissues: 364 ± 9 vs. 372 ± 9 cpm/200 μl and 456 ± 10 vs. 443 ± 13 cpm/200 μl; $P > 0.05$, $n = 6$, paired; relative paracellular resistance of control vs. SP: *phase 1*, 103 ± 10 vs. 115 ± 11 Ω·cm^2; *phase 2*, 109 ± 8 vs. 113 ± 9 Ω·cm^2; *phase 3*, 107 ± 9 vs. 110 ± 10 Ω·cm^2, $P > 0.05$, $n = 6$, paired). These results indicate that the SP-induced decrease of transepithelial resistance in human colon is due to increased transcellular conductance. However, we cannot exclude minor changes of paracellular permeability, which may not be detected by mannitol flux studies and the approach used to determine paracellular resistance.

Ionic Basis of SP-Induced I_{sc} Increase

Our results showed that the SP-induced I_{sc} increase was associated with a decrease of colonic PD, indicating an increase of negative charges on the luminal side that could be attributed to either enhanced movement of positive charges from the luminal to the serosal side of

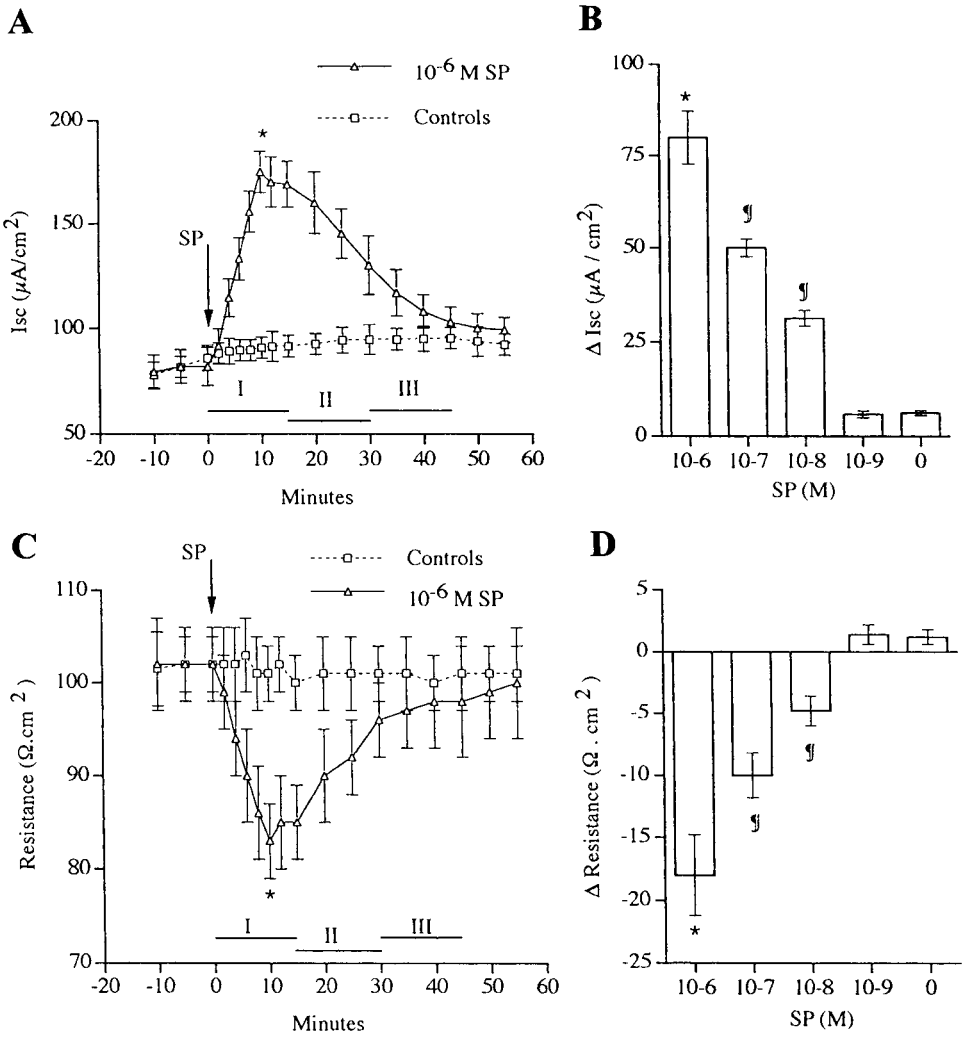

Fig. 1. Effect of substance P (SP) on human colonic electrophysiology. Time course effect of SP on human colonic short-circuit current (I_{sc}; *A*) and resistance (*C*). Concentration-dependent effect of SP on human colonic I_{sc} (ΔI_{sc}; *B*) and resistance (Δresistance; *D*). Colonic mucosae were mounted in Ussing chambers and incubated in serosal presence or absence (controls) of various SP concentrations. Resistance was calculated from potential difference (PD) and I_{sc} as described in *Methods*. I_{sc} or resistance was obtained after serosal administration of SP for 10 min. Values are means ± SE; n = 7/group. *$P < 0.001$ and ¶$P < 0.01$ vs. controls. I, II, III in *A* and *C* refer to *phases 1, 2, and 3* for determination of relative paracellular resistance (see *Methods*).

the mucosa (i.e., Na^+) (4, 22, 45) or movement of negative charges into the lumen (i.e., Cl^- or bicarbonate) (22). To test the ionic basis of SP-induced I_{sc} increase, we measured SP-mediated I_{sc} changes in the presence or absence of Cl^- in the incubation buffer as previously described (53). In Cl^--free buffer baseline levels of I_{sc} were reduced by 52% [110 ± 13 vs. 51 ± 8 μA/cm² (SE) control vs. Cl-free; $P < 0.01$, n = 6 paired], indicating that a major part of the I_{sc} is Cl^- dependent. SP-induced I_{sc} increase was reduced by 95 ± 1.4% in the presence of Cl^--free buffer (n = 6, $P < 0.001$). Cl^--free buffer also abolished the SP-induced decrease of human colonic resistance (−16 ± 3 vs. +1.1 ± 0.8 Ω·cm² in control vs. Cl^- free; n = 6, $P < 0.001$). We also investigated the involvement of ion transport pathways required for Cl^- secretion in epithelial cells (22). Preincubation of human colonic mucosa with either 10^{-7}, 10^{-6}, or 10^{-5} M of the inhibitor of the Na^+-K^+-$2Cl^-$ cotransporter bumetanide (14, 41, 54) or 10^{-7} or 10^{-6} M of the inhibitor of Na^+-K^+-ATPase ouabain (35) or 10^{-7} and 10^{-6} M of the inhibitor of basolateral K^+ channels charybdotoxin (44) inhibited the SP-induced I_{sc} increase by 48, 70, 92, 68, 94, 74 and 91%, respectively, compared with controls ($P < 0.01$, n = 6/group). Pharmacological inhibition of the SP-induced I_{sc} increase was always paralleled by respective inhibition of the SP-evoked resistance decrease (data not shown). Taken together our data indicate that the SP-induced I_{sc} increase largely depends on Cl^-.

Effect of TTX

Because previous in vitro studies showed that SP-induced secretion in guinea pig and dog colon was inhibited by pretreatment with the neuronal blocker TTX (29, 42), we investigated the effect of neuronal blockers on SP-induced changes in electrical parameters in human colonic mucosa. We found that the SP-induced I_{sc} increase was inhibited by 12.4 ± 3.6% ($P < 0.05$), 41 ± 2.1% ($P < 0.01$), 84 ± 1.5% ($P < 0.001$), and 98 ± 1.2% ($P < 0.001$) after preincubation of the colonic mucosa with 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M of TTX, respectively (n = 6/group). However, neither pretreatment with 10^{-5}, 10^{-6}, or 10^{-7} M of the muscarinic-receptor antagonist atropine nor with the nicotinic-receptor antagonist hexamethonium changed the SP-mediated I_{sc} increase in the human colon (I_{sc} of 73.2 ± 2, 68 ± 4, and 75.2 ± 5.4 μA/cm² after 10^{-5}, 10^{-6}, or 10^{-7} M atropine, 67.2 ± 3, 71 ± 3, and 70.4 ± 6.2 μA/cm² after 10^{-5}, 10^{-6}, or 10^{-7} M hexamethonium, and 72.5 ±

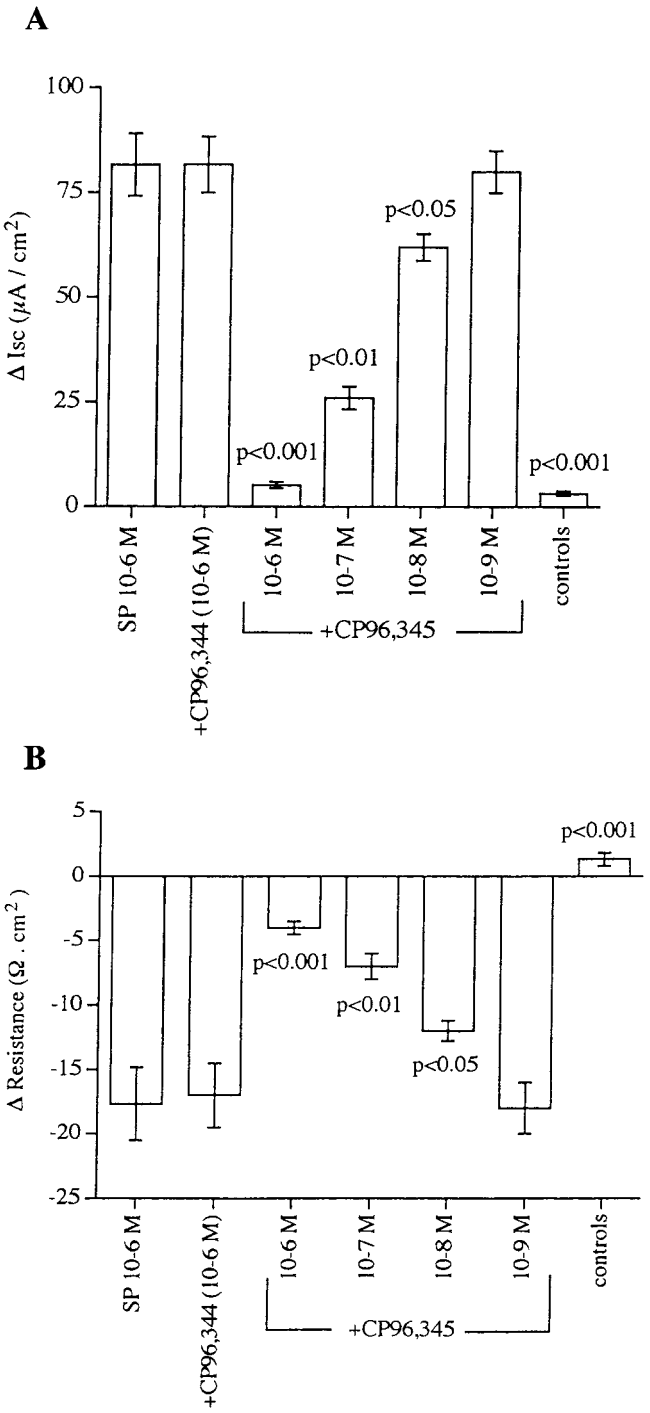

Fig. 2. Effect of neurokinin 1 (NK_1)-receptor antagonist. Concentration-dependent effect of specific NK_1 (SP)-receptor antagonist CP-96345 on human colonic I_{sc} (ΔI_{sc}; *A*) and resistance (Δ resistance; *B*). Human colonic mucosae were mounted in Ussing chambers and incubated 30 min before serosal SP (10^{-6} M) administration in serosal presence or absence of 10^{-9} to 10^{-6} M CP-96345 or 10^{-6} M of its inactive enantiomer CP-96344. Controls were incubated with vehicle alone and received serosal vehicle instead of SP. Resistance was calculated from PD and I_{sc} as described in *Methods*. I_{sc} or resistance was obtained after serosal administration of SP for 10 min. Values are means ± SE; n = 7/group. P values were calculated vs. SP alone.

2 μA/cm² after SP alone, n = 6/group). These data indicate that in the human colon mucosal nerves are involved in SP-induced I_{sc} increase and that this effect is not mediated via muscarinic or nicotinic receptors.

Involvement of Mast Cells and Histamine

Several studies indicate that mast cells and histamine participate in SP-mediated ion secretion in small and large intestine (29, 33, 53). Thus we tested the effect of different concentrations of the mast cell stabilizer lodoxamide and the H_1- and H_2-receptor antagonists pyrilamine and ranitidine, respectively, on SP-induced I_{sc} increase. We found that the SP-induced I_{sc} increase was inhibited by 97.4 ± 1.0% ($P < 0.001$), 73.2 ± 3.1% ($P < 0.01$), and 38.6 ± 4.5% ($P < 0.05$) after 10^{-6}, 10^{-7}, and 10^{-8} M pyrilamine, respectively, and by 98 ± 1.6% (P, 0.0001), 86 ± 2.4% ($P < 0.001$), 48 ± 3.2% ($P < 0.01$), and 17.0 ± 3.2% ($P < 0.05$) after 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M lodoxamide, respectively (n = 6/group). Administration of 10^{-5}, 10^{-6}, and 10^{-7} M ranitidine inhibited SP-induced I_{sc} increase by 22.0 ± 4.4% ($P < 0.05$), 16.0 ± 2.6% ($P < 0.05$), and 9.0 ± 3.2% (not significant), respectively. These results indicate involvement of mast cells and histamine in SP-induced changes in electrical parameters in human colon.

SP-Induced Histamine Release

The results with the H_1-receptor antagonist pyrilamine and the mast cell-stabilizer lodoxamide indicated that histamine is involved in the mediation of SP-induced I_{sc} increase. We therefore tested whether SP was able to induce histamine release from human colonic mucosa mounted in Ussing chamber. As shown in Fig. 3, serosal administration of 10^{-6} M of SP caused a significant release of histamine into the serosal bathing solution, whereas histamine was not detectable in the luminal bath (n = 3, data not shown). In contrast, preincubation of tissues with 10^{-6} M TTX or lodoxamide completely inhibited SP-induced histamine release. Serosal administration of vehicle alone did not change histamine concentration in the serosal bath (Fig. 3). These data indicate that SP-induced histamine release is mediated via nerves and that mast cells appear to be the major source of histamine in this response.

Effect of Indomethacin

Mast cells, fibroblasts, and inflammatory cells of the lamina propria are major sources of PGs, which are potent stimulators of intestinal secretion (1, 5, 13, 22). Thus we tested the concentration-dependent effect of the PG synthesis inhibitor indomethacin on SP-induced I_{sc} increase. We found that the SP-induced I_{sc} increase was inhibited by 71 ± 2.0% ($P < 0.001$), 48 ± 2.5% ($P < 0.01$), 22 ± 2.3% ($P < 0.05$), and 3.0 ± 1.2% ($P > 0.05$) after preincubation of the colonic mucosa with 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M indomethacin, respectively (n = 6/group). These data indicate that PGs are involved in the mediation of SP-induced secretion.

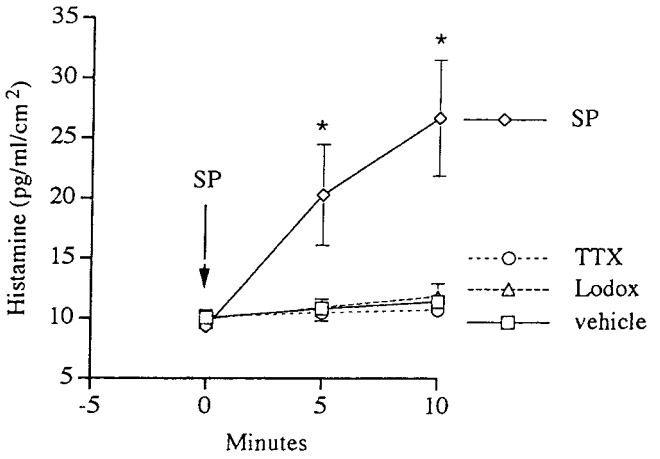

Fig. 3. Histamine release from human colonic mucosa in vitro. Human colonic mucosae were mounted in Ussing chambers and incubated 30 min before serosal SP (10^{-6} M), in serosal presence or absence of 10^{-6} M of neuronal blocker TTX or mast cell stabilizer lodoxamide (Lodox). Controls were incubated with vehicle alone and received serosal vehicle instead of SP. Histamine concentration in serosal bath was determined before and 5 and 10 min after SP administration as described in *Methods*. Values are means ± SE; n = 6 separate experiments each with quadruplicate determinations. *$P < 0.05$ vs. vehicle treated.

Effect of Genistein

Because tyrosine phosphorylation has been demonstrated to be involved in SP-mediated signal transduction in human astrocytoma cells in vitro (30), we investigated the effect of the tyrosine kinase inhibitor genistein on SP-induced changes in electrical parameters. Serosal administration of genistein before and during SP exposure concentration dependently inhibited SP-induced I_{sc} increase and resistance decrease. Although SP-induced I_{sc} increase was completely blocked by 10^{-6} M genistein ($P < 0.0001$, $n = 8$) (Fig. 4*A*), serosal administration of 10^{-7} and 10^{-8} M genistein before and during SP exposure inhibited SP-induced I_{sc} increase by 47% ($P < 0.01$) and 20% ($P < 0.05$), respectively. Administration of 10^{-9} M genistein had no effect on the SP-induced I_{sc} increase (Fig. 4*A*). To exclude that genistein blocked SP-stimulated I_{sc} increase by inhibiting Cl^- secretion from colonic epithelial cells, we next investigated the effect of genistein on cAMP- and Ca^{2+}-mediated Cl^- secretion using the secretagogues forskolin and carbachol, respectively (41, 44, 52). As shown in Fig. 4*B*, pretreatment with genistein (10^{-6} M) did not have an effect on forskolin (2×10^{-6} M)- or carbachol (2×10^{-5} M)-induced I_{sc} increase, compared with controls ($P > 0.05$, $n = 4$, paired). These data indicate that although genistein inhibits SP-induced changes in electrical parameters genistein at this concentration does not inhibit forskolin- and carbachol-stimulated I_{sc} increases in human colon in vitro.

Distribution of NK_1 (SP)-Receptors in Human Colonic Mucosa

Using an antibody directed against the COOH terminus of the SP receptor, we determined the distribution of SP receptor in human colonic mucosa by confocal microscopy. As shown in Fig. 5*A*, SP-receptor immunoreactivity was abundant in cells of the colonic lamina propria but was not observed in the epithelial cell layer. Preincubation of the SP-receptor antiserum with an excess of the COOH-terminal 15-amino acid peptide of the SP receptor before incubation with the colonic

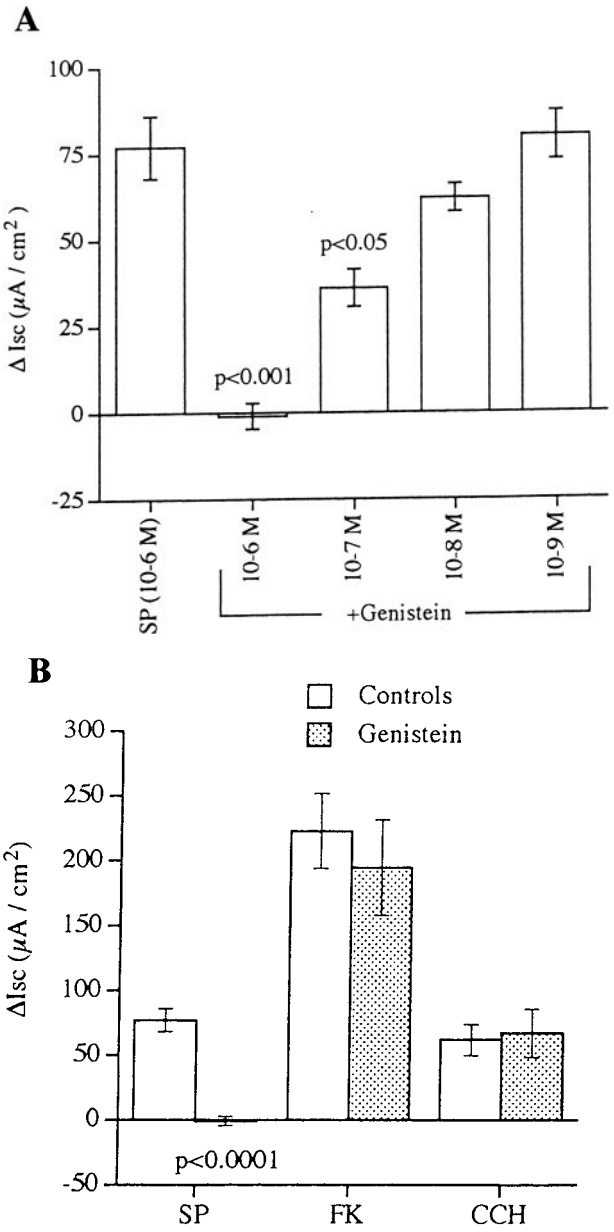

Fig. 4. Effect of genistein. *A*: concentration-dependent effect of tyrosine kinase inhibitor genistein on SP (10^{-6} M)-induced I_{sc} increase. Human colonic mucosae were mounted in Ussing chambers and incubated in serosal presence or absence of tyrosine kinase inhibitor genistein (10^{-9} to 10^{-6} M) 60 min before and during serosal administration of SP (10^{-6} M). *B*: effect of genistein (10^{-6} M) on SP (10^{-6} M)-, forskolin (FK, 2×10^{-6} M)-, or carbachol (CCH, 2×10^{-5} M)-induced I_{sc} increase. Ussing-chambered human colonic mucosae were incubated in serosal presence or absence (controls) of tyrosine kinase inhibitor genistein 60 min before and during serosal administration of SP, forskolin, or carbachol. ΔI_{sc} was obtained 10 min after administration of SP, FK, and CCH. Values are means ± SE, each with 5 determinations in parallel; $n = 8$ (*A*) and $n = 6$ paired tissues (*B*). *P* values were calculated vs. SP alone.

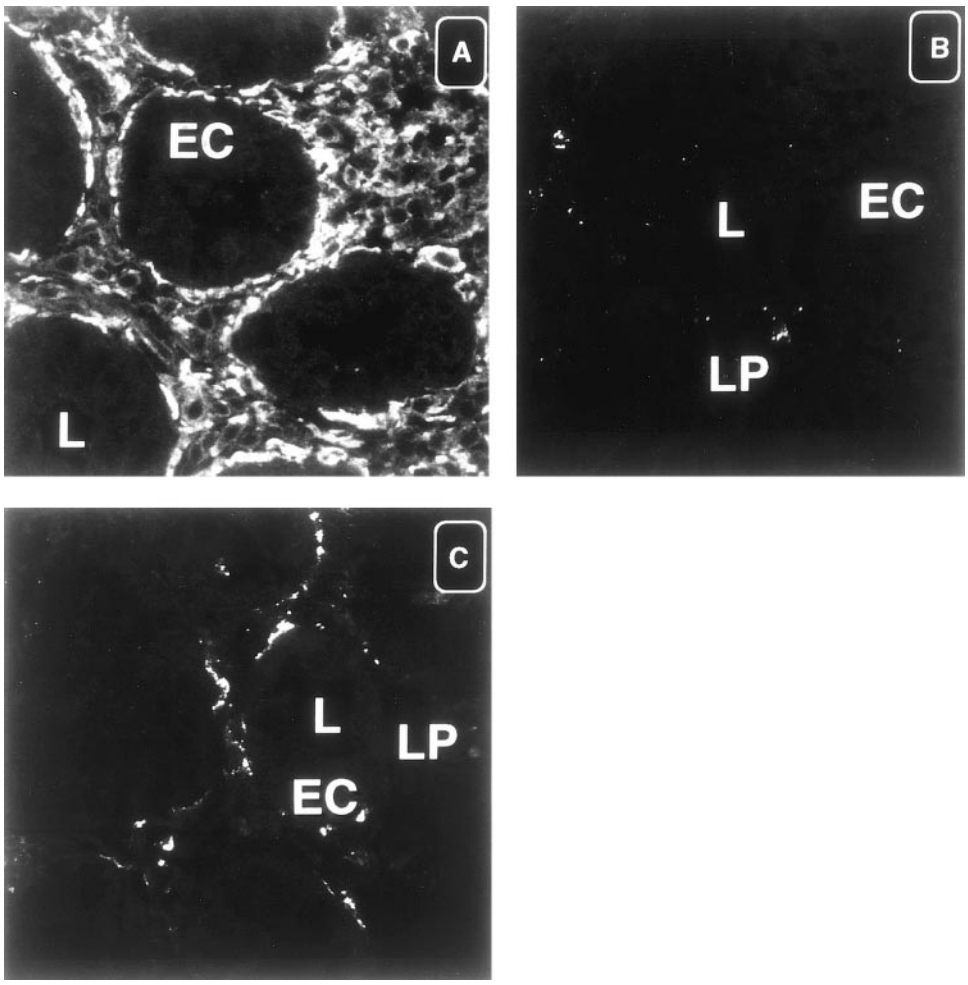

Fig. 5. Distribution of SP (NK_1) receptor in human colonic mucosa. Fixed normal human colonic mucosal sections cut in parallel to surface epithelium were incubated for 1 h at room temperature with rabbit polyclonal antiserum directed against COOH terminus of human NK_1 (SP) receptor (1:200 dilution; *A* and *B*) or with preimmune rabbit serum (*C*). Sections were processed for immunohistochemical detection by confocal microscopy as described in *Methods*. *A*: NK_1-receptor immunoreactivity is detected in mucosal lamina propria. *B*: normal human mucosa exposed to NK_1-receptor antiserum, which was preincubated with excess of COOH-terminal 15 amino acid peptide from which antiserum was generated. Note almost complete disappearance of staining. *C*: note almost complete absence of staining in tissue exposed to preimmune serum. EC, epithelial cells; L, lumen; LP, lamina propria. Magnification, ×220.

sections showed almost complete disappearance of staining (Fig 5*B*). Furthermore, tissues exposed to control rabbit antiserum showed very little immunoreactivity (Fig. 5*C*). SP-receptor immunoreactivity on nerve cells was determined using double-staining and two-photon microscopy (Fig. 6). Sections of human colonic mucosa were stained with SP-receptor antiserum (Fig. 6*A*) or anti-neurofilament antibody (Fig. 6*B*) and imaged with a two-photon fluorescence microscope. Marked area in Fig. 6*D*, showing whole tissue fluorescence, corresponds to the areas shown at higher magnification in Fig. 6, *A–C*. As shown in Fig. 6*C*, the merged images show colocalization of SP receptor on lamina propria nerve cells underlying the epithelium.

DISCUSSION

The findings of this study indicate that SP induces Cl^--dependent secretory response in normal human colon in vitro, which is mediated via NK_1 receptors, mucosal nerves and mast cells, and the mast cell product histamine. Furthermore, a tyrosine kinase(s) may be involved in the mediation of SP-induced secretion. Our evidence that SP induces a Cl^--dependent I_{sc} increase is in agreement with data obtained in guinea pig (29) and dog colon (42) and guinea pig (21, 23) and mouse (53) small intestine in vitro. We understand that ion flux studies using radiolabeled $^{22}Na^+$ and $^{36}Cl^-$ represent a more accurate method to measure transepithelial ion movement. However, because for radiosafety reasons we cannot perform these experiments in our laboratory, we used indirect pharmacological studies to assess the ionic basis of the SP-induced I_{sc} increase.

In the human colon SP-induced I_{sc} increase was inhibited by the specific neuronal blocker TTX, which is in keeping with data obtained in animal small and large intestine in vitro (7, 21, 23, 29, 38, 42, 53). In contrast, the nicotinic and muscarinic ACh-receptor antagonists hexamethonium and atropine, respectively, did not inhibit SP-induced secretion, in agreement with results obtained in dog colon (42). However, Kuwahara and Cooke (29) previously showed that atropine inhibits SP-induced secretion in guinea pig colon. These different responses could be attributed to species differences in tissues used in our study and those of Kuwahara and Cooke (29). Together, our data suggest that in human colonic mucosa ACh-containing neurons are not involved in the mediation of SP-induced neuronal reflexes.

The mast cell "stabilizer" lodoxamide and the H_1-receptor antagonist pyrilamine profoundly inhibited SP-induced changes in electrical parameters in human colon. SP caused a lodoxamide-sensitive release of

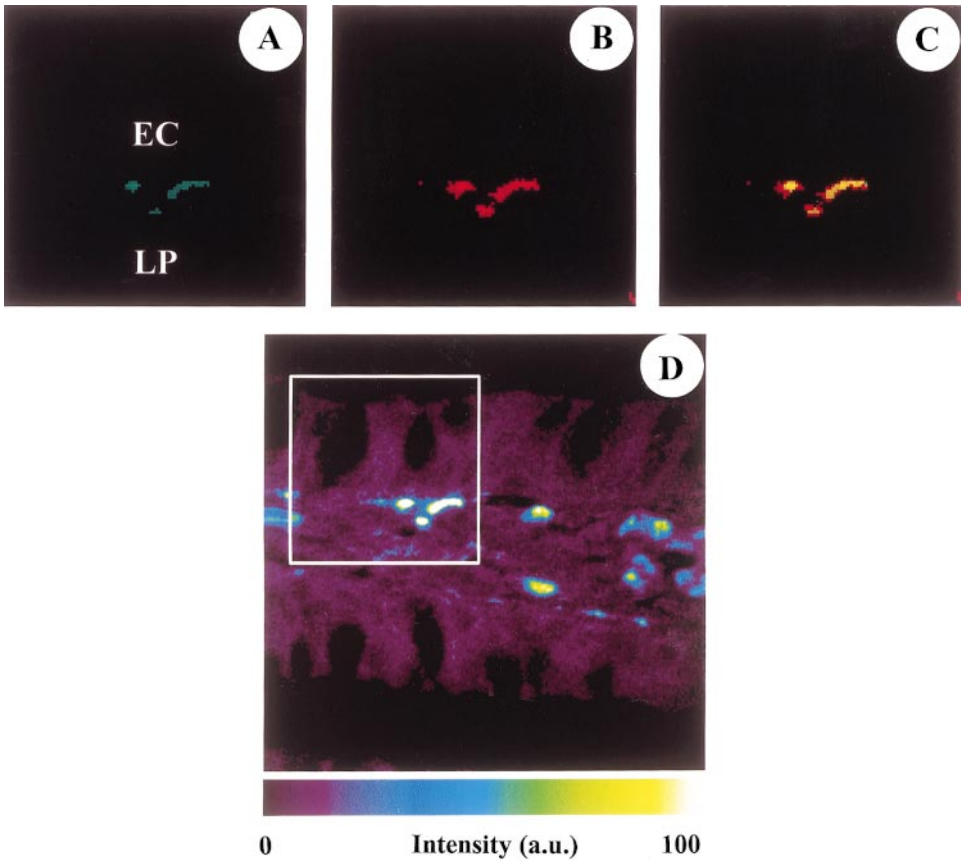

Fig. 6. Distribution of SP receptor on nerve cells in human colonic mucosa. Fixed normal human colonic mucosal sections cut in parallel to crypt-surface epithelial axis were stained with rabbit polyclonal antiserum directed against COOH terminus of human NK_1 (SP) receptor (FITC-conjugated, green fluorescence in *A*) and mouse monoclonal IgG1 antibody directed against rat brain neurofilament (tetramethylrhodamine isothiocyanate-conjugated, red fluorescence in *B*), or section was visualized for whole tissue fluorescence (*D*) and imaged with 2-photon fluorescence microscope as described in *Methods*. Marked area in *D* is shown at higher magnification in *A–C*. EC and LP indicate epithelial cell layer and lamina propria, respectively. Merged images (*A* and *B*) show colocalization of SP receptor on nerve cells (yellow fluorescence in *C*). Magnification is ×200 in *D* and ×400 in *A–C*.

histamine from human colonic mucosa (Fig. 3). Wang et al. (53) showed that mast cell-deficient mice exhibit a reduced ileal secretory response to SP, and Kuwahara and Cooke (29) showed that pyrilamine inhibited SP-induced I_{sc} increase in guinea pig colon in vitro. Using mast cell-deficient mice, we showed that intestinal secretion and inflammation mediated by *C. difficile* toxin A involves a SP mast cell-dependent pathway (57). The fact that histamine directly stimulates colonic secretion via H_1 receptors is well established (22, 24, 54, 56). We also found that, on an equimolar basis, the H_2-receptor antagonist ranitidine caused a smaller but significant inhibition of SP-induced I_{sc} increase in human colon compared with the effect of the H_1-receptor antagonist pyrilamine. Frieling and co-workers (16) showed that in the guinea pig colon histamine acts on enteric nerves via H_2 receptors. Taken together findings in this and the studies discussed above (16, 22, 24, 29, 53, 54, 56) indicate that in normal human colon SP-induced I_{sc} increase is mediated via histamine acting on H_1 receptors on colonic epithelial cells and probably on H_2 receptors on nerve cells.

Our results indicate that PGs are involved in the mediation of SP-induced colonic secretion in line with previous findings in guinea pig (29) and dog (42) colon in vitro. Although the cellular sources of PGs in our experimental system cannot be defined, it is well established that they can be released from lamina propria cells, including basophils, fibroblasts, and mast cells (5, 13, 58). Furthermore, there is evidence that histamine induces increased synthesis of PGE_2 in guinea pig colon (54), and PGE_2 itself causes a transient Cl^- secretory response in human and rat colon in vitro (46, 55). Using a coculture system, Berschneider and Powell (2) demonstrated that fibroblasts amplify histamine-induced secretion in T84 cell monolayers and that this effect is inhibited by indomethacin.

Results in this paper indicate that SP induces changes in electrical parameters by acting on NK_1 receptors on lamina propria nerve cells of human colonic mucosa (Fig. 2), in keeping with studies in guinea pig (29) and canine (42) colon. Other laboratories also provided evidence that several cell types in the animal colonic mucosa may express SP receptors, including nerves (6, 39), endothelial cells (39), and mast cells (47). In contrast, Cooke et al. (14) showed that luminal administration of the SP analog [Sar^9, Met $(O_2)^{11}$]SP causes an I_{sc} increase in guinea pig colon in vitro. Recent studies demonstrate expression of mRNA for NK_1 (SP) receptor in dog crypt colonocytes (26) and in isolated guinea pig colonic epithelial cells (14). Different ligands (SP vs. SP analog), experimental approaches (immunohistochemistry vs. in situ hybridization), and species (human vs. guinea pig and dog colon) may account for the differences observed in this study and the study of Cooke et al. (14) and Khan et al. (26).

We report here that genistein inhibited SP-induced I_{sc} increase in the human colon (Fig. 4*A*), indicating that tyrosine kinases are involved in this response. Several studies also indicate participation of tyrosine

kinases in the signal transduction pathway following binding of SP to its receptor in nonepithelial cells (20, 30). We also show that genistein blocked the SP-mediated I_{sc} increase without impairing secretory responses to forskolin and carbachol (Fig. 4*B*). Thus genistein may inhibit tyrosine kinases required for SP-activated signal transduction and does not alter secretagogue-stimulated secretion from colonic epithelial cells.

Our results indicate that nerve cells, mast cells, and histamine are involved in the secretory effects of SP. Because mast cells represent the major source of colonic histamine (5, 17), we conclude that in the human colon mast cells release histamine on SP-induced nerve cell stimulation. However, based on our results we cannot determine whether SP directly activates mast cells to release histamine. This would seem unlikely because previous studies showed that SP used at 30 μM failed to stimulate release of histamine from human intestinal mucosal mast cell preparations (12). According to the findings obtained in this and previous studies discussed above, the following sequence of events appears to mediate the SP effects in normal human colon. When NK_1 receptor binding occurs, SP activates nerve cells to release a noncholinergic mediator(s), which in turn stimulates mast cells to release histamine and probably PGs. These secretagogues in turn directly activate Cl^- secretion from colonic epithelial cells (2, 24, 46, 55, 56). However, involvement of mediators released from other cells of the colonic lamina propria, including endothelial cells, basophils, and fibroblasts, in the mediation of these SP effects cannot be excluded (2, 5).

In conclusion, our findings indicate that in the normal, noninflamed human colon SP responses are processed and amplified via "cross talk" between enteric nerves and mast cells, lamina propria immune cells, and fibroblasts and mediators (histamine, PGs) released from these cells. Our results are in keeping with the notion that enteric nerves and mast cells act as a functional unit in sensing, processing, and transducing SP-induced stimuli to epithelial cells and cells of the lamina propria that result in the modulation of colonic secretion. In addition, results in this paper may be relevant to the participation of SP in the pathophysiology of human colonic secretory disorders (25, 32).

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