5-HT activates nitric oxide-generating neurons to stimulate chloride secretion in guinea pig distal colon

ATSUKAZU KUWAHARA,[1] HIROFUMI KURAMOTO,[2] AND MAKOTO KADOWAKI[3]
[1]*Laboratory of Environmental Physiology, Institute for Environmental Sciences, University of Shizuoka, Shizuoka 422-8526;* [2]*Department of Applied Biology, Kyoto Institute of Technology, Matsugasaki, Sakyou-ku, Kyoto 606; and* [3]*Gastrointestinal Research Group, Pharmacological Research Laboratories, Fujisawa Pharmaceutical Company, Osaka 532, Japan*

Kuwahara, Atsukazu, Hirofumi Kuramoto, and Makoto Kadowaki. 5-HT activates nitric oxide-generating neurons to stimulate chloride secretion in guinea pig distal colon. *Am. J. Physiol.* 275 (*Gastrointest. Liver Physiol.* 38): G829–G834, 1998.—The participation of nitric oxide (NO) in serotonin (5-hydroxytryptamine; 5-HT)-evoked chloride secretion in guinea pig distal colon was examined. Submucosal/mucosal segments were mounted in Ussing flux chambers, and an increase in short-circuit current (I_{sc}) was used as an index of secretion. Addition of 5-HT to the serosal side produced a concentration-dependent (10^{-7}–10^{-5} M) increase in I_{sc} caused by chloride secretion. N^G-nitro-L-arginine (L-NNA) significantly reduced the 5-HT-evoked early (P-1) and late (P-2) responses to 61.1 and 70.6% of control, respectively. Neurally evoked response was also inhibited by L-NNA. The NO donor sodium nitroprusside (SNP, 10^{-4} M) increased basal I_{sc} mainly because of chloride secretion. The SNP-evoked response was significantly reduced by tetrodotoxin but was unchanged by atropine or indomethacin. These results suggest that the 5-HT-evoked increase in I_{sc} is associated with an NO-generating mechanism. Atropine significantly reduced the 5-HT (10^{-5} M)-evoked P-1 and P-2 responses to 71.8 and 19.7% of control, respectively. Simultaneous application of atropine and L-NNA further decreased the 5-HT-evoked responses more than either drug alone; application of L-NNA and atropine decreased the 5-HT-evoked P-1 and P-2 responses to 68.5 and 39.2% of atropine-treated tissues, respectively. These results suggest that noncholinergic components of P-1 and P-2 responses are 71.8 and 19.7% of control, respectively, and that NO components of P-1 and P-2 responses are 32 and 61%, respectively, of the noncholinergic component of the 5-HT-evoked responses. The results provide evidence that NO may participate as a noncholinergic mediator of 5-HT-evoked chloride secretion in guinea pig distal colon.

serotonin; ion transport; gastrointestinal; short-circuit current

SEROTONIN (5-hydroxytryptamine; 5-HT) is contained within subsets of myenteric neurons as well as in endocrine cells in the gastrointestinal tract (11, 13). The presence of 5-HT in the neurons suggests that 5-HT functions as a neurotransmitter of the enteric nervous system (ENS). Serotonergic myenteric neurons are interneurons in the ENS, and they possess a single long axon that projects aborally to neurons located in ganglia of either the submucous or the myenteric plexus (13, 29). Several studies showed that exogenous 5-HT promotes the mucosal secretion of water and electrolytes in the intestine of rats, guinea pigs, pigs, and humans (4, 5, 14, 15, 18, 24). In the guinea pig distal colon, 5-HT causes a biphasic increase in short-circuit current (I_{sc}), primarily due to chloride secretion (5, 18). The secretory response to 5-HT is partially inhibited by atropine and abolished by tetrodotoxin (TTX) (5, 18), suggesting that 5-HT-evoked chloride secretion consists of both cholinergic and noncholinergic components. 5-HT-evoked chloride secretion is mediated in part by 5-HT_3 and 5-HT_4 receptors located on neurons in guinea pig distal colon (19).

Recent immunohistochemical studies in the gastrointestinal tract indicated various roles of nitric oxide (NO) in the ENS of mammalian species, including monkeys and humans (1, 3, 6–9, 23). NO is a neurotransmitter involved in the control of intestinal motility, and recently it has been suggested that 5-HT-induced relaxation of intestinal smooth muscle involves NO (2, 25). Like 5-HT, NO appears to participate in mucosal function as well as in gastrointestinal motility; for example, NO induces electrolyte secretion in the rat intestine in vitro (21, 28, 30). Furthermore, the NO synthesis inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) prevents castor oil-induced diarrhea (22). In the guinea pig colon, an immunohistochemical study has shown that NO synthase (NOS) immunoreactivity is found in myenteric neurons as well as in the submucosa (23). A reasonable hypothesis based on these findings is that 5-HT-evoked chloride secretion might be linked to an NO pathway similar to the serotonergic/nitrergic mechanisms involved in regulation of intestinal motility. Support for such a hypothesis is the reported evidence that 5-HT-induced diarrhea is inhibited by the NOS inhibitor L-NAME in fasted mice (17).

The present study was therefore undertaken to test the idea that there is a neuronal nitrergic component to 5-HT-evoked ion transport in the distal colon of guinea pigs.

MATERIALS AND METHODS

Male albino guinea pigs (300–550 g; Hartley-Hazleton, Nippon SLC, Hamamatsu, Japan) were allowed food and water ad libitum. The animals were killed by a blow to the head and exsanguinated. Segments of distal colon 5–10 cm proximal to the anus were removed, flushed with Krebs-Ringer solution, and cut open along the mesenteric border. The tissues were pinned flat with the mucosal side down in a Sylgard-lined petri dish. The entire muscularis externa, including the myenteric plexus, was removed by blunt dissection. Four of these stripped preparations were obtained from one animal. Flat sheets of distal colon with intact submucosal ganglia were mounted between halves of Ussing flux chambers in which the total cross-sectional area was 0.64 cm^2.

The experimental methods for studying mucosal transport were similar to those previously described (20). Mucosal and

0193-1857/98 $5.00

serosal surfaces of tissues were incubated with 10 ml of buffer solution by recirculation from a reservoir maintained at 37°C for the duration of the experiment. Mucosal and serosal Krebs-Ringer solutions were identical and 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 solutions were gassed with 95% O_2-5% CO_2 and buffered to pH 7.2. The chambers were equipped with a pair of Ringer-agar bridges and calomel half-cells for the measurement of transmural electrical potential differences (PD). A pair of Ag-AgCl electrodes was connected to a voltage-clamp apparatus (SS-1335, Nihon-Kohden, Tokyo, Japan) that automatically compensated for the solution resistance between PD-sensing bridges. Tissue conductance was measured by calculating the ratio of I_{sc} to open-circuit values of PD or by determining the current necessary to change the transmural PD by 10 mV.

Submucosal neurons were electrically stimulated by passing current from an electronic stimulator (Nihon-Kohden, SEN-7203) through a pair of aluminum foil electrodes placed on the submucosal surface of the tissue. Electrical stimuli consisting of bipolar, rectangular pulses (0.5 ms, 10 V, 5 Hz) were applied for 30 s. The electrical stimulus was followed by the addition of atropine (10^{-5} M) to inhibit the cholinergic components of the response. N^G-nitro-L-arginine (L-NNA; 10^{-4} and 3×10^{-4} M) and its vehicle were added to the serosal bath, and the neurons were electrically stimulated again. The tissue responses were continuously recorded on a chart recorder (Recti-Horitz-8K, Nihon-Denki Sanei, Tokyo, Japan) or a Macintosh computer (MacLab/8 system, Analog Digital Systems, Castle Hill, Australia). Currents generated by the tissues both before and after the electrical stimulation were compared for control (no drug) and experimental tissues.

Tissues were paired on the basis of similar conductance. Noncumulative concentration-response curves for 5-HT were established. These concentration-response curves were constructed by adding 5-HT, at a single concentration, to the serosal bathing solution in the absence or presence of the NOS inhibitor L-NNA. L-NNA (10^{-4} M) was added to the serosal bathing solution 20–25 min before the addition of 5-HT. The experiments using sodium nitroprusside (SNP) in the presence of atropine, TTX, or indomethacin were carried out in the same way. In the combination studies (atropine, L-NNA, and atropine + L-NNA), the drugs were added together to the serosal bathing solution 20–25 min before addition of 5-HT (10^{-5} M).

All data are presented as means ± SE. Paired and unpaired Student's *t*-test and one-way ANOVA with pairwise comparisons by the Bonferroni method were used to determine the statistical significance of differences between control and experimental groups. Probability values <0.05 were considered statistically significant.

Drugs. The following chemicals were purchased from the suppliers indicated: 5-hydroxytryptamine creatinine sulfate (E. Merck, Darmstadt, Germany); L-NNA, bumetanide, indomethacin, and TTX (Sigma, St. Louis, MO); L-arginine monohydrochloride (L-Arg), D-arginine monohydrochloride (D-Arg), and SNP (Nacalai Tesque, Kyoto, Japan); and atropine sulfate (Wako, Osaka, Japan).

Bumetanide, indomethacin, and SNP were dissolved in dimethyl sulfoxide, 90% ethanol, and distilled H_2O, respectively. Other drugs were made up in Krebs-Ringer solution. The volume of drug added to the bath solutions did not exceed 100 μl/10 ml. Control tests of vehicles demonstrated that neither dimethyl sulfoxide nor ethanol (<0.5%) had an effect on baseline I_{sc}.

RESULTS

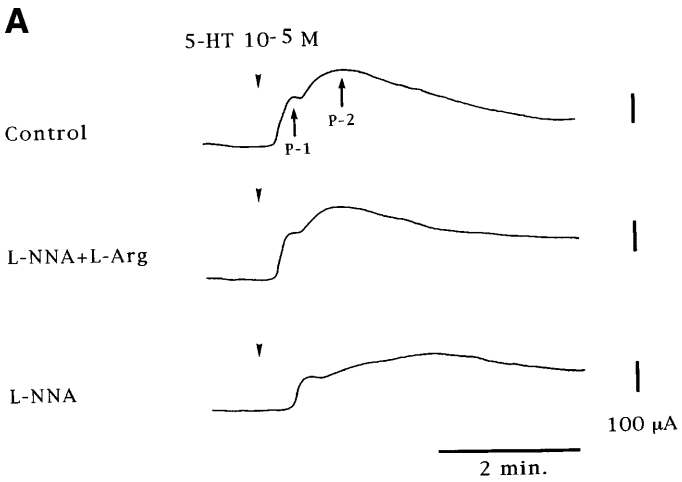
Effect of 5-HT on basal I_{sc} in presence of L-NNA. The effect of 5-HT on I_{sc} is shown in Fig. 1. The control 5-HT-evoked response consisted of two components, an early response and a late response, designated P-1 and P-2, respectively (Fig. 1*A*). 5-HT added to the serosal bath solution evoked a concentration-dependent increase in both P-1 and P-2 components of the I_{sc} response (Fig. 1, *B* and *C*). At 10^{-5} M 5-HT, the maximum P-2 response averaged 364.9 ± 34.7 μA/cm² (Fig. 1*C*, $n = 7$ animals) and was significantly greater than the maximum P-1 response (265.7 ± 37.8 μA/cm², $P < 0.01$, $n = 7$ animals, Fig. 1*B*).

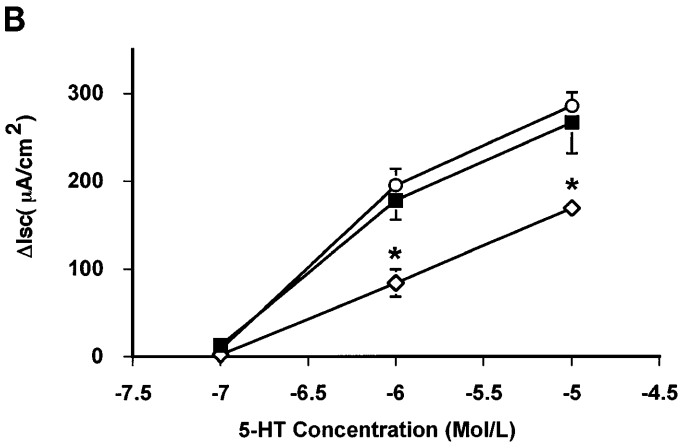
To determine whether the epithelial response evoked by 5-HT was mediated through the release of NO, the selective NOS inhibitor L-NNA was used. Addition of L-NNA (10^{-4} M) to the serosal bathing solution significantly reduced the electrogenic secretory P-1 and P-2 responses to 5-HT (10^{-5} M); the P-1 response was reduced to 63.5% of control (Fig. 1*B*; 168.8 ± 8.0 μA/cm², $n = 7$ animals, $P < 0.05$), and the P-2 response was reduced to 68.4% of control (Fig. 1*C*; 249.5 ± 18.1 μA/cm², $n = 7$ animals, $P < 0.05$). Furthermore, the addition of L-Arg (10^{-3} M) to the serosal bathing solution completely reversed the inhibitory action of L-NNA (10^{-4} M) on the 5-HT-evoked responses (Fig. 1). On the other hand, D-Arg (10^{-3} M) produced no change in the inhibitory effect of L-NNA; L-NNA still reduced the maximum 5-HT-evoked increases in P-1 and P-2 I_{sc} from 281.5 ± 35 to 199 ± 24 μA/cm² ($n = 9$ animals, $P < 0.05$) and from 340 ± 47 to 214.6 ± 29.2 μA/cm² ($n = 9$ animals, $P < 0.05$), respectively, in the presence of D-Arg. Neither L-NNA nor D-Arg altered the basal I_{sc} in any of the preparations (data not shown).

To determine the ionic basis for the increase in I_{sc} induced by 5-HT, we treated the tissues with bumetanide (5×10^{-4} M). 5-HT (10^{-5} M)-evoked increase in I_{sc} was greatly reduced by the treatment with bumetanide; bumetanide reduced the 5-HT-evoked P-1 and P-2 responses from 296.5 ± 27.4 to 211.4 ± 22.9 μA/cm² ($n = 6$ animals, $P < 0.05$) and from 356.6 ± 44.3 to 29.2 ± 5.2 μA/cm² ($n = 6$ animals, $P < 0.001$), respectively. The results confirmed that the 5-HT-evoked increase in I_{sc} is primarily caused by chloride secretion, as had been previously reported (5, 15, 19).

Effects of NO donor SNP on basal I_{sc} in presence of atropine, TTX, or indomethacin. To test whether NO itself affects baseline I_{sc}, the NO donor SNP was added to the serosal bathing solution. In preliminary experiments, we checked the effect of SNP on baseline I_{sc}, and at concentrations $<1 \times 10^{-6}$ M, SNP did not have a significant effect on I_{sc}. However, at higher concentrations (5×10^{-6}–10^{-4} M), SNP evoked an increase in I_{sc} in a concentration-dependent manner and SNP (10^{-4}M) reached maximum response at 48.3 ± 15 μA/cm² from baseline ($n = 12$ from 8 animals). Thus we used 10^{-4} M SNP for the subsequent experiments.

Addition of SNP (10^{-4} M) to the serosal bathing solution produced a gradual transient increase in baseline I_{sc} beginning within 1 min of the addition. SNP evoked an increase in I_{sc} of 24.7 ± 5.0 μA/cm² from the





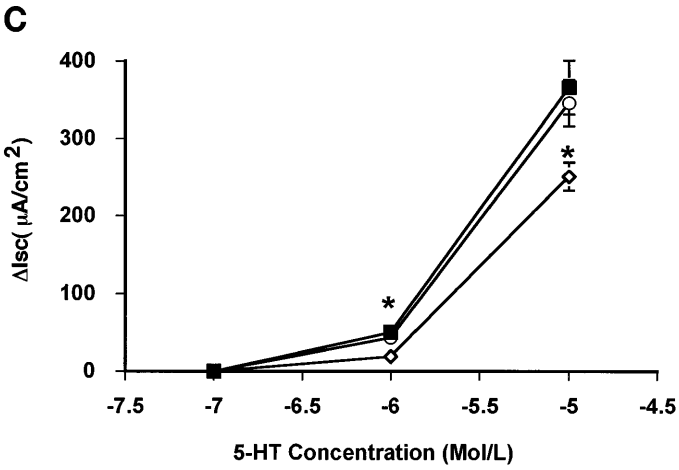


Fig. 1. Effects of N^G-nitro-L-arginine (L-NNA) on serotonin (5-hydroxytryptamine; 5-HT)-evoked changes (Δ) in short-circuit current (I_{sc}) in guinea pig distal colon. *A*: representative traces to illustrate effects of L-NNA and L-arginine (L-Arg) on 5-HT-evoked changes in I_{sc}. 5-HT (10^{-5} M)-evoked change in I_{sc} (Control) is made up of early (P-1) and late (P-2) phases (arrows) in guinea pig distal colon. Vertical and horizontal bars are I_{sc} and time, respectively. *B* and *C*: ■, control; ◇, L-NNA; ○, L-NNA + L-Arg. *B*: concentration-response curves for 5-HT-evoked P-1 responses in presence or absence of L-NNA and/or L-Arg. *C*: concentration-response curves for 5-HT-evoked P-2 responses in presence or absence of L-NNA and/or L-Arg. Each tissue received a single concentration of 5-HT. Noncumulative concentration-response curves of 5-HT were established. Nitric oxide (NO) synthase (NOS) inhibitor L-NNA (10^{-4} M) reduced amplitude of both P-1 and P-2 responses to 5-HT but did not alter basal I_{sc}. NOS substrate L-Arg (10^{-3} M) reversed inhibitory action of L-NNA on 5-HT-evoked responses but did not affect basal I_{sc}. Values are means ± SE for 7 animals. *Significantly different from control response to 5-HT ($P < 0.05$).

baseline I_{sc} (Fig. 2; $n = 7$ animals). Like 5-HT, chloride ion appears to be the major charge carrier in this SNP-evoked current, because bumetanide (5×10^{-4} M) significantly reduced the change in I_{sc} from 42.9 ± 6.5 to 17.5 ± 5.0 μA/cm² in another experiment ($n = 8$ animals, $P < 0.05$).

To determine whether the responses to SNP were caused by direct or indirect actions on the epithelium, TTX, atropine, or the cyclooxygenase inhibitor indomethacin was added to the serosal bathing solution at least 20 min before the addition of SNP. TTX (2×10^{-7} M) significantly reduced the SNP-evoked response to 10.6 ± 1.7 μA/cm² from 24.7 ± 5.0 μA/cm² (Fig. 2; $n = 7$ animals, $P < 0.05$). Neither atropine (10^{-6} M) nor indomethacin (10^{-4} M) significantly decreased SNP-evoked responses (from 24.7 ± 5 to 18.5 ± 4.3 and 16.3 ± 5.4 μA/cm², respectively, $n = 7$ animals; Fig. 2).

Cumulative effect of L-NNA and atropine on 5-HT-evoked response. If NO contributes to the 5-HT-evoked responses as a noncholinergic component, then the application of atropine and L-NNA together would be expected to decrease the 5-HT-evoked responses to a greater extent than would pretreatment with atropine or L-NNA alone. We therefore examined the effects of atropine (10^{-6} M) and L-NNA (10^{-4} M) together on 5-HT-evoked chloride secretion in guinea pig distal colon. 5-HT (10^{-5} M)-evoked responses were significantly decreased by pretreatment with atropine, L-NNA,

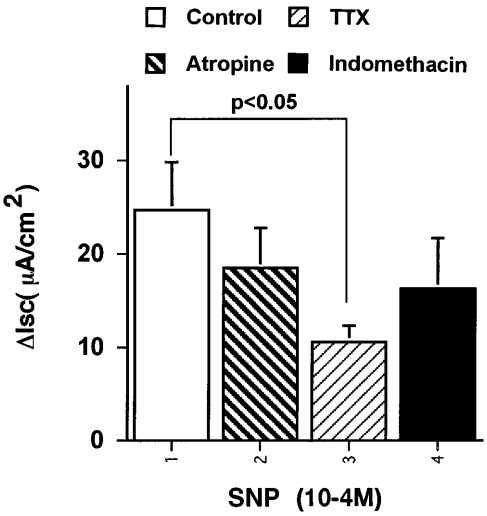


Fig. 2. Effects of atropine, tetrodotoxin (TTX), or indomethacin on sodium nitroprusside (SNP)-evoked increases in I_{sc} in guinea pig distal colon. Tissues were treated with TTX (2×10^{-7} M), atropine (10^{-6} M), or indomethacin (10^{-4} M) 15 min before addition of SNP (10^{-4} M). TTX significantly reduced SNP-evoked increase in I_{sc}. Although amplitudes of peak currents were reduced by both atropine and indomethacin, neither effect was statistically significant. Values are means ± SE for 6–7 animals. *Significantly different from control response to SNP ($P < 0.05$).

or atropine and L-NNA together, as shown in Fig. 3. The 5-HT-evoked P-1 and P-2 responses in the presence of atropine were decreased to 177.5 ± 35.3 μA/cm^2 from the control value of 247.1 ± 37.8 μA/cm^2 and to 53.1 ± 10.8 μA/cm^2 from the control value of 270.1 ± 59.4 μA/cm^2, respectively [Fig. 3, *B* and *C* (n = 5 animals); $P < 0.05$ and $P < 0.01$, respectively]. The result showed that the noncholinergic components of 5-HT-evoked P-1 and P-2 responses were 71.8 and 19.7%, respectively. A combination of atropine and L-NNA further decreased the 5-HT-evoked P-1 and P-2 responses to 121.5 ± 24.5 and 20.8 ± 7.2 μA/cm^2, respectively [Fig. 3, *B* and *C* (n = 5 animals); $P < 0.05$ and $P < 0.01$, respectively]. 5-HT-evoked P-1 and P-2 responses in the presence of atropine and L-NNA together were statistically different from those of the pretreatment with atropine or L-NNA alone (Fig. 3, *B* and *C*; $P < 0.05$). These results showed that NO components of P-1 and P-2 responses were 32 and 61% of the 5-HT-evoked noncholinergic responses, respectively.

Effect of L-NNA on noncholinergically mediated changes in I_{sc} by electrical field stimulation. To determine whether NO was involved in noncholinergic secretory response elicited by electrical field stimulation of submucosal neurons of the guinea pig distal colon, submucosa/mucosa preparations were electrically stimulated in the presence of atropine (10^{-5} M). The addition of atropine to the bath ensured that the neurally stimulated secretory response did not contain a muscarinic component. L-NNA significantly reduced the neurally evoked response in comparison to that of control, as shown in Fig. 4. The I_{sc} response to electrical field stimulation was decreased to 112.6 ± 16.4 from 132.8 ± 17.6 μA/cm^2 and to 90.9 ± 10.8 from 116.1 ± 10.8 μA/cm^2 in tissues exposed to 1 and 3×10^{-4} M L-NNA, respectively (Fig. 4; n = 7 animals, $P < 0.05$) However, the neurally evoked responses in different concentrations of L-NNA were not statistically different from each other.

DISCUSSION

The submucous plexus regulates intestinal chloride secretion in mammalian species. The idea that 5-HT is involved in the modulation of electrolyte transport in the gastrointestinal tract is supported by a considerable body of evidence. Functional postsynaptic 5-HT receptors on submucosal neurons in the guinea pig small intestine and colon have been electrophysiologi-

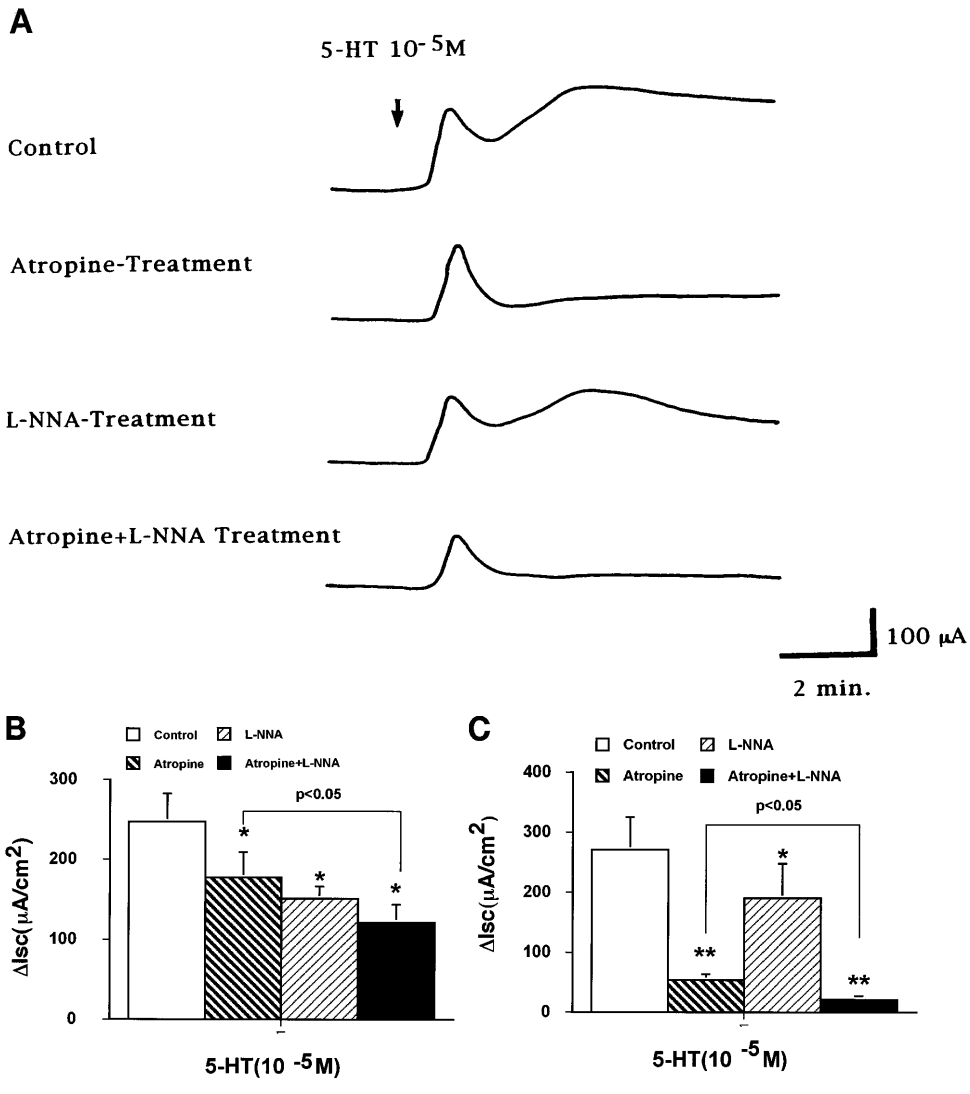


Fig. 3. Cumulative effects of atropine and L-NNA on response to 5-HT in guinea pig distal colon. *A*: representative traces to illustrate effects of atropine, L-NNA, and atropine + L-NNA on 5-HT-evoked changes in I_{sc}. 5-HT (10^{-5} M)-evoked responses (P-1 and P-2) were reduced by pretreatment with atropine or L-NNA alone or atropine + L-NNA in guinea pig distal colon. Simultaneous application of atropine and L-NNA further decreased 5-HT-evoked responses. Vertical and horizontal bars are I_{sc} and time, respectively. *B*: cumulative effects of atropine and L-NNA on 5-HT-evoked P-1 responses. *C*: cumulative effects of atropine and L-NNA on 5-HT-evoked P-2 responses. 5-HT (10^{-5} M)-evoked P-1 (n = 5 animals, $P < 0.05$; *B*) and P-2 (n = 5 animals, $P < 0.01$; *C*) responses were significantly reduced by pretreatment with 10^{-6} M atropine or 10^{-4} M L-NNA alone. 5-HT-evoked P-1 and P-2 responses in presence of atropine were further decreased by presence of atropine + L-NNA (*B* and *C*; n = 5 animals, $P < 0.05$). Values are means ± SE for 5 animals. Statistically different from control: * $P < 0.05$, ** $P < 0.01$.

cally demonstrated by the intracellular recording of depolarizing responses to the application of exogenous 5-HT (5, 27). Morphological investigations have demonstrated that 5-HT immunoreactivity is found in nerve fibers within submucous plexuses (29). The mucosal response to exogenous 5-HT in guinea pig distal colon is neurally mediated, because TTX abolishes this 5-HT-evoked response (5, 18). Muscarinic-receptor blockade with atropine also reduces the mucosal response to exogenous 5-HT, although a significant atropine-insensitive component remains (5, 16, 18). Taken together, these findings suggest that 5-HT stimulates mucosal electrolyte transport in the guinea pig by acting at receptors located on cholinergic as well as noncholinergic enteric neurons.

Among candidates as noncholinergic transmitters, NO has been shown to be a neurotransmitter of intestinal motility (25). Recent evidence also suggests a functional relationship between 5-HT and NO in the regulation of intestinal motility (2). NO also induces electrolyte secretion in the rat intestine in vitro (21, 28, 30).

5-HT-evoked increase in I_{sc} appears to be a chloride secretory response. In the present studies, we have confirmed this by demonstrating concentration-dependent 5-HT-evoked increases in I_{sc} that were greatly reduced by pretreatment of tissues with bumetanide, as reported previously (19). Pretreatment with the NOS inhibitory substance L-NNA reduced the 5-HT-evoked biphasic increase in I_{sc}; moreover, the reduction was reversed by the NOS substrate L-Arg but not by its D-enantiomer. Our data therefore support the idea that the 5-HT-evoked chloride secretion is, at least in part, linked with the NO pathway. In addition, neither L-Arg nor L-NNA altered the baseline I_{sc} in the guinea pig distal colon. This result is consistent with a previous study in rats in vivo (22), indicating that under normal conditions, NO is not produced within the intestinal wall in an amount sufficient to exert a tonic influence on the intestinal secretomotor function and that the supply of L-Arg for NO synthesis is not rate limiting.

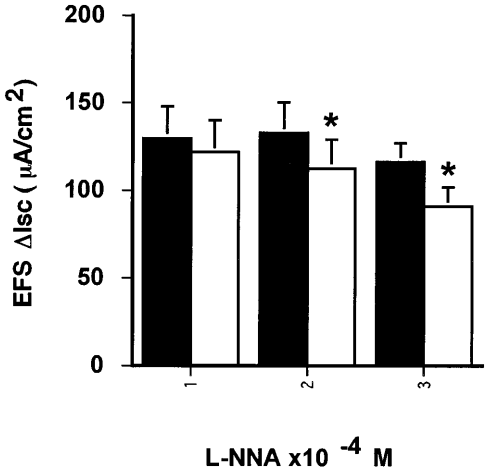


Fig. 4. Effect of L-NNA on neurally evoked secretion in presence of atropine. Submucosa/mucosa preparations of guinea pig distal colon were electrically stimulated in presence of atropine (10^{-5} M). Repetitive-stimulus pulses of 0.5-ms length, 10-Hz strength, and 5-Hz requency were used. Stimulus duration was 30 s. L-NNA (1 and 3×10^{-4} M) reduced neurally evoked noncholinergic responses. Filled bars, before addition of vehicle or L-NNA; open bars, after addition of vehicle or L-NNA. Values are means ± SE; $n = 7$ animals. *$P < 0.05$ significantly different from control responses (filled bars). EFS, electrical field stimulation.

We have examined whether NO itself can affect mucosal ion transport in guinea pig distal colon using the NO-donating compound SNP. In the present experiments, 10^{-4} M SNP evoked an increase in baseline I_{sc} in the guinea pig distal colon. This finding is consistent with the previous data that NO donors stimulate secretion of electrolytes in guinea pig and rat small intestine and colon in vitro (21, 28, 30). We have also determined that the ionic basis for SNP-evoked increase in I_{sc} is a chloride conductance, because the response to SNP as well as to 5-HT was reduced by bumetanide. In the present study, TTX significantly reduced the SNP-evoked response but did not abolish it. This finding is in good agreement with studies in the rat colon (28, 30), indicating that NO can affect the crypt cells through both neural and nonneural pathways. In contrast, TTX has no effect on SNP-evoked increase in I_{sc} in guinea pig small intestine (21). Possible explanations for these conflicting data may be the regional differences between the small and large intestine, or to species differences. Indeed, we have previously demonstrated that 5-HT-evoked chloride secretion is entirely linked with the enteric nervous system through 5-HT_3 and 5-HT_4 receptors in the guinea pig distal colon (19), whereas in the guinea pig ileum, the 5-HT-evoked chloride secretion contains a TTX-insensitive component mediated through 5-HT_4 receptors (26). Atropine did not reduce the SNP-evoked chloride secretion, but TTX significantly reduced it in the present experiments. These results suggest that NO also acts within the neural microcircuitry to alter epithelial transport. It is well known that prostaglandins can stimulate colonic fluid secretion by direct action on enterocytes and by the indirect activation of submucosal neurons (10, 12). In the present study, indomethacin tended to inhibit SNP-evoked responses, but the decreases in our experiments were not significant. However, Tamai and Gaginella (28) have reported that piroxicam, another cyclooxygenase inhibitor, greatly inhibited the SNP response in rat colon. This discrepancy probably reflects the inherent difficulties encountered in comparing functional effects of different preparations. Therefore, NO can directly stimulate the epithelium and, moreover, activate some neuronal pathways to secrete the chloride ion, but neither cyclooxygenase nor acetylcholine dependent pathways appear to be involved in the action of NO in guinea pig distal colon.

The present study has shown that 5-HT-induced chloride secretion was reduced by L-NNA and that the SNP-evoked response was not significantly blocked by atropine in guinea pig distal colon. These results support the idea that NO may be a noncholinergic transmitter in an enteric neural circuit that is activated by 5-HT. To further define the relationship between cholinergic and nitrergic neurons in 5-HT-evoked chloride secretion, additive effects were tested by the simultaneous administration of L-NNA and atropine. In the present experiments, the inhibition of

5-HT-evoked chloride secretion was greater when L-NNA and atropine were administered simultaneously than that observed when either atropine or L-NNA was used alone; simultaneous application of L-NNA and atropine further decreased the 5-HT-evoked P-1 and P-2 responses to 68 and 39% of atropine treatment tissues, respectively. In the present experiments, atropine significantly reduced the 5-HT (10^{-5} M)-evoked P-1 and P-2 responses to 71.8 and 19.7% of control (no drug), respectively. Taken together, the results suggest that noncholinergic components of P-1 and P-2 responses are 71.8 and 19.7% of control, respectively, and NO components of P-1 and P-2 responses are 32 and 61%, respectively, of the noncholinergic component of the 5-HT-evoked responses. The simultaneous application of L-NNA and atropine did not completely block the 5-HT-evoked responses, also suggesting that other mediators might be involved in 5-HT-evoked chloride secretion.

Finally, in the present experiments, L-NNA reduced electrical field stimulation-evoked response in the presence of atropine. The results clearly indicate that NO can function as a noncholinergic neurotransmitter. This is supported by morphological data that NOS-positive neurons are located in submucous plexus (8, 9).

In conclusion, the results provide strong evidence that 5-HT-induced chloride secretion in the guinea pig distal colon is, at least in part, mediated by an NO pathway and that NO may function as a noncholinergic transmitter in the submucosal plexus.

This work was supported by a Monbusyo International research grant, the Salt Foundation, and Health Science Foundations (A. Kuwahara). Part of this study was presented at the Third IUPHAR Satellite Meeting on Serotonin in Chicago, IL, in 1994, and was published in the proceedings (*Behav. Brain Res.* 73: 293–296, 1996).

Address for reprint requests: A. Kuwahara, Lab. of Environmental Physiology, Inst. for Environmental Sciences, Univ. of Shizuoka, Shizuoka 422–8526, Japan.

Received 4 September 1997; accepted in final form 11 May 1998.

REFERENCES

1. **Bredt, D. S., P. M. Hwang, and S. H. Snyder.** Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature* 347: 768–770, 1990.
2. **Briejer, M. R., L. M. A. Akkermans, A. L. Meulemans, R. A. Lefebvre, and J. A. J. Schuurkes.** Nitric oxide is involved in 5-HT-induced relaxations of the guinea-pig colon ascendens in vitro. *Br. J. Pharmacol.* 107: 756–761, 1992.
3. **Brookes, S. J. S., B. N. Chen, W. H. M. Hodgson, and M. Costa.** Characterization of excitatory and inhibitory motor neurons to the guinea pig lower esophageal sphincter. *Gastroenterology* 111: 108–117, 1996.
4. **Cooke, H. J., and H. V. Carey.** Pharmacological analysis of 5-hydroxytryptamine actions on guinea-pig ileal mucosa. *Eur. J. Pharmacol.* 111: 329–337, 1985.
5. **Cooke, H. J., Y.-Z. Wang, T. Frieling, and J. D. Wood.** Neural 5-hydroxytryptamine receptors regulate chloride secretion in guinea pig distal colon. *Am. J. Physiol.* 261 (*Gastrointest. Liver Physiol.* 24): G833–G840, 1991.
6. **Costa, M., J. B. Furness, S. Pompolo, S. J. H. Brookes, D. S. Bredt, and S. H. Snyder.** Projections and chemical coding of neurons with immunoreactivity for nitric oxide synthase in the guinea-pig small intestine. *Neurosci. Lett.* 148: 121–125, 1992.
7. **Dawson, T. M., D. S. Bredt, M. Fotuhi, P. M. Hwang, and S. H. Snyder.** Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues. *Proc. Natl. Acad. Sci. USA* 88: 7797–7801, 1991.
8. **De Giorgio, R., J. E. Parodi, N. C. Brecha, F. C. Brunicardi, J. M. Becker, V. L. W. Go, and C. Sternini.** Nitric oxide producing neurons in the monkey and human digestive system. *J. Comp. Neurol.* 342: 619–627, 1994.
9. **Ekblad, P., P. Alm, and F. Sundler.** Distribution, origin and projections of nitric oxide synthase-containing neurons in gut and pancreas. *Neuroscience* 63: 233–248, 1994.
10. **Frieling, T., C. Rupprecht, G. Dobreva,and M. Schemann.** Prostaglandin E_2 (PGE_2)-evoked chloride secretion in guinea-pig colon is mediated by nerve-dependent and nerve-independent mechanisms. *Neurogastroenterol. Mot.* 6: 95–102, 1994.
11. **Furness, J. B., and M. Costa.** Neurons with 5-hydroxytryptamine-like immunoreactivity in the enteric nervous system: their projections in the guinea-pig small intestine. *Neuroscience* 7: 341–349, 1982.
12. **Gaginella, T. S.** Eicosanoid-mediated intestinal secretion. In: *Secretory Diarrhea*. New York: Raven, 1990, p. 15–30.
13. **Gershon, M. D., A. L. Kirchgessner, and P. R. Wade.** Functional anatomy of the enteric nervous system. In: *Physiology of the Gastrointestinal Tract* (3rd ed.), edited by L. R. Johnson. New York: Raven, 1994, p. 381–422.
14. **Hansen, M. B.** ICS205–930 reduces 5-methoxytryptamine-induced short-circuit current in stripped pig jejunum. *Can. J. Physiol. Pharmacol.* 72: 227–232, 1994.
15. **Hardcastle, J., P. T. Hardcastle, and J. S. Redfern.** Action of 5-hydroxytryptamine on intestinal ion transport in the rat. *J. Physiol. Lond.* 320: 41–55, 1981.
16. **Johnson, P. J., J. C. Bornstein, J. B. Furness, D. J. Woollard, and S. L. Orrman-Rossiter.** Characterization of 5-hydroxytryptamine receptors mediating mucosal secretion in guinea-pig ileum. *Br. J. Pharmacol.* 111: 1240–1244, 1994.
17. **Kadowaki, M., M. D. Gershon, and A. Kuwahara.** Is nitric oxide involved in 5-HT-induced fluid secretion in the gut? *Behav. Brain Res.* 73: 293–296, 1996.
18. **Kuwahara, A., and Y. Kuwahara.** 5-Hydroxytryptamine (5-HT)-induced anion secretion in isolated guinea-pig distal colon. *J. Physiol. Lond.* 446: 197P, 1992.
19. **Kuwahara, A., Y. Kuwahara, and M. Kadowaki.** Analysis of FK1052, a potent 5-HT3 and 5-HT4 receptor dual antagonist, on ion transport of guinea pig distal colon. *Biomedical Res.* 15: 205–210, 1994.
20. **Kuwahara, A., Y. Kuwahara, T. Mochizuki, and N. Yanaihara.** Action of pituitary adenylate cyclase-activating polypeptide on ion transport in guinea pig distal colon. *Am. J. Physiol.* 264 (*Gastrointest. Liver Physiol.* 27): G433–G441, 1993.
21. **MacNaughton, W. K.** Nitric oxide-donating compounds stimulate electrolyte transport in the guinea pig intestine in vitro. *Life Sci.* 53: 585–593, 1993.
22. **Mascolo, N., A. A. Izzo, G. Autore, F. Barbato, and F. Capasso.** Nitric oxide and castor oil-induced diarrhea. *J. Pharmacol. Exp. Ther.* 268: 291–295, 1994.
23. **McConalogue, K., and J. B. Furness.** Projection of nitric oxide synthesizing neurons in the guinea-pig colon. *Cell Tissue Res.* 271: 545–553, 1993.
24. **Munck, L. K., A. Mertz-Nielsen, H. Westh, K. Bukhave, E. Beubler, and J. Rask-Madsen.** Prostaglandin E_2 is a mediator of 5-hydroxytryptamine induced water and electrolyte secretion in the human jejunum. *Gut* 29: 1337–1341, 1988.
25. **Sanders, K. M., and S. M. Ward.** Nitric oxide as a mediator of nonadrenergic noncholinergic neurotransmission. *Am. J. Physiol.* 262 (*Gastrointest. Liver Physiol.* 25): G379–G392, 1992.
26. **Scott, C. M., K. T. Bunce, and C. F. Spraggs.** Investigation of the 5-hydroxytryptamine receptor mediating the "maintained" short-circuit current response in guinea pig ileal mucosa. *Br. J. Pharmacol.* 106: 877–882, 1992.
27. **Surprenant, A., and J. Crist.** Electrophysiological characterization of functional distinct 5-hydroxytryptamine receptors on guinea-pig submucous plexus. *Neuroscience* 24: 283–295, 1988.
28. **Tamai, H., and T. S. Gaginella.** Direct evidence for nitric oxide stimulation of electrolyte secretion in the rat colon. *Free Rad. Res. Commun.* 19: 229–239, 1993.
29. **Wardell, C. F., J. C. Bornstein, and J. B. Furness.** Projection of 5-hydroxytryptamine-immunoreactive neurons in guinea-pig distal colon. *Cell Tissue Res.* 278: 379–387, 1994.
30. **Wilson, K. T., Y. Xie, M. W. Musch, and E. B. Chang.** Sodium nitroprusside stimulates anion secretion and inhibits sodium chloride absorption in rat colon. *J. Pharmacol. Exp. Ther.* 266: 224–230, 1993.