Full Paper

Nitric Oxide and Carbon Monoxide Act as Inhibitory Neurotransmitters in the Longitudinal Muscle of C57BL/6J Mouse Distal Colon

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Abstract. The present study was designed to identify the inhibitory neurotransmitters mediating nonadrenergic noncholinergic relaxation in the longitudinal muscle of C57/BL mouse distal colon. Relaxation induced by electrical field stimulation (EFS) was recorded isotonicly in the presence of atropine and guanethidine. Cyclic guanosine-3',5'-monophosphate (cyclic GMP) content was measured by radioimmunoassay. EFS-induced relaxation was inhibited by nitro-L-arginine (L-NNA) and Sn (IV) protoporphyrin dichloride IX (SnPP-IX), a nitric oxide (NO) and carbon monoxide (CO) synthase inhibitor, respectively. A combination of both inhibitors produced an additive effect. ODQ, a soluble guanylate cyclase inhibitor, inhibited EFS-induced relaxation. NOR-1, a NO donor, and carbon monoxide-releasing molecule-2 (CORM-2), a CO donor, treatment relaxed the distal colon and increased cyclic GMP content. The effects of NOR-1 and CORM-2 were inhibited by ODQ. KT5823, a cyclic GMP-dependent protein kinase inhibitor, inhibited EFS-induced relaxation. EFS-induced relaxation in the presence of KT5823 was further inhibited by L-NNA, but not by SnPP-IX. In addition, KT5823 inhibited CORM-2-induced relaxation, but not NOR-1-induced relaxation. H89, a cyclic AMP-dependent protein kinase inhibitor, inhibited EFS-induced relaxation, and EFS-induced relaxation in the presence of H89 was further inhibited by L-NNA. These results suggested that NO and CO function as inhibitory neurotransmitters in the longitudinal muscle of C57BL mouse distal colon.

Keywords: mouse distal colon, nonadrenergic noncholinergic (NANC) relaxation, nitric oxide (NO), carbon oxide (CO), cyclic guanosine-3',5'-monophosphate (cyclic GMP)-dependent protein kinase

Introduction

Neurotransmitters mediating relaxation in the gastrointestinal tract are nonadrenergic noncholinergic (NANC), and many substances have been reported as putative candidates of the inhibitory neurotransmitters (1). The relative extent of these substances to mediate relaxation varies and is dependent upon the animal species, the strains, and the regions examined (2). In addition, multiple neurotransmitters are utilized in relaxation studies with the relative importance of each varying with the region and species. In the dog, vasoactive intestinal peptide (VIP) and nitric oxide (NO) were reported to be

inhibitory neurotransmitters in the stomach (3) and NO alone was the inhibitory neurotransmitter in the ileum (4) and colon (5). In the rabbit, VIP and NO were relaxant mediators in the stomach (6) and NO alone was the relaxant mediator in the colon (7). In the guinea pig, VIP and pituitary adenylate cyclase activating peptide (PACAP), NO and adenosine-5'-triphosphate (ATP), and PACAP and NO were shown to be inhibitory mediators in the stomach (8), ileum (9), and colon (10), respectively. In the rat, relaxation of the stomach, ileum, and colon was mediated by NO, VIP, and peptide histidine isoleucine (PHI) (11); ATP and NO (12, 13); and VIP, PACAP, and NO (14), respectively. Furthermore, there are reports indicating interactions between inhibitory mediators (15, 16). Recently, differences were reported in the inhibitory mediators and the mechanisms of action between circular and longitudinal muscle layers in the mouse ileum (17).

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Therefore, detailed experiments taking species, region, and tissues into consideration are necessary to elucidate the role of mediator(s) that control relaxation in the gastrointestinal tract.

Mice are becoming increasingly important animal models for investigating physiological function on account of the ability to produce knockout (KO) strains and the advent of gene-targeting technology. Gene KO mice have been used to clarify inhibitory neurotransmission in the gastrointestinal tract. However, reports that examined the inhibitory transmission in the mouse gastrointestinal tract are minimal. NO was shown to act in the duodenum (18), jejunum (19), and ileum (20). On the other hand, involvement of NO in the relaxation of the longitudinal muscle of colon was shown to be controversial; in some reports, a NO synthase (NOS) inhibitor was shown to inhibit the relaxant response through the activation of intrinsic neurons (21), whereas it had no effect in another report (22). Thus, the role of NO as an inhibitory neurotransmitter in the relaxation of the longitudinal muscle of mouse colon requires re-estimation. Carbon monoxide (CO), synthesized by heme oxygenase-2 (HO-2) which is expressed within cell bodies of the myenteric ganglia and nerve fibers (23), has been reported to regulate the motility of the gastrointestinal tract (24). In HO-2-knockout mice, the transit of content through the intestine and colon was slower than that in wild-type mice, and NANC relaxation in ileal segments induced by electrical stimulation was small, compared with that of wild-type mice (23). Exogenously added CO produced relaxation in mouse gastric fundus and jejunum (25). Thus, CO may function as an inhibitory neurotransmitter in some gastrointestinal regions. However, the role of CO in relaxation of mouse colon has not been studied.

In the present study, we examined mediators of NANC relaxation induced by electrical field stimulation (EFS) in the longitudinal muscle of C57BL/6J mouse distal colon, specifically the role of NO and CO in NANC relaxation. NO and CO cause relaxations of gastrointestinal smooth muscle by activating soluble guanylate cyclase, thereby stimulating the formation of cyclic guanosine-3',5'-monophosphate (cyclic GMP) in part of the smooth muscle tissues of the gastrointestinal tract (24, 26). We also show the involvement of soluble guanylate cyclase-cyclic GMP pathways in NANC relaxation of C57BL/6J mouse distal colon.

Materials and Methods

Eight- to twelve-week-old male C57BL/6J mice obtained from JCL (Osaka) were lightly anesthetized with diethyl ether, stunned by a blow to the head, and bled via the carotid arteries. Animal maintenance and experimen-

tal procedures were performed under the approval of the guidelines of the ethics committees of Osaka Prefecture University. Segments of the colonic region were removed and placed in Tyrode solution consisting of 137 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl_2 , 1.1 mM MgCl_2 , 0.42 mM NaH_2PO_4 , 11.9 mM NaHCO_3 , and 5.6 mM glucose. The contents of the excised segments were gently flushed out with Tyrode solution. The upper portion (1.5–2.0 cm in length) of the colon away from the anus by 1.5 cm was defined as the distal colon. Whole segments of the distal colon were used.

Recording of responses of longitudinal muscle to EFS and agonists

As described in a previous study (27), segments of distal colon were suspended in an organ bath containing 4 ml Tyrode solution aerated with 5% CO_2 and 95% O_2 and maintained at 37°C. Since the segments were suspended in a Magnus apparatus in the direction of the longitudinal axis, the response of longitudinal muscle was recorded. The longitudinal muscle of each segment was subjected to a load of 0.5 g to obtain the most reproducible responses and a stable resting tone. The anal end of each segment was attached to a transducer (TD-112S; Nihon Kohden, Tokyo) and the oral end was mounted on an anodal electrode placed at the bottom of the bath. Atropine (1 μM) and guanethidine (5 μM) were added throughout the experiment to record NANC responses. After an equilibration period of 30 min, responses of the longitudinal muscle to EFS for 10 s with trains of 1–100 pulses of 0.5 ms width at 30 V, 1–10 Hz frequencies were recorded isotonicly via a preamplifier (JD-112S, Nihon Kohden) with a 10-min interval between tests. Time control experiments showed no decay in the response of EFS for several hours. The extent of relaxation was expressed as the area under the line of resting tone that was drawn on the bottom of resting spontaneous contractile activity (broken lines in Fig. 1) as described elsewhere (28). Relaxing responses to NOR-1, a NO donor, and carbon monoxide-releasing molecule-2 (CORM-2) were conducted before and after the application of different antagonists used. In addition, the responses to purinergic agonists were recorded. The relaxations induced by all the agonists were expressed as a percentage of the maximal relaxation induced by 30 μM papaverine. Antagonists were applied for at least 10–30 min before EFS or the addition of agonist.

Measurement of cyclic GMP content of longitudinal muscle cells of distal colon

Small longitudinal muscle strips (3–6 mg wet weight) prepared from the distal colon were equilibrated for 30 min with Tyrode solution containing 1 μM atropine

and 5 μ M guanethidine at 37°C and aerated with 5% CO₂ and 95% O₂. The preparations were then incubated with various concentrations of NOR-1 (1, 10 μ M) or CORM-2 (50, 100 μ M) for 5 min and then quickly frozen within a few seconds by placing them into liquid nitrogen. Frozen preparations were then homogenized in 2 ml of 6.0 N trichloroacetic acid (TCA) solution. After removal of TCA with ether, the cyclic GMP content was determined with a cyclic GMP assay kit (Amersham Japan, Tokyo). All determinations of cyclic GMP were carried out in the absence of phosphodiesterase inhibitor.

Statistical analyses

Only one preparation was made from each animal. Thus *n* in the results indicates the number of animals. All values are expressed as means $\pm$ S.E.M. Results were analyzed statistically by the paired *t*-test or evaluated by ANOVA and Student's *t*-test or the Welch test (if significant differences were indicated by ANOVA). *P*-values <0.05 were regarded as statistically significant.

Drugs

The following reagents were obtained from the indicated commercial sources: Sn (IV) protoporphyrin di-

chloride IX (SnPP-IX; Frontier Scientific, Inc., Logan, UT, USA); tricarbonyldichlororuthenium (II) dinmer (CORM-2; Johnson Matthey, London, UK); 1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one (ODQ; ALEXIS Biochemicals, Lausen, Switzerland); 2'-deoxy-*N*⁶-methyladenosine 3',5'-diphosphate diammonium (MRS2179), adenosine 3'-phosphate 5'-phosphosulfate (A3P5PS), α -chymotrypsin, *N*^ω-nitro-L-arginine (L-NNA), and ATP (Sigma Chemical Co., St. Louis, MO, USA); atropine sulfate, guanethidine, L-arginine, 12-epoxy-1*H*-diindolo-[1,2,3-*fg*:3',2',1'-*kl*]pyrrolo[3,4-*i*][1,6]benzodiazocine-10-carboxylic acid methyl ester (KT5823), and papaverine hydrochloride (Wako Pure Chemical Industries, Osaka); ($\pm$)-(*E*)-4-methyl-2-[(*E*)-hydroxyimino]-5-nitro-6-methoxy-3-hexenamide (NOR-1; Dojin, Kumamoto); Rp-8-bromoguanosine-3',5'-cyclic monophosphorothioate (Rp-8-br-cGMPS), *N*-[2-[P-bromocinnamylamino]ethyl]-5-isoquinolinesulfonamide (H89) (Biolog Products, Bremen, Germany). All other chemicals were of analytical grade.

Drugs were added to the organ bath in volumes of less than 1.0% of the bathing solution. The volume of vehicle of the drugs, redistilled water, did not affect either the spontaneous contractile activity or the muscle tone. SnPP-

Fig. 1. L-NNA or SnPP-IX on EFS-induced relaxation of longitudinal muscle of C57BL/6J mouse distal colon. Upper panels: relaxations were induced by EFS (10 Hz, 30 V, 0.5 ms for 10 s) in the presence of 30 μ M L-NNA (A) or 10–30 μ M SnPP-IX (B). The muscle preparations were treated with inhibitors for 10 min before EFS. Small bars indicate the 10-s duration of EFS. The lines indicate the presence of the indicated drugs. After recording normal spontaneous movement, the chart speed was increased from immediately before the stimulation to make the relaxant response clearer during the period indicated by dashed lines. Lower panels: histograms showing the effects of L-NNA (A) and SnPP-IX (B) on the relaxation induced by EFS. Relaxations are expressed as percentages of those before addition of the inhibitors (control). Values are means $\pm$ S.E.M. for five experiments. *Significantly different from control value, *P* < 0.05; [#]Significantly different from the value in the presence of L-NNA, *P* < 0.05.

Fig. 2. Additive effects of L-NNA and SnPP-IX on EFS-induced relaxation of longitudinal muscle of C57BL/6J mouse distal colon. Upper panel: relaxations were induced by EFS (10 Hz, 30 V, 0.5 ms for 10 s) in the presence of 30 μ M L-NNA and 30 μ M SnPP-IX. The muscle preparations were treated with inhibitors for 10 min before EFS. Small bars indicate the 10-s duration of EFS. The lines indicate the presence of the indicated drugs. After recording normal spontaneous movement, the chart speed was increased from immediately before the stimulation to make the relaxant response clearer during the period indicated by dashed lines. Lower panel: histograms showing the effects of L-NNA and SnPP-IX on the relaxation induced by EFS. Relaxations are expressed as percentages of those before addition of the inhibitors (control). Values are means $\pm$ S.E.M. for 4–5 experiments. *Significantly different from control value, $P < 0.05$; [#]Significantly different from the value in the presence of L-NNA, $P < 0.05$.

IX, CORM-2, NOR-1, and ODQ, H89 were dissolved in dimethyl sulfoxide and 100% ethanol, respectively, as stock solutions. The final dimethyl sulfoxide and ethanol concentrations were 0.1% and did not have any effect on the response of the preparations.

Results

Relaxant response induced by EFS in the longitudinal muscle of C57BL/6J mouse distal colon

EFS induced a rapid relaxation and a subsequent rebound contraction in the presence of atropine (1 μ M) and guanethidine (5 μ M) in the longitudinal muscle of the mouse distal colon segments (Fig. 1). These responses were completely inhibited by tetrodotoxin (1 μ M). L-NNA (30 μ M), a NOS inhibitor, inhibited EFS-induced relaxation (about 40% inhibition) (Fig 1A). Addition of L-arginine (1 mM) to the bath solution reversed the inhibitory effect of L-NNA. L-NNA at 100 μ M produced a stronger inhibition than at 30 μ M, and this inhibition was not reversed by the addition of L-arginine, suggesting that 100 μ M L-NNA cause effects other than NOS inhibition (Data not shown). SnPP-IX, a heme oxygenase inhibitor, inhibited EFS-induced relaxation in a concentration-dependent manner. The removal of SnPP-IX by washout reversed EFS-induced relaxation inhibited by 30 μ M to the control level, but did not that at 100 μ M. Thus, SnPP-IX (30 μ M) produced about 30% inhibition (Fig 1B) and was used in all other experiments. EFS-induced relaxation inhibited by L-NNA (30 μ M) treatment was further inhibited by the addition of SnPP-IX, indicating that the effects of both drugs were additive (Fig. 2). In contrast, treatment with α -chymotrypsin (3 units/ml)

Fig. 3. Relaxant responses induced by EFS (1–10 Hz) of longitudinal muscle of C57BL/6J mouse distal colon and the effects of 30 μ M L-NNA (A), 30 μ M SnPP-IX (B), and both drugs (C). Relaxations are expressed as percentages of those before addition of the inhibitors (control). Values are each the mean $\pm$ S.E.M. for five experiments. *Significantly different from the control value of the corresponding frequency, $P < 0.05$; [#]Significantly different from the value in the presence of L-NNA of the corresponding frequency, $P < 0.05$.

Fig. 4. Effects of ODQ on EFS-induced relaxation of longitudinal muscle of C57BL/6J mouse distal colon. A) Concentration-inhibition curve for ODQ. B) Effect of 30 μ M L-NNA on residual relaxation induced by EFS in the presence of 10 μ M ODQ. Relaxations were induced by EFS in the presence of ODQ at the indicated concentrations. Relaxations are expressed as a percentage of that before the inhibitors (control). Values are means $\pm$ S.E.M. for five experiments. *Significantly different from the control value, $P < 0.05$.

for 30 min had no effect on EFS-induced relaxation ($127.3 \pm 13.8\%$ of control, $n = 5$).

We then examined the inhibitory effects of L-NNA and SnPP-IX on relaxation induced by different frequencies of EFS. EFS-induced relaxation increased in a frequency-dependent manner (Fig. 3A). L-NNA (30 μ M) inhibited relaxation induced by EFS at 1, 3, and 10 Hz to a similar extent (1 Hz, $46.4 \pm 7.3\%$; 3 Hz, $41.7 \pm 8.7\%$; 10 Hz, $43.7 \pm 10.6\%$). SnPP-IX (30 μ M) inhibited EFS-induced relaxation at all frequencies to a similar extent (Fig. 3B). An additive effect of both inhibitors on relaxation was seen at all frequencies (Fig. 3C). Based on these results, subsequent experiments were carried out using the stimulation frequency of 10 Hz; the responses at EFS at this frequency were distinct and reproducible.

Fig. 5. Effects of ODQ on NOR-1 (A)– or CORM-2 (B)–induced relaxation in longitudinal muscle of C57BL/6J mouse distal colon. A) Concentration–relaxation curves to NOR-1 showing the responses in the absence (control) and the presence of 1 μ M ODQ. B) The relaxation induced by 50 μ M CORM-2 was measured in the absence (control) and the presence of 1 μ M ODQ. Relaxations are expressed as percentages of the maximal relaxation induced by 30 μ M papaverine. Values are means $\pm$ S.E.M. for four experiments. *Significantly different from the control value, $P < 0.05$.

Involvement of soluble guanylate cyclase in EFS-induced relaxation

ODQ, a soluble guanylate cyclase inhibitor, inhibited EFS-induced relaxation in a concentration-dependent manner (Fig. 4A). Maximal inhibition of ODQ, at a concentration of 10 μ M, produced about 70% inhibition compared to the control. Residual relaxation in the presence of ODQ was unaffected by the addition of L-NNA (Fig. 4B).

NOR-1 produced a significant relaxation in longitudinal muscle of mouse distal colon in a concentration-dependent manner (Fig. 5A). The relaxation induced by NOR-1 was significantly inhibited by 1 μ M ODQ treatment. The addition of CORM-2 (50 μ M) induced relaxation of the preparation (Fig. 5B), which was inhibited by ODQ.

Table 1. Effects of NOR-1 and CORM-2 on cyclic GMP contents of the longitudinal muscle of mouse distal colon

Treatment	Concentration (μM)	cGMP content (fmol/mg wet tissue per min)	
		Control	1 μM ODQ
No drug		7.1 $\pm$ 0.4 (28)	5.6 $\pm$ 0.5 (5)
NOR-1	1	12.0 $\pm$ 2.3 (15)*	
	10	19.3 $\pm$ 3.6 (8)*	4.9 $\pm$ 0.9 (4) [#]
CORM-2	50	9.6 $\pm$ 1.1 (14)*	6.1 $\pm$ 0.7 (5)
	100	21.2 $\pm$ 3.9 (8)*	9.7 $\pm$ 0.8 (3) [#]

The preparations were incubated for 5 min with the indicated concentrations of NOR-1 or CORM-2 in the absence or presence of 1 μM ODQ, and then cyclic GMP contents were measured by radioimmunoassay. Values are means $\pm$ S.E.M. for the numbers of experiments shown in parentheses. *Significantly different from the value of no drug in the absence of ODQ, $P < 0.05$. [#]Significantly different from the value of corresponding control, $P < 0.05$.

Effects of NOR-1 and CORM-2 on cyclic GMP contents in the longitudinal muscle of C57BL/6J mouse distal colon

The content of cyclic GMP in the absence of NOR-1 and CORM-2 was 7.1 $\pm$ 0.4 fmol/mg tissue per min. The addition of NOR-1 produced a significant increase in cyclic GMP level in a concentration-dependent manner (Table 1). Cyclic GMP content was also increased by 50 μM CORM-2 (50 μM). When the concentration of CORM-2 was increased to 100 μM , a further increase in the cyclic GMP content was obtained. ODQ (1 μM) did not affect the basal level of cyclic GMP content, but significantly inhibited the concentration-dependent effect of NOR-1 and CORM-2 on cyclic GMP levels (Table 1).

Involvement of cyclic GMP-dependent protein kinase (PKG) in NO- and CO-mediated relaxation

KT5823, a PKG inhibitor, inhibited EFS-induced relaxation in a concentration-dependent manner (Fig. 6, inset). At a concentration of 1 μM KT5823, EFS-induced relaxation was 65.3 $\pm$ 7.3% of the control (Figs. 6A and 7A). The PKG inhibitor Rp-8-Br-cGMPS also significantly inhibited EFS-induced relaxation (46.7 $\pm$ 3.4% inhibition). The remaining relaxation in the presence of KT5823 was further inhibited by L-NNA (Fig. 6A), but not by SnPP-IX (Fig. 6B). In addition, a remaining relaxation in the presence of Rp-8-Br-cGMPS was also further inhibited by L-NNA (38.0 $\pm$ 10.6% inhibition).

NOR-1-induced relaxation was unaffected by KT5823 (1 μM) (Fig. 7A). In contrast, CORM-2-induced relaxation was significantly inhibited by KT5823 (1 μM) (Fig. 7B) and Rp-8-Br-cGMPS (30 μM) (60.8 $\pm$ 9.3%

Fig. 6. Effects of KT5823 on EFS-induced relaxation in the absence and the presence of L-NNA (A) or SnPP-IX (B) in longitudinal muscle of C57BL/6J mouse distal colon. A) Histograms showing the effects of 1 μM KT5823 in the absence and the presence of 30 μM L-NNA. Inset: Effects of KT5823 on EFS-induced relaxation. B) Histograms showing the effects of 1 μM KT5823 in the absence and the presence of 30 μM SnPP-IX and effect of further addition of 30 μM L-NNA. Relaxations are expressed as percentages of those before addition of the inhibitors (control). Values are means $\pm$ S.E.M. for five experiments. *Significantly different from the control value, $P < 0.05$; [#]Significantly different from the value in the presence of KT5823, $P < 0.05$.

inhibition).

Since it was reported that the NO-cyclic GMP pathway relaxed tracheal smooth muscles through an activation of a cyclic AMP-dependent protein kinase (PKA) (29), we examined effects of a PKA inhibitor on EFS-induced relaxation. H89, the PKA inhibitor, inhibited EFS-induced relaxation in a concentration-dependent manner (Fig. 8A). A combination of H89 and L-NNA had an additive inhibitory effect on EFS-induced relaxation (Fig. 8B).

Fig. 7. Effects of KT5823 on NOR-1 (A)– or CORM-2 (B)–induced relaxation in longitudinal muscle of C57BL/6J mouse distal colon. A) Upper panels: Relaxations were induced by NOR-1 in the presence of 1 μM KT5823. The muscle preparations were treated with KT5823 for 10 min before EFS. Small bars indicate the 10-s duration of EFS. The lines indicate the presence of 1 μM KT5823. After recording normal spontaneous movement, the chart speed was increased from immediately before the stimulation to make the relaxant response clearer (dashed lines). Lower panels: Concentration–relaxant responses of NOR-1 in the absence (control) and the presence of 1 μM KT5823. B) The relaxation induced by 50 μM CORM-2 was measured in the absence (control) and the presence of 1 μM KT5823. Relaxations are expressed as percentages of the maximal relaxation induced by 30 μM papaverine. Values are means ± S.E.M. for five experiments. *Significantly different from the control value, $P < 0.05$.

Effects of P2Y₁-receptor antagonists on EFS-induced relaxation

A3P5PS, a P2Y₁-receptor antagonist, inhibited EFS-induced relaxation in a concentration-dependent manner (Fig. 9). A3P5PS at 100 μM had maximal effects ($33.8 \pm 4.2\%$ inhibition). MRS2179, a selective P2Y₁-receptor antagonist, inhibited EFS-induced relaxation to a similar extent as A3P5PS (1 μM, $37.6 \pm 1.7\%$ inhibition). The remaining relaxation in the presence of A3P5PS was significantly inhibited by L-NNA ($59.6 \pm 1.7\%$ inhibition). The further addition of SnPP-IX in the presence of both inhibitors almost completely inhibited EFS-induced relaxation ($7.7 \pm 1.6\%$ of control) (Fig. 9).

The addition of ATP and ADP to longitudinal muscle of mouse distal colon produced variable responses with the preparation and concentration studied. Thus, we were unable to obtain data about the effects of ATP and ADP on the longitudinal muscle of mouse distal colon.

Effect of H89 on P2Y₁ receptor-mediated relaxations

EFS-induced relaxation ($23.8 \pm 3.4\%$ of control) that was inhibited by ODQ (10 μM) was further inhibited by the addition of A3P5PS ($15.6 \pm 2.5\%$ of control, $P < 0.05$ vs. value in the presence of ODQ). H89 (1 μM) had no

effect on EFS-induced relaxation in the presence of A3P5PS (Fig. 10). EFS-induced relaxation in the presence of H89 ($62.3 \pm 3.5\%$ of control) was inhibited by L-NNA and more strongly inhibited by further addition of SnPP-IX (Fig. 8B).

Discussion

In the present study, NANC relaxation induced by EFS was produced by the activation of intrinsic neurons, probably myenteric neurons, in the longitudinal muscle layers of C57BL/6J mouse distal colon. We previously demonstrated that VIP and PACAP are neurotransmitters of the relaxant response in the longitudinal muscle of rat distal colon (14), but it now seems unlikely that peptidergic substances are involved in EFS-induced relaxation of C57BL/6J mouse distal colon, as judged by the lack of effect of α-chymotrypsin. In the distal colon of the ICR mouse, PACAP was reported to partially mediate EFS-induced relaxation (21). On the other hand, EFS-induced relaxation was inhibited to about 60% of the control by L-NNA, a NOS inhibitor, regardless of the stimulation frequencies. NOR-1, a NO donor, relaxed the preparation in a concentration-dependent manner. It has been reported that a NOS inhibitor was effective in inhibiting NANC

Fig. 8. Effects of H89 on EFS-induced relaxation of longitudinal muscle of C57BL/6J mouse distal colon. A) Relaxations were induced by EFS in the presence of H89 at the indicated concentrations. B) Effects of combined treatment with H89, L-NNA, and SnPP-IX on EFS-induced relaxation. Relaxations were induced by EFS at 10 Hz for 10 s. The preparation was successively treated with 1 μ M H89, 30 μ M L-NNA, and 30 μ M SnPP-IX. Note that relaxation was partially inhibited by H89 and L-NNA and almost completely abolished by the further treatment with SnPP-IX. Relaxations are expressed as a percentage of that before drugs (control). Values are each the mean $\pm$ S.E.M. for 3–5 experiments. *Significantly different from the control value, $P < 0.05$; #Significantly different from the value in the presence of H89, $P < 0.05$; †Significantly different from the value with the treatment of H89 and L-NNA, $P < 0.05$.

relaxation of longitudinal muscle of ICR and C57BL/10nJ mice (21), whereas it was not in BL/6 $\times$ DBA mouse (22). These results suggest that the participation of NO in NANC relaxation of the distal colon is different in discrete strains of mice, as has been previously described in rats (30). Furthermore, SnPP-IX, an inhibitor of HO, the enzyme that catalyzes the degradation of heme to biliverdin, iron, and CO, produced about 30% inhibition of the relaxation induced by EFS at all stimulation frequencies. The longitudinal muscle of mouse distal colon was relaxed by the addition of CORM-2. Thus, our re-

Fig. 9. Effects of combined treatment with A3P5PS, L-NNA, and SnPP-IX on EFS-induced relaxation in the longitudinal muscle of C57BL/6J mouse distal colon. Relaxations were induced by EFS at 10 Hz for 10 s. The preparation was successively treated with 100 μ M A3P5PS, 30 μ M L-NNA, and 30 μ M SnPP-IX. Note that relaxation was partially inhibited by A3P5PS and L-NNA and almost completely abolished by the further treatment with SnPP-IX. Inset: EFS-induced relaxation in the presence of A3P5PS at the indicated concentrations. Relaxations are expressed as a percentage of that before drugs (control). Values are each the mean $\pm$ S.E.M. for four experiments. *Significantly different from the control value, $P < 0.05$; #Significantly different from the value in the presence of A3P5PS, $P < 0.05$; †Significantly different from the value with the treatment of A3P5PS and L-NNA, $P < 0.05$.

Fig. 10. Effects of H89 on residual relaxation induced by EFS in the presence of 100 μ M A3P5PS in the longitudinal muscle of C57BL/6J mouse distal colon. EFS-induced relaxation was measured in the absence (control) and the presence of H89. Relaxations are expressed as a percentage of that before the addition of H89 (control). Values are each the mean $\pm$ S.E.M. for four experiments. *Significantly different from the control value, $P < 0.05$.

sults indicate for the first time the involvement of CO in NANC relaxation of the C57BL/6J mouse distal colon. A combination of L-NNA and SnPP-IX resulted in an additive inhibition of EFS-induced NANC relaxation, namely about 70% inhibition. NOS and HO-2, the constitutive isoform of HO, were present in enteric neurons and interstitial cells of Cajal (ICC) of mouse gastrointestinal tract (24, 31), and the double deletions of nNOS and HO-2 induced additional effects on the membrane potential of mouse jejunum (32). Taken together, endogenous NO and CO appear to mediate EFS-induced NANC relaxation in the longitudinal muscle of C57BL/6J mouse distal colon.

It was reported that both NO and CO activate a soluble guanylate cyclase in smooth muscle. (26, 33, 34) Thus, when the effects of ODQ, a soluble guanylate cyclase inhibitor, were examined, EFS-induced relaxation was inhibited in a concentration-dependent manner. Maximal inhibition was about 70% of the control, which was equal to the inhibition obtained with a combination of L-NNA and SnPP-IX. L-NNA did not affect EFS-induced relaxation in the presence of ODQ. In addition, NOR-1- and CORM-2-induced relaxations were significantly inhibited by ODQ. The activation of soluble guanylate cyclase results in increased cyclic GMP production (35). In the mouse distal colon, the content of cyclic GMP was increased by both NOR-1 and CORM-2. The increasing effects on cyclic GMP content seen with both donors did not occur in the presence of ODQ. Thus, it appears that endogenous NO and CO induce relaxation through the increase in cyclic GMP content following the activation of soluble guanylate cyclase in the longitudinal muscle of C57BL/6J mouse distal colon. These results are in accord with a report suggesting the involvement of soluble guanylate cyclase in NO-induced relaxation of the gastrointestinal tract (26). Recently, it was shown that CO and CORM-2 produced relaxation via activation of soluble guanylate cyclase in mouse gastric fundus and jejunum (22, 25).

PKG is the main intracellular mediator in smooth muscle relaxation induced by an increase in cyclic GMP (36). In the present study, EFS-induced relaxation was significantly inhibited by the PKG inhibitors, KT5823 and Rp-8-Br-cGMPS. However, the maximal inhibitory effect of these PKG inhibitors was only 35% – 45%, and the residual EFS-induced relaxation in the presence of KT5823 or Rp-8-Br-cGMPS was further inhibited by L-NNA, but not by SnPP-IX. These results indicate that PKG may involve a CO-cyclic GMP pathway, but do not involve a NO-cyclic GMP pathway. This finding was supported by the demonstration that KT5823 did not change NOR-1-induced relaxation, whereas PKG inhibitors inhibited CORM-2-induced relaxation. Some studies

have also suggested that PKG is not involved in the NO-induced responses (37 – 39). Despite the finding that NO- and CO-mediated relaxations were cyclic GMP-dependent, it is surprising that the pathways after the increase in cyclic GMP differ between them. One possible suggestion is as follows: ICC are thought to play a role in nitrergic neurotransmission of the gastrointestinal tract (40). Recently, ICC, in addition of smooth muscle cells, were innervated by nitrergic neurons and shown to express NO-sensitive soluble guanylate cyclase (41). On the other hand, there is no report indicating a relationship between ICC and CO-mediated neurotransmission, although ICC isolated from the mouse colon has been shown to express HO-2 (24). Further experiments, however, are needed to clarify the difference in the involvement of cyclic GMP-PKG between NO- and CO-mediated relaxations.

The NO-cyclic GMP pathway has been reported to produce relaxation through activation of PKA (29). Although H89 inhibited EFS-induced relaxation in the present study, the residual relaxation in the presence of H89 was further inhibited by L-NNA. Thus, it seems unlikely that PKA is involved in the relaxation of NO.

The combination of L-NNA and SnPP-IX did not produce complete inhibition of EFS-induced relaxation. Then when we attempted to identify inhibitory neurotransmitters other than NO and CO, we found that the P2Y₁-receptor antagonists A3P5PS and MRS2179 also significantly inhibited EFS-induced relaxation. In addition, EFS-induced relaxation was almost completely inhibited in the presence of L-NNA, SnPP-IX, and A3P5PS. An interplay between NO and the purinergic system has been reported in the mouse gastrointestinal tract (42). In the present study, the inhibitory effects of A3P5PS and L-NNA were additive; A3P5PS further inhibited EFS-induced relaxation in the presence of ODQ. These results indicate that an ATP-like endogenous P2Y₁ purinoceptor ligand is the inhibitory neurotransmitter involved in EFS-induced relaxation of longitudinal muscle in mouse distal colon. In the circular muscle of murine distal colon, ATP was reported to mediate the fast component of the nerve-evoked inhibitory junction potentials through activation of P2Y receptors (18). Recently, it was shown that ATP contributes to excitatory neurotransmission in the longitudinal muscle of mouse distal colon (43). The discrepancy, when compared with our results, may be due to differences in the experimental conditions. Unlike the previous report, our experiment was conducted in the presence of atropine and guanethidine (under NANC condition). Since ATP partially induced contraction through the activation of the P2Y purinoceptor that stimulated cholinergic neurons (43), the presence or absence of atropine seems likely to influence the results. As

described above, PKA is involved in the component of EFS-induced relaxation that is not mediated by NO and CO. When the relation between PKA and ATP-mediated component was studied, H89 did not further affect EFS-induced relaxation inhibited by A3P5PS. Therefore, activation of the P2Y₁ purinoceptor appears to produce relaxation via a mechanism involving PKA. The cyclic AMP system participates in the hyperpolarization evoked by P2Y-receptor activation in the circular muscle of mouse distal colon (44).

In the present study, ATP and ADP produced no clear responses. It was reported that the responses to purinergic agonists were different in the position of the basal tone in the longitudinal muscle of mouse distal colon, namely, ATP induced relaxation on the precontracted preparation and contraction in the basal tone (42). The basal tone of the preparation in the isotonic method is due to an intrinsic characteristic of this preparation. Thus, the basal tone differs among the preparations. This may explain the lability of the response to ATP and ADP.

In conclusion, NO and CO function as inhibitory neurotransmitters in the longitudinal muscle of C57BL mouse distal colon. Although both NO and CO activated soluble guanylate cyclase and produced an increase in cyclic GMP content, the subsequent mechanisms for relaxation were different. CO-mediated relaxation was dependent on PKG, whereas NO-mediated relaxation was not. In addition, the ATP-like substance that activated the P2Y₁-receptor appears responsible for relaxation in the mouse distal colon. The results support the previous data indicating that the type and relative degree of the inhibitory neurotransmitters in the relaxation vary between animal species, animal strains, and gastrointestinal regions (2). Further study is necessary to clarify the detailed mechanisms involved in the relaxation mediated by NO, CO, and the ATP-like substance in the longitudinal muscle of C57/BL mouse distal colon.

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