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Nonadrenergic, noncholinergic relaxation mediated by nitric oxide with concomitant change in Ca^{2+} level in rectal circular muscle of rats

Tadayoshi Takeuchi [a,b], Satomi Niioka [a], Masami Kishi [a], Toshiaki Ishii [a,b], Hideaki Nishio [a], Fumiaki Hata [a,b,*], Tadashi Takewaki [c], Koichi Takatsuji [d]

[a] *Department of Veterinary Pharmacology, College of Agriculture, Osaka Prefecture University, Sakai 599-8531, Japan*
[b] *Department of Molecular Physiology and Biochemistry, Research Institute for Advanced Science and Technology, Osaka Prefecture University, Sakai 599-8531, Japan*
[c] *United Graduate School of Veterinary Sciences, Gifu University, Gifu 501-11, Japan*
[d] *Osaka Prefectural College of Nursing, Habikino, Osaka 583-0872, Japan*

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Abstract

The mediators of nonadrenergic, noncholinergic (NANC) relaxation of the circular muscle of rat rectum were examined in vitro. In the circular muscle of rat rectum, N^G-nitro-L-arginine (L-NOARG) at 10 μM did not affect electrical field stimulation-induced relaxation but at 100 μM it inhibited electrical field stimulation-induced relaxation by about 75% and 1-mM L-arginine reversed the inhibition. Exogenous nitric oxide (NO) (1–10 μM) concentration dependently relaxed the circular muscle. Electrical field stimulation increased the cyclic GMP content of the circular muscle to about twice its resting level. L-NOARG, even at 10 μM, completely inhibited the electrical field stimulation-induced elevation of cyclic GMP content. However, L-arginine at 1 mM did not reverse the inhibition in cyclic GMP content. Inhibitory junction potentials (i.j.ps) induced by electrical field stimulation in the circular muscle cells were not affected by L-NOARG, 100 μM. Apamin ($\leq$ 1 μM) did not affect the electrical field stimulation-induced relaxation, but almost completely inhibited electrical field stimulation-induced i.j.ps. NO (0.3–10 μM) induced relaxation of the circular muscle with a concomitant decrease in intracellular Ca^{2+} level ($[Ca^{2+}]_i$). Abundant immunoreactivity of NO synthase was found in the circular muscle layer, in addition to myenteric and submucosal plexus. The results suggest that NO induces NANC relaxation with a concomitant change in $[Ca^{2+}]_i$ in the circular muscle of rat rectum. However, the involvement of changes in cyclic GMP level and in membrane potentials in the mechanism was not shown in the present experimental conditions.

Keywords: NANC (nonadrenergic noncholinergic) relaxation; Rectal circular muscle; Nitric oxide (NO); Relaxation; $[Ca^{2+}]_i$ decrease

1. Introduction

The mediators of nonadrenergic, noncholinergic (NANC) inhibitory responses in the gastrointestinal tract have been studied intensively, especially in the last 5 years. In 1990, nitric oxide (NO) was first suggested to mediate NANC inhibition in the gastrointestinal tract, e.g., in the longitudinal muscle of the ileocolonic junction (Bult et al., 1990; Boeckxstaens et al., 1990) and the duodenum (Toda et al., 1990) of the dog and the gastric fundus of the rat (Li and Rand, 1990). We also suggested an essential role of NO in the descending inhibition in the rat proximal colon (Hata et al., 1990). In studies subsequent to these, NO was suggested to mediate NANC inhibition in a number of gastrointestinal regions of the rat (Suthamnatpong et al., 1993a; Irie et al., 1991; Postorino et al., 1995; Martins et al., 1995; Niioka et al., 1997; Kanada et al., 1992, 1993). In spite of many such studies, the intracellular mechanism of NO for mediating NANC inhibition in the gastrointestinal tract has not been clarified. We previously suggested that changes in cyclic GMP content or in membrane potentials of the smooth muscle cells are not associated with NO-mediated relaxation in the proximal colon of rats (Takeuchi et al., 1996; Suthamnatpong et al., 1994). Association of changes in intracellular calcium level ($[Ca^{2+}]_i$) with contractile responses of various kinds

* Corresponding author. Tel.: +81-722-54-9479; Fax: +81-722-54-9480.

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of smooth muscle preparations were extensively studied, but involvement with relaxant responses, especially NO-mediated ones, has been little studied.

In the colon of the rat, vasoactive intestinal polypeptide (VIP) was suggested to mediate some component of the inhibition (Suthamnatpong et al., 1993a; Grider and Makhlouf, 1986; Grider and Rivier, 1990). Recently, pituitary adenylate cyclase activating peptide (PACAP) was also suggested to be a candidate as mediator of the relaxation (Grider et al., 1994; Kishi et al., 1996). Thus, it was of interest to examine the mediators for NANC inhibition in various regions of the rat gastrointestinal tract. There have been only few reports on the mediator of NANC inhibition in the rectum of mammals including the rat (Stebbing et al., 1996a,b, 1997). We now studied the mediators of NANC inhibition and the intracellular mechanism of the relaxation in circular muscle of the rectum of rats.

2. Materials and methods

Male Wistar-ST rats (250–350 g) purchased from Japan SLC (Shizuoka, Japan) were used. They were lightly anaesthetized with ether and then stunned by a blow on the head and bled via carotid arteries. Segments of the rectum were removed and placed in Tyrode solution consisting of (in mM): NaCl 137; KCl 2.7; $CaCl_2$ 1.8; $MgCl_2$ 1.1; NaH_2PO_4 0.42; $NaHCO_3$ 11.9; and glucose 5.6. The contents of the excised segments were gently flushed out with Tyrode solution.

2.1. Recording of responses of rectal circular muscles to electrical field stimulation

Strips (5 mm in width, 10 mm in length) were prepared from the rectum by cutting transversely to the longitudinal axis of the tract to record responses of the circular muscle (mucosa was not removed). The strips were suspended by connecting one end to the bottom of the organ bath and the other end to an isotonic transducer (TD-112A, Nihonkohden, Tokyo, Japan), in an organ bath containing 5 ml of Tyrode solution maintained at 37°C and bubbled with 95% O_2:5% CO_2. After an equilibration period of 30 min, responses of the circular muscle to electrical field stimulation with trains of 100 pulses of 0.5 ms width and supramaximal voltage (30 V) at a frequency of 10 Hz were recorded isotonically, with 10-min intervals between tests. The circular muscle was subjected to a resting load of 200 mg. Atropine (1 μM) and guanethidine (5 μM) were added to the bathing fluid throughout the experiment to record NANC responses. The extent of relaxation was expressed as the area under the line of resting tone that was drawn through the center of resting spontaneous contractile activity.

2.2. Measurement of cyclic GMP content of isolated circular muscle from rat rectum

Circular muscle preparations (0.4-mm wide, 0.2- to 0.3-mm thick and 10-mm long) without longitudinal muscle layer were excised from the rat rectum. After an equilibration period of 30 min, the preparations were incubated for 10 s at 37°C in the absence or presence of various concentrations of NO. To determine the effect of electrical field stimulation on cyclic GMP content, the preparations were mounted between two plate electrodes in an organ bath: the parameters for electrical field stimulation were the same as those used for the mechanical response. When the effects of N^G-nitro-L-arginine (L-NOARG) and L-arginine were examined, these compounds were also added during the equilibration period. After the incubation period, preparations were quickly frozen by putting them between two metal plates frozen on dry ice, and their cyclic GMP content was determined with a cyclic GMP assay kit (Amersham Japan, Tokyo). Every determination of cyclic GMP was carried out in the presence of 1-mM isobutylmethylxanthine to protect cyclic GMP from hydrolysis. Atropine (1 μM) and guanethidine (5 μM) were present throughout the experiment.

2.3. Recording of inhibitory junction potentials (i.j.ps) induced in circular muscle cells of rectum by electrical field stimulation

The segments of rectum were mounted in a 1.5-ml organ bath maintained at 30°C and perfused continuously with Tyrode solution at a rate of 3 ml min^{-1}. Atropine (1 μM) and guanethidine (5 μM) were added to the bathing solution throughout the experiment to block cholinergic and adrenergic responses, respectively. Membrane potentials were recorded with a conventional glass microelectrode filled with 3 M KCl with a resistance of 50–80 MΩ. Electrode impalements were made into the circular muscle cells of the deep layer from the serosal side (Takewaki and Ohashi, 1977). Intramural nerves within the segment were stimulated by a pair of Ag wire electrodes, one on the serosal surface 1–2 mm from the impaled glass microelectrode and the other in the solution. The distance between the two electrodes was approximately 20 mm.

2.4. Immunohistochemical study of NO synthase

Segments of the rectum were dissected and immersed in Zamboni solution for 72 h and rinsed for 24 h at 4°C in 0.1-M phosphate buffer (pH 7.4) containing 30% sucrose. NO synthase immunoreactive structure were visualized by means of the avidin–biotin peroxidase complex (ABC) method. Sections (20-μm thick) were cut longitudinally on a cryostat and were detached onto poly-L-lysine-coated glass slides. The sections were rinsed with 0.1-M phosphate buffered saline (PBS) and were incubated in 10%

normal goat serum in 0.1-M PBS for 1 h before incubation with the first antiserum against NO synthase. NO synthase antiserum was purchased from Sigma (Lot No. N2280) and diluted 1:1000 in 0.1-M PBS containing 0.3% Triton X-100, 1% normal goat serum and 1% bovine serum albumin. After 72 h of incubation with diluted antiserum at 4°C, the sections were rinsed in 0.1-M PBS at 4°C for 1 h and then incubated for 24 h in biotinylated anti-rabbit immunoglobulin (Vector) diluted 1:200 and finally incubated for 24 h at 4°C in ABC reagent (Vector), in order to obtain sections with low background staining. NO synthase immunoreactivity was visualized by incubation at room temperature for 20 min in 0.05 M Tris–HCl buffer containing 3,3-diaminobenzidine (20 mg/100 ml), ammonium nickel (II) sulfate hexahydrate (600 mg/100 ml) and 30% hydrogen peroxide (10 μl/10 ml).

2.5. Simultaneous measurement of relaxation and changes in intracellular Ca^{2+} level of rectal circular muscle

Small strips (about 1.0-mm-wide and 5-mm-long) were excised from the rectal circular muscle layer. The muscle strips were incubated in Tyrode solution containing 5 μM fura-2 acetoxymethylester (fura-2 AM) and 0.02% cremophor EL for 5 h at room temperature under 95% O_2–5% CO_2. After washing, the fura-2 loaded muscle was mounted on a strain gauge transducer (MINEBEA, UL-2GR) to record isometrically the muscle tension. Changes in $[Ca^{2+}]_i$ level were measured simultaneously in a Nihonbunko CAF-100 spectrofluorometer (Nihonbunko, Tokyo), using 340/380 excitation with emission at 510 nm. A load of 0.5 g was applied as resting tension. After the equilibration period, the strips were first contracted with 10 μM carbachol every 10 min. When the contractile responses became constant, NO was added into the bathing fluid. Antagonists were added 10 min before the addition of carbachol. The strips were continuously perfused with Tyrode solution at a rate of 2 ml min^{-1}.

2.6. Drugs

Apamin, glibenclamide, L-arginine, D-arginine, N^G-nitro-L-arginine (L-NOARG) and VIP-(10–28) were purchased from Sigma, St. Louis, USA. Charybdotoxin, PACAP, PACAP-(6–38) and VIP were from the Peptide Institute, Osaka, Japan. Rp-8 bromo cyclic GMPS was from BIOLOG Life Sci. Inst., Bremen, Germany. Fura-2 acetoxymethyl ester (fura-2AM) was from Dojin-do, Kumamoto, Japan. Tetrodotoxin was from Wako, Osaka, Japan. Gaseous NO was dissolved in Tyrode solution just before experiments, as described by Gillespie and Sheng (1988) and added to the organ bath in volumes of 0.3–300 μl. Drugs were added to the organ bath as solutions in redistilled water in volumes of less than 1.0% of the bathing solution. A similar volume of redistilled water alone had no effect on the muscle.

2.7. Statistical analysis

The data are expressed as means ± S.E.M. The statistical significance of differences between two mean values was assessed by Student's *t*-test. For comparison of one control with several experimental groups, the significance of differences was assessed by one-way analysis of variance followed by the Bonferroni/Dunn test and a P value of < 0.05 was regarded as significant.

3. Results

3.1. Responses of circular muscle of rat rectum to electrical field stimulation

At rest, circular muscle of the rectum exhibited a spontaneous small contraction at low frequency in comparison to that of ileum (Kanada et al., 1992). All preparations showed only contraction in response to electrical field stimulation examined every 10 min during about the initial 1 h. However, the resting tone of the circular muscle gradually increased during successive trials of electrical field stimulation. When a higher resting tone was acquired in this way, the preparation began to exhibit a rapid transient relaxation followed by a contraction (Fig. 1). The relaxation was observed only in the presence of 1 μM atropine. In the absence of atropine, the response was always contraction. Tetrodotoxin (1 μM) abolished all responses to electrical field stimulation.

3.2. Effects of L-NOARG and L-arginine on NANC relaxation in circular muscle of rat rectum

L-NOARG (100 μM) and L-arginine (1 mM) had no significant effect on spontaneous contractile activity or on the tone of circular muscle of the rat rectum. L-NOARG at 10 μM also had no effect on the relaxation induced by electrical field stimulation, but at 100 μM it significantly inhibited the electrical field stimulation-induced relaxation. The inhibitory effect was pronounced within 20 min after its application. Addition of L-arginine (1 mM) to the bathing fluid gradually reversed the inhibitory effect of L-NOARG, causing complete reversion in 20–30 min (Fig. 1A and Table 1). D-Arginine (1 mM) had no significant effect.

Exogenously-added NO (1–10 μM) elicited relaxation of the muscle (Fig. 1B).

3.3. Effects of L-NOARG, L-arginine and exogenous NO on cyclic GMP content of circular muscle of rat rectum

L-NOARG at 10 or 100 μM significantly decreased the resting content of cyclic GMP of the circular muscle preparations of rat rectum. Electrical field stimulation almost doubled the cyclic GMP content of the preparations.

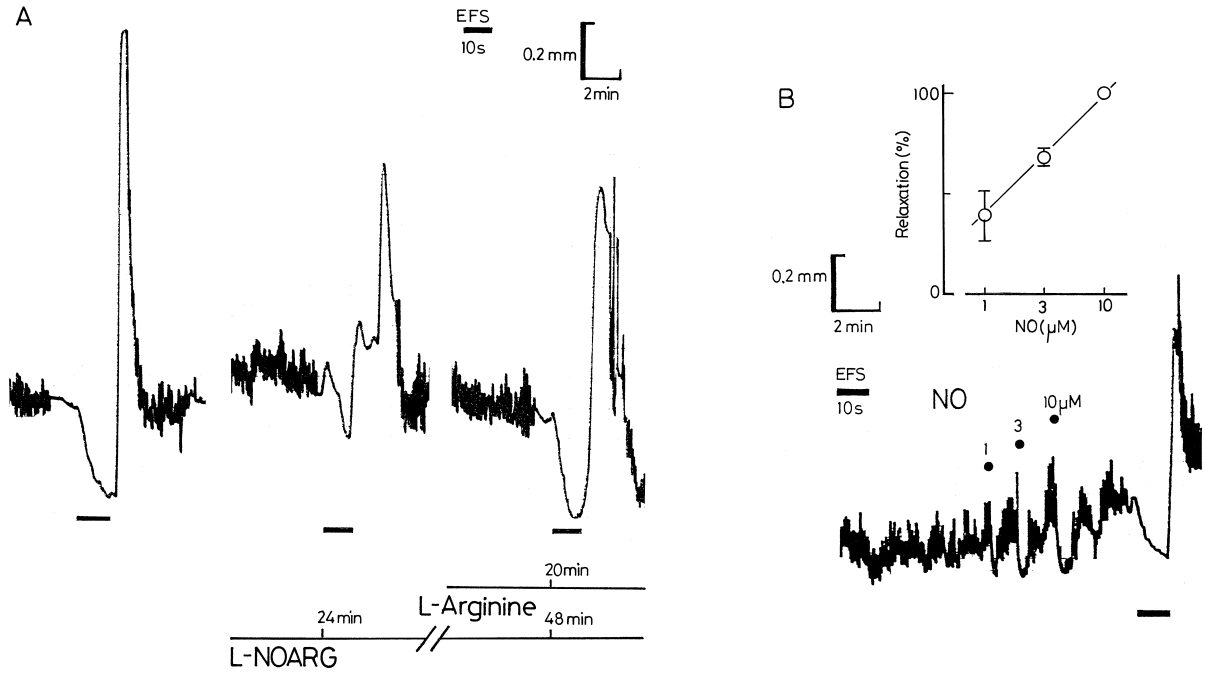

Fig. 1. Effects of L-NOARG and L-arginine on electrical field stimulation-induced relaxation of circular muscle of rat rectum and relaxations in response to exogenous NO. (A) The continuous lines indicate the presence of L-NOARG (100 μM) and L-arginine (1 mM) in the bathing fluid. Times noted on the lines indicate the time after addition of the drugs. Bold black bars indicate 10-s electrical field stimulation at 10 Hz. After recording of normal spontaneous movements, the chart was run fast immediately before the stimulation to make the relaxant response clear. (B) Various concentrations of NO were added at the times indicated by dots. Relaxation was expressed as a percentage of 10 μM NO-induced relaxation and summarized in an inset ($n = 4$). Atropine (1 μM) and guanethidine (5 μM) were present throughout.

Even at 10 μM, L-NOARG completely inhibited the electrical field stimulation-induced increase in cyclic GMP content to the lower level which had been obtained with L-NOARG alone (Fig. 2). The results indicate dissociation of the cyclic GMP level from relaxation of the circular muscle, since 10-μM L-NOARG did not affect electrical field stimulation-induced relaxation (Table 1). L-Arginine at 1 mM reversed the inhibitory effect of 100-μM L-NOARG on electrical field stimulation-induced relaxation (Table 1), but did not reverse the inhibitory effect on the electrical field stimulation-induced increase in cyclic GMP content (Fig. 2).

Table 1
Effects of L-NOARG, L-arginine, receptor antagonists and K^+ channel antagonists on electrical field stimulation-induced relaxation of circular muscle of rat rectum

Drug treated	Percentage of control
L-NOARG (10 μM)	111.2 ± 11.9 (3)
L-NOARG (100 μM)	25.0 ± 6.1 (4)[a]
L-Arginine (1 mM) after L-NOARG (100 μM)	94.3 ± 6.0 (4)[b]
Rp-8 bromo cyclic GMPS (30 μM)	103.2 ± 8.6 (5)
Apamin (100 nM)	99.5 ± 5.2 (4)
Charybdotoxin (100 nM)	110.2 (2)
Glibenclamide (10 μM)	111.4 (2)
VIP-(10–28) (3 μM)	100.0 ± 5.1 (4)
PACAP-(6–38) (10 μM)	114.3 (2)

Relaxations induced by electrical field stimulation in the presence of indicated drugs are expressed as percentages of those obtained before addition of drugs (control).
Values are means ± S.E.M. for the numbers of experiments shown in parentheses or means of two values.
Significantly different from the value for the corresponding control, Student's paired *t*-test, [a]$P < 0.05$ and from the value with 100-μM L-NOARG, [b]$P < 0.05$.

Rp-8 bromo cyclic GMPS at 30 μM, an antagonist of cyclic GMP-dependent protein kinase, which completely inhibited the 8-bromo cyclic GMP-induced relaxation of the rat proximal colon (Takeuchi et al., 1996), did not affect the electrical field stimulation-induced relaxation (Table 1). In another series of experiments, electrical field stimulation at 1 or 3 Hz, instead of 10 Hz, induced small or medium relaxation, respectively. Rp-8 bromo cyclic GMPS also did not affect these relaxations (data not shown; $n = 2$).

Furthermore, exogenous NO at 10 μM significantly increased the cyclic GMP content of the circular muscle preparations (from 25.0 ± 2.6, $n = 12$ to 74.2 ± 8.1 fmol/mg tissue, $n = 6$; $P < 0.01$). However, NO at concentrations lower than 3 μM did not significantly increase cyclic GMP (at 3 μM, 32.3 ± 3.7, $n = 6$). Thus, higher concentrations of NO were needed for enhancing the cyclic GMP content than were needed for relaxing the circular muscle (Fig. 1B). These results also indicate dissociation of the cyclic GMP level from relaxation of the circular muscle.

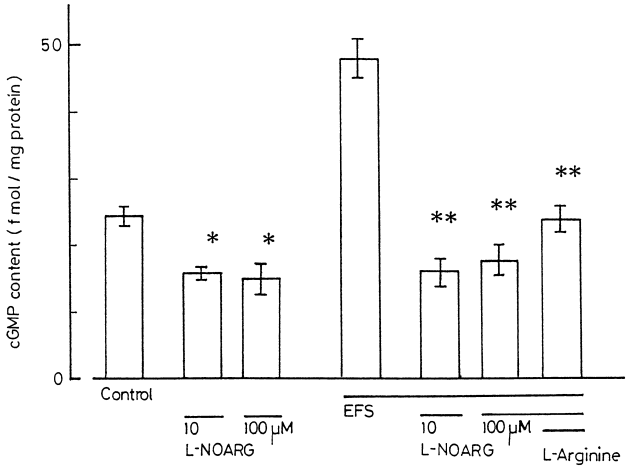

Fig. 2. Effects of L-NOARG and L-arginine on cyclic GMP contents of circular muscle preparations of the rat rectum. Effects of 10 or 100 μM L-NOARG without or with 1-mM L-arginine (L-Arg) on the resting (Control) and electrical field stimulation-elevated level of cyclic GMP were examined. Columns and bars represent means with standard errors from 5–18 experiments. Values significantly different at $P < 0.05$ from the value of control (*) and from the value of electrical field stimulation (**). For further details, see Section 2.

3.4. Effects of NO on the membrane potential and of L-NOARG on electrical field stimulation-induced i.j.ps of circular muscle cells

The resting membrane potentials of circular muscle cells of rat rectum was -57.1 ± 0.8 mV ($n = 83$). Significant relaxations of the circular muscle were observed in the micromolar range of NO (Fig. 1B). However, NO at concentrations of up to 100 μM induced no detectable change in the membrane potential of the circular muscle cells (data not shown; $n = 11$). In the presence of atropine (1 μM) and guanethidine (5 μM), electrical field stimulation induced i.j.ps of the membrane of the circular muscle cells. L-NOARG at concentrations up to 100 μM did not have any significant effect on the resting membrane potentials or electrical field stimulation-induced i.j.ps ($n = 7$, Fig. 3A).

3.5. Effects of K^+ channel blockers on NANC relaxation and electrical field stimulation-induced i.j.ps of circular muscle cells

Apamin, an antagonist of small conductance Ca^{2+}-activated K^+ channels, did not show any significant effect on NANC relaxation in the rectal circular muscle (Table 1). Apamin also did not affect the slight or medium relaxation induced by electrical field stimulation at 1 or 3 Hz, respectively (data not shown; $n = 3$). Charybdotoxin and glibenclamide, antagonists of large conductance Ca^{2+}-activated and ATP-sensitive K^+ channels also did not affect the relaxation (Table 1). In contrast, apamin concentration dependently inhibited the electrical field stimulation-induced i.j.ps and almost completely inhibited them at 1 μM ($n = 7$, Fig. 3B).

3.6. Effect of NO on intracellular calcium level of circular muscle of rat rectum

To study the relationship of NO-mediated relaxation with $[Ca^{2+}]_i$, changes in muscle tension and $[Ca^{2+}]_i$ were measured simultaneously. Small strips of the rectal circular muscle which had been loaded with fura-2 AM were pre-contracted with 10-μM carbachol. Following treatment with carbachol, the muscle strips exhibited a rapid contraction and subsequent spontaneous contractile activity. Exogenous NO inhibited the spontaneous activity and induced relaxation with concomitant decrease in $[Ca^{2+}]_i$ of the circular muscle. Both changes were dependent on the concentration of NO (Fig. 4). Rp-8 bromo cyclic GMPS at 30 μM and apamin at 1 μM did not affect the decrease in $[Ca^{2+}]_i$ or the relaxation induced by 1 and 3 μM NO ($n = 3$, Fig. 5).

3.7. Immunohistochemical observation of NO synthase

NO synthase immunoreactive neurons were observed in the myenteric plexus and there were many NO synthase

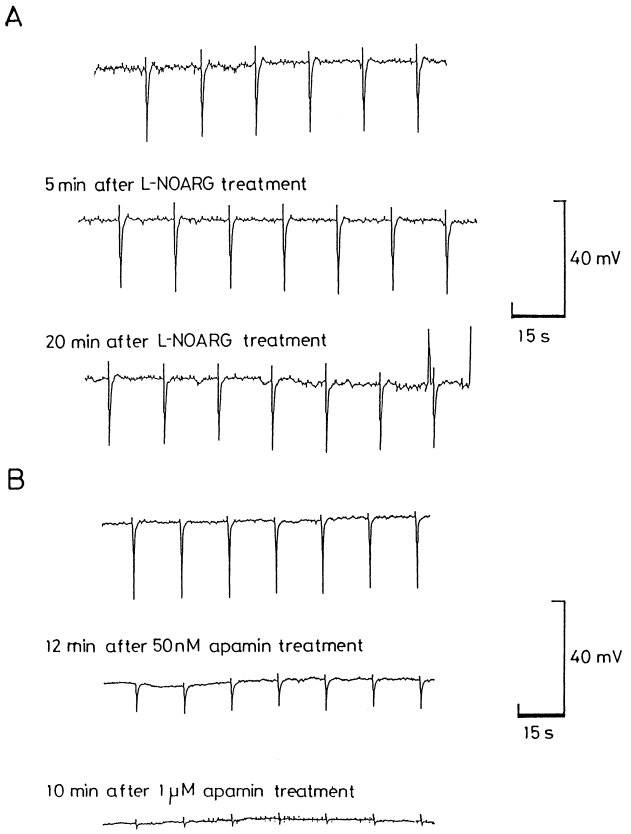

Fig. 3. Effects of L-NOARG and apamin on i.j.ps induced by electrical field stimulation in circular muscle cells of the rat rectum. I.j.ps induced by electrical field stimulation at 0.1 Hz were recorded before and after application of 100-μM L-NOARG (A), or before and after application of apamin (B). Atropine (1 μM) and guanethidine (5 μM) were added to the bathing fluid throughout the experiment.

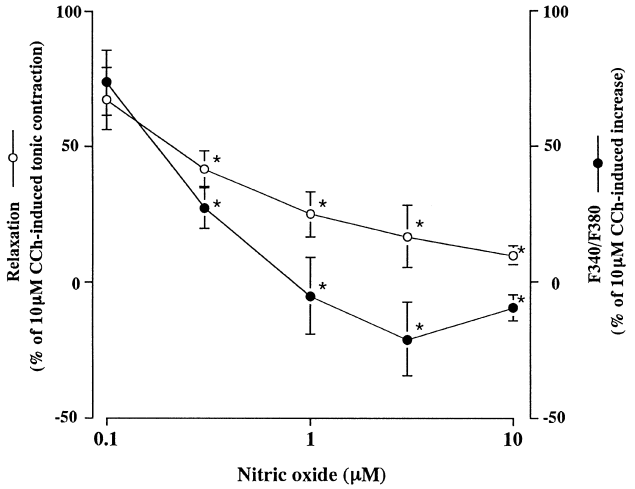

Fig. 4. Effects of NO on muscle tension and intracellular calcium level. Changes in muscle tension and intracellular calcium level ($[Ca^{2+}]_i$) were recorded simultaneously. Muscle strips which had been preloaded with fura-2 AM were first contracted with 10-μM carbachol. When the contraction reached a constant level, effects of NO were examined. Relaxation and decrease in $[Ca^{2+}]_i$ are expressed as a percentage of 10-μM carbachol- induced tension and $[Ca^{2+}]_i$ level (control), respectively. Note that higher concentrations of NO decreased $[Ca^{2+}]_i$ to under the resting level. Points are means ± S.E.M. for 3–4 experiments. * Significantly different from control, $P < 0.05$.

immunoreactive fibers in the circular muscle layer (Fig. 6). These fibers were seldom observed in the longitudinal muscle layer.

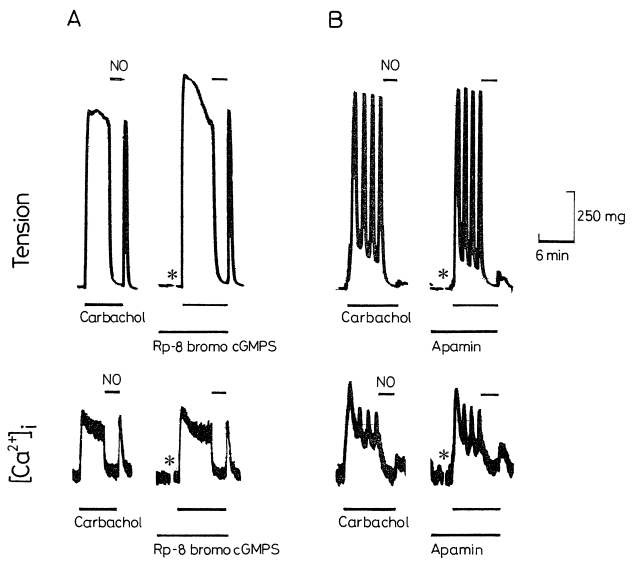

Fig. 5. Effects of Rp-8 bromo cGMPS and apamin on NO-induced relaxation and decrease in intracellular Ca^{2+} level. Relaxant responses and $[Ca^{2+}]_i$ were recorded simultaneously. Muscle strips which had been preloaded with fura-2 AM were first contracted with 10-μM carbachol and then effects of NO were examined before and 10 min after the treatment with 30-μM Rp-8 bromo cGMPS (A) or 1-μM apamin (B). Concentration of NO was 1 or 3 μM in A or B, respectively. Lines indicate the presence of drugs noted. * Point at which run of the chart was temporarily stopped. Records are typical of those from 2 (A) and 3 (B) preparations.

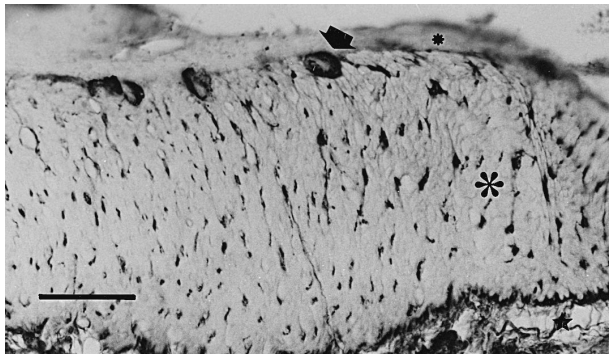

Fig. 6. NO synthase immunoreactive structures in the rat rectum. NO synthase immunoreactivity was seen in the circular muscle layer (large asterisk) and the myenteric plexus (arrow). Upper and lower parts are longitudinal muscle layer (small asterisk) and submucosal layer (star mark), respectively.

3.8. Effects of VIP-(10–28) and PACAP-(6–38) on electrical field stimulation-induced relaxation in circular muscle of rat rectum

VIP at concentrations up to 10 μM and PACAP up to 10 μM did not induce any appreciable relaxation of the rectal circular muscle, although these concentrations of the agonists induced significant relaxation in the distal colon (Kishi et al., 1996). VIP-(10–28) (3 μM) and PACAP-(6–38) (10 μM) did not inhibit electrical field stimulation-induced relaxation (Table 1). These concentrations of the antagonists inhibited the electrical field stimulation-induced NANC relaxation by about 50% in the distal colon (Kishi et al., 1996).

4. Discussion

Inhibition of NANC relaxation by an inhibitor of NO synthesis and reversal of the inhibition by an excess amount of the substrate, L-arginine, are the most popular and reasonable criterion for identification of NO as mediator of NANC relaxation. In our present experiments with circular muscle of rat rectum, L-NOARG inhibited electrical field stimulation-induced relaxation and L-arginine reversed the inhibitory effect of L-NOARG. Exogenous NO induced a concentration-dependent relaxation. Immunohistochemistry revealed an abundant distribution of NO synthase in the rectum. Thus, an important role of NO in NANC relaxation in the circular muscle was suggested. But L-NOARG at 100 μM did not have any significant effect on the spontaneous contractile activity. Therefore, it seems likely that nitrergic neurones participate mainly in the NANC relaxant response, but not in the modulation of spontaneous activity.

There have been many reports of an association of the cyclic GMP level with NO-mediated relaxation in preparations of gastrointestinal tract, such as the lower esophageal

sphincter of dogs (Shikano et al., 1988), the gastric fundus of guinea-pigs (Jin et al., 1993), ileum (Kanada et al., 1992, 1993) and proximal colon (Suthamnatpong et al., 1993b) of rats, the taenia coli of rabbits (Shikano et al., 1988) and guinea-pigs (Shikano et al., 1988; Jin et al., 1993) and dispersed gastric muscle cells (Murthy et al., 1993; Murthy and Makhlouf, 1995). Nevertheless, we previously reported dissociation of the cyclic GMP level from relaxation in the distal colon of rats: exogenous NO concentration dependently increased the cyclic GMP content of the tissue, but did not induce any appreciable relaxation of the segments (Suthamnatpong et al., 1993b; Maehara et al., 1994). Recently, we also reported that NO-mediated relaxation of rat proximal colon is not associated with changes in cyclic GMP content of the tissue (Takeuchi et al., 1996). In the present study, although L-NOARG at 10 μM completely inhibited electrical field stimulation-induced cyclic GMP elevation in circular muscle of rat rectum, it did not affect the relaxation of the segments. It is noteworthy that this concentration of L-NOARG lowered the cyclic GMP content to less than the control level. Therefore, the possibility that a slight rise in cyclic GMP above the background participates in the relaxation seems unlikely. And also, L-arginine reversed the inhibitory effect of L-NOARG at 100 μM on electrical field stimulation-induced relaxation, but not the inhibitory effect on elevation of the cyclic GMP content. These findings suggest that NO-mediated relaxation is not associated with changes in cyclic GMP content in the circular muscle of the rectum, although we do not completely rule out the possibility of a localized elevation of cyclic GMP within the smooth muscle cells. An increase in cyclic GMP level in response to electrical field stimulation remains as an interesting problem. That is, electrical field stimulation increased the level and L-NOARG completely counteracted the increase, suggesting synthesis of cyclic GMP by NO in the rectal circular muscle. However, the role of cyclic GMP in this preparation was not clarified.

We previously suggested that NO-mediated relaxation of rat proximal colon is not associated with i.j.ps of the cell membrane (Suthamnatpong et al., 1994). The present findings with circular muscle of the rat rectum also suggest independence of NO-mediated relaxation from changes in the membrane potentials, because electrical field stimulation-induced i.j.ps were not affected by L-NOARG and exogenous NO did not affect the membrane potentials of the circular muscle cells. Furthermore, apamin did not affect the relaxation, but almost completely inhibited the i.j.ps.

NANC relaxation was usually followed by a contraction which was abolished by TTX but not by atropine. Although the mediator of the response was not studied in the present work, direct association of the contraction with NANC relaxation seems unlikely because L-NOARG and apamin did not show any significant effect on the contractile response.

Although VIP has been suggested to mediate NANC inhibition in longitudinal muscle of the distal colon (Kishi et al., 1996), its participation in NANC inhibition was not shown in the rectal circular muscle. Namely, VIP did not relax the circular muscle of the rectum. A VIP receptor antagonist did not inhibit electrical field stimulation-induced relaxation of the muscle. PACAP has also been suggested to mediate NANC relaxation in longitudinal muscle of the distal colon via activation of apamin-sensitive K^+ channels (Kishi et al., 1996). In the rectal circular muscle, however, PACAP is not associated with NANC inhibition: PACAP did not cause relaxation, a PACAP receptor antagonist did not inhibit electrical field stimulation-induced NANC relaxation and apamin did not inhibit the electrical field stimulation-induced relaxation. Thus, there are clear differences in NANC inhibitory mediators between longitudinal muscle of the distal colon and circular muscle of the rectum of rat, in spite of their being in adjoining regions.

The relationship between contraction and changes in $[Ca^{2+}]_i$ of smooth muscle cells has been much studied. In contrast, there are few reports on the relation between relaxation and changes in $[Ca^{2+}]_i$, especially with respect to the NO-mediated relaxation of intestinal smooth muscle. In the present study, we measured simultaneously changes in muscle tension and $[Ca^{2+}]_i$ level in segments of the circular muscle layer of the rectum which had been preloaded with fura-2 AM. The segments were first precontracted with carbachol and changes in the tension had to be measured isometrically due to the apparatus used. Thus, experimental conditions were not necessarily identical to those for all other experiments in the present study, but the concentration range of NO necessary for the relaxation was similar in both experiments. NO induced muscle relaxation with a concomitant decrease in $[Ca^{2+}]_i$ in a concentration dependent manner. The decrease in $[Ca^{2+}]_i$ as well as muscle tension was not affected by an antagonist of cyclic GMP-dependent protein kinase or apamin, suggesting that NO induced decreases in $[Ca^{2+}]_i$ were not associated with inhibition of the cyclic GMP-cyclic GMP-dependent protein kinase system or apamin-sensitive K^+ channels.

Thus, the present findings suggest that NO mediates NANC relaxation in rat rectal circular muscle by decreasing $[Ca^{2+}]_i$, the mechanism of which may be independent of changes in cyclic GMP level and in membrane potentials of the smooth muscle cells. The relationship of decreased $[Ca^{2+}]_i$ to relaxation of rectal circular muscle induced by nitrergic neurons requires further study.

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