Nitric oxide has been suggested to mediate nonadrenergic, noncholinergic (NANC) relaxation in many regions of the gastrointestinal tract [22, 23]. However, accumulated evidence suggests that the role of nitric oxide in NANC relaxation is not uniform throughout the gastrointestinal tract, but quite variable in region to region. Wistar-ST rat intestine is a case in point. That is, an essential role of nitric oxide in the relaxation was suggested in circular [11] and longitudinal [24, 26] muscles of the proximal colon, and in circular muscle of the rectum [27], while only minor or no role was suggested in longitudinal muscle of the jejunum [19] or the distal colon [16, 24], respectively. In addition to regional difference, we recently found a strain difference in the mediator of NANC relaxation of the distal colon of rats between the Sprague Dawley and Wistar-ST strains: nitric oxide was suggested to partially mediate NANC relaxation of the Sprague Dawley but not Wistar-ST strain [21].

In addition to nitric oxide, vasoactive intestinal peptide (VIP) is another candidate for the mediator of NANC relaxation in several gastrointestinal regions: lower oesophageal sphincter of opossums [5] and rabbits [2], stomach of dogs [1], guinea pigs [6, 10] and rats [13], taenia coli of guinea pigs [7, 10], and colon of rats [9, 24]. Pituitary adenylate cyclase activating peptide (PACAP) was also suggested to mediate NANC relaxation in some regions: lower oesophageal sphincter of cats and humans [20], stomach of guinea pigs [14], and colon of guinea pigs [12] and rats [8, 15]. We suggested that VIP and PACAP mediate NANC relaxation of longitudinal muscle of the distal colon of Wistar-ST rats via opening of charybdotoxin- and apamin-sensitive K⁺ channels, respectively [15, 25]. However, participation of the peptides as well as nitric oxide in NANC relaxation does not seem to be uniform throughout the gastrointestinal tract. For example, neither role of VIP in longitudinal muscle of the proximal and mid colon [24], and circular muscle of the rectum [19] of Wistar-ST rats, nor role of PACAP in circular muscle of the rectum of Wistar-ST rats [19] was suggested. We also found differences of participation of VIP among strains of the rat: VIP partially mediates the relaxation of longitudinal muscle of the distal colon in Wistar-ST but not in Sprague Dawley rats and relaxation of circular muscle in Sprague Dawley but not in Wistar-ST rats [21].

We have also studied regional differences of the participation of nitric oxide in NANC relaxation in Wistar rats [28]. NANC mediators in Sprague Dawley rats has not been studied in detail. Therefore, the present study is designed to in detail and totally elucidate the participation of nitric oxide, VIP and PACAP in NANC relaxation in every region throughout the intestine of the Sprague Dawley rat. On the basis of the combined results of the present and our previous studies, differences in participation of nitric oxide, VIP and PACAP in NANC relaxation among strains of the rat were discussed.

MATERIALS AND METHODS

Sprague Dawley and Wistar-ST rats were purchased from Nippon SLC (Shizuoka, Japan). Wistar rats were also purchased from JCL Inc. (Osaka, Japan). Male 8-week-old rats (280–330 g) were lightly anaesthetized with diethyl ether and then stunned by a blow on the head and bled via carotid arteries. Segments of the jejunum, ileum, proximal and distal colon, and rectum were removed and placed in Tyrode solutions consisting of (in mM): NaCl 137, KCl 2.7, CaCl₂ 1.8, MgCl₂ 1.1, NaH₂PO₄ 0.42, NaHCO₃ 11.9 and glucose 5.6.
The contents of the excised segments were gently flushed out with Tyrode solution. Whole segments of each intestinal region except ileum were used. Ileal segments, 2–3 cm in length, were excised from central part of the ileum. The narrow part formed by sphincter on the ascending colon defined the boundary between proximal and middle regions. The portion of the colon which is attached by mesentery with the small intestine was defined as the distal region.

Recording of responses of longitudinal muscle to electrical field stimulation (EFS): Intestinal segments were suspended in an organ bath filled with Tyrode solution aerated with 5% CO₂ in O₂ and maintained at 37°C. Atropine (1 μM) and guanethidine (5 μM) were present throughout the experiment to block cholinergic and adrenergic responses, respectively. Responses of the longitudinal muscle to EFS for 10 s with trains of 1–300 pulses of 0.5 msec width at 30 V were recorded isotonically with a 10-min interval between tests. Tetrodotoxin (1 μM) inhibited the EFS-induced responses of the preparations. The longitudinal muscle of each segment was subjected to a load of 1.0 g to obtain the most reproducible responses and stable resting tone. The preparations were equilibrated for at least 30 min before the experiments. 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. Drugs were added to the organ bath (5 μl) in volumes of less than 1.0% of the bathing solution. These volumes of the drugs, redistilled water, did not affect the spontaneous contractile activity or the muscle tone.

Drugs: Apamin, VIP fragment VIP10–28 and N⁵-nitro-L-arginine (N⁵-nitro-amidino-L-2,5-diamino-pentanoic acid: L-NOARG) were purchased from Sigma Chemical Co., ST. Louis, U.S.A. VIP, charybdotoxin, PACAP and its fragment PACAP6–38 were purchased from The Peptide Institute, Osaka, Japan. Gaseous nitric oxide was dissolved in Tyrode solution just before experiments, as described by Gillespie and Osaka, Japan. Gaseous nitric oxide was dissolved in Tyrode solution aerated with 5% CO₂ in O₂ and maintained at 37°C. Atropine (1 μM) induced a rapid transient relaxation in the presence of atropine (1 μM). The spontaneous contractile activity and pattern of EFS (10 Hz, 10 s)-induced relaxation in ileal segments of Sprague Dawley rats was similar to those observed in the jejunum. L-NOARG at 10 μM slightly inhibited the EFS-induced relaxation and at 100 μM exhibited the maximal inhibitory effect, 52% inhibition, on the relaxation and L-arginine at 1 mM completely reversed the inhibition (Table 1). L-NOARG at 100 μM also inhibited the relaxation induced by EFS at 0.1 and 1 Hz by 71.2 ± 2.6% (n=3) and 70.2 ± 11.2% (n=3), respectively.

VIP10–28 and PACAP6–38 at 3 μM did not have any effect on the relaxation (Table 1).

RESULTS

 Participation of nitric oxide, VIP and PACAP in NANC relaxation of longitudinal muscle of several intestinal regions of Sprague Dawley rats

Jejunum: Jejunal segments exhibited spontaneous and frequent contractile activity with wide amplitude. EFS at 10 Hz for 10 s induced a rapid transient relaxation in the presence of atropine (1 μM) and guanethidine (5 μM) followed by a contraction which always occurred immediately after turning off the EFS. The pattern of the responses was similar to that of jejunal segments obtained from Wistar and Wistar-ST rats. N⁵-Nitro-L-arginine (L-NOARG) at 10 μM did not affect the muscle tone and the spontaneous contractile activity, but it inhibited EFS-induced NANC relaxation by about 45% within 10–20 min after application (Fig. 1 and Table 1). Addition of an excess of L-arginine (1 mM) completely reversed the inhibition within 20–30 min (Fig. 1). A higher concentration of L-NOARG, 100 μM, inhibited the relaxation to a greater extent than 10 μM concentration. However, such inhibition was only partially or scarcely reversed by L-arginine (data not shown). In another series of experiment, the effects of L-NOARG on the relaxations induced by EFS at different frequencies were also examined. A single pulse induced a transient relaxation of the longitudinal muscle. With an increase in the frequency, greater responses were induced. EFS at 0.1–30 Hz was used in the following experiments. Extents of the inhibitory effects of L-NOARG on the relaxations induced by EFS remained substantially unchanged even at different frequencies of stimulation used (Fig. 2).

Either a VIP antagonist, VIP10–28 or a PACAP antagonist, PACAP6–38, at 3 μM exhibited the maximum inhibition against the EFS-induced relaxation in Wistar-ST rat distal colon [15]. However, the same concentration of the two antagonists did not have any effect on the relaxation in the Sprague Dawley rat jejunum (Table 1).

Ileum: Frequency and amplitude of the spontaneous contractile activity and pattern of EFS (10 Hz, 10 s)-induced relaxation in ileal segments of Sprague Dawley rats were similar to those observed in the jejunum. L-NOARG at 10 μM exhibited the maximal inhibitory effect, 52% inhibition, on the relaxation and L-arginine at 1 mM completely reversed the inhibition (Table 1). L-NOARG at 100 μM also inhibited the relaxation induced by EFS at 0.1 and 1 Hz by 71.2 ± 2.6% (n=3) and 70.2 ± 11.2% (n=3), respectively.

VIP10–28 and PACAP6–38 at 3 μM did not have any effect on the relaxation (Table 1).

Proximal colon: The proximal colonic segments exhibited frequent and spontaneous activity with the largest amplitude among the segments from all regions examined in the present study. EFS induced relaxation in the proximal colon regardless of the presence or absence of atropine, although EFS induced the relaxation only in the presence of atropine in all the rest of intestinal regions. These properties of the proximal colon of Sprague Dawley rats were common to those of the other two strains studied.

L-NOARG at 10 μM significantly inhibited the EFS (10 Hz, 10 s)-induced relaxation and L-arginine at 1 mM completely reversed the inhibition. Higher concentrations of L-NOARG than 10 μM did not show any further inhibition. Participation of nitric oxide in EFS-induced NANC relaxation in the proximal colon of rat irrespective of the strains was most significant in comparison to other regions, accounting for 69% participation in the relaxation (Table 1). L-NOARG at 10 μM also inhibited the relaxation induced by EFS at 0.1 and 1 Hz by 40.1 ± 5.0% (n=4) and 41.2 ± 8.1% (n=4), respectively.

VIP10–28 and PACAP6–38 at 3 μM did not have any effect on the relaxation (Table 1).

Distal colon: Since partial participation of nitric oxide and absence of participation of VIP in the relaxation was reported in the previous study [21], participation of PACAP was examined in the present study. PACAP6–38 at 3 μM inhibited the EFS (10 Hz, 10 s)-induced relaxation of longitudinal muscle of the distal colon by 43% (Table 1). Apamin at 1 μM, which
exhibited the maximal inhibitory effect on the EFS (10 Hz, 10 s)-induced relaxation of the Wistar-ST rat distal colon [15], also inhibited the relaxation of the Sprague Dawley rat distal colon by 76.5 ± 9.1 % (n=5). Bath application of exogenous PACAP (10–100 nM) induced slow gradual relaxation of longitudinal muscle of the distal colon. Apamin at 1 µM significantly inhibited 100 nM PACAP-induced relaxation (67.2 ± 11.4% inhibition, n=5).

The relaxation of the distal colon which remained after PACAP6–38 treatment was very significantly inhibited by L-NOARG (10 µM) treatment (Fig. 3A; 71.9 ± 3.9% inhibition, n=3). PACAP (100 nM)-induced relaxation was not affected significantly by L-NOARG at 10 µM (Fig. 3B; n=4). These results suggest that nitric oxide and PACAP separately mediate NANC relaxation in the Sprague Dawley rat distal colon.

**Rectum**: Characteristics of small spontaneous contractile activity, gradual increase in tone during successive EFS and the pattern of EFS-induced relaxation followed by contraction of the rectal segments were very similar to those of the distal colon and to those in the other two strains studied.

Significant participation (66 %) of nitric oxide in the EFS-induced relaxation at 10 Hz for 10 s was shown by using 10 µM L-NOARG and 1 mM L-arginine (Table 1). L-NOARG

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Fig. 1. Effects of L-NOARG and L-arginine on EFS-induced relaxation of longitudinal muscle of jejunum of Sprague Dawley rats. Relaxations were induced by EFS at 10 Hz for 10 s. Lines indicate the presence of L-NOARG (10 µM) and L-arginine (1 mM) in the bathing fluid. Times noted on the lines indicate the time after addition of the drugs. Short lines indicate 10 s EFS at 10 Hz. After recording normal spontaneous movements, the chart was run at a fast speed immediately before the stimulation to make the relaxant response clear. 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 (Dotted lines).

Fig. 2. Inhibition of the relaxations induced by EFS at different frequencies by L-NOARG treatment. Data are expressed as a percent inhibition of the relaxation. Values are means with standard errors for 4–7 experiments. For further detail, see Methods and legend of Fig. 1.
Participation of VIP and PACAP was studied in Wistar rats. Mediators of NANC relaxation of longitudinal muscle of various intestinal regions of Sprague Dawley rats

Responses of longitudinal muscle of the segments obtained from various intestinal regions of Sprague Dawley rats to EFS at 10 Hz for 10 s were recorded before (control) and after treatment with 10 μM L-NOARG. The component of the relaxation which was inhibited by L-NOARG and completely reversed by 1 mM L-arginine was defined as nitric oxide-mediated component and expressed as a percentage of inhibition to the control. Result obtained with 100 μM L-NOARG is shown in the case of ileum, since only in the ileum 100 μM L-NOARG further inhibited the relaxation and 1 mM L-arginine reversed the inhibition completely (see the text). The responses were also recorded before and after the treatment with 3 μM VIP10–28 or PACAP6–38. The component of the relaxation which was inhibited by 3 μM VIP10–28 or PACAP6–38 was defined as VIP- or PACAP-mediated component, respectively. Values are means ± s.e.m. for the numbers of experiments shown in parentheses. For further details, see Methods. a) Data are cited from our previous study, Okishio et al. [21].

Mediators of NANC relaxation of longitudinal muscle of various intestinal regions of Wistar rats

Participation of VIP and PACAP was studied in Wistar rats. VIP10–28 at 3 μM did not show any effect on EFS (10 Hz, 10 s)-induced relaxation of longitudinal muscle of the jejunum, ileum, proximal colon and rectum of Wistar rats (n=3). However, the VIP receptor antagonist inhibited the relaxation in the distal colon by 36.4 ± 3.4% (n=14). Charybdotoxin at 100 nM, which exhibited the maximal inhibitory effect on EFS-induced relaxation of the Wistar-ST rat distal colon [15], also inhibited the relaxation by 37.2 ± 5.1% (n=3).

PACAP6–38 at 3 μM also did not show any effect on the relaxation of the jejunum, ileum, proximal colon and rectum of Wistar rats (n=3). However, PACAP6–38 at 3 μM and apamin at 1 μM inhibited the relaxation in the distal colon by 41.1 ± 7.2% (n=6) and 76.7 ± 3.2% (n=3), respectively.

In the distal colon, the relaxation which remained after PACAP6–38 treatment was further inhibited by VIP10–28 treatment. The remaining relaxation after both the treatments was completely inhibited by L-NOARG treatment (Fig. 4). VIP (3 μM)-induced relaxation was not affected by PACAP6–38 at 3 μM or L-NOARG at 10 μM (n=3, data not shown). PACAP (100 nM)-induced relaxation was not affected by VIP10–28 at 3 μM or L-NOARG at 10 μM (n=3, data not shown). These results suggest that VIP, PACAP and nitric oxide separately mediate NANC relaxation in the Wistar rat distal colon.

DISCUSSION

The present study revealed that nitric oxide partially (40–70%) participates in NANC relaxation in the every intestinal region studied in Sprague Dawley rats, and that PACAP partially (43%) participates only in the distal colon. The roles of nitric oxide, VIP and PACAP in the NANC relaxation were also studied in some intestinal regions in Wistar and Wistar-ST rats. We recently reported that mediators of NANC relaxations of the distal colon of Sprague Dawley and Wistar-ST rats are different [21]. Data obtained in the present study in addition to our previous studies enabled us to further clarify the strain-difference in the mediator of NANC relaxation of the rat intestine (Table 2). Frequency and amplitude of the spontaneous contractile activity and the EFS-induced responses were different among the intestinal regions, but those observed in each region were not so significantly different among the strains. The regional differences in participation of nitric oxide in the NANC relaxation were shown in every strain. Namely, among the strains, the most significant participation of nitric oxide throughout the intestine was shown in the Sprague Dawley strain. Among the regions irrespective of the strain, the most significant participation was
shown in the proximal colon. However, there are many regions where nitric oxide has no role in NANC relaxation (Table 2). Although it has been suggested that the extent of participation of the mediators in NANC relaxation depends on the frequencies of EFS under some experimental conditions, there was no appreciable difference in the extents of nitric oxide-participation in NANC relaxation among the frequencies of EFS used in every intestinal region studied. Thus, it seems that nitric oxide participates to a certain extent in NANC relaxations induced by EFS with frequencies over a wide range tested.

There are many reports describing the role of VIP in NANC relaxation in the peripheral organs. However, only a few reports of that in rat intestine are present: the mid colon of Sprague Dawley rats [9] and the distal colon of Wistar-ST rats [24]. Recently, we showed that VIP mediates NANC relaxation via charybdotoxin-sensitive K+ channels in the distal colon of Wistar-ST rats [15]. This was the case in the distal colon of Wistar rats in the present study. However, no participation of VIP in the relaxation was suggested in the distal colon of Sprague Dawley rats and in any regions other than the distal colon of all the three strains. In the present study, effect

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**Fig. 3.** Effects of PACAP<sub>6–38</sub> and L-NOARG on relaxation induced by EFS in the distal colon of Sprague Dawley rats. (A) Relaxations were induced by EFS at 10 Hz for 10 s. Lines indicate the presence of PACAP<sub>6–38</sub> at 3 µM and L-NOARG at 10 µM in the bathing fluid. Times noted on the lines indicate the time after addition of the drugs. Short lines indicate 10 s EFS at 10 Hz. (B) Relaxations were induced by 100 nM PACAP in the absence or presence of 10 µM L-NOARG. Representative results of separate 3 and 4 experiments are shown in Fig. 3 A and B, respectively.
of VIP10-28 was examined on the relaxation induced by EFS only at 10 Hz, because VIP10-28 inhibited relaxations of longitudinal muscle of the Wistar-ST rat distal colon induced by EFS at 0.1 and 10 Hz by a similar extent about 45% [15] and the antagonist did not affect the relaxation in Sprague Dawley rats induced by EFS at 10 Hz for longer duration, 20–90 s [21]. The present results indicate that VIP participates NANC relaxation only in the distal colon of Wistar and Wistar-ST, but not Sprague Dawley rats.

It has been suggested that PACAP induces the relaxation via opening of apamin-sensitive K⁺ channels in the colon of rats [8] and the tenia coli of guinea pigs [12] and mediated i.j.ps via opening of the channels in the caecum of guinea pigs [17]. We also suggested that PACAP mediates about half NANC relaxation in the distal colon of Wistar-ST rats via opening of apamin-sensitive K⁺ channels [15, 25]. A few reports also suggest association of ATP-mediated relaxation in the human colon [3] and ATP-mediated i.j.ps in the guinea pig colon [29] with apamin-sensitive K⁺ channels. In all three strains studied, participation of PACAP in the relaxation was not shown in any regions other than the distal colon in the present study. However, in the distal colon of all three strains, PACAP was suggested to partially participate in the relaxation via apamin-sensitive K⁺ channels. Thus, roles of VIP and PACAP as a mediator of NANC relaxation were suggested only in the distal colon. NANC relaxation was mediated by PACAP and nitric oxide separately in Sprague Dawley rats, by VIP, PACAP and nitric oxide in Wistar rats, and VIP and PACAP in Wistar-ST rats.

Murthy and colleagues suggested a serial pathway in which nitric oxide and VIP released from myenteric neurons relax the colonic smooth muscle, VIP simultaneously producing nitric oxide in the muscle cells and nitric oxide further stimulating VIP release from the neurons [18]. It was also suggested that the nitric oxide-mediated mechanism might be present upstream of the VIP-mediated one in descending relaxation (circular muscle) of the distal colon of Sprague Dawley rats [21]. However, it is unlikely that such a serial pathway of the two or three mediators is involved in relaxation of longitudinal muscle of the distal colon of the three strains studied in the present study.

In summary, extent of participation of nitric oxide in NANC relaxation of longitudinal muscle of the Sprague Dawley rat intestine differs among regions of the intestine and the participation was more significant throughout the intestine than that in Wistar and Wistar-ST rats. Participation of VIP and PACAP was suggested only in the distal colon and the extent of their participation also differs among the strains.
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Table 2. Comparison of nitric oxide-mediated component in NANC relaxation in various intestinal regions among Sprague Dawley (SD), Wistar and Wistar-ST rats

<table>
<thead>
<tr>
<th></th>
<th>Nitric oxide-mediated component (%)</th>
<th>SD</th>
<th>Wistar&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Wistar-ST</th>
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<tbody>
<tr>
<td>Jejunum</td>
<td></td>
<td>45.5 ± 5.9 (7)</td>
<td>3.5 ± 3.5 (4)</td>
<td>25.5 ± 4.5 (5)&lt;sup&gt;11&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ileum</td>
<td></td>
<td>52.2 ± 12.1 (6)</td>
<td>43.3 ± 12.5 (3)</td>
<td>31.3 ± 4.8 (3)</td>
</tr>
<tr>
<td>Proximal colon</td>
<td></td>
<td>69.3 ± 12.2 (3)</td>
<td>88.2 ± 6.8 (4)</td>
<td>87.3 ± 6.7 (11)&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td>Distal colon</td>
<td></td>
<td>38.8 ± 5.6 (3)&lt;sup&gt;11&lt;/sup&gt;</td>
<td>37.5 ± 12.9 (4)</td>
<td>3.3 ± 6.3 (9)&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rectum</td>
<td></td>
<td>65.5 ± 13.4 (3)</td>
<td>3.7 ± 3.7 (6)</td>
<td>2.6 ± 11.3 (4)</td>
</tr>
</tbody>
</table>

Data are cited from our previous studies: a) Okishio et al. [21], b) Takeuchi et al. [28], c) Nioka et al. [19], d) Suthamnatpong et al. [24]. Data of Sprague Dawley rats were noted again from Table 1. For further details, see legend of Table 1 and Methods.

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