Review

Mediators and Intracellular Mechanisms of NANC Relaxation of Smooth Muscle in the Gastrointestinal Tract

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Tension of the skeletal muscle is solely controlled by excitatory cholinergic motor neurons. By contrast, the peripheral smooth muscle tissues are dual-controlled by nonadrenergic noncholinergic (NANC) inhibitory neurons as well as excitatory neurons. In the gastrointestinal tract, many possible candidates, including peptides, amino acids, nucleotides and gaseous compounds, for the inhibitory mediator and mechanisms of their inhibitory control have been extensively studied. This article introduces the diversity of inhibitory mediators and their probable intracellular mechanisms on the basis of recent findings on this theme, many of which are unexpected interesting findings.

1. NANC relaxation in the gastrointestinal tract.

1) Mediators of NANC relaxation

Nitric oxide was first suggested to be a possible mediator of NANC relaxation in the longitudinal muscle of the ileocolonic junction (1, 2) and the duodenum (3) of the dog and the gastric fundus of the rat (4). The role of nitric oxide was also suggested in the descending inhibition in the proximal colon of the rat (5). In studies subsequent to these, nitric oxide was suggested to mediate NANC relaxation in a number of gastrointestinal regions of many species of animals (see for the review; 6, 7). Since there have been numerous studies suggesting the role of nitric oxide as an NANC inhibitory mediator in the gastrointestinal tract up to date, nitric oxide seems to be the most important mediator of NANC relaxant response. However, the fact does not necessarily mean a universal role of nitric oxide throughout the gastrointestinal...
nal tract as described in the later sections.

Another candidate of the mediator, vasoactive intestinal peptide (VIP) has been studied extensively. There has been many reports of VIP-induced relaxation of smooth muscle of gastrointestinal tract, such as the gastric muscle of the cat (8) and guinea pig (9), the internal anal sphincter of the opossum (10) and the sphincter of Oddi of the dog (11). The role of VIP as a mediator of NANC relaxation was more strongly suggested from the studies in which the neurally-mediated relaxation was inhibited by anti-VIP serum and VIP receptor antagonists in the lower oesophageal sphincter of the opossum (12) and rabbit (13), the gastric muscularis mucosae of the dog (14), the gastric fundus of the guinea pig (15, 16) and rat (4), the tenia coli of the guinea pig (16, 17) and the middle to distal (16) and distal (18) colon of the rat. Thus, VIP seems to widely participate in the NANC relaxation in the gastrointestinal tract. However, so far as the intestine of the rat is concerned, the colon is only one region where VIP participates.

Pituitary adenylate cyclase activating peptide (PACAP), found in the digestive tract as well as in the brain and other peripheral tissues in rats by radioimmunoassay (19), was shown to be present in nerve cell bodies and nerve fibers in the gut of several species of animals by immunohistochemistry (20–24). Exogenously added PACAP induced relaxation of smooth muscle of the gastrointestinal tract, such as the lower oesophageal sphincter of the cat and human (25), the pylorus of the rabbit (26), the tenia coli of the guinea pig (27) and the colon of the human (28). Involvement of PACAP in the NANC relaxation was further suggested by using selective PACAP receptor antagonists and anti-PACAP serum in the gastric fundus of the guinea pig (29), the ileum of the dog (30), the colon of the rat (31, 32), the tenia coli of the guinea pig (33) and the internal anal sphincter of the opossum (34, 35).

Neurotensin is also a candidate for the mediator of NANC relaxation. A relaxant effect of neurotensin was first reported in the rat duodenum (36). Subsequent studies also show neurotensin to induce relaxation of the smooth muscle of the dog gastric corpus (37), the rat ileum (38) and proximal colon (39), and the guinea pig ileum (40) and colon (41). The role of neurotensin as a mediator of NANC relaxation was first suggested by Goedert et al. (40) according to indirect evidence that apamin, an antagonist of Ca2+-activated K+ channel, inhibits both the neurotensin- and nerve stimulation-induced relaxation of the ileal smooth muscle of guinea pigs (40). Neurotensin was also suggested to be the mediator in the jejunum and ileum of the rat by using desensitization method and selective antagonists of the neuroten-
sin receptor (42).

Calcitonin gene related peptide (CGRP) was reported to inhibit the contraction of smooth muscle cells from the guinea pig stomach (43) or relax the segments prepared from the guinea pig ileum (44, 45) and colon (46), guinea pig gastric corpus (47), rabbit colon (48), rat fundus (47) and opossum internal anal sphincter (49). Participation of CGRP as a sensory transmitter in ascending contraction and descending relaxation was suggested in the rat middle to distal colon (50). Thus, evidence is not sufficient to indicate CGRP as a mediator of NANC relaxation in the gastrointestinal tract at present.

Of nucleotides ATP is only one purinergic candidate for the NANC mediator suggested so far. Its role as the mediator was first suggested from the evidence that ATP was released
from the guinea pig and toad stomachs on stimulation of vagus neurons (51). Coexistence of ATP and nitric oxide synthase in NANC inhibitory neurons in the rat ileum, colon and anococcygeus muscle was shown (52). Electrical field stimulation (EFS)-induced NANC relaxation of the longitudinal muscle-myenteric plexus preparation of the rat ileum was partially inhibited after the desensitization of ATP receptors in the preparation (53). The relaxation of the guinea pig taenia coli was partially inhibited by a selective P2 purinoceptor antagonist, pyridoxal phosphate-6-azophenyl-2',5'-disulfonic acid (54). The antagonist and desensitization to α, β-methylene ATP also reduced NANC relaxations to EFS in the rat gastric fundus (55). Furthermore, apamin-sensitive inhibitory effects of ATP were suggested in the human (56) and guinea pig (57) colon. The fast inhibitory junction potential (i.e., p.) induced by electrical stimulation was also suggested to be mediated by ATP in an apamin-sensitive manner in the circular muscle of the guinea pig ileum (58, 59) and the rat colon (60). Thus, ATP seems to be the mediator of NANC relaxation in these tissue preparations, while its role was not confirmed in the rat ileum (61), proximal colon (5) and caecum (62), and the guinea-pig gastric fundus (63).

2) Diversity of inhibitory nerve control in the gastrointestinal tract
   (i) Regional difference

   Accumulated evidence indicates that the mediator of inhibitory control is not uniform throughout the gastrointestinal tract, but is quite variable from region to region. For example, if we carefully examine the many reports on the role of nitric oxide as the mediator of inhibitory responses in the rat gastrointestinal tract, it is seen that the contribution of nitric oxide to the inhibitory response is not uniform. Although an essential role of nitric oxide in NANC relaxation was suggested in the proximal colon of rats (5, 18, 64), regions where NANC inhibitory responses seem to be mediated solely by nitric oxide are rather rare, but its participation is partial and extent of the participation varies from region to region: the rat gastric fundus (4), duodenum (65, 66), jejunum (67), ileum (53) and rectum (68). However, these studies were not necessarily carried out under the same experimental conditions. Therefore, it is preferable to study extent of participation of nitric oxide in every intestinal region under the same experimental conditions. Exact comparison planned under the same experimental conditions in Wistar-ST rats showed significant differences in extent of participation of nitric oxide among the regions and also no participation in the distal colon or rectum (69; Table 1 left column). The exact comparison also showed an extremely localized roles of VIP and PACAP for the NANC relaxation only in the distal colon (69).

   (ii) Species difference

   Evidence also suggests that there are differences among species of animals in the mediator of NANC relaxation. For example, in the distal colon, no role of nitric oxide for NANC relaxation is seen in Wistar-ST rats as described above. However, a role of nitric oxide as the mediator was suggested in the distal colon of the guinea pig (70), rabbit (71), cat (72) and mouse (73). The species difference in addition to the regional difference seems very interesting in comparison to the universal contribution of an excitatory neurotransmitter, ACh, to the contractile response throughout the gastrointestinal tract and over all mammalians studied so
Table I. Comparison of nitric oxide-mediated component in NANC relaxation in various intestinal regions among Wistar-ST, Wistar and Sprague Dawley (SD) rats

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

Relaxations of longitudinal muscle of the segments obtained from various intestinal regions of Wistar-ST, Wistar and Sprague Dawley rats to EFS at 10 Hz for 10 s were recorded before (control) and after treatment with 10 μM N⁶-nitro-L-arginine (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. Values are mean ± S.E.M. for the numbers of experiments shown in parentheses. Data are cited from the reference, Okishio et al. 2000 (69).

(iii) Strain difference

In the distal colon of Wistar-ST rats, EFS-induced NANC relaxation was not affected by an inhibitor of the nitric oxide synthesis, N⁶-nitro-L-arginine (L-NOARG) and exogenously added nitric oxide did not induce any relaxant response (18, 32, 74), suggesting the absence of role of nitric oxide in NANC relaxation. Other groups of investigators have reported the participation of nitric oxide, in addition to that of VIP, in the descending relaxation in the mid and distal colon (75, 76), and longitudinal muscle of the distal colon (77) of Sprague Dawley rats. Tonic inhibition of spontaneous contractile activity by nitric oxide was also suggested in the distal colon of Wistar rats (78). The recent study in which extent of the participation of nitric oxide was exactly compared under the same experimental conditions first clearly showed the differences in mediator of NANC relaxation among strains of the rat (69, 79). Surprisingly, participation of nitric oxide in a certain intestinal region is significantly different among strains of the rat (Table 1). In addition, there were some notable tendencies: among the strains, the most significant participation throughout the intestine was seen in the Sprague Dawley strain. Among the regions, the most significant participation was seen in the proximal colon irrespective of the strain. Other interesting findings are also shown in Wistar and Wistar-ST strains: nitric oxide does not have any essential role in mediating the NANC relaxation in many regions except the proximal colon (Table 1). The study also showed differences in the participation of VIP between Wistar-ST and Sprague Dawley strain and between longitudinal and circular muscle. Namely, the extent of VIP-mediated component was approximately 40% in longitudinal muscle of Wistar-ST strain and also in circular muscle of Sprague Dawley strain, whereas this component was absent from circular muscle of Wistar-ST strain and longitudinal muscle of Sprague Dawley strain (79).
(iv) Changes with age

The influence of aging on nitric innervation has been suggested in the gastrointestinal tract. Importance of the nitric innervation increased during development of animals (80). The relaxant response to sodium nitroprusside decreased with age in the rat gastric fundus (81). Contribution of nitric innervation to NANC relaxation was slightly decreased with age in the rat ileum (82). There are also a few reports on the change in the population of nitric oxide synthase with age. Although the number of NADPH-diaphorase-positive neurons in the myenteric plexus did not significantly change with age in the rat small intestine (83), it increased in the rat proximal colon with age (84). Nitric oxide synthase immunoreactive neurons gradually increased during the first month of postnatal life, suggesting the neurochemical differentiation at neonatal stage (85). Thus, the results are insufficient to know the influence of aging on the role of nitric oxide as a mediator for NANC relaxation.

Change in the number of immunoreactive VIP neurons with age were studied in the gastrointestinal tract: the concentration of immunoreactive VIP increases with age and reaches adult level by day 28 after birth in Sprague Dawley rats (86), and the concentration is maximum in 3-month-old and then decreases with age in the mouse intestine (87). Immunoreactive nerve fibers increase with age in the Wistar rat (88) and decrease in senile rats (89). However, there is little finding on the change with aging in the role of VIP as a mediator for NANC relaxation in the rat intestine.

Recently, it has been extensively studied whether participation of nitric oxide, VIP and PACAP in NANC relaxation in the Wistar rat intestine changes with age. The results suggest that NANC relaxation in every region of the intestine at 2-week-old is almost solely mediated by nitric oxide, and its significance as an inhibitory mediator gradually or rapidly decreases with age (90) (Table 2). In other words, rates of the decrease are different in different intestinal regions. Therefore, if we examine at a certain age, extent of the participation of

<table>
<thead>
<tr>
<th>Table 2.</th>
<th>Nitric oxide-, VIP- and PACAP-mediated components in NANC relaxation in 2-, 4-, 8- and 50-week-old Wistar rats</th>
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<tbody>
<tr>
<td></td>
<td>2-week-old</td>
</tr>
<tr>
<td>Jejunum</td>
<td>70.2±9.1 (6)</td>
</tr>
<tr>
<td>Ileum</td>
<td>90.4±4.6 (5)</td>
</tr>
<tr>
<td>Proximal colon</td>
<td>—</td>
</tr>
<tr>
<td>Distal colon</td>
<td>89.3±3.1 (4)</td>
</tr>
<tr>
<td>Rectum</td>
<td>96.7±3.3 (3)</td>
</tr>
<tr>
<td></td>
<td>VIP-mediated component (%)</td>
</tr>
<tr>
<td>Distal colon</td>
<td>10.0±4.2 (14)</td>
</tr>
<tr>
<td></td>
<td>PACAP-mediated component (%)</td>
</tr>
<tr>
<td>Distal colon</td>
<td>40.5±9.9 (7)</td>
</tr>
</tbody>
</table>

Relaxations of longitudinal muscle of the segments obtained from various intestinal regions of Wistar rats to EFS at 10 Hz were recorded. The components of the relaxation which were inhibited by L-NOARG, 3 μM VIP₁₀⁻₂⁸ or 3 μM PACAP₁⁻₃₈ were defined as nitric oxide-, VIP- or PACAP-mediated component, respectively. For further details, see legend of Table 1. Data are cited from the references, Takeuchi et al. 1998 (90), 1999 (92) and 2000 (91).
nitric oxide differs among all intestinal regions as mentioned in a preceding section. By contrast, the role of VIP in mediating NANC relaxation, which was found only in the distal colon of the Wistar rat, is very little at neonatal stage, but it increases with age (91). Although there was no report on the influence of aging on the role of PACAP as the mediator, a recent paper reported 40–60% participation of PACAP in the NANC relaxation irrespective of age in the distal colon of Wistar rats (92). These results were also summarized in Table 2. As judged from the data of Table 2, even if no association of nitric oxide or VIP with NANC relaxation is shown in some region of the gastrointestinal in some species of animals, the possibility of their roles at a younger or elder age in the corresponding region should not be excluded.

2. Intracellular mechanism of NANC relaxation of gastrointestinal smooth muscle

1) cyclic GMP–cyclic GMP dependent protein kinase (PKG) pathway

Nitric oxide and some nitroso compounds had been reported to increase the content of cyclic GMP in various tissue preparations (93). These compounds were also reported to increase cyclic GMP content of the tissue preparations obtained from various regions of gastrointestinal tract, where nitric oxide was suggested to mediate relaxation, such as the ileum (94) and proximal colon (74, 95) of rats, and the fundus of canines (96). EFS induced nitric oxide–mediated relaxation with a concomitant increase in the cyclic GMP content in the proximal colon of rats (95). EFS also increased cyclic GMP content in the lower oesophageal sphincter of opossums (97) where nitric oxide–mediated relaxation was suggested (98, 99). Furthermore, blockade of soluble guanylate cyclase resulted in inhibition of nitric oxide–mediated inhibitory responses in the canine fundus (96), guinea pig colon (100), rat (101) and canine (102) proximal colon, and guinea pig tenia coli (103). Nitric oxide–mediated relaxation via cyclic GMP–PKG pathway was also suggested in the dispersed cells from gastric muscle of guinea pigs (104, 105) and rabbits (105). These results strongly suggest that nitric oxide mediates inhibitory responses in these tissue and cell preparations via cyclic GMP–PKG pathway.

However, the following results clearly deny involvement of cyclic GMP–PKG pathway in the nitric oxide–mediated inhibitory response. Cystamine, an inhibitor of soluble guanylate cyclase, inhibited an EFS–induced increase in cyclic GMP content, but it did not affect EFS–induced relaxation in the opossum lower oesophageal sphincter (106). Inhibition of soluble guanylate cyclase by cystamine also did not affect the nitrergic relaxation of the rat duodenum (107). Inhibition by LY83583, an another inhibitor of the enzyme also did not affect nitrergic relaxation of the rat proximal colon, even though it decreased cyclic GMP content to the level lower than the resting (108). Furthermore, EFS and exogenous nitric oxide significantly increased cyclic GMP content of the rat distal colon, but exogenous nitric oxide did not induce any appreciable relaxation (95). Similar results were obtained using atrial natriuretic peptide (ANP) in the same tissue (95, 109).

Although an exact reason for such discrepancy is still unknown, the fact must not be overlooked that every report, except that using dispersed cells, which suggest nitric oxide–
mediated relaxation via cyclic GMP–PKG pathway as noted above used 1H-[1,2,4]oxadiazolo [4,3-a]quinoxalin-1-one (ODQ) to inhibit soluble guanylate cyclase (96, 100–103). Recently, ODQ was shown to inhibit the NANC relaxation in a cyclic GMP–PKG independent manner (110). It was also suggested that inducible nitric oxide synthase, possibly induced during preparation of the dispersed smooth muscle cells, is involved in relaxant effect of nitric oxide in isolated smooth muscle cells but not in segments of gastrointestinal tract (111). If much more cyclic GMP is produced in the cells under such conditions, some kind of side effect by higher concentration of cyclic GMP might be appeared. Indeed, in the permeabilized muscle preparations from the rat distal colon where nitric oxide and ANP did not induce any relaxation (95, 109), higher concentrations of cyclic GMP did induce moderate relaxation (, although the magnitude was small compared to that in the proximal colon) (74). Therefore, it seems likely that cyclic GMP itself, especially at higher concentrations, has some relaxant effect on the gastrointestinal smooth muscle under certain experimental conditions. However, involvement of cyclic GMP–PKG pathway in nitric oxide–mediated NANC relaxation of the gastrointestinal tract remains uncertain.

2) cyclic AMP–cyclic AMP dependent protein kinase (PKA) pathway

It is well known that norepinephrine induces significant relaxation in various smooth muscle tissues via activation of adenylate cyclase, production of cyclic AMP and in turn, activation of PKA. In the NANC relaxation of the gastrointestinal tract, a few intestinal peptides were suggested to activate the pathway.

(i) Activation of cyclic AMP–PKA pathway by VIP

VIP was first suggested to relax the isolated smooth muscle cell by increasing cyclic AMP content of the cells from the guinea pig stomach (112). VIP–induced relaxation via cyclic AMP was also suggested in the lower esophageal sphincter of opossums (113) and in the isolated cells from the rabbit gastric antrum (114). Cyclic AMP, which had been loaded into smooth muscle cells as caged compound, intracellularly relaxed the rabbit distal colon (115). Subsequently, association of activation of PKA with VIP–cyclic AMP sequence was suggested by using selective inhibitors of PKA in the tissue preparations from the lower esophageal sphincter of opossums (116), and the isolated cells from the stomach of the rabbit (105, 117) and guinea pig (104, 105), and from the ileum of guinea pig (118). Recently, VIP–cyclic AMP–PKA pathway was also reported in NANC relaxation of the rat distal colon (119).

(ii) Association of PACAP–induced relaxation with cyclic AMP–PKA pathway

PACAP was first isolated from ovine hypothalamus using its adenylate cyclase stimulating activity (120). PACAP was suggested to mediate NANC relaxation in several regions of gastrointestinal tract of several species of animals as mentioned in the preceding chapter. However, it is not clear yet whether its role in the NANC relaxation is associated with adenylate cyclase stimulating activity. Indeed PACAP–induced relaxation is accompanied by an increase in cyclic AMP content of the tissue preparations from the guinea pig tenia coli (33, 121), cat and human lower oesophageal sphincter (25), and of the dispersed cells from the rabbit gastric muscle (122), but there is no evidence for coupling of PACAP–elevated cyclic AMP content with NANC relaxation in the gastrointestinal tract. An inhibitor of PKA, H–89
did not affect PACAP-induced relaxation of the guinea pig tenia coli, while VIP-induced relaxation was significantly inhibited (33). Furthermore, PACAP at the concentration, which induced a maximal relaxant response in the rat distal colon, did not affect the cyclic AMP content of the tissue (119). Thus, in contrast to the VIP-mediated response noted above, PACAP-mediated one seems unlikely to be associated with its adenylate cyclase stimulating activity.

(iii) Possible association of CGRP-increased cyclic AMP with NANC relaxation

Participation of CGRP in the inhibitory responses in the gastrointestinal tract was mentioned in the preceding section. Among such studies, a few reports suggest that CGRP-induced responses are mediated by increased cyclic AMP in guinea pig gastric muscle cells (123), the opossum internal anal sphincter (124) and the guinea pig ileum (125).

3) Hyperpolarization of the membrane

When the relationship between NANC relaxant response and change in membrane potentials of smooth muscle cells in a certain tissue is discussed, there may be two viewpoints. Namely, one is whether neurogenic NANC relaxant response which is mostly induced by EFS is associated with the change in the membrane potentials. The other is whether exogenous added compounds that were suggested as the mediator for NANC relaxant response induce change in the membrane potentials. In the ordinary study on the role of a certain mediator it is examined whether selective antagonists for the mediator can block both the responses induced by EFS and the exogenous possible mediator itself.

(i) Association of nitric oxide-mediated relaxation with changes in membrane potentials

Excitation of myenteric neurons results in changes in the membrane potentials of smooth muscle cells, excitatory and inhibitory junction potentials. Inhibitory junction potentials (i.j.ps) can be recorded mostly in the presence of atropine which blocks dominant transmission, excitatory one. Therefore, it has been studied in the presence of atropine what mediator is involved in EFS-induced i.j.ps in various regions of gastrointestinal tract of various species of animals. Inhibitors of nitric oxide synthesis moderately or significantly diminished EFS-induced i.j.ps. recorded in circular smooth muscle cells of the opossum esophagus (126, 127) and lower esophageal sphincter (128), the guinea pig (59, 129) and hamster (130) ileum, the canine proximal colon (131), the guinea pig proximal and distal colon (132) and the guinea pig internal anal sphincter (133). These inhibitions by the inhibitors always reversed by addition of excess amount of a substrate for nitric oxide synthesis, L-arginine. Nitric oxide is suggested to mediate slow i.j.ps, which is more clearly produced by repetitive electrical pulses, but not fast i.j.ps, which is produced by a single pulse, in many tissue preparations (59, 60, 130, 134, 135). Exogenously added nitric oxide or nitroso compounds induced hyperpolarization of the membrane of circular smooth muscle cells of the opossum esophageal sphincter (128) and esophagus (126, 127), the canine jejunum (136), the hamster ileum (130), the rat caecum (62), the human colon (134), the guinea pig proximal (132, 137) and distal (132) colon. Exogenous nitric oxide increased outward K⁺ current in the patch clamped smooth muscle cells isolated from the canine (138) and rabbit (139) colon. Many studies which report EFS-induced i.j.ps with concomitant muscle relaxation or inhibitory response are also present: the opossum
esophagus (140), the canine small intestine (140), the human and canine jejunum (136, 141), the human (134) and rat (60) colon, the rat caecum (62), the canine proximal colon (142) and the guinea pig internal anal sphincter (133). Thus, these numerous reports strongly suggest that NANC inhibitory pathway involves nitric oxide-mediated i.j.ps in the various regions of gastrointestinal tract.

Interestingly, many reports among these indicate that the NANC inhibitory pathway is blocked by apamin, a blocker of small conductance Ca\(^{2+}\)-activated K\(^+\) channels (SK channels) (59, 127, 129, 130, 132, 134, 140). Some reports suggest involvement of cyclic GMP in nitric oxide mediated-slow i.j.ps (127, 128, 130, 139). Although it seems likely that these results indicate the pathway, nitric oxide-cyclic GMP-i.j.ps, in the NANC inhibitory transmission in the gastrointestinal tract, intracellular mechanism of nitric oxide in inducing NANC relaxation is not necessarily clarified throughout the gastrointestinal tract. For example, nitric oxide-mediated i.j.ps recorded in the rat colonic circular muscle (60), the rat caecum (62), the opossum esophagus (140) and the guinea pig anal sphincter (133) was suggested to be apamin resistant. The i.j.ps in the guinea pig proximal and distal colon is partly apamin resistant (132). Moreover, cyclic GMP- and membrane potential-independent nitric oxide mediated NANC relaxation was shown in longitudinal and circular muscle of the rat proximal colon (64). In the studies, i.j.ps were recorded only in the presence of atropine but relaxations of both muscles were induced even in the absence of atropine. Apamin completely abolished the i.j.ps, but never had an effect on the relaxations. L-NOARG inhibited the relaxations, but did not affect the i.j.ps. Exogenously added nitric oxide (0.1-10 μM) induced relaxations, but did not affect the membrane potentials at these concentrations. Soluble guanylate cyclase inhibitors completely inhibited the stimulatory effect of EFS on the cyclic GMP content, but did not affect the relaxations to EFS (108).

(ii) Association of i.j.ps with relaxation induced by other candidates for the mediator

Some candidates for the NANC mediator were also suggested to be associated with changes in the membrane potentials of the smooth muscle cells.

Although PACAP was often suggested as a mediator of NANC relaxation in various regions of various species of animals noted in the preceding paragraph, electrophysiological study of the actions of PACAP is not present so many. EFS-induced i.j.ps were significantly reduced after the desensitization of the preparations to PACAP, and EFS-induced i.j.ps and exogenous PACAP-induced hyperpolarization were blocked by apamin in longitudinal muscle of the tenia caecum of the guinea pig (143). PACAP was also suggested to induce apamin-sensitive i.j.ps with concomitant apamin-sensitive relaxation of the longitudinal muscle of the rat distal colon. Exogenous PACAP-induced hyperpolarization and relaxation were inhibited by apamin in the tissue (32). Interestingly, it was suggested that activation of tyrosine kinase is involved in the PACAP-mediated apamin-sensitive inhibitory pathway (144). On the other hand, it was reported that PACAP-induced hyperpolarization was apamin-sensitive but PACAP-induced relaxation was apamin-resistant in the circular muscle of the guinea pig proximal colon (145) and the rat colon (146). These results strongly suggest that PACAP-mediated relaxation is independent of apamin-sensitive i.j.ps in these tissue preparations. Thus, clear difference among the tissues is present in the action mechanism of PACAP in
inducing relaxation of smooth muscle of the gastrointestinal tract.

There are a few reports to suggest association of VIP-induced i.j.ps with NANC relaxation in the gastrointestinal tract. Exogenous VIP hyperpolarized the membrane of circular muscle of the chicken rectum (147) and guinea pig ileum (148). Slow i.j.ps induced by repetitive electrical pulses in the guinea pig ileum were inhibited by a VIP antagonist (148). VIP-mediated slow i.j.ps with concomitant slow NANC relaxation were suggested in the longitudinal muscle of the rat proximal colon (32, 119). Interestingly, these hyperpolarizations and relaxations induced by VIP were apamin resistant.

Exogenously added ATP or P_2 purinoceptor agonists induced hyperpolarization of the membrane of circular smooth muscle cells in the human (149) and canine (150) jejunum, the guinea pig ileum (59, 148) and proximal colon (145), the porcine ileum (151), and the human (134) and rat colon (60), and the guinea pig internal anal sphincter (133). Antagonists for P_2 purinoceptors inhibited i.j.ps induced by an electrical pulse. α, β-Methylene ATP desensitization also resulted in inhibition of the i.j.ps (59, 60, 133, 145, 148, 151, 152). All of the suggested ATP-mediated i.j.ps are apamin-sensitive. ATP was suggested to mediate fast i.j.ps instead of slow one in several reports (59, 60, 134, 145, 148, 149). Moreover, ATP-mediated relaxation or inhibition of contraction was induced concomitant with i.j.ps (60, 145, 151). Thus, it seems that ATP mediates apamin-sensitive fast i.j.ps with concomitant relaxation in these tissue preparations.

4) Change in intracellular Ca^{2+} ion concentration

A reduction in intracellular Ca^{2+} ion concentration ([Ca^{2+}]_i) leads to smooth muscle relaxation. Based on current understanding of regulation of [Ca^{2+}]_i, several mechanisms are proposed for a reduction in [Ca^{2+}]_i. That is, activation of K^+ channels by which membrane is hyperpolarized results in a decrease in Ca^{2+} influx through voltage-dependent Ca^{2+} channels; inhibition of membrane voltage-dependent Ca^{2+} channels; activation of plasma membrane Ca^{2+}-ATPase by which efflux of Ca^{2+} is increased; activation of sarcoplasmic reticulum Ca^{2+}-ATPase by which sequestration of cytoplasmic Ca^{2+} into SR is increased. There have been many reports to suggest involvement of these proposed mechanisms in the reduction of [Ca^{2+}]_i in many kinds of tissue and isolated cells including smooth muscle of several kinds of tissue (see for review, 153).

Association of a reduction in [Ca^{2+}]_i with nitric oxide–induced inhibitory response of smooth muscle was first suggested in the canine gastric smooth muscle. Namely, nitric oxide or its-related compounds inhibited increases in [Ca^{2+}]_i and tension via inhibition of the voltage–dependent Ca^{2+} channels that participate in the plateau phase of slow waves of the tissue (154). Exogenous nitric oxide induced relaxation with a concomitant decrease in [Ca^{2+}]_i in the rat rectal circular muscle (68). Nitric oxide was also suggested to increase [Ca^{2+}]_i via Ca^{2+} release from sarcoplasmic reticulum (SR) in interstitial cells and decrease [Ca^{2+}]_i via an unknown mechanism in smooth muscle cells isolated from the canine colon (155). Treatment of strips of the mouse gastric fundus with ryanodine, an activator of SR Ca^{2+} release channel, resulted in partial inhibition of nitric oxide–mediated relaxation (156). Ryanodine also inhibited EFS–induced relaxation of the rabbit corpus cavernosum (157).
These results indicate importance of the function of SR in nitrergic relaxation via regulation of intracellular Ca\(^{2+}\) dynamics. The importance of the function of SR was also suggested by using thapsigargin and cyclopiazonic acid, selective inhibitors of SR Ca\(^{2+}\)-ATPase. Thapsigargin and cyclopiazonic acid as well as ryanodine inhibited both the EFS-induced i.j.ps and nitric oxide donor-induced hyperpolarization in the opossum oesophagus. It was also suggested in the study that an elevation of subplasmalemmal Ca\(^{2+}\) released from the superficial SR is important in inducing relaxation and i.j.ps (158). Recently, results obtained in simultaneous measurements of effects of thapsigargin on changes in muscle tension and [Ca\(^{2+}\)]\(_i\) in the rat proximal colon suggest that nitric oxide induces the relaxation of the muscle preferentially by activating the Ca\(^{2+}\) uptake by SR and in turn decreasing [Ca\(^{2+}\)]\(_i\), (159).

It was also reported that PACAP-induced relaxation of longitudinal muscle of the rat distal colon occurred with a concomitant decrease in [Ca\(^{2+}\)]\(_i\), (144). Interestingly, although involvement of cyclic GMP-PKG pathway in a reduction in [Ca\(^{2+}\)]\(_i\), was suggested in many kinds of tissue and isolated cells (153), there is no such report for the gastrointestinal smooth muscle to date. The final step to induce NANC relaxation seems a decrease in [Ca\(^{2+}\)]\(_i\), which must result in a decrease in phosphorylation of myosin light chain. However, evidence to prove it is still insufficient at present.

5) Decrease in sensitivity of contractile machinery to Ca\(^{2+}\)

To date NANC relaxation without changes in [Ca\(^{2+}\)]\(_i\) has not been reported. However, if sensitivity of the contractile machinery to Ca\(^{2+}\) decreases (Ca\(^{2+}\) desensitization), the muscle will relax despite a constant Ca\(^{2+}\) level. Only one report suggested that nitric oxide caused Ca\(^{2+}\) desensitization by which contractile activity of canine gastric antrum was substantially inhibited (154). In permeabilized smooth muscle preparations from the rat proximal and distal colon, one of the second messengers of NANC inhibitory transmission cyclic GMP did relax at a fixed Ca\(^{2+}\) level, indicating Ca\(^{2+}\) desensitization by cyclic GMP (74). A decrease in phosphorylation of myosin light chain via activation of myosin light chain phosphatase is proposed for the desensitization mechanism by cyclic GMP in longitudinal muscle of the rabbit ileum (160). However, it is still unknown whether Ca\(^{2+}\) desensitization is involved in the mechanism of NANC relaxation of gastrointestinal smooth muscle.

3. NANC relaxation and peristaltic movement

Experiments to study the mediators and intracellular mechanisms of NANC relaxation were mostly carried out by examining responses of longitudinal muscle. Several reports described above also studied the mediators involved in the descending relaxation, in which responses of circular muscle were recorded. Localized stimulation elicited by inflation of a small balloon or mechanical radial stretch results in relaxation of the circular muscle anal to the distended region. Although relaxation of longitudinal muscle is important for the motility of the intestine, descending relaxation is more important especially for the intestinal peristalsis.

Intestinal peristalsis which is initiated by radial distension and mechanical stimulation by
intestinal contents consists of a synchronous ascending contraction and descending relaxation of both longitudinal and circular muscle (161). The ascending and descending neural pathways contain afferent sensory neurons, interneurons, and excitatory or inhibitory motor neurons. Inhibitory neural pathway was suggested to play a crucial role for setting the threshold at which peristaltic emptying is triggered (162). Inhibitory motorneurons to the circular muscle are involved in an accommodation mechanism which reflexes the circular muscle (163). The role of nitric oxide for peristalsis was suggested from the effects of nitric oxide synthase inhibitors and nitric oxide donors on it (162). A dual excitatory and inhibitory role of nitric oxide was also suggested (164). Furthermore, it was shown that nitric oxide is an important neuromodulator within the myenteric intrinsic nervous pathways (165), and that it facilitates and depresses release of ACh from interneurons in ascending and descending nervous pathways, respectively (166). Inhibition of nitric oxide synthesis resulted in inhibition of transit of intestinal contents and inhibition of descending relaxation in the rat colon in vivo (167). VIP was also suggested to be involved in the peristaltic reflex: the velocity of propulsion of synthetic fecal pellets which were inserted at the proximal end of the segment was slowed down by a VIP receptor antagonist in the guinea pig colon (168). ATP and PACAP as well as nitric oxide were also suggested to have an important role for gastric emptying in the rat stomach in vivo and in vitro studies (169).

Thus, inhibitory neural control is essential for the peristalsis of the intestine. Nitric oxide, the most probable candidate for the mediator of NANC relaxation seems to have a role not only as a transmitter of inhibitory motor neurons but also a modulator of neurons within ascending and descending neural pathways.

4. Overview

There have been numerous studies on the NANC inhibitory transmission in the gastrointestinal tract. However, since an essential role of nitric oxide in the transmission was suggested in 1990, interesting findings which directly indicate the mediator of NANC relaxation and its intracellular mechanism have been rapidly accumulated in the last decade. The unexpected interesting finding is diversity of inhibitory mediators in the gastrointestinal tract. Especially, in addition to differences in participation of nitric oxide in NANC relaxation among intestinal regions and species of animals, differences among the strains of animals are surprising. However, the most important finding is the change in participation of nitric oxide with age. That is, nitric oxide has an essential role for the NANC relaxation in every intestinal region at neonatal stage but the role gradually or rapidly decreases with age. The rates of the decrease are different in different intestinal regions, different species of animals and different strains of animals. Therefore, if we examine the role of nitric oxide in a certain intestinal region in a certain animal, the extent of its participation shown differs in study to study. So far as nitric oxide is concerned, one of the most interesting theme is to answer the question of why its participation in NANC relaxation in the gastrointestinal tract decreases with age. In contrast, participation of VIP in NANC relaxation in the rat distal colon is scarce in the neonatal stage and increases with age, whereas participation of PACAP does not change with
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...age in the same tissue. Although roles of nitric oxide and other several possible mediators for NANC relaxation have been extensively studied, diverse aspects of mediators of NANC relaxation mean complicated nature of the inhibitory transmission and some part probably large part, beyond the imagination, of the detail of the nature may remain unknown.

Action mechanisms of nitric oxide and others suggested as a mediator of NANC relaxation are not sufficiently clarified yet. Suggested action mechanisms of a few mediators are tentatively summarized in the scheme (Fig. 1). A decrease in $[\text{Ca}^{2+}]_i$ of smooth muscle cells which must couple to a decrease in phosphorylation of the myosin light chain seems the final step of the intracellular mechanism in the NANC inhibitory pathway, because NANC relaxation without any change in $[\text{Ca}^{2+}]_i$ has not been reported. Although several second messengers such as cyclic GMP, cyclic AMP and tyrosine kinase were suggested to be activated by receptor activation by the NANC inhibitory mediators, coupling of these second messengers and decrease in $[\text{Ca}^{2+}]_i$ has not been elucidated. Another interesting unclarified question is a relationship between the change in the membrane potentials of smooth muscle cells and decrease in $[\text{Ca}^{2+}]_i$. Hyperpolarization of the membrane links to a decrease in cell excitability. Indeed, some kinds of $K^+$ channels such as SK channels, large conductance $Ca^{2+}$-activated $K^+$ channels (BK channels) and ATP-activated $K^+$ channels were suggested to be associated with the NANC relaxations. However, there is no report which shows directly relationship between the mechanism of activation of such $K^+$ channels and the decrease in $[\text{Ca}^{2+}]_i$ after the second messenger activates its action targets. Since SK and BK channels are activated by $Ca^{2+}$ and relaxation of the smooth muscle results from a decrease in $Ca^{2+}$, dual changes in $[\text{Ca}^{2+}]_i$ might occur in the smooth muscle cells. In this respect, an idea of localized increase and decrease of $[\text{Ca}^{2+}]_i$, seems likely and $Ca^{2+}$ released from the superficial $Ca^{2+}$ store of SR for the $Ca^{2+}$-activated $K^+$ channels seems important. Role of SR via regulation of $Ca^{2+}$ ion dynamics in the smooth muscle cells is now one of the highlight points in this theme. However, it should be reminded that i,j,p's does not necessarily link to the relaxation in many cases especially in nitric oxide-mediated NANC relaxation.

The most important physiological significance of NANC relaxant response should be discussed in relation to peristaltic movements. Descending relaxation as well as ascending contraction is essential for the peristaltic movements, because blockade of inhibitory motor neuron results in blockade of descending pathway and peristaltic movements. Furthermore, the roles of nitric oxide in the ascending and descending pathways as an modulator of interneurons within myenteric plexus were suggested. Therefore, mediators of NANC relaxant response may have crucial role for the peristaltic movements of the gastrointestinal tract.
Fig 1. Possible mechanisms of NANC relaxation in longitudinal smooth muscle of the Wistar-ST rat intestine. (A) Nitric oxide induces the relaxation by decreasing $[Ca^{2+}]_i$ via activation of $Ca^{2+}$ uptake by SR (pathway 1) in the proximal colon. Increased cyclic GMP (cGMP) may also contribute to the relaxation by decreasing $Ca^{2+}$ sensitivity (pathway 2). (B) VIP and PACAP induce the relaxation by decreasing $Ca^{2+}$, via activation of calcium activated $K^+$ channels in the distal colon. VIP activates charybdo toxin (ChTx)-sensitive $K^+$ channels via cyclic AMP-PKA pathway (pathway 1). PACAP activates apamin-sensitive $K^+$ channels via the tyrosine kinase pathway. The activation of $K^+$ channels may result in hyperpolarization of the resting membrane potential and in turn closes the voltage dependent $Ca^{2+}$ channels (VDCC). Cyclic AMP (cAMP) may also contribute to the relaxation probably at a high concentration by decreasing $Ca^{2+}$ sensitivity (pathway 2). Receptors for VIP (VIP-R) and PACAP (PACAP-R) are currently classified into three types; PAC1 exhibits a high affinity for PACAP, but a low affinity for VIP, and VPAC1 and VPAC2 exhibit similar affinity for them.
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