Properties of acetylcholine-induced relaxation of smooth muscle isolated from the proximal colon of the guinea-pig

Youhei Kodama¹, Satoshi Ino², Yuhsuke Shigemasa¹ and Hikaru Suzuki¹

¹Department of Cell Physiology, Nagoya City University Medical School, Japan; ²Department of Anatomy, Faculty of Medicine, Fukui University, Japan

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Abstract

The properties of mechanical responses elicited by stimulation with acetylcholine (ACh) were investigated in circular smooth muscle preparations isolated from the proximal colon of guinea-pig. Application of ACh (10⁻⁸–10⁻⁶ M) for 3–5 min produced a biphasic response, with an initial contraction followed by a relaxation. Atropine inhibited the initial contraction, while Nω-nitro-L-arginine (L-NA) inhibited the relaxation, suggesting that the former was produced by activation of muscarinic receptors while the latter was produced by an elevated production of nitric oxide (NO). In the presence of atropine, the ACh-relaxation was attenuated by removal of the mucosa and abolished by removal of both submucosal and mucosal layers. The ACh-induced relaxation was also attenuated by either tetrodotoxin (TTX, 3 × 10⁻⁷ M) or hexamethonium (10⁻⁶ M). In the presence of atropine, transmural nerve stimulation (TNS) elicited a biphasic response, with an initial phasic contraction followed by a relaxation. The amplitude of TNS-induced relaxation was significantly reduced by hexamethonium or L-NA and was abolished by TTX. Both ACh and TNS produced relaxation in preparations isolated from the proximal colon, but not in those from the middle part of colon. Immunohistochemistry for neuronal nitric oxide synthase revealed no difference in the distribution of nitrergic nerves between the proximal and middle part of the colon, with nitrergic nerves in both the mucosal and submucosal layers as well as in the smooth muscle and myenteric layers. These results suggest that ACh induces NO production by excitation of postganglionic nerves distributed mainly in the mucosal and submucosal layers. In circular smooth muscle preparations isolated from the middle part of colon, ACh or TNS produced contractile responses alone, with no associated relaxation, suggesting that the ACh-activated postganglionic nitrergic nerves are distributed in the mucosal and submucosal layers of the proximal colon but not in the middle part of the colon.

Key words: acetylcholine, relaxation, postganglionic nitrergic nerves, submucosal layer, proximal colon

Correspondence to: Hikaru Suzuki, Ph.D., Department of Cell Physiology, Nagoya City University Graduate School of Medical Sciences, Mizuho-ku, Nagoya 467-8601, Japan
Phone: +81-52-853-8131  Fax: +81-52-842-1538  e-mail: hisuzuki@med.nagoya-cu.ac.jp
Introduction

Acetylcholine (ACh) is an excitatory transmitter substance of cholinergic enteric nerves distributed in gastrointestinal tissues, and this amine depolarizes the membrane and produces contraction in gastrointestinal smooth muscle cells, through activation of atropine-sensitive muscarinic receptors (Bolton, 1979; Kuriyama et al., 1998). The cellular mechanism of the ACh-induced depolarization is controversial. In single smooth muscle cells isolated from the guinea-pig ileum, ACh depolarizes the membrane by the activation of non-selective cation channels which are sensitive to flufenamic acid or Ni²⁺ (Inoue et al., 1990; Inoue, 1995; Chen et al., 1993; So and Kim, 2003). However, in circular smooth muscle tissues isolated from the guinea-pig stomach antrum, the ACh-induced depolarization is not inhibited by chemicals which are known to inhibit non-selective cation channels, possibly due to heterogeneous distribution of muscarinic receptors in the intestinal wall (Hotta et al., 2005). The ACh-induced contraction responses of intestinal smooth muscles are produced mainly by activation of trans-membrane signaling pathways including the elevated production of inositol 1,4,5-trisphosphate (IP₃) and elevated Ca-sensitivity of contractile proteins due to inhibition of myosin phosphatase within the rho-kinase pathways (Kuriyama et al., 1998).

Most gastrointestinal smooth muscles are spontaneously active with generation of slow waves generated by interstitial cells of Cajal (ICC) distributed in the wall (Sanders, 1996; Huizinga et al., 1997). The distribution and role of ICC in the gastrointestinal tract are heterogeneous. In the stomach, ICC distributed in the myenteric layer of the antrum (ICC-MY) serve as the pacemakers of the slow waves generated in smooth muscle cells, while ICC distributed in intramuscular layers (ICC-IM) have a role in the transmission of neural signals to smooth muscle cells (Sanders, 1996; Ward and Sanders, 2001). In the proximal colon there are two types of ICC. Firstly the ICC distributed in the myenteric layer (ICC-MY) and secondly those distributed in the submucosal layer (ICC-SMP). It is the combined activity of these two types of ICC that induce the different activities of the circular smooth muscle in the dog (Smith et al., 1987; Nahar et al., 1998) and rat (Pluja et al., 2001; Kato et al., 2009). Spontaneous rhythmic contractions of circular muscle triggered by ICC-SMP have also been noted in the proximal colon of the guinea-pig (Kobayashi et al., 1995; Kobayashi et al., 1996).

In circular smooth muscle preparations isolated from the proximal colon of the rat, an analysis of the mechanical responses elicited by transmural nerve stimulation (TNS) indicates an innervation by at least 4 different nerve types, i.e. cholinergic excitatory, peptidergic excitatory, nitrergic inhibitory and non-adrenergic non-cholinergic non-nitrergic (NANCNN) inhibitory nerves, with the phasic contractions which originate from the activity of ICC-MY being strongly inhibited by nitrergic nerves, although the neural regulation of the phasic contractions triggered by ICC-SMP is very weak (Kato et al., 2009). Thus, although the physiological functions and roles of these ICC distributed in the proximal colon remain unclear, the regulatory mechanisms of their activity vary between the different types of ICC.

Attempts were made to investigate the properties of the mechanical response produced by exogenously applied ACh in smooth muscle preparations isolated from the proximal colon of the guinea-pig. In view of the heterogeneous distribution of muscarinic receptors on ICC or smooth
ACh-induced relaxation of proximal colon (Hotta et al., 2005), together with the contribution of the different types of ICC on the rhythmic activity of the colon (Kobayashi et al., 1995; Nahar et al., 1998; Pluja et al., 2001; Kato et al., 2009), stimulation with exogenously applied ACh was expected to produce different responses in smooth muscle cells and ICC. Experiments were thus carried out to investigate the properties of the mechanical responses produced by ACh in circular smooth muscle preparations isolated from the proximal colon of the guinea-pig. The results indicate that ACh produced a biphasic mechanical response in smooth muscle preparations, with an initial contraction produced by activation of muscarinic receptors followed by a relaxation which was mediated by an elevated production of nitric oxide (NO). The ACh-induced relaxation response was inhibited by tetrodotoxin or hexamethonium, but not by atropine, suggesting the possible involvement of an elevated production of NO through excitation of postganglionic nitrergic nerves in this tissue. A part of these results have been reported briefly at the 20th Annual Meeting of the Japanese Pathophysiological Society held in Nara (Kodama et al., 2010).

Materials and Methods

Male guinea-pigs, weighing 200–300 g, were anesthetized with fluoromethyl 2,2,2-trifluoro-1-(trifluoromethyl) ethyl ether (sevoflurane, Maruishi Pharm., Co. Ltd., Osaka, Japan), and exsanguinated by decapitation. All animals were treated ethically according to the guiding principles for the care and use of experimental animals in the field of basic sciences, approved by The Experimental Animal Committee of the Nagoya City University Medical School. The proximal and middle parts of the colon was excised, and opened by cutting vertically. Three types of circular smooth muscle preparation (width 1 mm, length 15 mm) were isolated from the proximal colon: preparations with intact longitudinal muscle layers and submucosal layers attached (intact muscle preparations), preparations which had the mucosal layer removed but the longitudinal muscle layer still attached (mucosal layer removed preparations), and preparations with an intact longitudinal muscle layer but with the submucosal layer and mucosal layers removed (submucosal layer removed preparations). Circular muscle preparations with attached mucosal and submucosal layers (mid-colon preparations) were also isolated from the middle part of the colon, to compare the properties with those of the proximal colon preparations.

Both ends of each of these preparations were tied with fine threads, and suspended vertically in a cylindrical recording chamber (diameter 10 mm, 25 mm depth). Preparations were superfused with oxygenated Krebs solution (warmed to 36.5°C), at a constant flow rate of about 3 ml/min. One thread was anchored to the bottom of the chamber, while the other end was connected to the lever of a force-transducer (TB-612T, Nihon-Kohden, Tokyo, Japan). The isometric force changes produced by the preparations were recorded through a pre-amplifier (AP-621G, Nihon Kohden, Tokyo, Japan), digitized using P-clamp (Axon Instruments, Foster City, CA, USA) and stored on a personal computer for later analysis. A pair of silver plates (width, 0.5 mm) was placed on either side of the recording chamber, along the length of the muscle segment, and brief electrical current stimuli (0.05 ms duration, 10 V intensity) were applied to the muscle through the plates, using an electric stimulator (SEM-3013, Nihon-Kohden, Tokyo, Japan).

The ionic composition of the Krebs solution was as follows (mM): Na⁺ 137.4, K⁺ 5.9, Ca²⁺ 2.5,
Mg²⁺ 1.2, HCO₃⁻ 15.5, H₂PO₄⁻ 1.2, Cl⁻ 134 and glucose 11.5. The solution was aerated with O₂ containing 5% CO₂, and the pH of the solutions was maintained at between 7.2–7.3.

Chemicals used were acetylcholine chloride (ACh), atropine sulphate, hexamethonium bromide, Nω-nitro-L-arginine (L-NA) and tetrodotoxin (TTX). All chemicals were purchased from Sigma-Aldrich Chemicals (St. Louis, MI, USA). These chemicals were dissolved first in distilled water at concentrations which were more than 1,000 times higher than those used in the experiments, and then further diluted with Krebs solution to prepare the desired concentrations. The addition of these drugs did not alter the pH of the Krebs solution.

Tissue segments (20 mm long) were isolated from the proximal and middle regions of the colon, and kept in Krebs solution in a dissecting chamber at room temperature and lightly stretched before being immobilized on the Sylgard plate covering the bottom of the dissection chamber using tiny pins. Each preparation was fixed with Zamboni’s fixative (Iino et al., 2007) for 1 h, and then kept in phosphate-buffered saline at 4°C. The preparation was embedded in OCT compound (Sakura Finetek, Tokyo, Japan), and sections (12 μm) cut in a cryostat. Sections were collected on glass slides and pre-incubated with 5% normal donkey serum in 0.01 M phosphate buffered saline (PBS) before being incubated with rabbit anti-neuronal nitric oxide synthase (nNOS) antibody (sc-651, Santa Cruz, CA, USA, 1:1000 in PBS) and mouse monoclonal anti-α-smooth muscle actin (A5228, Sigma, USA, 1:1000 in PBS). After sections had been incubated with primary antibodies at room temperature overnight, they were washed with PBS for at least 1 hour, before incubation in secondary antibodies (Alexa Fluor-coupled donkey anti-IgG, 1:500 in PBS) for 1 hour at room temperature. After washing with PBS, specimens were counterstained with 4’,6-diamidino-2-phenylindole (DAPI; Invitrogen, CA, USA) and mounted with PermaFluor Aqueous Mounting Medium (Thermo Electron Corporation, MA, USA). Preparations were examined with a TCS-SP2 confocal microscope (Leica, Wetzlar, Germany) and images collected and measured using Leica Confocal Software.

Experimental values were expressed as the mean value ± standard error of the mean (SEM). Statistical significance was tested using the Student’s t-test, and probabilities of less than 5% (P<0.05) were considered to be significant.

**Results**

*Mechanical responses produced by acetylcholine (ACh)*

Circular muscle preparations isolated from the proximal colon of guinea-pig were spontaneously active with an irregular generation of phasic contractions which were superimposed on the irregular change of resting tension levels by 1 to up to 20 mN (Fig. 1A). The spontaneous phasic contractions tended to decrease in frequency and amplitude with time, and in most preparations examined (23 out of 27 tissues) there were only irregular changes in the resting tension with no phasic contractions after a period of 2 hours from the start of the experiments (Fig. 1B). The mean values of the amplitude and frequency of the phasic contractions were 5.8 ± 1.0 mN and 13.3 ± 1.4 times min⁻¹, respectively (n=19). Smooth muscle preparations then began to produce small and regular rhythmic contractions, which appeared usually 3–4 hours after the start of experiments (Fig. 1C). The mean values of the amplitude and frequency of these rhythmic
contractions were 1.1 ± 0.6 mN and 20.8 ± 0.7 times min⁻¹ (n=19), respectively. These rhythmic contractions were not observed in preparations in which the submucosal layers had been removed (Y. Kodama, unpublished observation), which suggests that these activities were produced by cells distributed in the submucosal layer, as was the case in the rat proximal colon (Kato et al., 2009).

Experiments were carried out to record the mechanical responses produced by increasing concentrations of ACh (10⁻⁹ – 10⁻⁵ M) in intact muscle preparations isolated from the proximal colon. Experiments were carried out in preparations which had been incubated in the recording chamber for more than 2 hours, and this allowed similar results in different preparations. Low concentrations of ACh (<10⁻⁷ M) elicited a relaxation which developed slowly and reached a peak value at about 4–5 min during the stimulation with ACh (Fig. 2, A–C). The amplitudes of contraction and relaxation were increased depending on the concentration of ACh. Higher concentrations of ACh (>10⁻⁷ M) elicited a biphasic response, with an initial contraction (elevation of resting tension) and a following relaxation (Fig. 2, C–E). A transient relaxation was often elicited before producing the initial contraction response (Fig. 2D). The relaxation response commenced about 1–2 min after stimulating with ACh and the peak response appeared following the removal of ACh.

The effects of atropine and Nω-nitro-L-arginine (L-NA) on the ACh-induced responses were investigated in intact smooth muscle preparations isolated from the proximal colon. The effects of these antagonists were observed on the typical biphasic mechanical responses produced by 10⁻⁷ M ACh. This concentration of ACh was also chosen because of the reproducibility of responses (data not shown). The biphasic mechanical response produced by ACh (Fig. 3A) was changed to a
relaxation response alone in the presence of atropine (10^{-6} M) (Fig. 3B). Additional application of 10^{-4} M L-NA resulted in an attenuated relaxation response to ACh stimulation (Fig. 3C). Similar experiments were repeated in different preparations (n=9), and the summarized results (Fig. 3D) confirmed the typical responses shown in Fig. 3. In separate experiments, a low concentration (10^{-5} M) of L-NA on the ACh-induced relaxation resulted in a weak inhibition, with the amplitude of the ACh-induced relaxation reduced by about 70%. The effects of L-NA and atropine, applied in either order, on the ACh-induced mechanical responses were also examined in the isolated proximal colon preparations. When L-NA (10^{-4} M) was applied first, there was a sustained contraction during stimulation with 10^{-7} M ACh. If atropine (10^{-6} M) was then applied, the mechanical response was abolished during stimulation with ACh (data not shown). These results indicate that the ACh-induced initial contraction was produced by the activation of muscarinic receptors while the later relaxation was produced by an elevated production of nitric oxide (NO).
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Experiments were carried out to investigate the effects of removal of both of the mucosal and submucosal layers on the mechanical responses produced by ACh in smooth muscle preparations isolated from the proximal colon. Application of ACh (10⁻⁷ M) for 5 min produced a biphasic responses in intact (Fig. 4A) and mucosal layer removed preparations (Fig. 4B). In preparations in which both the mucosal and submucosal layers had been removed, ACh produced a contraction without a following relaxation response (Fig. 4C). The summarized data indicated that the removal of mucosal layer could significantly attenuate the relaxation response produced by ACh (Fig. 4E). These results indicate that production of nitric oxide (NO) by ACh requires the presence of both the mucosal and submucosal layers in the proximal colon of the guinea-pig, with the role of the relaxing effects of NO on the circular smooth muscle being much stronger with the NO produced by the submucosa than it was with the NO production from the mucosa alone.

Effects of removal of mucosal and submucosal layers on the ACh-induced responses

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Effects of ACh on circular smooth muscle preparations isolated from the middle part of colon

Experiments were carried out to measure the mechanical responses of circular smooth muscle preparations isolated from the middle part of colon, and the results were compared with those
measured in preparations isolated from the proximal colon. In intact preparations isolated from the middle part of colon, ACh (10^{-7} M) elicited an excitatory response, with no associated relaxation response (Fig. 4D). The elevation of resting tension produced by ACh was much smaller than that observed in the proximal colon (Fig. 4E). Thus, these results indicate that the submucosal and mucosal layer-dependent relaxation produced by ACh was a characteristic of preparations isolated from the proximal colon.

**Effects of TTX and hexamethonium on the ACh-induced responses**

Attempts were made to observe the effects of TTX and hexamethonium on the ACh-induced responses in intact preparations isolated from the proximal colon of the guinea-pig. Experiments were carried out in the presence of atropine, to facilitate the measurement of the ACh-induced relaxation responses. Typical responses of the ACh-induced response produced in the presence of
TTX (3 × 10⁻⁷ M) and hexamethonium (3 × 10⁻⁷ M) are shown in Figs. 5B and 5E, respectively. The amplitude of the ACh-induced relaxation was decreased by either TTX or hexamethonium. These were confirmed in 5 preparations, and the pooled data summarized in Fig. 5, C and F. These results suggest that ACh produced an increase in NO production via the excitation of postganglionic nerves and the activation of nicotinic receptors.

**Mechanical responses produced by transmural nerve stimulation (TNS)**

Experiments were carried out to investigate the properties of the mechanical responses produced by direct excitation of intramural nerves using transmural application of brief electric pulses (i.e., transmural nerve stimulation, TNS). Selective excitation by TNS of intramural nerves was confirmed by a reversible inhibition of the evoked responses with 3 × 10⁻⁷ M tetrodotoxin (TTX) (data not shown). In intact preparations isolated from the proximal colon, TNS (10 stimuli at 10 Hz frequency) elicited a phasic contraction with 20–30 s duration and 50–150 mN amplitude, followed by a relaxation with 2–3 min duration and 2–10 mN amplitude (Fig. 6A). Similar responses were always elicited by TNS, irrespective of the presence or absence of spontaneous phasic contractions (data not shown). In the presence of 10⁻⁶ M atropine, TNS produced a complex response with three main components: an initial rapid relaxation, with a following phasic contraction, which were followed by a slow relaxation (Fig. 6B). Although the amplitude of the
contraction was variable between preparations, the amplitude and duration of the relaxation elicited after TNS was the same in all preparations examined. Additional application of $10^{-4}$ M L-NA resulted in an attenuated relaxation response, with an associated reduction of both the initial relaxation and contraction responses (Fig. 6C). The effects of L-NA were measured only on the relaxation responses produced by TNS (Fig. 6D), and the results suggested that they were produced by NO. In separate experiments, the effects of low concentration ($10^{-5}$ M) of L-NA were tested, and the results indicated that this low concentration of L-NA tended to reduce the amplitude of relaxation, but the change was not statistically significant (data not shown).

The effects of removal of both the mucosal and submucosal layers on the TNS-induced responses were also observed in preparations isolated from the proximal colon. The TNS-induced responses were similar between intact (Fig. 7A) and mucosal layer removed preparations (Fig. 7B). However, no relaxation response was elicited by TNS in preparations in which both mucosal and submucosal layers had been removed (Fig. 7C). In intact preparations isolated from the middle part of the colon, no relaxation was again elicited by TNS (Fig. 7D). Pooled data obtained from

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**Fig. 6.** Mechanical responses were produced by transmural nerve stimulation (TNS) in intact circular smooth muscle preparation isolated from the proximal colon. TNS (10 stimuli at 10 Hz frequency) was applied at the arrow, in the absence (A, Control) and presence of $10^{-6}$ M atropine (B) and atropine with $10^{-4}$ M L-NA (C). D, mean value ($\pm$ SEM) of the peak amplitude of relaxation produced by TNS observed in different conditions. *, significantly different to Control ($P<0.05$). #, significantly different to responses obtained in the presence of atropine alone ($P<0.05$).
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different preparations (n=5–21) again indicated that the TNS-induced relaxation was elicited by nitrergic nerves distributed in the mucosal and submucosal layers of the proximal colon but not in the middle part of the colon (Fig. 7E).

**Effects of TTX and hexamethonium on the TNS-induced responses**

The effects of TTX (3 × 10⁻⁷ M) and hexamethonium (3 × 10⁻⁷ M) on the mechanical responses produced by TNS were investigated in intact preparations isolated from the proximal colon of the guinea-pig. The relaxation responses produced by TNS (10 stimuli at Hz frequency), evoked in the presence of atropine, were abolished by TTX (Fig. 8B) and were attenuated by about 50% by hexamethonium (Fig. 8D). Similar experiments were repeated in different preparations, and the summed data for TTX and hexamethonium were shown in Fig. 8, C and E, respectively. These results confirmed that the excitation of nitrergic nerves was required to produce NO for the
TNS-induced relaxation.

**Distribution of nitrergic nerves in the mucosal and submucosal layers**

Distribution of nitrergic nerves in the wall of colon was visualized by immunostaining of neuronal nitric oxide synthase (nNOS). The results indicated that nNOS-immunopositive nerves were distributed in the mucosal and submucosal layers, in addition to the myenteric layers and within smooth muscle bundles (both circular and longitudinal muscles). In addition, nNOS-immunopositive nerve cell bodies were observed in the submucosal and myenteric layers. The distribution of nNOS-immunopositive structures was identical in preparations isolated from both the proximal (Fig. 9A) and middle (Fig. 9B) part of the colon.

**Discussion**

The present experiments have revealed that in isolated circular smooth muscle preparations of the proximal colon of the guinea-pig, ACh produced a biphasic response, with an initial contraction
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followed by relaxation. The initial contraction was abolished by atropine, suggesting that it was produced by activation of muscarinic receptors, while the relaxation was inhibited by L-NA, suggesting that it was produced by NO. Removal of the mucosal layer significantly attenuated this response, while removal of both the submucosal and mucosal layers abolished the ACh-induced relaxation. From these results, it is reasonable to consider that ACh enhances the production of NO in the mucosal and submucosal layers. Furthermore, the ACh-induced relaxation could be inhibited by either TTX or hexamethonium, an inhibitor of the excitation of nerves by blocking the voltage-gated Na⁺ channels or an antagonist of nicotinic receptors, respectively. These findings indicate that the ACh-induced elevation of NO production requires an excitation of postganglionic nerves (possibly nitrergic nerves) distributed in the submucosal layer. The removal of the mucosal layer was also effective in reducing the ACh-induced relaxation, suggesting further that the ACh-excited nitrergic nerves extend axons to the mucosal layers. The distribution of nitrergic nerves was examined in the proximal colon by immunohistochemical staining of nNOS, and the results supported a wide distribution of nNOS-positive nerves in the mucosal and submucosal layers, in addition to a rich distribution of nitrergic nerves in both smooth muscle and myenteric layers.

Experiments were also carried out to measure the mechanical responses elicited by excitation of enteric nerves, including nitrergic nerves, by transmural nerve stimulation (TNS) with electrical pulses. Application of TNS for a brief period of time (10 pulses at 10 Hz frequency) elicited a biphasic response, with an initial contraction followed by a relaxation, which was an L-NA-sensitive NO component. The possible contribution of ACh released from cholinergic nerves and the generation of action potentials produced during generation of excitatory junction potentials (e.j.p.) in smooth muscle cells in the initial contraction is considered, since this contraction

![Fig. 9. Distribution of nNOS-immunopositive cells in the proximal (A) and middle (B) part of the guinea-pig colon. nNOS immunoreactivity, smooth muscle actin immunoreactivity and nuclei were revealed with green, red and blue colors, respectively. Longitudinal sections of the colon, with the serosal membrane at the bottom and the mucosal layer at the top. Arrows indicate nNOS-immunopositive nerve cell bodies. CM: circular smooth muscle, LM: longitudinal smooth muscle, MM: muscularis mucosae, SM: submucosa. Calibration Bars: 100 μm.](image-url)

response is partially inhibited by atropine (Hoyle and Burnstock, 1989; Kuriyama et al., 1998). However, the mechanism of the inhibition of the initial transient contraction by hexamethonium remains unclear. TTX abolished both the initial contraction and the following relaxation response, confirming that TNS indeed elicited a selective excitation of enteric nerves.

Thus, the present experiments have suggested a distribution of nitrergic nerves which have cholinergic synaptic inputs in both the mucosal and submucosal layers. The distribution of myenteric nerves in the proximal colon indicates that most of the nitrergic nerves which innervate the circular muscle layer have their cell bodies in the myenteric layer. It has been shown that some of the neurons such as the Dogiel type II neurons distributed in the myenteric layer send their axons to the mucosal layer (Neunlist and Schemann, 1997; Kunze and Furness, 1999; Furness, 2000; Lomax and Furness, 2000). We, therefore, would like to propose that in the proximal colon of the guinea-pig, there is a population of nitrergic nerves whose cell bodies are located in the submucosal layer, that receive cholinergic inputs and which send axons to circular smooth muscle cells. The proximal colon is rich in ICC-SMP (Kobayashi et al., 1995; Kobayashi et al., 1996; Nahar et al., 1998; Pluja et al., 2001), and these cells periodically generate plateau-type action potentials (Yoneda et al., 2002; Yoneda et al., 2003; Hotta et al., 2005). While the physiological functions of ICC-SMP remain unclear, we consider that their activity is possibly involved in the special digestive movements found in the proximal colon, such as in anti-peristaltic movements (Hukuhara and Neya, 1968). Alternatively, it is also considered that the special movements which occur in the proximal colon are produced by a combination of the activity between nitrergic nerves and ICC-SMP.

However, the observation that nNOS-immunopositive nerves are distributed rather homogeneously in the submucosal layer of both the proximal and middle part of the colon of the guinea-pig, does not support the regional difference in the ACh-induced or TNS-induced relaxation responses observed in the proximal colon. The wide distribution of nitrergic nerves is histologically confirmed in the intestine including the colon (Kunze and Furness, 1999; Furness, 2000). These results could be interpreted if NO released from these nitrergic nerves is capable of inducing relaxation in the proximal colon but not in the middle part of the colon. At the moment, it remains unclear why there is such a heterogeneous distribution of ACh-induced relaxation in the colon. However, it is important to point out that the absence of NO-induced relaxation by TNS does not necessarily indicate the absence of the distribution of nitrergic nerves in the colon. We consider the possibility that there may be an absence of effective nerve terminals in the circular muscle or that there is a lack of ICC-IM which transmit the neural signals to smooth muscle cells (Ward and Sanders, 2001) in the middle part of colon, but not in the proximal colon.

In summary, in the isolated proximal colon of the guinea-pig, ACh produces relaxation by an elevated production of NO through excitation of postganglionic nerves, possibly distributed in both the mucosal and submucosal layers. Excitation of nitrergic nerves by TNS also produces an NO-induced relaxation in this preparation. In circular smooth muscle preparations isolated from the middle part of the colon, ACh or TNS produced contraction responses alone, with no associated relaxation, suggest that the ACh-activated release of NO is effective to induce dilation in the proximal colon but not in the middle part of colon. Nitrergic nerves activated by ACh or TNS may be distributed in the submucosal and mucosal layers.
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References


