Modulation of the activity of two pacemakers by transmural nerve stimulation in circular smooth muscle preparations isolated from the rat proximal colon

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Abstract

Circular smooth muscle preparations isolated from the rat proximal colon periodically generated two different amplitudes and frequencies of phasic contractions: large phasic contractions (LPC) with a frequency of about 1.5 times/min and small phasic contractions (SPC) with a frequency of about 9 times/min. Preparations with no attached longitudinal smooth muscle layer (and also myenteric layer) generated SPC alone, while those with no attached submucosal layer generated only LPC, indicating that the pacemakers of the LPC and SPC are distributed in the myenteric and submucosal layers, respectively. In intact preparations, transmural nerve stimulation (TNS) applied for 1–2 min with different frequencies (0.2–2 Hz) inhibited the phasic contractions. The amplitude of LPC was reduced at >0.25 Hz and abolished at >0.3 Hz, while the amplitude but not the frequency of SPC was reduced at >0.5 Hz (in a frequency-dependent way). The TNS-induced inhibitory responses were augmented by atropine and attenuated by Nω-nitro-L-arginine (L-NA). In the presence of L-NA and atropine, TNS elicited biphasic (inhibitory and following excitatory) responses. The former were not antagonized by apamin, guanethidine or suramin, while the latter were antagonized by capsaicin, suggesting an innervation by non-adrenergic non-cholinergic non-nitrergic (NANCNN) inhibitory and peptidergic excitatory nerves, respectively. In preparations with the longitudinal muscle layer removed, TNS inhibited only the amplitude of SPC, which was augmented by atropine and antagonized by L-NA. In intact preparations, muscarinic stimulation with acetylcholine increased the frequency of LPC, while nitricergic stimulation with sodium nitroprusside reduced the amplitude and frequency of LPC, and also the amplitude but not the frequency of SPC. These results indicate that the rat proximal colon has two types of pacemaker cells. Myenteric pacemaker cells which receive predominantly nitricergic, but also cholinergic, peptidergic and NANCNN innervation, and submucosal pacemaker cells that are not markedly influenced by intramural nerves.

Key words: proximal colon, P-region, phasic contraction, pacemaker cells, enteric nerves
Introduction

Gastrointestinal smooth muscle cells are spontaneously active with rhythmic generation of electrical responses such as slow waves and spike potentials (Tomita, 1981), which are triggered mainly by a group of cells, the interstitial cells of Cajal (ICC), that are distributed in the gastrointestinal wall (Sanders, 1996; Huizinga et al., 1997; Sanders et al., 1999). There are many types of ICC distributed in the gastrointestinal wall, but the ICC distributed in the myenteric layer (ICC-MY) trigger the rhythmic activity of intestinal smooth muscle (Ward et al., 1994; Sanders, 1996; Huizinga et al., 1997). ICC and smooth muscle cells as well as ICC themselves are connected by gap junctions (Nakamura and Shibata, 1999; Komuro et al., 1999), so that ICC distributed within smooth muscle bundles of the stomach (intramuscular ICC, ICC-IM) or those distributed in the mucosal side of circular muscle of the intestine (deep muscular ICC, ICC-DMP) contribute to the conduction of pacemaker potentials generated in ICC-MY to smooth muscle cells (Sanders et al., 1999; Hirst and Ward, 2003).

In the circular smooth muscle of the small intestine of W/W' mice which lack both ICC-MY and ICC-DMP due to the partial mutation of c-kit gene (Sanders, 1996), junction potentials evoked by transmural nerve stimulation (TNS) are strongly attenuated, together with an impaired generation of slow waves in smooth muscle cells (Ward et al., 2000). In addition, the responses of smooth muscle to exogenously applied acetylcholine (ACh) as well as sodium nitroprusside (SNP), cholinomimetic and nitromimetic agents, are also attenuated in these mutant mice (Ward et al., 2000). Based on these observations, Ward and Sanders (2001) proposed that either ICC-IM or ICC-DMP have a role in the transmission of neural signals to smooth muscle cells, i.e., cholinergic and nitrergic nerves innervate ICC-IM directly, and junction potentials generated in ICC-IM in response to transmitter substances are conducted to smooth muscle cells in an electrotonic manner through gap junctions.

The proximal colon of some laboratory animals has a distribution of ICC-MY and, in addition, a group of ICC distributed in the submucosal layer (ICC-SM) (in the guinea-pig, Kobayashi et al., 1996; Nahar et al., 1998: in the dog, Kobayashi et al., 1995; Horiguchi et al., 2003: in the mouse, Yoneda et al., 2002: in the rat, Plujà et al., 2001: in the rabbit, Lentle et al., 2008). These two types of ICC have a functional connection with circular smooth muscle cells, and elicit periodic electrical and mechanical responses of smooth muscle independently. In the proximal colon of guinea-pigs, a causal relationship has been shown to exist between contractions with a frequency of 10–12 cycles per min and ICC-SM (Nahar et al., 1998). Smooth muscle of the rat colon produces two types of contraction, with large amplitude contractions at a low frequency (1–2 times/min) and small amplitude contractions at a high frequency (10–12 times/min) both of which are triggered by periodic excitation of smooth muscle cells (Plujà et al., 2001). The physiological significance of the co-localization of two types of pacemaker cells in the proximal colon, however, remains unclear, with a possible role considered in the “anti-peristaltic movement” (Hukuhara and Neya, 1968) which appears specifically in this region (Kobayashi et al., 1996; Yoneda et al., 2002).

In the canine colon, two types of pacemaker cells are involved in inhibitory neural regulation (Smith et al., 1989), with nitrergic nerves taking the major role in this inhibition (Dalziel et al.,
Neural modulation of pacemaker activity in P-region (1991; Keef et al., 2002). However, it remains unclear whether this also occurs in the rat colon. The present experiments were designed to investigate how the two types of pacemaker cells are regulated by intramural nerves in the rat proximal colon. Experiments were carried out to measure the mechanical responses produced by nerve stimulation in circular smooth muscle preparations isolated from the rat proximal colon (P-region). Intramuscular nerves were stimulated transmurally by a train of brief electrical pulses for a long period of time (1–2 min), and the responses produced by sustained stimulation of the smooth muscle with transmitter substances were measured. A brief report of part of this study was presented to the 51st Annual Meeting of the Japan Society of Smooth Muscle Research in Nagoya (Kato et al., 2009).

Materials and Methods

Male Wistar King rats, weighing 200–300 g, were anesthetized with fluoromethyl 2,2,2-trifluoro-1-(trifluoromethyl) ethyl ether (sevoflurane, Maruishi Pharm., 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 physiological sciences, approved by The Experimental Animal Committee of the Nagoya City University Medical School. The proximal colon was excised, and opened by cut open along its length. The mucosal layer was removed using fine scissors, and a circular smooth muscle strip about 1 mm wide was prepared. Three types of circular smooth muscle strip were prepared: those with intact longitudinal smooth muscle and submucosal layers (intact tissue), those with the longitudinal smooth muscle layer removed but with an intact submucosal layer, and those in which the submucosal layer had been removed but with the longitudinal smooth muscle layer intact. Both ends of each preparation were tied with fine threads, so that they could be suspended vertically in the cylinder-shaped recording chamber (diameter 10 mm, 20 mm depth). The preparations were superfused with oxygenated Krebs solution (warmed to 36.5°C), at a constant flow rate of about 3 ml/min. The thread at one end was anchored to the bottom of the chamber, and the other end connected to the lever of a mechano-transducer (TB-612T, Nihon-Kohden, Tokyo, Japan). The isometric forces produced by the preparations were recorded through a pre-amplifier (AP-621G, Nihon Kohden, Tokyo, Japan) and stored on a personal computer for later analysis. A pair of silver plates (width, 0.5 mm) was placed one on either side of the recording chamber, along the muscle segment, and a brief electrical current stimulus (0.05 ms duration, 10 V intensity) applied to the muscle through the plates.

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 7.2–7.3.

Chemicals used were acetylcholine chloride (ACh), atropine sulphate, guanethidine sulphate, neostigmine bromide, Nω-nitro-L-arginine (L-NA), nifedipine, sodium nitroprusside (SNP), phentolamine mesylate and tetrodotoxin (TTX). Phentolamine mesylate was purchased from CIBA Geygy (Switzerland) while the other chemicals were purchased from Sigma-Aldrich Chemicals, St. Louis, MI (USA). Nifedipine was dissolved first with dimethyl sulphoxide (DMSO) at a concentration of 10⁻² M, and further diluted to 10⁻⁶ M with Krebs solution just
before use. The other chemicals were dissolved first with distilled water at concentrations which were 1,000 times higher than those used in the experiments. All chemicals were further diluted with Krebs solution to prepare the desired concentrations.

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

**Results**

*Properties of spontaneous phasic contractions*

Intact preparations produced two types of phasic contractions periodically, large and small amplitude of phasic contractions (LPC and SPC, respectively), each with different frequencies and amplitudes (Fig. 1A). The amplitude of LPC was 2–5 times larger than SPC, while the frequency of SPC was several times higher than LPC. In the 16 preparations examined, the amplitudes of LPC and SPC were $15.6 \pm 1.9$ mN and $5.4 \pm 0.9$ mN, respectively (Fig. 1D).
frequency of LPC was about 1.5 times min⁻¹, while it was about 9 times min⁻¹ for SPC (Fig. 1E). In preparations with the submucosal layers removed, only LPC were generated periodically (Fig. 1B), at the same amplitude and frequency seen in intact preparations (Fig. 1, D and E, respectively). Preparations in which the longitudinal smooth muscle layer had been removed showed rhythmic generation of SPC alone (Fig. 1C), at the same amplitude and frequency seen in intact preparations (Fig. 1, D and E, respectively). These results indicate that the cells which pace the generation of LPC and SPC are distributed in the longitudinal smooth muscle layer (including the myenteric layer) and the submucosal layer, respectively. In intact preparations, LPC with two peaks (indicating superimposition of LPC and SPC) were often observed, suggesting that the activity of myenteric and submucosal pacemaker cells was independent. The evidence that the frequency of LPC generated in the submucosal layer-removed preparations and that of SPC in longitudinal smooth muscle layer-removed preparations were similar to those generated in intact preparations (Fig. 1, D and E), also supported the concept that the activity of the pacemaker cells for these two types of phasic contraction were independent.

Both LPC and SPC were abolished by 10⁻⁶ M nifedipine, in a reversible manner (data not shown), suggesting that these contractions were produced through activation of voltage-sensitive L-type Ca-channels.

Mechanical responses produced by transmural nerve stimulation (TNS)

Experiments were carried out to stimulate circularly-cut smooth muscle preparations isolated from the rat proximal colon with a train of electrical pulses (0.5 ms duration, 10 V intensity) at 0.5–1 Hz frequency for 1–2 min. These stimulations elicited an inhibitory response, i.e., a reduction in the amplitude of both LPC and SPC. The electrical stimulation did not elicit any mechanical responses in the presence of 0.3 µM tetrodotoxin (TTX), but the inhibitory response recovered when TTX was removed from the superfusate (data not shown). These results suggest that the electrical stimulation selectively excited intramural nerves, and thus it was a transmural nerve stimulation (TNS). When TNS was applied for a longer period of time (usually >5 min), the TNS-induced inhibitory responses were reversible.

TNS was applied with increasing frequency (0.2–2 Hz) for a constant period of time (2 min) to intact preparations isolated from the rat proximal colon. Application of TNS at 0.2 Hz frequency did not significantly alter the amplitude and frequency of either LPC or SPC (Fig. 2A), while the application of 0.5 Hz TNS abolished LPC and elicited a sustained generation of reduced amplitude SPC (Fig. 2B). The amplitude and frequency of phasic contractions generated during TNS were quantified and the changes plotted as a function of the stimulus frequency. During TNS, the amplitude of LPC were decreased at a frequency of 0.25 Hz and abolished at a frequency of 0.33 Hz, while the amplitude of SPC decreased at stimulus frequencies higher than 0.5 Hz but was not abolished even at a stimulus frequency of 2 Hz (Fig. 2C). The frequency of LPC tended to decrease at a stimulus frequency of both 0.2 and 0.33 Hz (but not significant from control), but disappeared at stimulus frequencies >0.5 Hz, while the frequency of SPC generated during TNS remained unchanged over a wide range of frequencies (0.2–2 Hz, Fig. 2D). Thus, in circular smooth muscle preparations of the rat proximal colon,
TNS mainly reduced the amplitude of phasic contractions, with no marked change in their occurrence. The results also indicate that the TNS-induced inhibition appeared to be much more marked in LPC than in SPC.

**Effects of atropine and Nω-nitro-L-arginine on phasic contractions**

The effects of inhibition of both muscarinic receptors and nitric oxide synthase with atropine and Nω-nitro-L-arginine (L-NA) respectively on LPC and SPC were investigated in intact preparations isolated from the rat proximal colon. Application of atropine (10^-6 M) did not produce any significant change in either the amplitude or frequency of spontaneously generated phasic contractions. Addition of L-NA (10^-5 M), in the presence of atropine, significantly increased both the amplitude and frequency of spontaneous LPC, and also the frequency of SCP (Fig. 3). When the order of application of these inhibitors was changed, similar changes were observed on both LPC and SPC, i.e., L-NA increased both the amplitude and frequency of LPC as well as the frequency of SPC, with no significant change in the amplitude of SPC, while addition of atropine in the presence of L-NA did not produce further change in either the amplitude or frequency of LPC and SPC (data not shown). These results suggest that endogenous NO produced a sustained inhibition of the activity of the pacemaker cells for both

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**Fig. 2.** Effects of transmural nerve stimulation (TNS) on phasic contractions of circularly-cut smooth muscle preparation of the P-region (intact preparation). In A and B, TNS was applied for 2 min at different frequencies (A, 0.2 Hz; B, 0.5 Hz). The amplitude (C) and frequency (D) of phasic contractions generated during TNS were plotted as a function of the frequency (0.2–2 Hz). ○, LPC; □, SPC. Mean ± S.D. (n=6–20). *, significant difference from control (P<0.05).
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LPC and SPC, but that this was not the case for endogenous acetylcholine.

The effects of both atropine (10^{-6} M) and L-NA (10^{-5} M) on the TNS-induced responses in intact circular smooth muscle preparations of the rat proximal colon were also investigated. Figure 4 shows typical effects of L-NA on the TNS-induced responses, followed by those of the effects of co-application of atropine with L-NA. In the absence of these inhibitors, TNS applied for 2 min at 1 Hz frequency caused a sustained inhibition of SPC and abolished LPC, in a reversible manner (Fig. 4A). In the presence of L-NA, TNS elicited weak inhibitory responses, with only the amplitude of LPC being smaller during TNS (Fig. 4B), but the frequency of LPC (Fig. 4E) and both the amplitude and frequency of SPC (Fig. 4, F and G, respectively) were not significantly changed. Addition of atropine, in the presence of L-NA, caused no marked change in the mechanical responses to TNS, although weak inhibitory responses were elicited (Fig. 4C). The quantified data for both LPC and SPC indicated that TNS abolished LPC in the absence of L-NA, while in the presence of L-NA the amplitude of LPC was reduced during TNS, with no significant change in the frequency. Application of atropine in the presence of L-NA did

![Image](image-url)

**Fig. 3.** Effects of atropine and N\(^\omega\)-nitro-L-arginine (L-NA) on spontaneously generating phasic contractions of intact circularly-cut smooth muscle preparations isolated from the rat P-region. The amplitude (D) and frequency (E) of phasic contractions were measured before (Control) and during application of 10^{-6} M atropine (Atropine) and the additional co-application of 10^{-5} M L-NA (L-NA). Mean value (+ S.D.) was shown for LPC (filled column) and SPC (open column). *, significant difference from Control (P<0.05).
not further modulate either the amplitude or frequency of LPC (Fig. 4, D and E, respectively). In the absence of L-NA and atropine, TNS also reduced the amplitude of SPC, with no change in the frequency, but was antagonized by L-NA (Fig. 4, F and G). Co-application of atropine with L-NA produced no further change in the SPC elicited during TNS (Fig. 4, F and G).

Experiments were carried out to observe the effects on the TNS-induced responses of the application of atropine followed by a subsequent application of L-NA (Fig. 5). The TNS-induced inhibitory responses generated in the absence of L-NA and atropine (Fig. 5A) were augmented by application of $10^{-6}$ M atropine, with generation of reduced amplitude SPC alone (Fig. 5B). Application of L-NA, in the presence of atropine, resulted in a strong attenuation of the TNS-induced inhibitory response, and an irregular generation of LPC but constant generation of SPC (Fig. 5C). Quantified data (Fig. 5, D–G) indicated that the TNS-induced inhibition of both LPC and SPC were antagonized by L-NA, while they were augmented by atropine. An important finding was the absence of a change in frequency of the SPC during TNS-induced inhibition, either in the absence or presence of either atropine or L-NA, or of both.

Fig. 4. Effects of Nω-nitro-L-arginine (L-NA, $10^{-5}$ M) and atropine ($10^{+4}$ M) on phasic contractions generated during TNS in intact circularly-cut smooth muscle preparations isolated from the rat P-region. TNS (1 Hz frequency for 2 min)-induced responses were recorded before (A, Control) and during application of L-NA (B) and the additional co-application of atropine (C). The amplitude (D) and frequency (E) of phasic contractions were measured before (Control) and during application of L-NA and the additional co-application of atropine. Mean value (+ S.D.) of phasic contractions was shown for LPC (D, amplitude; E, frequency) and SPC (F, amplitude; G, frequency). Filled column, before TNS; open column, during TNS. *, significant difference from values obtained before TNS ($P<0.05$).
These results indicate that in intact preparations, the TNS-induced inhibitory responses could be antagonized by L-NA, either in the presence or absence of atropine, suggesting that NO, produced possibly in response to nitrergic nerve excitation, was the main initiator of the TNS-induced inhibition. Although the inhibition occurred for both LPC and SPC, the inhibitory effects were more marked on the LPC than on the SPC. The effects of TNS on the frequency of LPC were unclear due to their abolition, while the reduction in amplitude of the SPC by TNS was not associated with any change in frequency. The results also indicated that an effective cholinergic innervation was minimal or absent in the circular smooth muscle of the rat proximal colon.

The possible involvement of a functional cholinergic innervation of the circular smooth muscle of the rat proximal colon was further examined by using neostigmine, an inhibitor of cholinesterase. In the preparation shown in Fig. 6, TNS-induced inhibitory responses were elicited in the absence of L-NA (Fig. 6A). In the presence of L-NA, TNS elicited an inhibition of
LPC and excitation of SPC (Fig. 6B). Co-application of $5 \times 10^{-8}$ M neostigmine, together with L-NA, resulted in an increase in both the amplitude and frequency of SPC, giving an unclear discrimination of the LPC (Fig. 6C). In the presence of both neostigmine and L-NA, TNS elicited an excitatory response with an increase in the amplitude of the SPC and an elevation of the resting tension (Fig. 6C). These TNS-induced excitatory responses were antagonized by atropine, and a weak inhibition and following excitation of phasic contractions was observed during TNS (Fig. 6D). Similar experiments were repeated in 8 preparations, and in all cases TNS elicited excitatory responses in the presence of neostigmine. These results suggest that the circular smooth muscle of the rat proximal colon does indeed receive a functional cholinergic excitatory innervation, which can only be visualized when the activity of acetylcholine esterase is inhibited by neostigmine.

The possible involvement of a distribution of adrenergic nerves in the circular smooth muscle of the rat proximal colon was examined using either guanethidine ($10^{-5}$ M) or phentolamine ($10^{-6}$ M). Application of either guanethidine or phentolamine for up to 60 min did not produce any change in the spontaneous phasic contractions or in the TNS-induced responses (data not shown), suggesting that the circular smooth muscle of the rat P-region does
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not have an effective adrenergic innervation.

Non-adrenergic non-cholinergic non-nitrergic responses

In most of the intact preparations examined, TNS applied in the presence of both L-NA and atropine produced weak inhibitory responses (Fig. 7A) or biphasic responses (initial inhibitory, following excitatory) (Fig. 7, C and E). Experiments were carried out to test the effects of apamin (10^{-7} M), suramin (10^{-4} M) and capsaicin (5 \times 10^{-6} M) on these TNS-induced responses in the presence of L-NA (10^{-5} M) and atropine (10^{-6} M). Apamin inhibits the non-adrenergic non-cholinergic (NANC) inhibitory responses in gastro-intestinal preparations (Shuba and Vladinirnova, 1980), and similar inhibitory actions are also found with suramin, possibly by inhibiting purinergic receptors (Dunn and Blakeley, 1988; Ohno et al., 1993; Ohno et al., 1996). Capsaicin has generally been applied to inhibit neuro-effector transmission of peptidergic nerves by depleting transmitter substances from nerve endings.

Apamin increased the amplitude of the SPC and also the frequency of the LPC, which
resulted in an unclear definition of both SPC and LPC, while the TNS-induced responses were not markedly altered (Fig. 7B). The TNS-induced biphasic responses produced in the presence of L-NA and atropine (Fig. 7C), were again not markedly changed by suramin (Fig. 7D). The TNS-induced excitatory responses appeared slowly during sustained stimulation, and were antagonized by capsaicin (Fig. 7F). The TNS-induced responses elicited in the presence of L-NA and atropine were all abolished by TTX ($3 \times 10^{-7}$ M), but not by guanethidine ($10^{-5}$ M) (data not shown). These results suggest that the TNS-induced excitatory responses produced in the presence of atropine and L-NA have a causal relationship with peptidergic nerves, while the inhibitory responses are produced by an as yet unidentified type of nerve.

**TNS-induced mechanical responses of preparations in which the submucosal or longitudinal muscle layers had been removed**

In smooth muscle preparations with the submucosal layer removed, TNS inhibited the generation of LPC (Fig. 8A). The TNS-induced inhibition was attenuated by adding L-NA to the superfusate (Fig. 8B). In the presence of L-NA, additional application of neostigmine increased the frequency of spontaneously generated LPC, and TNS produced an excitatory response with
an elevated resting tension (Fig. 8C). Additional application of atropine reduced both the amplitude and frequency of spontaneous LPC, and also reduced the amplitude of the LPC during TNS (Fig. 8D). These results indicate that preparations with no submucosal layer receive nitrergic and cholinergic innervation, and excitation of these nerves elicited the inhibition and facilitation of LPC generation, respectively.

The modulation of SPC generation during TNS was also examined in preparations in which the longitudinal muscle layer had been removed. In preparations without a longitudinal muscle layer, TNS elicited an inhibitory response, which was characterized by a reduced amplitude of SPC with no marked change in frequency (Fig. 8E). The TNS-induced inhibitory response was antagonized by L-NA (Fig. 8F), and was further changed to an excitatory response by co-application of neostigmine (Fig. 8G). Finally, co-application of atropine reduced the amplitude of the SPC and abolished the TNS-induced responses (Fig. 8H). Thus, distribution of cholinergic excitatory and nitrergic inhibitory innervation was again confirmed in preparations with no longitudinal muscle layer. The stimulation of these nerves modulated the amplitude, but not the frequency, of SPC.

**Mechanical responses produced by exogenously applied acetylcholine and sodium nitroprusside**

The effects of exogenously applied acetylcholine (ACh) and sodium nitroprusside (SNP), as cholinomimetic and nitromimetic agents respectively, were investigated on the mechanical responses of circular smooth muscle preparations isolated from the rat P-region. In intact preparations, application of ACh (10^{-6} M) increased both the amplitude and frequency of phasic contractions; the frequency of LPC increased with no marked change in amplitude, while the SPC increased in amplitude only, but mainly in the initial 1.5–2 min (Fig. 9A). The results of applying increasing concentrations of ACh (10^{-8}–10^{-5} M) are summarized in Figs. 9B and C, respectively. There was an increase in the frequency of LPC at higher concentrations of ACh (>10^{-6} M), with no marked change in the amplitude, while at these concentrations the amplitude of the SPC increased, with no significant change in frequency. In three of the ten experimental preparations, both the amplitude and frequency of the LPC were increased by high concentrations (>10^{-6} M) of ACh, while in the remainder there was only an increase in frequency of LPC. In the latter preparations, the amplitude of SPC was increased by high concentrations (>10^{-6} M) of ACh, with no significant change in the frequency.

In intact preparations, stimulation of the smooth muscle with increasing concentrations of SNP (10^{-9}–10^{-6} M) resulted in a concentration-dependent decrease in amplitude of both LPC and SPC (Fig. 10E). The amplitude of LPC tended to decrease at SNP concentrations above 10^{-8} M, and all had disappeared at higher concentrations (>10^{-7} M) of SNP (Fig. 10E). The amplitude of SPC was also decreased by SNP at concentrations higher than 10^{-7} M, and at 10^{-6} M SNP the amplitude was greatly reduced but could still be recognized (Fig. 10D). The frequency of LPC was reduced by SNP at concentrations above 10^{-8} M and was abolished above 10^{-7} M, while the frequency of SPC was not significantly changed by SNP up to 10^{-6} M (Fig. 10F). Thus SNP inhibits the amplitude of both LPC and SPC, with a decreased frequency for LPC but not for SPC.
Discussion

The present experiments revealed that circular smooth muscle preparations isolated from the proximal colon (P-region) of the rat were spontaneously active with the generation of two types of phasic contraction (LPC and SPC), and that application of transmural nerve stimulation (TNS) elicited inhibition of these phasic contractions. The TNS-induced inhibition was markedly attenuated by Nω-nitro-L-arginine (L-NA), suggesting that the main transmitter substance responsible for the inhibition was nitric oxide (NO). TNS applied in the presence of L-NA did not show clear atropine-sensitive components, and it required an inhibition of acetylcholine esterase activity by neostigmine to visualize them, suggesting a low density of cholinergic innervation in the P-region. Thus, nitricergic inhibitory innervation may be the major neural component in the P-region, which is comparable to the observation that colonic smooth muscle is mainly innervated by nitricergic inhibitory nerves in animals such as the dog (Nahar et al., 1996; Keef et al., 2002), guinea-pig (Rae and Muir, 1996) and rat (Plujà et al., 1999).

Recording the mechanical responses of preparations in which either the longitudinal muscle layer or submucosal layer had been removed, revealed that the pacemaker cells producing both
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LPC and SPC were distributed in the myenteric and submucosal layers, respectively. In laboratory animals including rats, the P-region has ICC-MY distributed in the myenteric layer and ICC-SM distributed in the submucosal layer (Kobayashi et al., 1995; Kobayashi et al., 1996; Nahar et al., 1998; Plujà et al., 2001). Generation of two types of rhythmic activity has been reported in smooth muscle isolated from the proximal colon (Smith et al., 1987a, 1987b; Plujà et al., 2001). Thus, it is considered that LPC and SPC are produced by ICC-MY and ICC-SM respectively. LPC occur 1–2 times/min, while SPC occur 10–12 times/min, with the amplitude of LPC being much larger than that of SPC. These parameters are comparable to those observed in the guinea-pig (Kobayashi et al., 1996) and mouse (Lyster et al., 1992; Lyster et al., 1995; Yoneda et al., 2004), but differ from those in the dog which has a much higher frequency activity in pacemaker cells distributed in the myenteric layer than in those of the submucosal layer (Smith et al., 1987a, 1987b).

Comparison of the frequency of LPC and SPC in the different smooth muscle preparations indicated that the frequency of each type of phasic contraction was identical, either in preparations containing both types of pacemaker cells or in preparations with only one type of pacemaker cell. This suggests that the activity of the pacemaker cells for LPC and those for SPC are independent and that they do not influence each other. It is reasonable to consider that

**Fig. 10.** Effects of sodium nitroprusside (SNP) on phasic contractions of intact circularly-cut smooth muscle preparations isolated from the rat P-region. Mechanical responses were recorded in the absence (A) and presence of SNP (B, 10^{-8} M; C, 10^{-7} M; D, 10^{-6} M). The amplitude (E) and frequency (F) of phasic contractions (●, large phasic contractions, LPC; ○, small phasic contractions, SPC) were plotted against the concentration of SNP. Mean ± S.D. (n=10–30). *, significant difference from control (SNP=0 M) (P<0.05).
ICC-MY and ICC-SM have gap junctional connections to circular smooth muscle cells, since smooth muscle contraction can be elicited by either type of pacemaker cell. Recording the electrical responses of circular smooth muscle cells using microelectrodes indicates that ICC-MY and ICC-SM elicit different types of activity, with the former initiating bursts of spike potentials and the latter plateau-type action potentials (or slow waves) (Plujà et al., 2001). The amount of Ca\(^{2+}\) influx produced during a burst of spike potentials may be larger than that produced during generation of slow waves, and this may be causally related to the difference in the amplitude of LPC and SPC. Both types of phasic contraction were abolished by nifedipine, suggesting that these two types of electrical activity induce muscle contraction by facilitating the influx of Ca\(^{2+}\) through voltage-sensitive L-type Ca-channels.

Experiments were carried out to investigate the neural modulation of spontaneous activity in the rat P-region, by applying a train of TNS for a period of time (1–2 min). It was expected that TNS could cause a sustained stimulation of the receptors on the effector cells with a relatively constant concentration of the transmitter substances released from intramural nerves. The results indicated that in circular smooth muscle of the rat P-region, TNS produced inhibitory responses, with predominantly LPC in comparison with SPC. The inhibitory responses produced by TNS were antagonized by L-NA, suggesting that the mediator was mainly NO, as was the case in the guinea-pig (Nahar et al., 1996) or dog (Keef et al., 2002). This was supported by the evidence that the phasic contractions were inhibited by SNP, an NO donor. However, the TNS-induced reduction in amplitude of the phasic contractions was not associated with a change in frequency, for both LPC and SPC. This is probably a very important finding, as the factors which modulate the frequency of spontaneous activity may be the result of direct actions on the pacemaker cells (Suzuki et al., 2006; Tanaka et al., 2009).

There are many subtypes of ICC distributed in gastrointestinal preparations (Sanders, 1996), and some types of ICC have a role in the mediation of signal transmission from nerves to smooth muscle cells (Ward and Sanders, 2001). Histological observation in the gastrointestinal tract indicates that functional nerve terminals in the form of varicosities are in close contact with ICC-IM or ICC-DMP but not with ICC-MY in the mouse (Komuro et al., 1999; Ward et al., 2000) and dog (Horiguchi et al., 2003). It is therefore reasonable to consider that TNS may modulate the activity of ICC, if they receive direct innervation, and that the modulated activity of ICC would be reflected in the activity of smooth muscle cells. TNS with increasing frequency of stimuli indicated that the inhibition of LPC appeared at a much lower frequency than that for SPC. This suggests that ICC-MY, but not ICC-SM, received a direct nitrergic innervation. The results also indicated that TNS modulated only the amplitude but not the frequency of the phasic contractions, which strongly suggests that both ICC-MY and ICC-SM do not receive direct innervation. It seems likely that NO released in response to intramural nitrergic nerve excitation diffuses to circular smooth muscle cells and inhibits their contractions. Exogenously applied NO, using SNP, reduced the amplitude and frequency of LPC, while it reduced the amplitude, but not the frequency of SPC. The reduction in amplitude of the phasic contractions may be caused by inhibition of the contractile mechanism in smooth muscle cells, while the change in frequency is likely to be related to the activity of pacemaker cells. Thus, it is summarized that NO inhibits the ICC-MY, the possible pacemaker cells for LPC, but not the
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ICC-SM, the possible pacemaker cells for SPC, i.e. the ICC-MY receive nitrergic innervation directly, while the ICC-SMP do not.

Cholinergic innervation appears to be very sparse in the rat P-region, as it requires inhibition of cholinesterase activity with neostigmine to visualize a TNS-induced cholinergic component. However, the frequency of both LPC and SPC was increased by neostigmine alone, in an atropine-sensitive manner. These results suggest that the spontaneous release of ACh from cholinergic nerves is extremely high in P-region, or alternatively, that neostigmine itself has a stimulating action on muscarinic receptors in this tissue. Either way, TNS applied in the presence of neostigmine and L-NA increased the amplitude of phasic contractions, with no marked change in their frequency. Stimulation of muscle with exogenously applied ACh indicated that the frequency of LPC was increased by much lower concentrations of ACh than that of SPC. Thus, the frequency of LPC, but not of SPC, was increased by stimulation of muscarinic receptors. Collectively, the results again suggest that the activity of ICC-MY, but not that of ICC-SM, can be modulated by cholinergic nerves.

Distribution of non-adrenergic, non-cholinergic and non-nitrergic (NANCNN) nerves has been reported in many types of smooth muscle including that of the colon (Hoyle and Burnstock, 1989). Apamin inhibits the inhibitory junction potentials evoked in response to stimulation of NANCNN nerves in gastrointestinal preparations of the guinea-pig (Shuba and Vladinivola, 1980; Komori and Suzuki, 1986), while suramin can block the hyperpolarization produced by purinergic agonists (Dunn and Blakeley, 1988), and also the inhibitory junction potentials evoked in both the stomach of the guinea-pig (Xue et al., 1998) and the rat (Xue et al., 1996; Xue et al., 1999), as well as the mouse vas deferens (Dunn and Blakeley, 1988). These results suggest that the transmitter substance responsible for the NANCNN inhibitory junction potentials is ATP (Xue et al., 1999; Zizzo et al., 2007; McDonnell et al., 2008). The present experiments showed that in the rat proximal colon, TNS-induced inhibitory responses generated in the presence of atropine and L-NA showed properties comparable to the responses produced by stimulation of NANCNN nerves. However, the responses were not markedly attenuated by apamin or suramin. Excitation of NANCNN nerves for a period of time with a high frequency often evokes a phasic contraction at the cessation of stimulation (Hata et al., 2000), which may be comparable to the off-responses evoked in the rat P-region. Neither of these agents significantly altered either the inhibitory responses or the off-responses, indicating a distribution of unidentified inhibitory nerves in the rat P-region. On the other hand, the NANCNN excitatory responses were inhibited by capsaicin, suggesting an involvement of peptidergic nerves. The excitatory responses appeared successively during sustained TNS, and although the present experiments could not identify these to be due to NANCNN excitatory nerves, their properties are comparable to the substance P neurons observed in gastrointestinal preparations of the guinea-pig (Hoyle and Burnstock, 1989).

It is summarized that in the proximal region of the rat colon, the circular smooth muscle produces two types of contractions periodically, with large phasic contractions (LPC) which are elicited by pacemaker cells distributed in the myenteric layer and small phasic contractions (SPC) which are elicited by pacemaker cells distributed in the submucosal layer. The absence of co-generation of LPC and SPC suggests that direct connection between these two types of
pacing cells may be weak. In response to TNS, the LPC are easily abolished while the SPC are reduced in amplitude with no marked change in frequency. These inhibitory responses are sensitive to L-NA, suggesting that the main contributor is nitricergic nerves. Excitation of cholinergic nerves elicits excitatory responses (increased amplitude and frequency of LPC) that are inhibited by atropine. However, the density of cholinergic innervation seems to be very low, and requires inhibition of cholinesterase activity to visualize cholinergic responses. There is a distribution of non-adrenergic, non-cholinergic and non-nitricergic inhibitory and excitatory nerves in the P-region of the rat; with the excitatory responses produced by capsaicin-sensitive peptidergic nerves, and the inhibitory responses produced by as yet unidentified nerves.

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