

ASCENDING EXCITATORY AND INHIBITORY MOTOR ACTIVITY OF COLONIC LONGITUDINAL AND CIRCULAR MUSCLES IN RAT MODEL

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Summary

In this experiment we studied the role of excitatory and inhibitory neurotransmissions in the ascending reflex pathways in isolated rat colon. Partitioned organ bath, electrical field stimulation (EFS), drugs and isolated preparations were used to evaluate motor activity of (LM) and circular muscles (CM). Ascending motor responses of LM and CM were frequency-dependent contraction, significantly more expressed in LM. Atropine (0.3 μ M) decreased ascending contractions of LM. During atropine treatment spantide (0.1 μ M) further suppressed ascending contractile motor responses. In the presence of atropine, L-NNA (0.5 mM) restored ascending contractions of LM, while contractions were strongly depressed after addition of L-arginine (0.5 mM). Ascending response in CM, caused by atropine, consisted of an initial relaxation followed by contraction. Spantide decreased the contraction. L-NNA reduced the relaxation and significantly restored the atropine-influenced contraction, while L-arginine induced a deep relaxation of CM. The presence of ChAT, SP-containing nerve cell bodies and fibers and NADPH-diaphorase-reactive cell bodies and processes in myenteric ganglia were detected. The results indicated that nitric oxide is an important modulator of ascending cholinergic and tachykininergic excitation in colonic region of the large intestine of rats.

Key words: ascending reflex, rat colon, atropine, L-arginine, L-NNA, spantide

Introduction

Motility of the colon is an object of experimental and clinical studies because of the medical and social significance of neutrally mediated diseases of the large intestine. The motor activity of the colon occurs in isolated preparations, indicating that the nerve structures that induce the processes of movement are within the wall of the intestine [1-5]. The intrinsic pacemaker mechanisms determining the generation and spread of motor complexes of the colon are preserved in *in vitro* conditions, despite the fact that the transit along the colon *in vivo* could be impaired [6]. The colonic rhythmic contractions observed in mammalian experimental models seem to be equivalent to the high amplitude propagating contractions, characterizing the human large intestines [7].

The specific role of longitudinal muscle (LM) or circular muscle (CM) in the coordinated colonic motor activity is a matter of debate. In rat colon, the LM contracted and relaxed in a fashion reverse to that of the CM during the peristaltic reflex [8]. The polarized neural reflex in the murine large intestine could be triggered by longitudinal stretch [9], while the ascending excitatory and descending inhibitory neural pathways in guinea pig distal colon depend on sensory elements within the CM layer [10]. In rat colon, an essential role in ascending reflex contractility was attributed to LM [3]. Obviously, although that knowledge the colon motility has advanced during the last years, the autonomic regulation of colonic motility and the mechanisms underlying ascending and descending neuromuscular communications are still not fully understood [11, 12] and require further elucidation.

In this study, we examined neurally-mediated colonic motility, using segment preparations isolated from rat colon. The motor activity was pharmacologically tested with cholinergic-, tachykininergic- and nitrergic-related drugs to explicate the role of excitatory and inhibitory neurotransmissions in the ascending reflex pathways.

Materials and Methods

Animals and preparations

The study was approved by the *Bulgarian Food Safety Agency* (Protocol № 36/18.06.2015). The experiments were performed in compliance with the national (Ordinance No 15, February 03, 2006) and international laws and policies (EEC Council Directive 86/609, December 12, 1987).

The experiment was carried out on 24 male Wistar rats (body weight 272.4 ± 23.5 g), purchased from the Research and Laboratory Animal Breeding, Slivnitsa (Bulgaria). The animals were kept at a temperature of $22 \pm 2^\circ\text{C}$ and humidity of $50 \pm 10\%$. They were fed with normal pelleted diet and water *ad libitum* was given. The rats were fasted overnight, stunned by a blow on the neck and decapitated. The abdominal cavity was opened and the distal part of the large intestine was removed. A 55-60 mm long section of colon with intact nerve plexuses and smooth muscle layers integrity was isolated

[13] and mounted horizontally in partitioned organ bath.

Experimental equipment

Partitioned organ bath for studying the peristaltic reflexes in isolated intestinal preparations was used [14]. The spontaneous or electrically-induced motor activity of LM and CM belonging to the proximal part of the colonic segment were *synchronously* registered with strain gauges after preloading of preparations in longitudinal and circular axis equivalent to 10 mN.

Electric field stimulation (EFS) with the following characteristics: 0.8 ms, 40 V, 2 or 5 or 10 Hz, 20 s was applied by two platinum electrodes (0.45 mm thick), placed diametrically opposite each other and 14 mm apart along the sides of organ baths at an interval of at least 5 min [14, 15].

The mechanographic on-line recording techniques were completed by strain gauges and amplifiers (Microtechna, Prague, Czech Republic), stimulators (Experimetria, Budapest, Hungary) and recorders TZ 4620 (Laboratorni pristroje, Prague, Czech Republic).

Protocol of the experiment

The colonic preparations were allowed to equilibrate for a period of 30 min before starting the registration of the spontaneous activity and electrically-induced responses.

Application of EFS to the oral compartment of the organ bath elicited 'local' motor responses of LM and CM of the proximal part of the colonic segment, provoked by excitation of local nerve networks lying in the site of stimulation [13]. The EFS applied in the anal compartment of the bath induced 'ascending' motor responses of both muscle layers in the proximal part of the preparation at a distance of 20 mm away from the site of application of EFS, due to propagation of excitation via orally directed reflex pathways supplying LM and CM. The pattern and amplitude of motor responses to 5 Hz-stimulation resembled the pattern and amplitude of the spontaneous contractions of rat colon [3, 13] and were considered to be appropriate for evaluation of drug effects.

Solutions and drugs for motor activity studies

The modified Krebs solution (NaCl 120 mM, NaHCO₃ 15.4 mM, KCl 5.9 mM, CaCl₂ 2.5 mM, NaH₂PO₄ 1.2 mM, MgCl₂ 1.2 mM, and glucose 11.5 mM), continuously aerated by 95 % O₂ and 5 % CO₂ (pH 7.2) at 36.5° C was used.

The following substances were used: atropine sulfate (Merck, Germany), [D-Arg¹, D-Trp^{7,9}, Leu¹¹]-Substance P (Spantide), L-arginine (L-arg) and NG-nitro-L-arginine (L-NNA) (Sigma Chemicals, USA), tetrodotoxin (TTX, Sankyo, Switzerland). The stock solutions were prepared with distilled water and diluted to their final concentration in the Krebs solution before treatment. The stock solutions of TTX and Spantide were stored at -20° C. The drugs were added in volumes not exceeding 0.5-1.0% of the volume of compartment. The concentration and the contact time of drugs were as follows: TTX (0.1 µM, 10 min), atropine (0.3 µM, 15 min), Spantide (0.1 µM, 15 min), NG-nitro-L-arginine (0.5 mM, 15 min) and L-arginine (0.5 mM, 15 min). When the drugs were added consecutively (atropine plus Spantide or atropine plus NG-nitro-L-arginine or atropine plus L-arginine) the time course of drug action was 30 min.

Immunohistochemical and histochemical assessment

The presence of substance P in myenteric plexus of the colonic segment was tested by immunohistochemistry according to Hsu et al (1981) [16], and the presence of nitric oxide by histochemistry for nicotinamide adenine

dinucleotide phosphate (NADPH)-diaphorase – according to Sherer-Singler et al. (1983) [17].

Statistical analysis

As a baseline for measuring the amplitude (in mN) of motor events, we accepted the lowest point of the amplitudes of spontaneous high-amplitude contractions. Data were processed statistically using one-way ANOVA, followed by post hoc LSD multiple comparison test and presented as mean values±S.E.M. We assumed that the differences between studied groups were statistically significant if p-value<0.05.

Results

Spontaneous motor activity

The spontaneous motor activity of the LM and CM were presented as irregular high-amplitude contractions as seen in the longitudinal or circular axis of the colonic segment preparations. The tissue tone did not change considerably over a period of about 150-180 min. The contractions of both muscles could be considered as moving in anal direction complex with a frequency 0.52 ± 0.07 cycles per min (n=24). The amplitudes of the contractions of the LM, 10.8 ± 1.9 mN (n=24) were higher than those of the CM, 5.1 ± 0.8 mN (n=24, p<0.05) – Figure 1A). The contractions of the LM and CM occurred synchronously, with a delay in appearance of the CM contractions (Figure 1B).

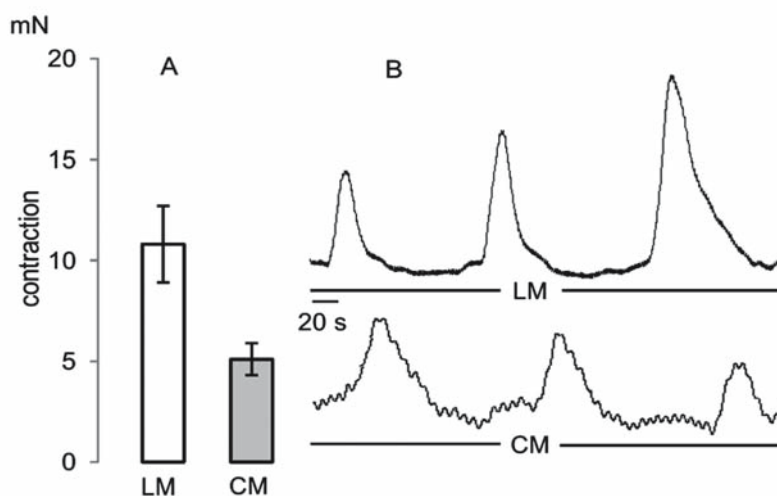


Figure 1. Typical mechanographic records showing spontaneous motor activity of smooth muscles in proximal part of isolated rat colonic segments (B) and the amplitude of contractions (A). Designations: longitudinal (LM) and circular (CM) muscles

Electrically-induced local motor responses

Local motor responses of both LM and CM were elicited by applying electrical stimulation to the proximal part of the colonic segments.

The local responses of LM were frequency-

dependent contractions, and the contractions induced by 2-Hz electrical stimulation were significantly less pronounced compared to those provoked by 5-Hz or 10-Hz stimulation (Figure 2 A, B, C).

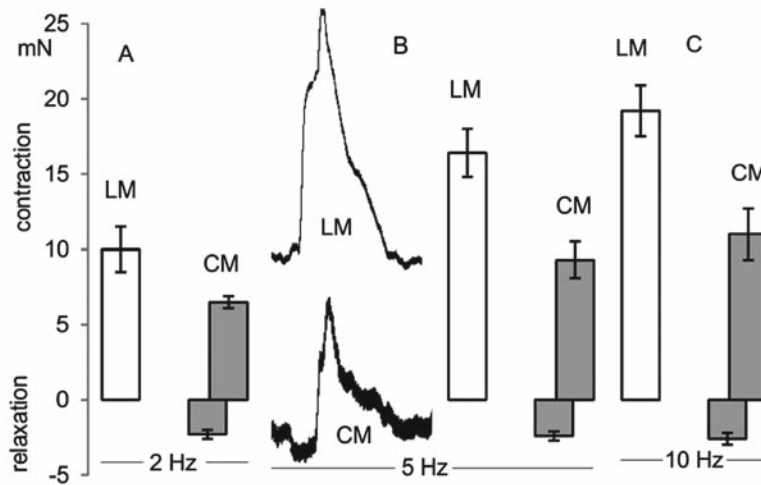


Figure 2. Local motor responses of isolated rat colonic segments to electric stimulation applied at frequencies of 2 Hz (A), 5 Hz (B) and 10 Hz (C). Designation: longitudinal (LM) and circular (CM) muscles

The responses of CM consisted of an initial relaxation followed by a contraction (Figure 2; B). The amplitudes of relaxation in the local responses of CM were not dependent on the frequency of EFS, while the contractions became markedly stronger with the increase of the frequency of the electrical stimuli (Figure 2; A, B, C).

Electrically-induced ascending motor responses

The ascending motor responses of LM and CM of the proximal part of the preparations were frequency-dependent contractions (Figure 3 A, B, C).

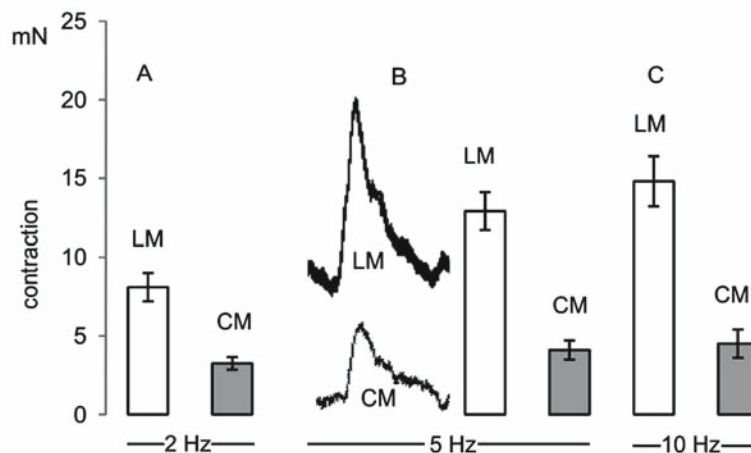


Figure 3. Ascending motor responses of isolated rat colonic segments to electric stimulation applied at frequencies of 2 Hz (A), 5 Hz (B) and 10 Hz (C). Designation: longitudinal (LM) and circular (CM) muscles

The amplitudes of the ascending contractile responses of LM were significantly higher comparing to the amplitudes of the ascending

responses of CM induced by all the frequencies of electrical stimulation used (at 5 Hz-stimulation, LM, 12.9 ± 1.2 mN and CM, 4.1 ± 0.6 mN, $n=12$,

$p < 0.05$) – Figure 3; B.

The amplitudes of the ascending contractions were considerably less expressed, as compared to the local contractile responses in both LM and CM.

Drug effects on the electrically-elicited ascending motor responses

Atropine ($0.3 \mu\text{M}$) decreased the ascending contractions of LM elicited by EFS, applied at a frequency of 5 Hz ($5.40 \pm 0.60 \text{ mN}$, $n=10$,

$p < 0.05$ vs. control). The addition of NK1 antagonist spantide ($0.1 \mu\text{M}$) on the background of atropine further suppressed the amplitudes of the ascending contractile motor responses ($3.2 \pm 0.3 \text{ mN}$, $n=10$, $p < 0.05$). In the presence of atropine, L-NNA (0.5 mM) restored to a great extent the ascending contractions of LM, while the amplitudes of contractions were strongly decreased after adding L-Arg (0.5 mM) – Figure 4.

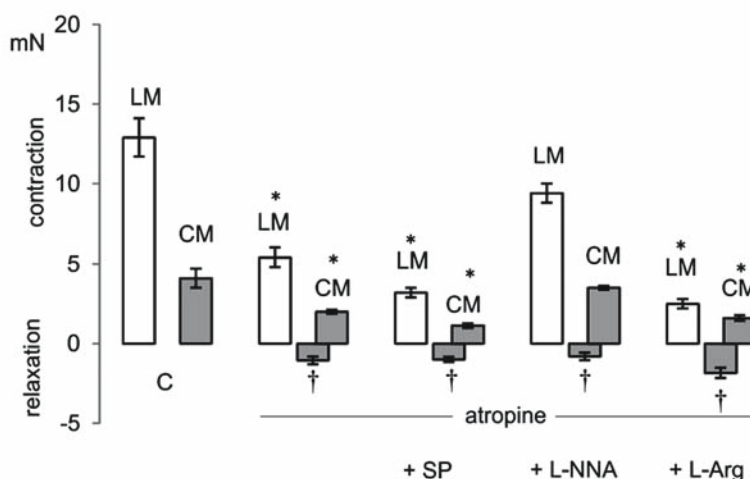


Figure 4. Ascending motor responses of longitudinal and circular muscles in isolated rat colonic segments to electric stimulation applied at frequencies of 5 Hz. Designation: control (C) and in the presence of atropine, atropine plus spantide (Sp), atropine plus L-NNA, atropine plus L-arginine. The values represent means \pm S.E.M. of at least eight experiments. Designation: longitudinal (LM), circular (CM) muscles; significant differences at $p < 0.05$ – (*) vs. controls, (†) opposite effects vs. controls.

In the presence of atropine ($0.3 \mu\text{M}$), the ascending responses in CM of proximal part of the colonic preparation induced by electrical stimulation of 5 Hz converted from contractions to responses, consisting of a relaxation ($1.05 \pm 0.12 \text{ mN}$, $n=12$) followed by contraction ($2.0 \pm 0.24 \text{ mN}$, $n=12$, $p < 0.05$ vs. ascending contractile responses). Spantide ($0.3 \mu\text{M}$), added on the background of atropine decreased the contraction to $1.12 \pm 0.16 \text{ mN}$ ($n=8$) without changing the relaxation. L-NNA (0.5 mM), a competitive inhibitor of nitric oxide synthase slightly reduced the amplitude of relaxation and restored the atropine-influenced contraction ($3.5 \pm 0.24 \text{ mN}$, $n=8$, $p < 0.05$). L-Arg (0.5 mM) deepened the relaxation in atropine-pretreated colonic preparations (Figure 4).

Effect of TTX

The addition of TTX ($0.1 \mu\text{M}$) for 10 min to oral or anal part of colon segments prevented the local and ascending motor responses of LM and CM, respectively.

Immunohistochemical and histochemical testing

The immunohistochemical studies showed the presence of SP-immunostained cell bodies and nerve fibers, which ran between the myenteric ganglia and the muscle layers (Figure 5 A, B).

NADPH-diaphorase-reactive multipolar nerve cell bodies and their processes outlined myenteric ganglia. NADPH-diaphorase-positive neurons and numerous varicose fibers in the ganglia and internodal strands were seen (Figure 5 C, D).

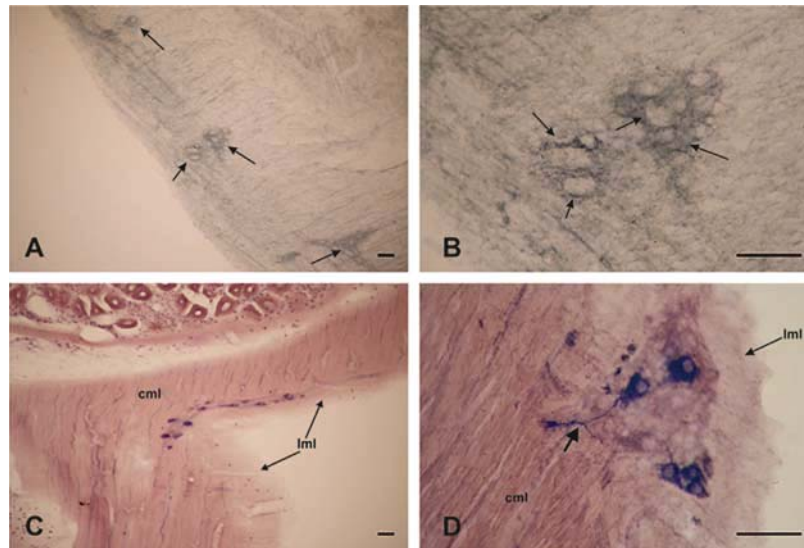


Figure 5. Photomicrographs illustrating substance P immunohistochemistry (A, B) and NADPH-d histochemistry (C, D) in proximal part of colonic preparation. A – SP-immunoreactive nerve fibers outlined myenteric ganglia (arrows) in proximal part of the colon. B – Detail from A. SP-immunonegative neurons are outlined (arrows) by immunopositive varicose nerve fibers. C – NADPH-diaphorase-positive nerve cell bodies in the myenteric ganglia and nerve fibers in the internodal strands. D – Myenteric ganglion containing NADPH-d-positive and negative neurons in the proximal part of colon. Large arrow points to varicose and branching positive axon in the myenteric ganglion. Scale bar=50 μ m

Discussion

This study evaluated motor responses in gut preparations isolated from rat colon. According to Dalziel et al. (2014) [18] segments of intact rat large intestine, and hence intact enteric neural circuitry, provide an ideal *in vitro* model for investigation of colonic motility. The partitioned organ bath used in this study allowed simultaneous recording of the motility of LM and CM of the segment-preparations as a display of enteric nerve-dependent functional coordination in the motor activity of both muscles.

The spontaneous high-amplitude contractions of LM and CM in isolated segments of rat proximal colon we established are in concert with the described high-amplitude contractions in rat proximal, middle and distal colon [3, 14, 19, 20]. The rhythmic high-amplitude contractions observed in the large intestine of many mammals seem to be equivalent to the propagating contractions in humans [7]. We found that the contractions of LM were significantly higher compared to contractions of CM and they appeared synchronously in both muscles layers. The fact that contractile activity was recorded in the proximal part of the segments suggests the propagation of the contractile events from the

proximal to the distal part of the rat colon. On the contrary, in the guinea pig, the distal colon is the most likely region to induce a peristaltic wave, if compared with the mid- or proximal colon [21]. Relaxations were not registered in the spontaneous motor activity of LM and CM that could be due to the lack of fecal material and filling the lumen of the segments with Krebs solution in such experimental conditions.

The application of EFS to the oral or to the distal part of the preparations elicited local or ascending motor responses of LM and CM belonging to the proximal part of the colonic segments. The effect of electrical stimulation resulted from the action of excitatory and/or inhibitory neurotransmitters, which are released as a consequence of propagation of action potential along the nerve terminals [15, 22]. The motor responses of rat colonic LM and CM induced by EFS were neurogenic by nature, since they were prevented by tetrodotoxin, an inhibitor of firing the action potential in neurons. TTX blocked the motor patterns in isolated segments of the guinea-pig proximal and distal colon [5].

The electrically-induced responses, as well as spontaneous high-amplitude contractions appeared synchronously in LM and CM,

suggesting co-activation of neural circuits supplying both muscles. The increase of stimulus frequency enhanced the amplitudes of local and ascending responses without changing the motor pattern, thus suggesting that the increase of the responses corresponded to the frequency-dependent release of the same neurotransmitters. It is known that more nerve terminals are involved in the process of the neurotransmitter release when electrical stimuli are applied at higher frequencies [23].

Our experiments showed that in the rat proximal colon the local motor responses of both muscles, which were considered to result from the local excitation of the modular nerve structures [24] were different. Comparison with the other tubular smooth muscle organs shows that a wide variety of motor patterns occurs in the large intestine. This variety suggests a relatively autonomic nervous system in the gut wall [25]. Although the stimulatory effects of pelvic nerve stimulation and inhibitory effects of inferior mesenteric ganglia could not be excluded from the regulation of colonic motility (guinea-pig distal colon [11], the neural mechanisms underlying the colonic motor patterns have now been identified to lie within the myenteric plexus and do not require inputs from the mucosa or submucosal ganglia for their generation [25]. We found that under local electrical stimulation the LM responded with contraction, while the response of CM consisted of an initial relaxation followed by contraction. The relaxation could be due to the prevalence of inhibitory innervation serving the CM of the rat colon. The neurally-evoked release of the inhibitory neurotransmitter nitric oxide in human colon is greater in CM than in LM [26].

We noticed that the electrically-activated ascending motor responses of LM and CM in rat colonic segments manifested as contractions. Similarly to both high-amplitude contractions observed in the spontaneous motor activity and contractile components of the local motor responses, the ascending contractions of CM were significantly less expressed as compared to the contractions of LM, most probably demonstrating a predominant role of the LM in the colonic contractile coordination mechanisms. More recently, ascending responses, different in pattern and resulting from nerve excitation,

were reported in murine distal colon, in which nerve stimulation produced both inhibition and excitation on CM and LM [12].

The ascending electrically-induced contractions of LM and CM were decreased by atropine, a blocker of muscarinic receptors and spantide, a tachykinin receptor antagonist thus demonstrating that excitatory cholinergic and tachykininergic neurotransmissions are involved in the ascending motor reflex pathways supplying the rat proximal colon. In the presence of atropine, a relaxation in the ascending contractile responses of CM revealed. L-NNA, an inhibitor of nitric oxide synthase decreased while L-arginine, a substrate of nitric oxide synthesis increased the ascending relaxations of CM. On the background of atropine the ascending contractile responses of LM were increased by L-NNA and were decreased by L-arginine suggesting that nitrergic neurotransmission plays role in ascending responses of both muscles of rat colon. The persistence of relaxing response in the presence of L-NNA confirms the existence and participation of other inhibitory neurotransmitters, in addition to nitric oxide effect on motor responses of CM [12]. Relaxation was not observed in the ascending LM responses when the ascending excitatory cholinergic and tachykininergic neurotransmissions were inhibited. This is most likely due to ascending nitric oxide-dependent reflex pathways, whose function is not so well expressed in LM as compared to CM in rat colon.

Cholinergic and tachykininergic neurotransmissions have been widely recognized as playing a key role in neural circuits, serving the motor events of the large intestine of several mammals, including colonic region of mouse [27] and guinea pig [28]. More recent observations have demonstrated that opioidergic [18] and GABAergic [29] neurotransmissions could modulate the motility of the colon, too. We found that nitric oxide-related drugs affected the ascending motor responses of the colonic LM and CM. Despite the assumption that nitric oxide is a principal neurotransmitter in descending inhibitory motor pathways [30, 31], our results indicated that nitric oxide is an important modulator of ascending cholinergic and tachykininergic excitation in colonic region of the large intestine of rats.

Conclusions

The partitioned organ bath used in this study allowed registration of excitatory and inhibitory activity of local as well as ascending reflex motor responses of LM and CM in the proximal part of colonic region in a rat experimental model. Cholinergic and tachykininergic neuronal and neuromuscular communications provided contractile responses of both muscles. A dominant contractility of LM was observed, suggesting an essential role in coordinated contractile processes. Nitrgergic mechanisms are involved in modulation of ascending cholinergic and tachykininergic excitation but nitric oxide-dependent reflex pathways supply rather CM than LM. The presence of Substance P- and NADPH-diaphorase-positive nerve structures proved the physiological significance of substance P and nitric oxide in nerve pathways in the rat colonic region.

Acknowledgments

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