Archives of the Balkan Medical Union
vol. 53, no. 1, pp. 14-22
March 2018

ORIGINAL PAPER

NEUROTRANSMITTER IMPLICATIONS IN DESCENDING MOTILITY OF LONGITUDINAL AND CIRCULAR MUSCLES IN RAT COLON

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Abstract

Introduction. The role of neurotransmitter systems in the motor activity of longitudinal or circular muscles in autonomic regulation of the motility of the colon by the nervous system is unclear.
The aim of the study was to investigate the neurotransmitter implications in descending motility of longitudinal and circular muscles in rat colon.
Methods. Electrically-induced (2, 5 or 10 Hz, 0.8 ms, 40 V, 20 s) local or descending motor responses of longitudinal and circular muscles in isolated preparations and drugs were used to define the neurotransmitters' role in colonic motility.
Results. The spontaneous activity of the distal part of preparations manifested as high-amplitude irregular contractions more expressed in the longitudinal muscles. The electrically-induced local responses differed considerably in the two muscles: in longitudinal muscle there were frequency-dependent contractions, while initial relaxation followed by contraction was observed in circular muscle. The descending motor response resembled the pattern of the local responses, but the amplitudes were significantly less expressed, as compared to the respective local responses. In

Résumé

Les implications des neurotransmetteurs dans la motilité descendante des muscles longitudinaux et circulaires dans le côlon du rat

Introduction. Le rôle des systèmes de neurotransmetteurs dans l'activité motrice des muscles longitudinaux ou circulaires dans la régulation autonome de la motilité du côlon par le système nerveux n'est pas clair.
Le but de l'étude est constitué par les implications des neurotransmetteurs dans la motilité descendante de ces mêmes muscles dans le côlon.
Méthodes. Des réponses motrices induites électriquement (2, 5 ou 10 Hz, 0,8 ms, 40 V, 20 s) locales ou descendantes de ces muscles dans des préparations isolées et des médicaments ont été utilisées pour définir le rôle des neurotransmetteurs dans la motilité colique.
Résultats. L'activité spontanée de la partie distale des préparations s'est manifestée par des contractions irrégulières de grande amplitude exprimées davantage dans les muscles longitudinaux. Les réponses locales induites électriquement différaient considérablement dans les deux muscles: le longitudinal, des contractions dépendantes de la fréquence, tandis qu'une relaxation initiale

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atropine-pretreated (0.3 μM) preparations, the contractions of longitudinal and circular muscle significantly decreased, and initial relaxation preceded the contractions of the longitudinal muscle. In the presence of atropine, spantide (0.1 μM) reduced more noticeably the contractile components of responses in both muscles. L-NNA (0.5 mM) restored to a great extent the descending contractions of the longitudinal and circular muscles, while L-Arginine (0.5 mM) strongly depressed the contractions and more significantly increased the relaxation in circular muscle.

Conclusions. The present results suggest the implication of both cholinergic and tachykininergic systems as excitatory neurotransmissions in the colonic descending reflex pathways and the involvement of nitrergic neurotransmitter systems in the descending inhibitory neuromuscular signalization predominantly serving the circular muscle.

Key words: atropine, L-arginine, NG-nitro-L-arginine, spantide, descending reflex pathways, rat colon.

Abbreviations:
CM – circular muscle
EFS –electrical field stimulation
L-NNA – NG-nitro-L-arginine
LM –longitudinal muscle
NO – nitric oxide

suivie d'une contraction était observée dans le muscle circulaire. La réponse motrice descendante ressemblait au schéma des réponses locales, mais les amplitudes étaient significativement moins exprimées, par rapport aux réponses locales respectives. Dans les préparations prétraitées à l'atropine (0.3 μM), les contractions des muscles longitudinaux et circulaires ont significativement diminué, et la relaxation initiale a précédé les contractions du muscle longitudinal. En présence d'atropine, la spantide (0.1 μM) a réduit plus sensiblement les composantes contractiles des réponses dans les deux muscles. L-NNA (0.5 mM) a rétabli largement les contractions descendantes des muscles longitudinaux et circulaires, tandis que la L-Arginine (0.5 mM) a fortement diminué les contractions et augmenté plus significativement la relaxation dans le muscle circulaire.

Conclusions. Les présents résultats suggèrent l'implication des systèmes cholinergiques et tachykininergiques comme neurotransmissions excitatrices dans les voies réflexes. descendantes du côlon et l'implication des systèmes de neurotransmetteurs nitrergiques dans la signalisation neuromusculaire inhibitrice descendante desservant principalement le muscle circulaire.

Mots-clés: atropine, L-arginine, NG-nitro-L-arginine, spantide, voies réflexes descendantes, côlon .

Abréviations:
CM – muscle circulaire
EFS – stimulation du champ électrique
L-NNA – NG-nitro-L-arginine
LM – muscle longitudinal
NO – oxyde nitrique

Introduction

The large intestine in humans is subject to various injuries such as tumor proliferation, pre- and postoperative traumas or inflammatory and neurodegenerative processes that result in motility disorganization. In recent years methodological advances have demonstrated that the colonic motor activity occurs in isolated preparations, thus indicating that motility-mediating autonomic nerve structures are contained in the gut wall[1-5]. The intrinsic pacemaker mechanisms, which are responsible for generation and propagation of the colonic motor complexes, are preserved in *in vitro* condition even in cases when the transit along the colon *in vivo* has been impaired[6].

The role of autonomic neurotransmitters involved in colonic ascending and descending reflex motility of the longitudinal (LM) and circular (CM) muscles remains incompletely understood[7] and requires further studies. The elucidation of the expression and function of the major neurotransmitters, which determine neuronal excitability within the elucidation of the participation of major neurotransmitters in the function of the enteric nerve system, could give a new therapeutic option for gastrointestinal disorders[8].

In the present study, we were particularly focused on evaluating the neurotransmitter implications in the descending motility of colonic LM and CM. To elucidate the role of excitatory and inhibitory neurotransmissions in the descending reflex pathways, the electrically-induced descending motor responses were tested by cholinergic-, tachykininergic- and nitrergic-related substances.

Materials and methods

The experiments were approved by the responsible national agency (Protocol No 36/18.06.2015) and conducted in accordance with the national laws

and policies and in conformity with the international guidelines.

Animals, preparations and experimental equipment

Male Wistar rats (n=24) weighing 278.5±17.6 g were kept at optimal temperature (22±2°C) and humidity (50±10%), given normal pelleted diet and water *ad libitum*. The animals were starved overnight, stunned by a blow on the neck and decapitated. Segments of distal colon with intact smooth muscle layers and nerve plexuses integrity, 55-60 mm in length were isolated and placed horizontally in partitioned organ bath[1]. The motor activity of LM and CM in the distal part of the preparations were simultaneously recorded by using strain gauges at an initial tension in longitudinal or circular axis equivalent to 10 mN[9].

Electrical field stimulation (EFS)[10] at intervals not less than 5 min (0.8 ms, 40 V, 2 or 5 or 10 Hz, 20 s)[9] was used to stimulate nerve structures of the colonic segments. The mechanographic on-line recordings were done using strain gauges and amplifiers (Microtechna, Prague, Czech Republic), stimulators (Experimetria, Budapest, Hungary) and TZ 4620 recorders (Laboratorni pristroje, Prague, Czech Republic).

Protocol design

The application of electrical stimulation to the anal compartment of the organ bath induced local motor responses of both muscle layers of distal part of the preparations, due to the excitation of local nerve networks in the site of stimulation[1]. EFS applied in the oral section of the bath induced motor descending motor activity of both muscles in the anal part of the colonic segments at a distance of 20 mm, due to spreading excitation via the distally directed reflex pathways. The pattern and amplitude of the motor responses to 5 Hz-stimulation were similar to the spontaneous high-amplitude contractions of rat colon and were considered suitable for evaluating the drug effects[1,3].

The composition of the modified Krebs solution was as follows (mM): NaCl 120, KCl 5.9, $NaHCO_3$ 15.4, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.5 and glucose 11.5. The solution was continuously aerated by 95 % O_2 and 5 % CO_2 (pH 7.2) at 36.5° C. Drugs used included: atropine sulphate (Merck, Darmstadt, Germany), [D-Arg[1], D-Trp[7,9], Leu[11]]-Substance P (Spantide), NG-nitro-L-arginine (L-NNA) and L-arginine (Sigma Chemicals, St. Louis, MO, USA). The volumes of added drug solutions were 0.5-1% of the compartment volume.

The timing of drug treatment and concentration of drugs were as follows: atropine (0.3 μM, 15 min), spantide (0.1 μM, 15 min), L-NNA (0.5 mM, 15 min) and L-arginine (0.5 mM, 15 min).

Immunohistochemical and histochemical tests

The presence and distribution of substance P and nitric oxide in neuronal structures of the myenteric plexus of the colonic preparations were tested by immunohistochemistry for SP[11] and NADPH-diaphorase histochemistry[12]. The following substances were used for immunohistochemistry and NADPH-diaphorase histochemistry: mouse anti-rat ATP Synthase, LifeSpan BioSciences Inc., Seattle, WA, USA; biotinylated goat anti-mouse IgG (LS Bio; 1:250); avidin-biotin complexes (Vector Labs, Burlingame, CA, USA); reduced β-NADP (Sigma, USA); nitroblue tetrazolium (NBT, Sigma, USA). Reichert Jung freezing microtome (Austria), light microscope Jenaval (Germany) and light microscope with digital camera Nikon (Japan) were used for morphological assessment.

Statistical analysis

The lowest point of the amplitudes of spontaneous high-amplitude contractions was assessed as a baseline for measuring the amplitude of the spontaneous motor activity and the contraction/relaxation events, characterizing the electrically-elicited motor responses as force in mN. Data are presented as mean values ± S.E.M. Statistical significance was evaluated by one-way ANOVA LSD post-hoc test at $p<0.05$. All analyses were performed with SPSS 14 statistical software.

Results

The spontaneous contractions of LM and CM could be considered as a motor complex moving towards the anal part at a frequency of 0.52±0.07 cycles per min. The amplitudes of LM contractions were higher than those of CM (9.9±2.8 mN and 5.5±1.5 mN; n=20, $p<0.05$) (Figure 1 A). The spontaneous motor activity of the anal part of the colonic segments represented irregular high-amplitude contractions that occurred at the same time in both muscle layers (Figure 1 B).

The local responses of LM were frequency-dependent contractions. The amplitudes of responses induced by 2-Hz EFS were lower as compared to the contractions elicited by EFS, applied at frequencies of 5 Hz or 10 Hz (n=10, $p<0.05$) (Figure 2 A, B, C). The contractile responses of CM were preceded by a relaxation. The amplitudes of relaxation in the local responses of CM were not dependent on the frequency of EFS (-2.42±0.3 mN at 2 Hz-stimulation vs. –2.58±0.4 mN at 10-Hz stimulation, n=10,

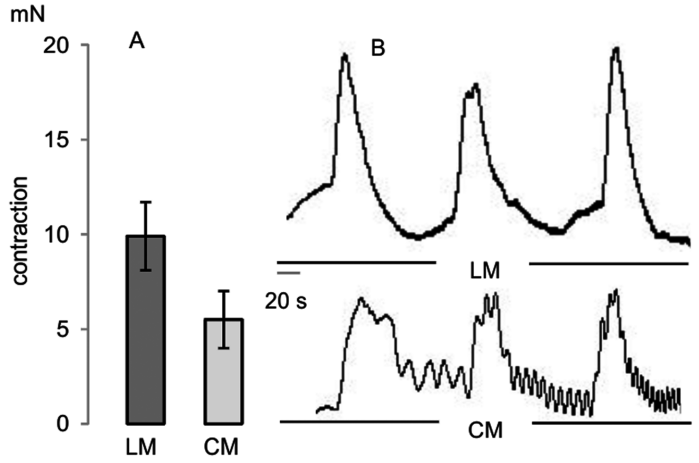

Figure 1. Amplitudes of contractions (A) and mechanographic records of spontaneous motor activity (B) of longitudinal and circular muscles in descending part of isolated segments from rat distal colon. The values represent means ± S.E.M.; n=20. Designations: circular (CM) and longitudinal (LM) muscles.

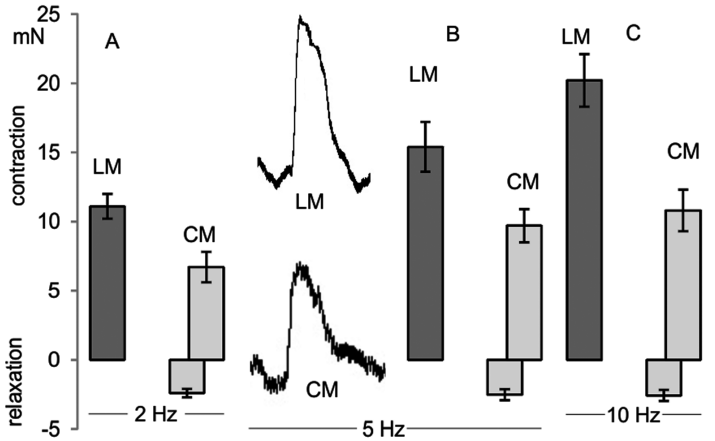

Figure 2. Local motor responses in segments isolated from rat distal colon to electrical stimulation applied at frequencies of 2 Hz (A), 5 Hz (B) and 10 Hz (C). The values represent means ± S.E.M; n=10. Designation: circular (CM) and longitudinal (LM) muscles.

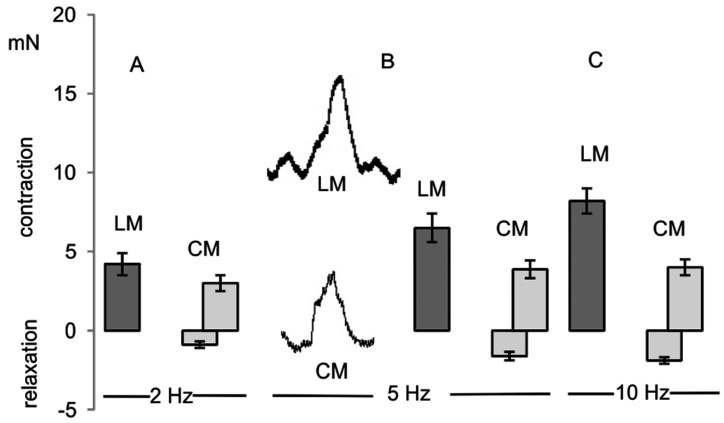

Figure 3. Descending motor responses in segments isolated from rat distal colon to electrical stimulation applied at frequencies of 2 Hz (A), 5 Hz (B) and 10 Hz (C). The values represent means ± S.E.M; n=10. Designation: circular (CM) and longitudinal (LM) muscles.

p>0.05), while the contractions progressively increased depending on the frequency of EFS (Figure 2 A, B, C).

EFS applied to the oral part of the colonic segments caused synchronous descending responses of LM and CM in the anal part of the preparations. The contractions of LM were frequency-dependent (Figure 3 A, B, C). The descending responses of CM consisted of an initial relaxation followed by a contraction (Figure 3 A, B, C). Both of these responses depended on the frequency of EFS and were significantly different at 2 Hz *vs.* 10-Hz stimulation (n=10, $p<0.05$). The amplitudes of the descending contractions of LM were significantly higher as compared to those of CM-induced by all frequencies of EFS (n=10, $p < 0.05$) (Figure 3).

The descending responses in both LM and CM were considerably less expressed by amplitude as compared to the respective local responses (Figure 2 and Figure 3).

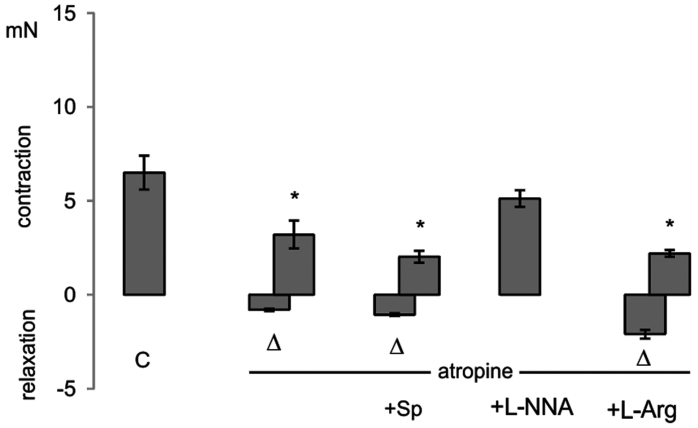

Figure 4. Drugs effect on descending motor responses of longitudinal muscle in segments isolated from rat distal colon to electrical stimulation applied at frequencies of 5 Hz. The values represent means ± S.E.M. of at least eight experiments. Designation: control (C), L-arginine (L-arg), NG-nitro-L-arginine (L-NNA), spantide (Sp), (*) – significant differences at p<0.05 *vs*. controls, (Δ) opposite effects *vs*. controls.

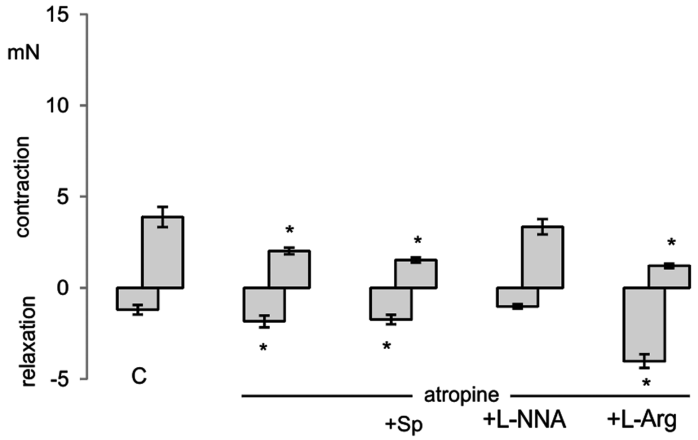

Figure 5. Drugs effect on descending motor responses of circular muscle in segments isolated from rat distal colon to electrical stimulation applied at frequencies of 5 Hz. The values represent means ± S.E.M. of at least eight experiments. Designation: control (C), L-arginine (L-arg), NG-nitro-L-arginine (L-NNA), spantide (Sp), (*) – significant differences at p<0.05 *vs*. controls.

Atropine (0.3 μM) converted the descending contractile responses of LM induced by 5-Hz-EFS to responses consisting of an initial relaxation followed by a contraction that was considerably reduced as compared to those in segments not treated with atropine (3.3±0.7 mN, n=10, p<0.05). On the background of atropine, spantide (0.1 μM) augmented the relaxation and further inhibited the ascending contractions (2.1±0.3 mN, n=8, p<0.05). In the presence of atropine, L-NNA (0.5 mM) abolished the relaxation and restored to a great extent the descending contractions of LM. L-Arginine (0.5 mM) significantly deepened the relaxations to –2.2±0.2 mN (n=8) and depressed the descending contractions to 2.2±0.2 mN (n=8, p<0.05) (Figure 4).

When atropine was added to Krebs solution, there was an increased initial relaxation (-1.8±0.3 mN, n=10), followed by decreased contraction (2.0±0.2 mN, n=10, p<0.05) in descending responses of CM at 5-Hz frequency EFS. Spantide (0.3 μM), added on atropine-pretreated preparations further decreased the contractions. L-NNA (0.5 mM) slightly reduced the relaxations and significantly restored the atropine-depressed contractions (3.3±0.4 mN, n=8, p< 0.05). L-Arginine (0.5 mM) induced a deep relaxation in atropine-pretreated colonic segments (4.0±0.4 mN) and inhibited the contractions to a greater extent (Figure 5).

The immunohistochemical studies revealed SP-containing nerve cell bodies and SP-immunostained nerve fibers that ran between the myenteric ganglia and the muscle layers (Figure 6 A, B). Myenteric ganglia were outlined by many NADPH-diaphorase-reactive nerve cell bodies and processes (Figure 6 C, D).

Discussion

This study evaluated the motor activity of intact segments isolated from rat colon. Partitioned organ baths were used to allow the simultaneous recording of the motor activity of LM and CM as a display of functional coordination of both these muscles in the colonic motility. Electrical stimulation was applied to excite the nerve structures.

The spontaneous motor activity of LM and CM of the anal part of the segment-preparations isolated from rat colon presented irregular high-amplitude contractions appearing synchronously in both muscles without changes in the tissue tone. These results

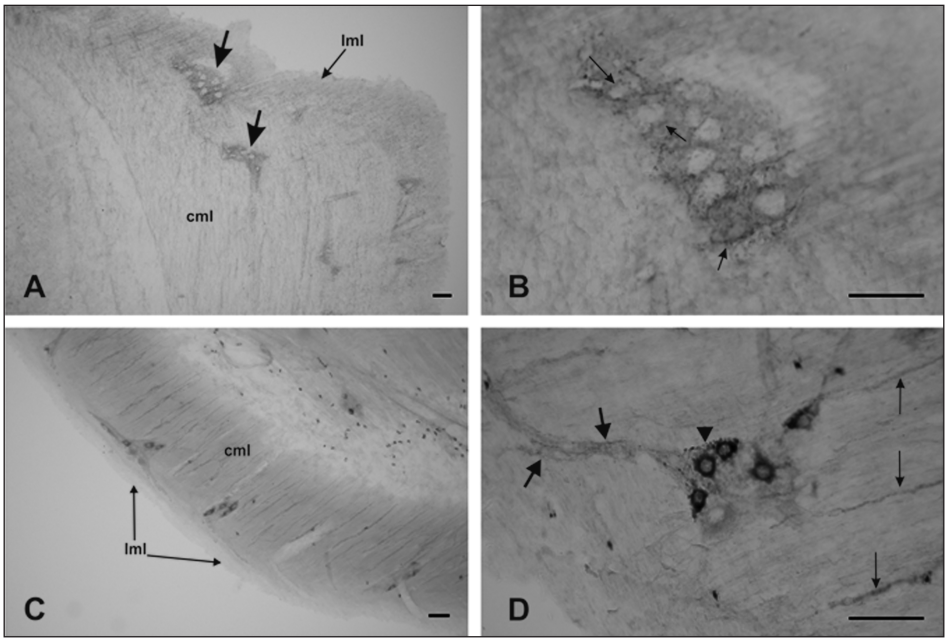

Figure 6. Substance P immunohistochemistry (A, B) and NADPH-d histochemistry (C, D) of muscle coat in descending rat colon. A – the myenteric ganglia (large arrows) are outlined by large number SP-immunoreactive nerve fibers. B – small arrows point to SP-immunopositive varicose axons around the unstained perikarya. C – general view of the myenteric plexus in the space between longitudinal muscle layer (lml) and circular muscle layer (cml). D – positive perikarya demonstrating high staining intensity in the myenteric ganglion (head arrow). Highly intensely stained varicose nerve fibers in the interganglionic bundle (small arrows). Large arrows point to varicose nerve fibers in the nerve bundles in the cml. Scale bar=50 μm.

were similar to observations characterizing the pattern of spontaneous motor activity in rat proximal and distal colon[1,3,13]. This activity seems to be typical for the rat colon, in view of the rich variety of high-amplitude contractility and colonic migrating motor complexes, occurring in mammalian large intestine[14]. We found that the contractions appeared synchronously in both muscles, and the contractions of LM were significantly higher by amplitude as compared to those of CM. It could be assumed that an essential contractility of LM in the distal part of colon similar to that described in the proximal colon[3]. The finding, that spontaneous contractile activity of both LM and CM was recorded in the anal part of the preparations, showed the existence of local pacemaker mechanisms that induced motor events. In guinea pig, the distal colon is the most likely site to elicit peristaltic waves[15]. Relaxations were not recorded in the spontaneous activity of both muscles that was most probably due to the experimental set-up, which required filling the lumen of the segments with bath nutrient solution. It seems that spontaneous rat colonic relaxations are dependent on the content of the gut lumen. More recently, it has been postulated that, in guinea pig colon, the formation and the propulsion of fecal content involve both content-independent migrating motor complexes and content-dependent mechanism[5].

EFS used in our experiments induced local or descending motor responses of LM and CM when applied either to the anal or to the oral part of the colonic segments, thus presenting action of excitatory and/or inhibitory neurotransmitters released as a consequence of propagation of action potential along the nerve terminals. Frequency-dependent increased amplitudes without changes in the pattern of the responses showed the involvement of more nerve terminals in the process of release of one and the same neurotransmitters, which is in agreement with results reported by other authors[10,16]. Both the spontaneous high-amplitude contractions and the electrically-induced motor events we observed appeared synchronously in LM and CM, indicating co-excitation of the nervous circuits in both muscle layers.

Our results showed that in the rat distal colon, the local and the descending motor responses had different patterns in LM and CM. Although stimulatory effects of pelvic nerve and inhibitory effects of inferior mesenteric ganglia were demonstrated in the regulation of colonic motility (guinea-pig distal colon) the great number of various motor patterns occurring in the large intestine was attributed to the relatively enteric nervous system[17]. The neural mechanisms underlying the colonic motility have now been identified to lie within the myenteric plexus[14]. We found that local or descending motor responses of

LM in the rat distal colon were contractions, while these of CM consisted of a relaxation followed by a contraction. The difference in response patterns of both muscles could indicate the prevalence of the inhibitory neuronal circuits in CM and predominant excitatory neurotransmissions in LM. The stimulation-induced generation of the inhibitory neurotransmitter nitric oxide in human colon is greater in CM than in LM[18]. Different by pattern motor responses were observed in murine distal colon, in which nerve stimulation produced inhibition of CM and excitation of LM[7]. Regarding amplitude, descending motor responses of LM and CM were less noticeable, as compared to the respective local responses. The latter corresponded with the view that the magnitude of electrically-evoked motor responses depends on the distance from which stimulation is applied[16]. The technical set-up used in our study made it possible to register the descending responses of LM and CM at a distance of 20 mm from the stimulation point, indicating a length of at least 20 mm of the descending reflex pathways. The patterns of the descending responses of both muscles, relaxation and contraction, indicated that inhibitory and excitatory neurotransmissions assisted the descending neuromuscular communications in the rat distal colon.

We observed that blocking the cholinergic receptors by atropine led to a considerable decrease in the amplitude of the descending contractions of LM and an initial relaxation. The contractions in the descending responses of CM were similarly suppressed, thus demonstrating an essential stimulating role of cholinergic neurotransmission in the descending motility of the rat distal colon. The elimination of the muscarinic receptors did not completely prevent the excitatory patterns in the descending motor responses in both muscles, which indicated the presence of other excitatory neurotransmitter(s) beside the cholinergic one. Spantide, a NK-1 tachykinin receptors antagonist further lowered the atropine-provoked decrease of the contractions of LM and CM, thus suggesting involvement of substance P in the descending excitatory neurotransmission in the rat distal colon. Cholinergic and tachykininergic neurotransmissions are believed to play a role in neuronal circuits that serve the motor events of the large intestine of several mammalians, including the colonic region of mouse[19] and guinea pig[20]. Recently, substance P has been proposed as an excitatory neurotransmitter of the rat recto-anal region[21]. Since the descending contractions of LM and CM were suppressed in the presence of cholinolytic and tachykininergic antagonist, the relaxations of the circular muscle were deeper and a relaxation was detected in the responses of LM, it could be suggested that elimination of the excitatory cholinergic and tachykininergic action unmasked the effects of inhibitory neurotransmission(s). More recently, purinergic[7], GABA-B receptor-induced[22] or serotoninergic[23,24] inhibition of the motility in the mammalian colon has been reported. This study demonstrated that substances, affecting nitrergic transmission influenced the descending motor activity in rat distal colon. In guinea pig distal colon, nitric oxide was shown to regulate the motor responses of the circular muscle[17] and modulated the electrically-evoked peristaltic contractions[15]. We found that, in the presence of atropine L-NNA, an inhibitor of nitric oxide synthase considerably restored the descending contractions of LM and CM and eliminated relaxation in the responses of LM, while L-Arginine, being a substrate of nitric oxide synthesis, strongly depressed the contractions in both LM and CM and significantly increased the relaxations which were much better expressed in CM. Nitric oxide has been accepted as the principal neurotransmitter of descending inhibitory motor pathways along the gut[25]. Our results indicated a role of nitric oxide as an important modulator of descending excitatory cholinergic and tachykininergic neurotransmissions in the rat colonic region. The L-NNA-provoked inhibition of the nitric oxide synthesis leads to suppression of the negative modulation of cholinergic reflex pathways to LM and CM in the guinea pig distal colon[26]. The increase of the contractions in the descending responses of both LM and CM during treatment with L-NNA on the background of atropine could be attributed to an increased release of substance P. Nitric oxide-dependent substance P-mediated neurotransmission has been demonstrated in the small intestine of guinea pig[27]. In fact, the nitric oxide-related drugs affected both contractile and relaxant events in the descending responses of rat distal colon. These findings make us assume that nitric oxide is an important contributor to the activity of both excitatory and inhibitory neurotransmitter systems involved in the descending reflex pathways in the colonic region of the large intestine. However, we observed that the contractile and relaxant events of the electrically-elicited descending responses occurred on the background of nitric oxide-related drugs, which indicates that other neurotransmitter systems, probably excitatory adrenergic and inhibitory purinergic are involved in the descending reflex pathways in the colonic LM and CM. Our drug treatment results and immuno- and histochemical data for the expression and distribution of NADPH-diaphorase and substance P-positive nerve structures in the myenteric ganglia give us grounds to assume an important role of substance P and nitric oxide in the neurotransmitter pathways and implications of these neurotransmitters in the regulation of motility in the colonic region of the large intestines.

Conclusions

In summary, this study evaluated the electrically-induced local and descending motor responses of the colonic LM and CM in a rat model-preparation. The different patterns of the responses displayed the role of neurotransmitters systems involved in the descending reflex pathways underlying the motility in both muscles. Most probably, cholinergic and tachykininergic neurotransmissions are involved in the manifestation of the contractile activity, while nitrergic neurotransmission, better expressed in the reflex pathways supplying CM than LM underlies the relaxant events. Testing of the responses by drugs and the availability and distribution of substance P- and NADPH-diaphorase-positive nerve structures indicated the implication and significance of acetylcholine, substance P and nitric oxide in the modulation of descending reflex pathways serving rat colonic region. Defining the role of neurotransmitter systems in the neuromuscular communications is important for elucidating the pathophysiology and the drug treatments of motility disorders, related to the distal regions of the intestines.

Acknowledgments: This study was supported by grants from Medical University of Pleven (Grant No. 13/2015) and Institute of Neurobiology of the Bulgarian Academy of Sciences, Sofia, Bulgaria.

Compliance with Ethics Requirements:

„The authors declare no conflict of interest regarding this article"

„The authors declare that all the procedures and experiments of this study respect the ethical standards in the Helsinki Declaration of 1975, as revised in 2008(5), as well as the national law."

„All institutional and national guidelines for the care and use of laboratory animals were followed"

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