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Region-related modular nerve-dependent motor activity in anorectum – cholinergic and nitrergic contribution to rat model

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Disturbances of enteric nerve-mediated anorectal evacuation mechanisms have medical and social impact. The study aimed at further eliciting the contribution of cholinergic and nitrergic neurotransmission systems to modular nerve networks in different regions of Wistar rat anorectum. Electrical field stimulation (EFS, 0.8 ms, 40 V, 2, 5 or 10 Hz, 20 s), computerized mechanographic on-line setup and drugs were used to evaluate the motor responses of isolated rings from circular muscle of rectum (proximal, middle, and distal part), internal anal sphincter, and anal canal. Twitch-like frequency-dependent contractions, more pronounced in rectal preparations, characterized the modular motor responses of rectal circular muscle rings and anal canal. Depending on the frequency of stimulation, the motor activity of internal anal sphincter varied from deep long-lasting relaxation to initial short-lasting relaxation, followed by a contraction. Electrically-evoked responses of anorectal preparations were tetrodotoxin (0.1 μM)-sensitive. In the presence of atropine (0.3 μM) the contractions of rectal rings decreased, relaxation of internal anal sphincter increased and inhibition of the contractions of the anal canal occurred, followed by relaxation. During atropine treatment, NG-nitro-L-arginine (0.5 mM) increased the contractile responses and suppressed internal anal sphincter relaxations. L-arginine (0.5 mM) decreased the contractions and extended the relaxations of internal anal sphincter and anal canal. Our results suggest that cholinergic and nitrergic systems are not equally involved in modular nerve networks of various regions of anorectum. Cholinergic transmission is more expressed in distal rectum, underlying its contractile potency, while nitric oxide-dependent transmission(s) control the relaxation ability of the internal anal sphincter and anal canal.

Key words: rat anal region, modular nerve networks, atropine, L-arginine, NG-nitro-L-arginine

INTRODUCTION

The peristaltic movements of the small and large intestine occur in *in vitro* conditions. The nervous structures that mediate ascending pathways inducing contraction and descending pathways eliciting relaxation of circular muscle are contained within the gut wall (Crema 1970, Costa and Furness 1976, Grider and Makhlouf 1986, Tonini and Costa 1990, Smith and McCarron 1998, Ivancheva and Radomirov 2002, Radomirov et al. 2009).

The motor behavior depends on the functional activity of the respective gut region. Mixing and propulsion are the basic contractile activities in the small intestine and the liquid content moves in a step-wise fashion. In the large intestine, the content consisted of solid fecal pellets that can be transmitted by giant propagated contractions (Sarna 1991, Shafik et al. 2001, Bassotti et al. 2005, Ono et al. 2005).

Because of the clinical and social relevance of the disturbances of evacuation in the large bowel, the nerve-mediated motor activity is a matter of experimental and clinical studies. In the recto-anal region various nerve pathways have been demonstrated, such as colorectal stimulatory (Van der Veek et al. 2004), recto-colonic inhibitory (Bampton et al. 2002, Law et al. 2002), colo-anal (Malcolm and Camilleri 2000), anorectal (Shafik et al. 2003a), and recto-anal excitatory (Sangwan et al. 1995, Radomirov et al. 2009). The bio-

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logical control of anorectal motility during evacuation is mainly attributed to the intrinsic recto-anal inhibitory reflex which consists of contraction of rectal circular muscle and synchronous internal anal sphincter relaxation, highlighting the importance of the motor activity in the circular axis of distal part of gut (Stebbing et al. 1996, De Lorijn et al. 2005, Kojima et al. 2006).

Although knowledge about the large intestine has considerably advanced over the last years, the mechanisms of motility of anorectum have remained largely obscure (Shafik et al. 2003b) and the role of local neuronal circuitry in the motor activity of distal rectum and anal sphincters is not well-studied. As small rings of circular muscle can contract independently, these rings and the associated enteric nerve networks can be regarded as functional modules. The spatiotemporal coordination of the modules is the determining factor for the generation of the rich repertoire of motor patterns (Costa and Brookes 1994, Costa et al. 2000).

The modular contractile and/or relaxant activity of anorectum requires elucidation. According to Bharucha (2006), both internal and external anal sphincters are involved in the maintenance of motility of the anal canal. To our best of knowledge, there are no experimental data in literature on the modular nerve-mediated motor evens in circular axis of rat anorectum.

We examined the motor activity of anorectum using experimental models, isolated from rat distal gut. We were particularly interested in evaluating the motor responses of isolated small ring model-preparations from circular muscle of distal rectum, internal anal sphincter and anal canal as display of excitation of modules of enteric networks in various regions of the anorectum. Electrical field stimulation was applied to activate the nerve structures. Mechanographic computerized on-line technique was used to register the electrically-elicited motor responses. To evaluate the contribution of cholinergic and nitrergic neurotransmissions to the modular nerve networks, the motor responses were tested in the presence of drugs that influence these transmissions.

METHODS

The experiments were carried out in the Laboratory of Peripheral synapses of Institute of Neurobiology of the Bulgarian Academy of Sciences and were approved by the Animal Care and Use Ethics Committee of the Institute of Neurobiology.

Animals

The study was carried out on 38 male Wistar rats (weight 250–280 g, age 4 months). The animals were obtained from the Research and Laboratory Animal Breeding Center of Slivnitsa (Bulgaria), and were housed at a temperature of 22 ± 2°C and humidity of 50 ± 10%, given normal pelleted diet and water ad libitum. The animals were fasted overnight, stunned by a blow on the neck and decapitated. The abdominal cavity was opened and the pubic symphysis was cut away thus exposing the large intestine. The perianal skin was excised and the rectum with the anal canal was removed and placed in modified Krebs solution at room temperature. The extrinsic blood vessels and nerves along the mesenteric border were then carefully trimmed away. A segment consisting of the distal part of the rectum and the anal canal with intact nerve plexuses-smooth muscle layers was isolated (Brading et al. 2008).

Isolated preparations

Rings, 2 mm in width were dissected in the circular axis from the distal part of the rectum. The anal canal preparations, 3–4 mm in width, including the internal and external anal sphincters, were isolated from the rectum. The rectal circular muscle rings, the anal canal, and the internal anal sphincter (1.5–2 mm in width) obtained by dissecting the external sphincter were mounted in organ baths. The motor activity was measured between two opposite sites of the ring circumference. All preparations were stretched under an initial tension of 10 mN. Computerized mechanographic on-line technique was used for registration of the motor responses of the isolated preparations.

Electrical stimulation

Electrical field stimulation (EFS) was used (Paton and Vizi 1969) to excite the nerve structures. EFS was applied by means of two platinum electrodes (0.45 mm thick) placed diametrically opposed and 14 mm apart along the sides of organ baths. Rectangular pulses with a duration of 0.8 ms and voltage of 40 V were delivered at a frequency of 2, 5 or 10 Hz for 20 s at an interval not shorter than 5 min (Ivancheva and Radomirov 2002).

Protocol design, drugs and solutions

The responses of the rings of circular muscle of rectum, internal anal sphincter and anal canal induced by EFS were considered as 'modular motor responses' resulting from excitation of the local modules of nerve networks lying in the field of electrical stimulation. The preparations were allowed to equilibrate for 30 min before starting the experiment.

The electrically-elicited modular motor responses were registered before and during drug treatment. Drugs were administered in volumes not exceeding 0.5–1 % of the bath volume. The contact time and the concentration of drugs were as follows: tetrodotoxin (TTX, Sankyo, Zurich, Switzerland; 0.1 μM, 10 min), atropine (atropine sulfate, Merck, Darmstadt, Germany; 0.3 μM, 15 min), NG-nitro-L-arginine (L-NNA, Sigma Chemicals, St. Louis, MO, USA; 0.5 mM, 15 min) and L-arginine (Sigma Chemicals, St. Louis, MO, USA; 0.5 mM, 15 min). When the drugs were added consecutively (atropine plus NG-nitro-L-arginine or atropine plus L-arginine) the time course of drug action was 30 min (Radomirov et al. 2009). Drugs were dissolved in distilled water and diluted to their final concentration in Krebs solution before use. The stock solution of TTX was stored at −20°C.

The composition of the modified Krebs solution was (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.

Statistical analysis

The spontaneous muscle tone of the preparations was considered as a baseline for measuring the amplitudes of electrically-evoked motor responses as force in mN. Data are presented as mean values ± SE. Statistical significance was evaluated by Student's *t*-test for paired data and one-way ANOVA, followed by the LSD as a post-hoc test at $P<0.05$. All analyses were performed using Statgraphics Plus 4.1 for Windows, SPSS 14 (Statistical Package for the Social Sciences) statistical softwares.

RESULTS

The spontaneous motor activity of circular muscle rings from distal rectum was characterized by irregular contractions of variable frequency and amplitude. The internal anal sphincter and the anal canal expressed rhythmic phasic contractions. The isolated preparations did not considerable change the tissue tone for period longer than 120 min.

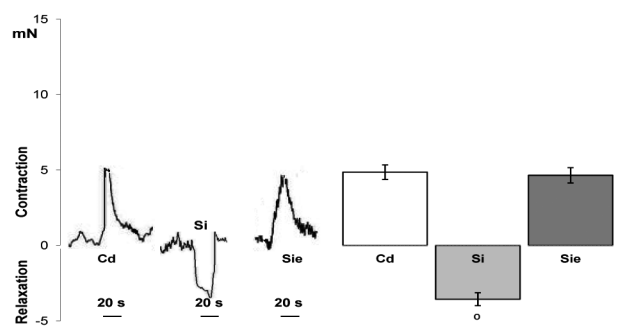

Fig. 1. Modular motor responses of ring-preparations isolated from different regions of rat anorectum. (A) typical mechanographic records and (B) graphs showing the modular motor responses of rings of circular muscle from distal rectum (Cd), internal anal sphincter (Si) and anal canal (Sie) induced by electrical stimulation (0.8 ms, 40 V, 20 s) applied at a frequency of 2 Hz. Bars represent the mean ± SEM of at least 8 experiments. Symbols indicate: significant differences at $P<0.05$ – (*) and opposite effects (o) vs. the responses of Cd.

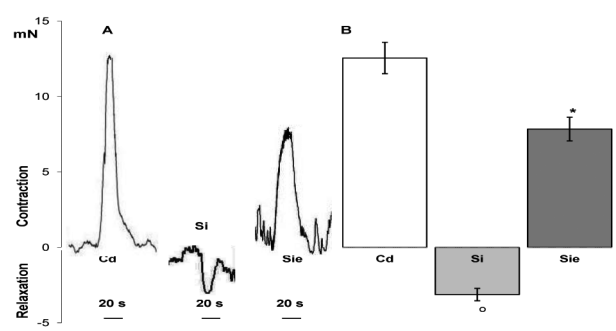

Fig. 2. Modular motor responses of ring-preparations isolated from different regions of rat anorectum.. (A) typical mechanographic records and (B) graphs showing the modular motor responses of rings of circular muscle from distal rectum (Cd), internal anal sphincter (Si) and anal canal (Sie) induced by electrical stimulation (0.8 ms, 40 V, 20 s) applied at a frequency of 5 Hz. Bars represent the mean ± SEM of at least 8 experiments. Symbols indicate: significant differences at $P<0.05$ – (*) and opposite effects (o) vs. the responses of Cd.

Modular motor responses of preparations isolated in circular axis of anorectum

EFS applied at a frequency of 2 Hz to the circular preparations isolated from the different regions of rat anorectum elicited fast twitch-like contractile responses in circular muscle rings of rectum and of anal canal and a deep long-lasting relaxation of the internal anal sphincter (Fig. 1A). The amplitudes of contractile responses of the circular muscle rings from distal rectum and the anal canal did not differed being 4.85 ± 0.48 mN and 4.64 ± 0.51 mN (n=14, P>0.05) respectively, although the contractions of rectal muscle rings were more pronounced. The relaxation of the internal anal sphincter reached a peak of 3.57 ± 0.43 mN (n=9) (Fig. 1B).

The modular contractions of rectal circular muscle rings and anal canal increased when EFS was applied at higher frequencies of 5 or 10 Hz (Figs 2A and 3A) and the values of amplitudes considerably exceeded those obtained at 2 Hz electrical stimulation. The contractile responses of the rectal circular muscle rings were significantly more expressed than the contractions of the anal canal, the greatest difference being in responses obtained when EFS was used at a frequency of 10 Hz (15.27 ± 1.22 mN vs. 9.24 ± 0.93 mN, respectively, n=13, P<0.05) (Figs 2B and 3B).

The pattern of the modular responses of internal anal sphincter to EFS with frequencies of 5 or 10 Hz was different. Relaxation did not persist throughout the period of electrical stimulation of 5 Hz (Fig. 2A). The relaxation (3.13 ± 0.41 mN) was less pronounced, as compared to relaxation induced by 2-Hz EFS (n=10, P<0.05) (Fig. 2B). Electrical stimuli with the frequency of 10 Hz produced modular response of internal anal sphincter, consisting of an initial short-lasting relaxation followed by a contraction with peak amplitude of 3.95 ± 0.19 mN (n=10) (Fig. 3A). The relaxation of 2.0 ± 0.31 mN at 10-Hz stimulation was significantly less pronounced than the relaxation induced by EFS at a frequency of 2 Hz or 5 Hz (n=10, P<0.05) (Fig. 3B).

Relaxations were not observed in the electrically-induced modular motor responses of rectal circular muscle rings and anal canal.

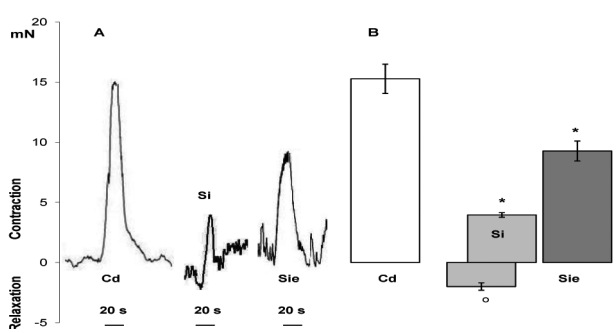

Fig. 3. Modular motor responses of ring-preparations isolated from different regions of rat anorectum. (A) typical mechanographic records and (B) graphs showing the modular motor responses of rings of circular muscle from distal rectum (Cd), internal anal sphincter (Si) and anal canal (Sie) induced by electrical stimulation (0.8 ms, 40 V, 20 s) applied at a frequency of 10 Hz. Bars represent the mean ± SEM of at least 8 experiments. Symbols indicate: significant differences at P<0.05 – (*) and opposite effects (o) vs. the responses of Cd.

Drug effects on the modular motor responses

The modular motor responses of the preparations isolated from different regions of anorectum were registered in the presence of drugs that exert an influence the cholinergic and nitrergic transmissions.

Atropine at a concentration of 0.3 μM decreased the modular contractions of rectal circular muscle rings evoked by 2 Hz EFS to 2.61 ± 0.53 mN (n=9, P<0.05), considerably increased the relaxation of the internal anal sphincter (4.62 ± 0.34 mN, n=8, P<0.05) (Fig.4A), and converted the contractile response of the anal canal to a two-component response, consisting of initial contraction followed by relaxation. During atropine treatment, the contraction was suppressed to 2.80 ± 0.28 mN (n=8, P<0.05) and inducing a relaxation of 1.26 ± 0.14 mN (n=8) was registered (Fig. 4A). In preparations pretreated with atropine, L-NNA (0.5 mM) increased the atropine-decreased contractions of circular muscle rings of rectum and anal canal, evoked a contraction of the internal anal sphincter and prevented the relaxation in the modular response of anal canal. L-arginine had the opposite effects. The amplitudes of contractions decreased, while the relaxations of the internal anal sphincter and anal canal extended to 5.28 ± 0.42 mN (n=8, P<0.05) and 3.42 ± 0.34 mN (n=8, P<0.05), respectively (Fig. 4 A).

The effects of atropine, L-NNA and L-arginine on the modular responses of the rectal circular muscle rings and anal canal were not considerably changed when the electrical stimulation was applied at a frequency of 5 or 10 Hz. The effects of atropine tended to increase, while those of L-arginine tended to decrease

with raising the frequency of the electrical stimuli (Figs 4B and 4C). In the presence of atropine, there was an increase of relaxations of internal anal sphincter induced by EFS at a frequency of 5 Hz or 10 Hz. The contraction in the response of internal anal sphincter to 10-Hz electrical stimulation was significantly decreased to 2.60 ± 0.22 mN (n=8, P<0.05) (Fig. 4C). When L-NNA (0.5 mM) was added to atropine-treated Krebs solution, the relaxation of the internal anal sphincter significantly decreased to 1.42 ± 0.16 mN (n=8, P<0.05) and 1.02 ± 0.08 mN (n=8, P<0.05) at 5-Hz and 10-Hz EFS, respectively. A secondary contraction (3.34 ± 0.36, n=8) in response to EFS applied at a frequency of 5 Hz was registered (Fig. 4B). L-arginine, when added to atropine, considerably increased the relaxations in response to EFS applied at frequencies of 5 Hz or 10 Hz and decreased the contraction in responses provoked by 10-Hz stimulation to 1.32 ± 0.18 mN (n=8, P<0.05) (Figs 4B and 4C).

The electrically-induced modular motor responses of the of rectal circular muscle rings, internal anal sphincter and anal canal were not registered when TTX at a concentration of 0.1 μM was added to the nutrient solution of the organ baths 10 min before stimulation (n=3, data not shown).

DISCUSSION

The present study showed that electrically-elicited motor responses of small circular rings that form functional modules (Costa et al. 2000) in rat anorectum were neurogenic in nature, since they were prevented by TTX, a blocker of neuronal conductance. The pattern of electrically-elicited activity in model-preparations of circular muscle of distal rectum, internal anal sphincter and anal canal differed indicating excitation of modular neuro-neuronal and/or neuro-muscular communications underlying specific modular contraction/relaxation events.

Electrical field stimulation caused local release of excitatory and/or inhibitory neurotransmitters underlying contraction and/or relaxation in isolated intestinal preparations (Paton and Vizi 1969, Kadlec et al. 1986). We observed that frequency-dependent contractions characterized the modular responses of circular muscle rings of distal rectum and anal canal. The pattern of responses was not changed by the increase of stimulus frequency. This could probably be explained by electrically-provoked release of one and the same excitatory neurotransmitter or co-release of various neurotransmitters with a complementary role in the motor activity and their interactions could produce the same contractile responses of circular rectal muscles. The amplitudes of modular contractile responses of the rectal rings exceeded those of the contractions of the anal canal. The role of rectal muscle layers in the contraction or relaxation motility is not fully understood.

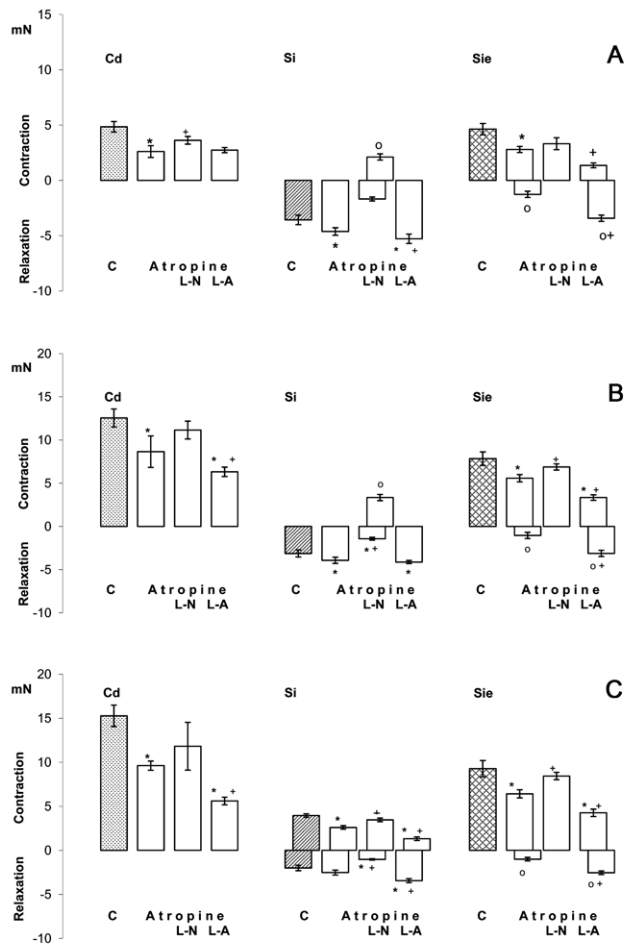

Fig. 4. Modular motor responses of ring-preparations, induced by electrical stimulation (0.8 ms, 40 V, 20 s) applied at a frequency of 2 Hz (A), 5 Hz (B) and 10 Hz (C). Graphs showing the modular motor responses of rings of circular muscle from distal rectum (Cd), internal anal sphincter (Si) and anal canal (Sie) Abbreviations: control responses in the absence (C) and in the presence of drugs – atropine, atropine plus L-NNA (L-N) and atropine plus L-arginine (L-A). Bars represent the mean ± SEM of at least 8 experiments. Symbols indicate: significant differences at P<0.05 – (*) vs. controls, (+) vs. atropine treatment and (o) opposite effects vs. the controls.

There exist controversial views regarding the propulsive function of the rectum as a conduit or as a reservoir organ (Shafik et al. 2006). More recently, it has been shown that in rat model-preparations the contractile activity increased from the colon to the rectum, while the relaxation remained uniform, thus indicating essential contractile potency than relaxation ability in colo-rectal-anal tube (Radomirov et al. 2006, Brading et al. 2008).The lack of relaxation and the dominant contractile activity that was registered in the present experiments most probably demonstrate a biologically reasonable higher contractile potency of distal rectum to overcome the resistance of contracted anal canal and indicate a role of rectum rather as a conduit than a storage organ.

The electrically-induced modular responses of the internal anal sphincter that were registered varied from a deep relaxation to short-lasting relaxation, followed by contraction. Relaxation was shown as electrically-induced response of internal anal sphincter (Matsufuji and Yokoyama 2003, Rattan 2005, Rattan et al. 2005), while the involvement of frequency-sensitive inhibitory/excitatory mechanisms in the autonomic activity of internal anal sphincter is not clear yet.

The electrically-provoked neurotransmitter release is dependent on the frequency of electrical stimuli (Paton 1955). During high-frequency stimulation, the number of excited nerve varicosities is greater than the number of varicosities releasing transmitter during stimulation with single-pulse electrical stimulation (Alberts and Stjarne 1982, Kadlec et al. 1984, 1986). Different substances can exist in a single ne\uron and can be liberated as co-transmitters to activate different receptors, thus causing a variety of effects (Allcoln et al. 1986, Bertaccini and Coruzzi 1987, Radomirov and Venkova 1988). Contractile responses of internal anal sphincter induced by electrical field stimulation or by adrenergic agonists were more recently described in dogs, monkeys and human (Matsufuji and Yokoyama 2003, Cobine et al. 2007), and Substance P was proposed as exciting mediator of rat internal anal sphincter (Yang et al. 2006). Opazo and colleagues (2009, 2011) showed a functional co-transmission for nitric oxide and purines with complementary role in inhibitory motor pathways to internal anal sphincter of rats (Opazo et al. 2011) and pigs (Opazo et al. 2009). The prevalence of effects of inhibitory or excitatory neurotransmitters closing up to the anal gut region remains scarcely investigated and is not fully understood (Nagano et al. 2004). Previous studies on entire rat anorectum showed that cholinergic excitatory ascending and descending pathways and nitric oxide-dependent inhibitory ascending neurotransmission to rectal circular muscle and inhibitory descending to internal anal sphincter and anal canal are involved in the control of motility of recto-anal region (Radomirov et al. 2009, 2010).

Our experiments showed that the electrically-elicited modular motor responses of circular preparations from different regions of anorectum were affected by cholinergic- and nitrergic-related drugs. The blocking of cholinergic receptors by atropine considerable decreased the contractions, thus indicating essential stimulating role of cholinergic neurotransmission in the motility in circular axis of anal region. Since in the presence of atropine the relaxation of the internal anal sphincter was increased and relaxation was observed in the responses of anal canal, it could be suggested that elimination of excitatory cholinergic neurotransmission unmasked action of inhibitory neurotransmission(s). Nitric oxide has been accepted as inhibitory neurotransmitter in the intestine (Rand and Li 1995, Stebbing et al. 1996). This study demonstrated that L-NNA, an inhibitor of nitric oxide synthase, increased the contractions or the contractile components of the responses during atropine treatment and decreased the relaxation. On the other hand, L-Arginine, a substrate of nitric oxide synthesis, decreased the contractile activity and increased the relaxation. These results are in agreement with the concept that nitric oxide could affect the transmitter release from motor nerve terminals. The L-NNA-resulting block of nitric oxide synthesis has been demonstrated to eliminate the inhibitory action of nitric oxide on acetylcholine release (Barthó and Lefebvre 1995, Smith and McCarron 1998), to provoke an increase of contraction of the rectum and prevent the relaxation of internal anal sphincter in the guinea pig recto-anal region (Yamanouchi et al. 2002). We found that the block of cholinergic receptors did not completely prevent the excitatory components of the modular motor responses, which indicated the presence of other excitatory neurotransmitter(s) in addition to the cholinergic one. More recently, substance P has been proposed as an excitatory mediator of the internal anal sphincter (Yang et al. 2006, Radomirov et al. 2010). Thus, the increase of contractile components in modular

responses of the rat anorectum resulting from treatment with L-NNA in the presence of atropine could be related to release of substance P. Nitric oxide-dependent substance P-mediated neurotransmission has been presented in guinea pig small intestine (Wiklund et al. 1993). In fact, the nitric oxide-related drugs affected both contraction and relaxation events of the modular responses we observed. Our results make us assume that nitric oxide contributes to excitatory and inhibitory neurotransmissions in circular axis of the rat anorectum. These observations could be not considered as unexpected since more recent findings have demonstrated the involvement of nitrergic system in a number of biological processes such as production of hydroxyl radicals (Thomas et al. 2007), alpha-2 adrenoreceptor-mediated effects (Gyires et al. 2007), epileptiform activity (Yildirim et al. 2011) or iron-induced cell loss (Gulturk et al. 2008). The action of nitric oxide was proposed as a new form of synaptic and nonsynaptic interactions (Vizi 2000, Kiss and Vizi 2001). The wide variety of effects of nitric oxide most probably plays a role in the modulation of motor activity in circular axis of anorectum.

CONCLUSIONS

In conclusion, the present study showed that application of electrical stimulation to excite modular nerve networks displayed differently-expressed region-related role of cholinergic and nitrergic systems in modulation of motor activity in circular axis of anorectum in rat model. It could be suggested that cholinergic excitatory neurotransmission is more expressed in nerve modules supplying circular muscle of distal rectum, and that nitric oxide-dependent inhibitory neurotransmission is presented mainly in nerve modules controlling the internal anal sphincter and the anal canal. The latter assumption indicates contribution of cholinergic neurotransmission to contractile activity and contribution of nitrergic neurotransmission to relaxation events, providing for consecutively coordinated propulsion and evacuation of anorectal content. An understanding of the motor activity and the role of neurotransmitters is important in the pathophysiology and complex treatment (including drugs and sacral or anal electric stimulation) of anorectal motility disorders, related to the dysfunction of the distal region of anorectum.

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