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Tachykinins mediate non-adrenergic, non-cholinergic excitatory neurotransmission to the hamster ileum via NK1 and NK2 receptors

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Abstract

The present study was designed to investigate Substance P (SP) and a related tachykinin, Neurokinin A (NKA), contributions to the excitatory neurotransmission to the circular smooth muscle of the hamster ileum. In the presence of atropine (0.5 μM), guanethidine (3 μM) and N^G-nitro-L-arginine methyl ester (L-NAME) (200 μM), electrical field stimulation (EFS) evoked a non-adrenergic, non-cholinergic (NANC) excitatory junction potential (EJP) and contraction of circular smooth muscle. Applications of SP and NKA produced depolarizing and contractile responses in a concentration-dependent fashion. The EJP and contraction were almost abolished by the non-specific tachykininergic antagonist, spantide (3 μM). Application of SP antagonist, L-732,138, (1 μM) markedly inhibited EJP (82.5%) and contraction (68.9%) and completely blocked excitatory responses produced by exogenous application of SP. While application of NKA antagonist, SR48968 (1 μM) completely blocked the depolarising and contractile responses to NKA, it only slightly inhibited those to EFS (17.2% and 31.4% respectively).These results provide evidence that, in the circular muscle of hamster ileum, endogenous tachykinins are the main NANC excitatory neurotransmitters and their action is mediated by both NK1 and NK2 receptors.

Keywords: EJP; Substance P; Neurokinin A; Tachykinins; Intestine

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Introduction

It has been indicated that tachykinins (TKs) may play a role in excitatory neurotransmission in the mammalian gut. In particular, substance P (SP) and neurokinin A (NKA) are present in neurons of the enteric nervous system where they appear to co-exist with acetylcholine (ACh) (Costa and Furness, 1987).

SP contracts nearly all parts of the gastrointestinal tract (Bartho and Holzer, 1985). A large part of SP-induced contraction of the guinea pig ileum can be attributed to direct action on smooth muscle, but a small part is mediated by cholinergic neurons and blocked by muscarinic antagonists (Holzer and Lembeck, 1980; Huidobro-Toro et al., 1982). NKA also produces contraction of intestinal smooth muscle while the relative potencies vary depending on the species and regions of the gut (Holzer-Petsche et al., 1985; Holzer-Petsche et al., 1987; Maggi et al., 1990a,b). Both NK1 (preferentially SP) and NK2 (preferentially NKA) receptors have been suggested to mediate the contractile response to TKs in guinea pig (Maggi et al., 1990a,b) and human (Maggi et al., 1990a,b) ileums. Moreover, it has been shown that in the circular muscle of the rat small intestine, the non-adrenergic, non-cholinergic (NANC) contraction to electrical field stimulation (EFS) involves TKs, acting via NK1 and NK2 receptors (Maggi and Giuliani, 1995).

SP causes depolarization of smooth muscle membrane and increases the spike frequency in guinea pig ileum (Bauer and Kuriyama, 1982; Niel et al., 1983). The mechanism by which SP produces depolarization is evidenced by decrease in K^+ conductance (Fujisawa and Ito, 1982; Holzer-Petsche, 1983). In addition, SP also activates Ca^{++}-activated K^+ channels presumably as a result of increase in Ca^{++} influx (Mayer et al., 1990).

Localization studies showed immunoreactivity for NK1 receptors on enteric neurons and Interstitial cells of Cajal (ICC) but not on intestinal muscle (Sternini et al., 1995; Portbury et al., 1996a,b). While immunoreactivity for NK2 receptors have been observed in both longitudinal and circular intestinal smooth muscle and on enteric neurons (Portbury et al., 1996a,b).

Species differences regarding the involvement of TKs in the regulation of gut motility, therefore, may exist. However, in spite of the studies that have been conducted on these different species and the establishment of TKs as mediators of excitatory responses, yet there is no available information about the possible contribution of TKs and their modulatory effect on membrane potential in the hamster. On the basis of this background, the present study was designed to characterize the non-cholinergic excitatory neurotransmission to the hamster ileal circular smooth muscle. The contributing roles of SP and a related TK, NKA, to NANC neurotransmission in the hamster ileum were also assessed.

Methods

Tissue preparation

Tissues were obtained from male Syrian hamsters weighing 80–120 grams. Under light ether anaesthesia, hamsters were killed by exsanguinations via carotids. Through an abdominal incision, a length of about 3–4 cm of intact ileum was removed and immediately immersed in physiological salt solution (PSS; see below) at room temperature. The ileal segment was placed in a dissection dish containing PSS and the intraluminal contents were flushed using a small cannula filled with PSS.

The surgical procedures and pre- and post-operative care of the animals conformed to the Gifu University Animal care and Use committee in accordance with Japanese Department of Agriculture guidelines and all efforts were made to minimize animal suffering and to reduce the number of animal used.

Electrophysiological recordings

One cm-length of the ileal segment was excised and pinned to the rubber floor of the experimental chamber of an organ bath of 4 ml capacity. The bath was constantly perfused with pre-warmed (35 °C) PSS containing 0.5 μM atropine, 3 μM guanethidine and 0.1 μM nifedipine at a flow rate of about 3 ml min^{-1}. The PSS was previously bubbled with 95% O_2 : 5% CO_2 gas mixture. Tissue preparations were allowed to equilibrate for approximately one hour before experiments were undertaken.

Membrane potential changes were recorded using conventional glass microelectrodes that had resistances of 50–80 MΩ when filled with 3 M KCl. The microelectrode insertions were made into the circular muscle cells of the deep layer through the serosal side. A successful insertion was confirmed when a sharp drop in the recorded voltage to a negative resting membrane potential of about −45 mV was established and remained stable. Application of electrical field stimulation (EFS) to the intramural nerves of the preparation was done by a pair of silver wire electrodes. The two electrodes were placed parallel to the longitudinal axis of the preparation so that one is passing through the lumen of the fixed tissue while the other wire outside the preparation in the PSS of the bath. The two electrodes were connected to square wave stimulator (Sen-2201, Nihon Koden, Tokyo, Japan).

Membrane potentials in response to EFS were recorded from circular smooth muscle cells located within 2 mm of the simulating electrode (inside the lumen). Excitatory junction potentials (EJPs) were evoked by EFS of intramural nerves of the preparation with square-wave pulses (1–5 pulses) of 0.5 ms duration at 15 V. Membrane potential changes were displayed on an oscilloscope (CS 4025, Kenwood, Tokyo, Japan). Analogue electrical signals were recorded on a thermal-array recorder (RTA-1100M, Nihon Kohden, Tokyo, Japan) for illustration and further analysis.

Mechanical recordings

Mechanical changes of the circular direction of the preparations were recorded isometrically with a force displacement transducer (Orientic T7-30-240, Japan), an AC amplifier (AS1202, Nihon Kohden, Tokyo, Japan), and a potentiometric pen recorder (Hitachi, 561, Japan). EFS was carried out by means of two platinum-wire rings which were connected to an electronic stimulator.

EFS was used to deliver rectangular pulses at different frequencies. The pulse duration was fixed at 0.5 ms for stimulation of intramural nerves. The stimulus intensities were used as supra-maximal voltages at any particular frequency. Unlike electrophysiological recordings, mechanical ones have been done in absence of nifedipine. Contractions were expressed as percentages of those produced by high (60 mM) K^+ concentration.

Physiological solutions

The physiological solutions used in this study had the following composition (mM): NaCl 137, KCl 4.0, $Na_2H_2Po_4$ 0.5, $NaHCo_3$ 11.9, $CaCl_2$ 2.0, $MgCl_2$ 1.0 and glucose 5.6. The pH of the solution was 7.4.

Drugs

Substance P, NKA and spantide were obtained from Peptide Institute, INC (Osaka, Japan). N^G-nitro-L-arginine methyl ester (L-NAME), guanethidine sulphate, and L-732,138 were obtained from Sigma (Ct Louis, MO, USA). SR48968 were a kind gift from Sanofi ~ Synthelabo (Montpellier Cedex 04-France). Drugs were dissolved in distilled water. Stock solutions were at more than 100 times higher concentrations than those used for experiments, further dilutions were made in PSS. Final concentrations of distilled water in the bathing solution were less than 0.01%; thus had no effect on either membrane potential or muscle tone.

Data presentation and statistical analysis

Data are expressed as mean ± S.E. Differences between the means were analyzed either by one-way analysis of variance, followed by the Dunnett's test for multiple group comparisons, or by student's t-test (paired or unpaired) for comparison of two groups. P value of less than 0.05 was considered significant.

Results

General

In the presence of atropine (0.5 μM), guanethidine (3 μM), L-NAME (200 μM) and nifedipine (0.1 μM), ileal circular muscle cells had an average resting membrane potential of − 48.3 ± 0.4 mV and displayed either electrical quiescence 85% (240/282 cells) or spontaneous electrical rhythmic potentials

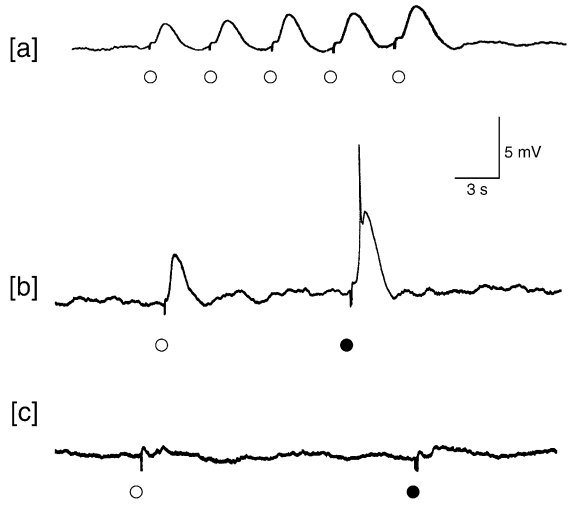

Fig. 1. EJPs produced by single and multiple stimulations of the intra-mural nerves in the ileal circular smooth muscle. a) Repetitive nerve stimulation by single pulses at 0.3 Hz resulted in facilitation. b) EJP produced by a single pulse and 5 pulse-train stimulations at 20 Hz. Spike was produced at stimulation by train of multiple stimuli. c) TTX blocked EJPs produced by both single and multiple stimulations. ○: denotes stimulation using single pulse. ●: denotes stimulation using train of 5 pulses.

15% (42/282) represented as slow waves. When occurred, slow waves had amplitude of 3.5 ± 0.2 mV and frequency of 9.6 ± 0.1 cycles min^{-1}.

EFS (0.5 ms duration, 15 V) of the intramural nerves with single pulses produced EJPs, which were abolished after tetrodotoxin (TTX) (0.1 μM), indicating that they are of neurogenic origin. Repeated

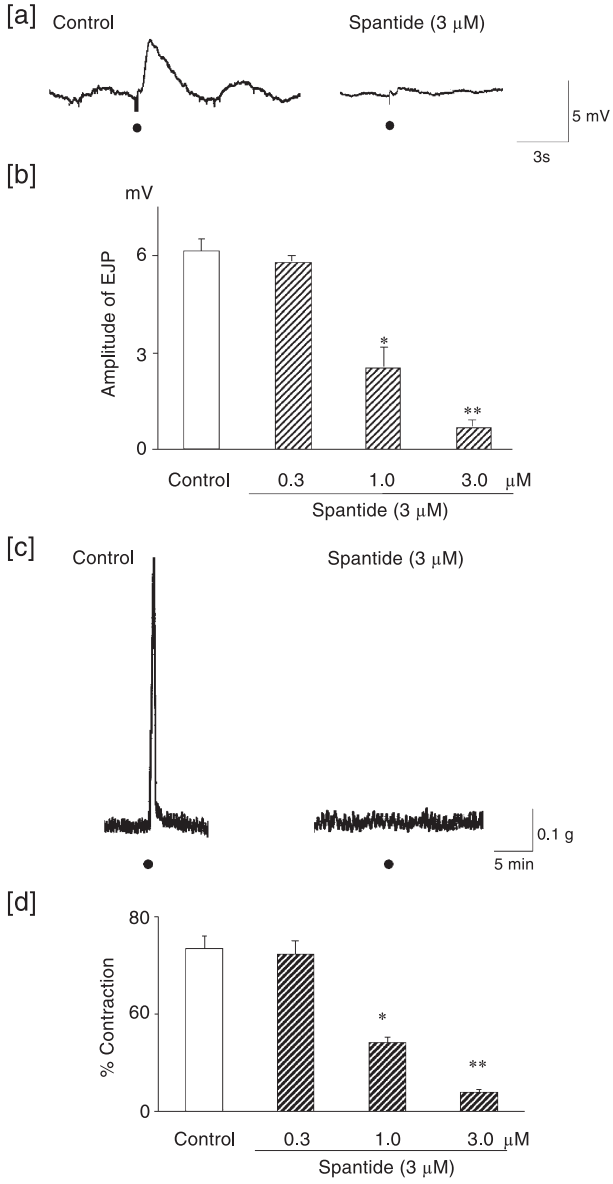

Fig. 2. Effects of spantide on the EJP and contraction. a) Control and after spantide (3 μM). b) Summary graph showing the concentration–dependent inhibition of spantide on EJP. c) Control contraction, as a % of KCl-induced contraction, and after spantide. d) Summary graph showing the concentration-dependent inhibition of spantide on EFS-induced contraction ●: denotes EFS using a train of 5 pulse-stimulation.

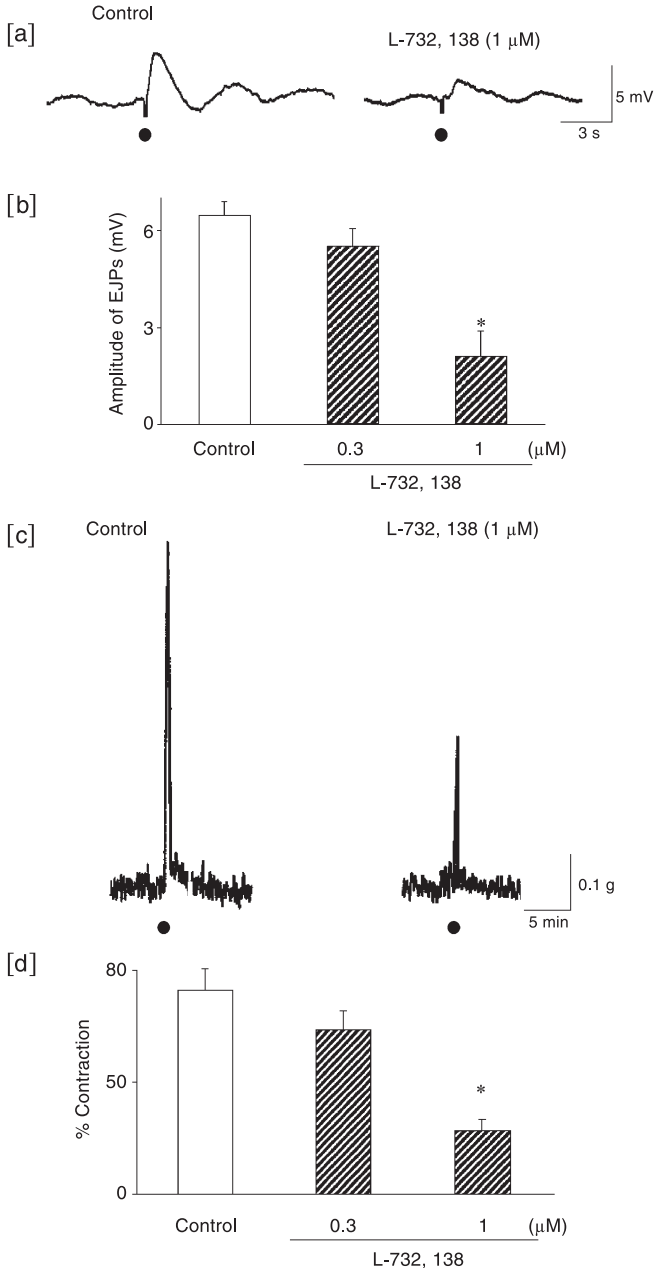

Fig. 3. Effects of L-732,138 on the EJP and contractile response of ileal circular muscle. a) Control EJP and after L-732,138 (1 μM). b) Summary graph showing the concentration-dependent inhibition of L-732,138 on EJP. c) Control contraction, as a % of KCl-induced contraction, and after L-732,138 (1 μM). d) Summary graph showing the concentration-dependent inhibition of L-732,138 on EFS-induced contraction. ●: denotes EFS using a train of 5-pulse stimulation.

single stimulation resulted in facilitation. EFS stimulation with trains each of five pulses evoked EJPs, sometimes with spikes, of circular smooth muscle cells of the hamster ileum (Fig. 1). The EJP had a duration of 2800 ± 81.0 ms, latency of 370 ± 4.5 ms, time-to-peak of 980 ± 13.0 ms and amplitude of 5.6 ± 0.5 mV (n = 27).

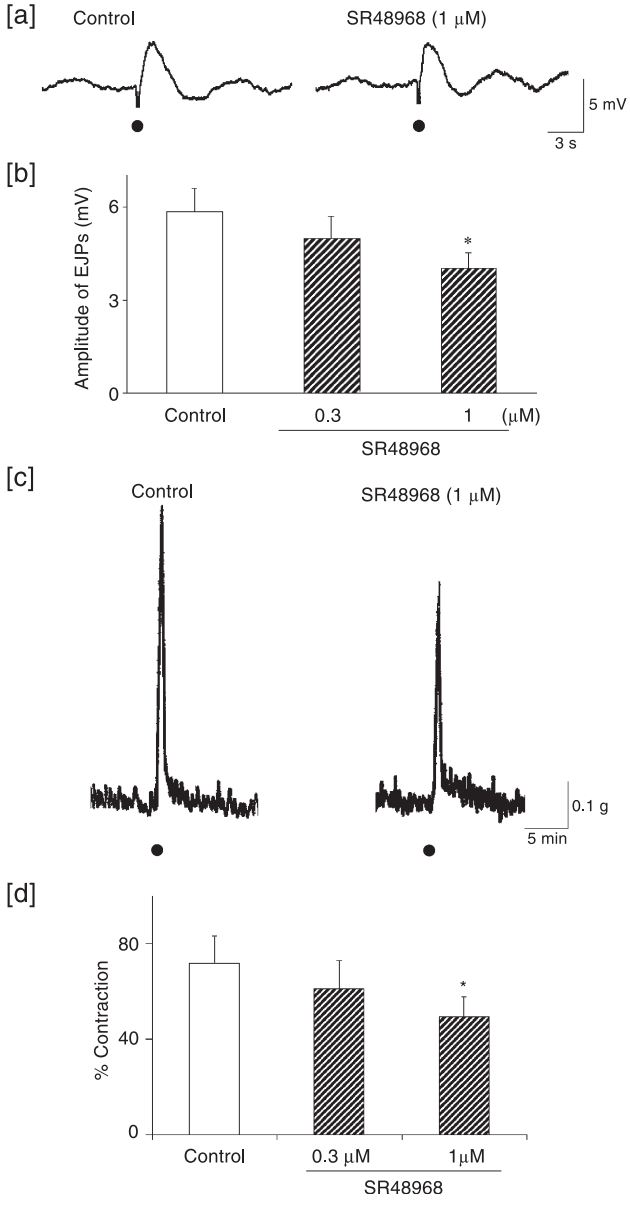

Fig. 4. Effects of SR48968, on the EJP and EFS-induced contraction. a) EJP before and after SR48968 (1 μM). b) Summary graph showing the concentration-dependent inhibition of SR48968 on EJP c) Contractile response before and after SR48968 (1 μM). d) Summary graph showing the concentration-dependent inhibition of SR48968 on EFS-induced contraction. ●: denotes EFS using a train of 5-pulse stimulation.

In the presence of atropine (0.5 μM), guanethidine (3 μM) and L-NAME (200 μM), EFS of intramural nerves of hamster ileal strips at circular direction with trains of stimuli (20 Hz, 5 s) evoked monophasic contractions.

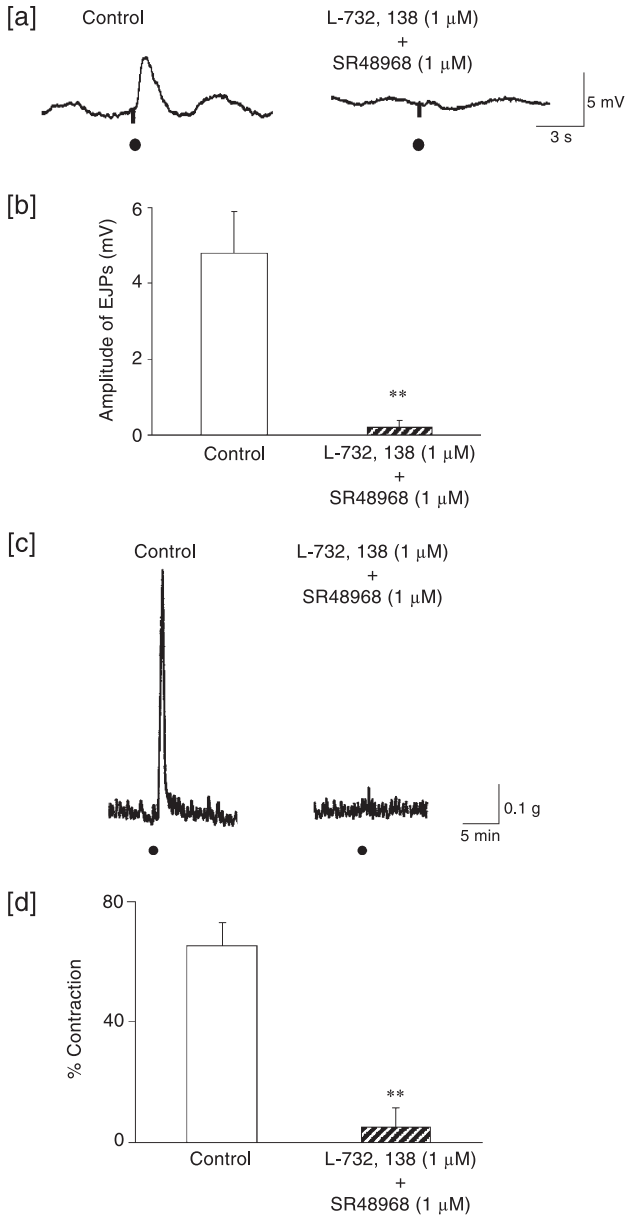

Fig. 5. Effects of combined application of L-732,138 and SR48968 on EFS-induced EJP and contraction. a) & b) Blot and graph showing control EJP and the abolishing effect of combined application of L-732,138 and SR48968 (1 μM each). c) & d) Blot and graph showing control contraction and the abolishing effect of combined application of L-732,138 and SR48968 (1 μM each). ●: denotes EFS using a train of 5-pulse stimulation.

Effects of NK1 and NK2 receptor antagonists on excitatory junction potential and contraction

Tachykininergic receptor antagonist, spantide, (1–3 μM) had no effect either on the resting membrane potential or resting contractile tone but it inhibited EJP and contraction in a concentration-dependent manner. Application of 3 μM of the antagonist for 20 min completely abolished the EJP and the contraction induced by EFS of intramural nerves of the hamster ileum (Fig. 2).

Application of a NK1 receptor antagonist, L-732,138, (1–3 μM), similarly had no effect on the resting membrane potential and resting contractile tone, but inhibited EJP and contraction in a concentration dependent fashion. A concentration of 1 μM for 20 min markedly inhibited both the EJP (82.5%) and the contractile response (68.9%) (Fig. 3).

Application of NK2 receptor antagonist, SR48968 (1 μM) for 20 min slightly inhibited both EJP (17.2%) and contraction (31.4%) (Fig. 4). SR48968 also had no effect on resting membrane potential and resting contractile tone of circular muscle.

Combined application of both L-732,138 and SR48968 (1 μM each) almost abolished EJP and contraction (96.1% and 91.6, respectively) (Fig. 5).

Depolarizing and contractile responses induced by exogenous applications of substance P and neurokinin A

In the presence of atropine (0.5 μM), guanethidine (3 μM), L-NAME (200 μM) and nifedipine (0.1 μM; in membrane potential recordings only), exogenous application of SP (1 ~ 10 μM) produced a slowly developing but sustained concentration-dependent depolarization. Within the used concentration

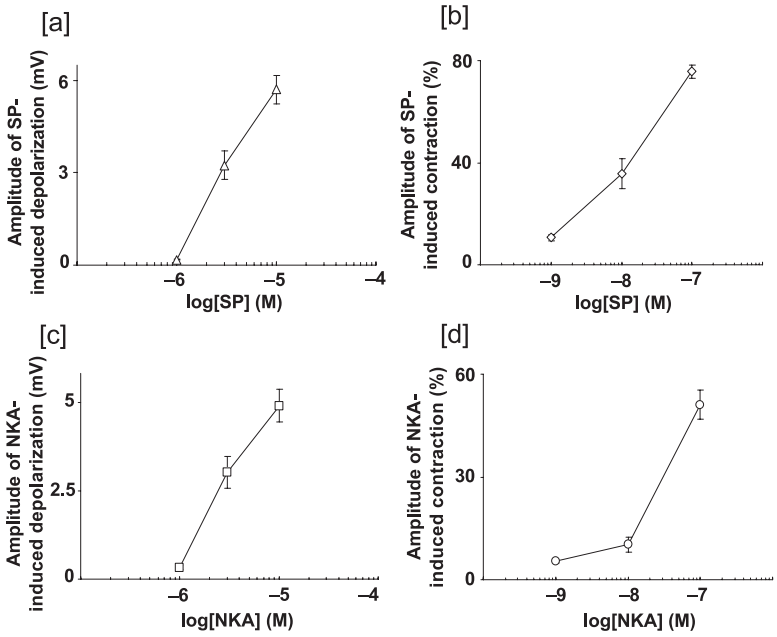

Fig. 6. Graphs a) & b) are showing the dose-dependent depolarizing and contracting responses to exogenously applied SP while graphs c) & d) are showing the dose-dependent depolarizing and contracting responses to exogenously applied NKA.

range, 10 μM SP produced a peak response of 6.5 ± 1.5 mV after 15000 ± 28.0 ms from application (n = 8) (Fig. 6a). Similar but relatively lesser responses were produced by application of NKA using the same concentration range. After 11300 ± 83 ms, a peak of 5.0 ± 0.8 mV was reached upon application of 10 μM NKA (n = 7) (Fig. 6c). Statistical analysis revealed that there was a significant difference between times to peak after exogenous applications of SP and NKA ($P < 0.01$).

Fig. 6b&d show the concentration-dependent contractile responses produced by exogenous applications of SP and NKA (1 ~ 100 nM range for each agonist).

SP induced depolarization (10 μM) and contraction (30 nM) were inhibited in a concentration-dependent manner by L-732,138. Ten and three μM of the latter completely abolished depolarization and contraction, respectively. While applications of 10 μM and 100 nM of SR48968 blocked depolarization (10 μM) and contraction (100 nM) produced by NKA (n = 8).

Discussion

The findings of the present study indicate that SP (mainly) and NKA (partially) are likely to be the NANC excitatory transmitters underlying the EJP and contraction of the hamster ileum. This view is supported by the following observations: (i) EJP was inhibited in a concentration-dependent manner by spantide, L-732,138 and SR48968; (ii) EFS-induced contraction was inhibited also in a concentration-dependent manner by the same antagonists; (iii) exogenous application of SP and NKA produced membrane depolarization and muscle contraction.

In the present study, spantide abolished the EJP and the contraction evoked by EFS; in addition, L-732,138 and SR48968 blocked the depolarizing and contracting responses to exogenously applied SP and NKA, respectively. These data suggest that the effects of SP and NKA were mediated by its own specific receptors. Tachykinin receptors has three subtypes; NK1 and NK2 receptors in smooth muscles which respond fully to SP and NKA with different potencies (Maggi et al., 1993); the third type is NK3 receptors in enteric neurons which also acted upon by SP and NKA producing neuronal excitation and release of other mediators including ACh, TKs and nitric oxide (NO) (Maggi et al., 1990a,b; Maggi et al., 1994).

Recently, evidence showed that NK1 receptor is found mainly on enteric neurons and on ICC not on the intestinal muscle. This leads to the hypothesis that the NK1 receptor-mediated excitation of the muscle may be indirect, via activation of ICC (Portbury et al., 1996a,b).The transmitter released from enteric motor neurons binds primarily to receptors expressed by ICC. Depolarization of neighboring smooth muscle cells occurs via conduction of excitatory junction potential via gap junctions between ICC and smooth muscle cells (Daniel and Posey-Daniel, 1984). On the other hand, NK2 receptor is expressed mainly on intestinal muscle and thus confirms the pharmacological experiments which had identified the muscle as site of excitatory effect mediated via NK2 receptor agonists (Portbury et al., 1996a,b).

This recent localization finding of NK1 and NK2 receptors may be parallel with data shown in the present study. We found that the exogenous application of SP to ileal circular muscle results in membrane depolarization that reaches its peak after about 15 s, while the depolarization after application of NKA reaches its peak relatively earlier, after about 11 s. This difference in time may be necessary for the conduction between ICC and intestinal muscle cells after application of SP. While in case of NKA, it acts directly on its receptor located on intestinal muscle itself. However, further investigations are needed for validating this hypothesis as the possibility of the presence of NK1 receptor on the smooth muscle can not be excluded.

Hou et al., 1989 suggested two pathways for action of SP to induce smooth muscle contraction depending on whether the nerves are functioning normally or not. SP releases ACh by acting preferentially on cholinergic neurons when the nerves are intact, but acts directly on the smooth muscle when conduction of the nerves is blocked. In another study, Martinez-Cuesta et al., 1996 indicated a role of endogenous NO in the modulation of spontaneous tone and motility in the rat duodenum; where, induction of NO synthase may result in a reduction in spontaneous motility of the tissue. However, in the present study the effects of ACh and NO were blocked by the addition of atropine and L-NAME, respectively, all over the experiments. Taken together, therefore, the excitatory response that may be attributed to ACh was discarded and at the same time, the possible masking effect of NO on the excitatory response of SP was also avoided indicating that the excitatory responses of both SP and NKA are mediated through their specific receptors. The interaction between NO and SP, however, seems to be more complex and, therefore, further experiments are being conducted in our laboratory to make clear the exact features of that interaction.

Application of NK2 receptor antagonist, SR48968 only slightly inhibited EFS-induced depolarization and contraction. This might be attributed to the relative poor distribution of NK2 receptors or NKA content in the hamster ileum if compared with NK1 or SP, respectively.

Our data is consistent with those of Zagorodnyuk et al., 1995 who stated that both NK1 and NK2 receptors mediate NANC EJP and contraction in the circular muscle of the guinea pig duodenum, and a co-operation of the signal(s) generated by the two receptors appears important to activate the effector mechanism (L-type calcium channels) which mediates excitation-contraction coupling at this level. Also, our results are consistent with those of Serio et al., 1998 who stated that NK2 receptor antagonists, SR48,968 and MEN 10,627 (up to 5 μM) produced a partial inhibition of the excitatory responses to EFS.

The difference in sensitivity for TKergic agonists in membrane potential and mechanical recordings; being relatively higher in the former and lower in the later; is likely coming from the nature of the mechanisms of excitatory responses of TKs that comprise pharmacomechanical (more sensitive) and electromechanical (less sensitive) couplings. The first mechanism is independent on the membrane potential changes and comes mainly from increasing the level of intracellular calcium. This mechanism seems to be far more sensitive to TKs than the second one. Thus, a considerable contractile response was recorded at the nanomolar concentrations of TKs used. On the other hand, the partial contribution of the electromechanical coupling mechanism (that is dependent on depolarization of the membrane and increasing action potential frequency) to the excitatory response mediated by TKs seems to be less sensitive and thus EJP was recorded only upon using micromolar concentrations of TKs.

It is also worthy to be noted that repetitive single stimulation of intramural nerves facilitates TKergic transmission and consequent contractions. This may play an important role in the ascending excitation limb of peristaltic reflex of hamster intestine.

Conclusion

In conclusion, the results of the present study provide evidence that, in the circular muscle of hamster ileum, endogenous TKs are the main mediators for NANC excitatory neurotransmission and their action is achieved by both NK1 and NK2 receptors.

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