Apamin-Sensitive Nitric Oxide- and ATP-Mediated Motor Effects on the Guinea Pig Small Intestine

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Abstract. The involvement of nitric oxide and ATP in both spontaneous and electrically-induced nonadrenergic noncholinergic (NANC) motor activity with special interest in the apamin-sensitive mechanisms was studied in a guinea pig ileum model. Depending on the concentration (0.1 or 1 μmol/l), apamin, a blocker of the calcium-activated potassium channels and antagonist of ATP action, induced either TTX (0.1 μmol/l)-resistant increase in tone or contractions. SNP, a nitric oxide donor, applied cumulatively (0.1–100 μmol/l) evoked a concentration-dependent relaxation, the EC_{50} value being 0.39 ± 0.12 μmol/l. At concentrations of 0.1 or 1 μmol/l, apamin decreased the SNP effects and shifted the concentration-response curves for SNP to the right. The EC_{50} value for SNP in the presence of apamin at a concentration of 0.1 μmol/l increased to 59.34 ± 36.53 μmol/l. ATP (1 or 50 μmol/l) induced TTX-resistant contractions. The effects of ATP were reduced by apamin (1 μmol/l). The contractile effect of ATP occurred in the presence of SNP. SNP provoked relaxation on the background of ATP. The NANC responses to electrical stimulation (0.8 ms, 40 V, 2 or 20 Hz, 20 s) consisted of an initial relaxation phase followed by a phase of contractions, twitch-like and tonic. L-NNA (0.5 mmol/l), an inhibitor of nitric oxide syntheses, abolished the relaxation phase. L-arginine (0.5 mmol/l) restored it. Apamin (0.1 or 1 μmol/l) completely eliminated the relaxation phase and concentration-dependently inhibited the tonic contraction of the phase of contractions. The present findings indicate that the apamin-sensitive nitric oxide-evoked relaxation could be realized by calcium-activated potassium channels and that the apamin-sensitive ATP-induced contraction is mediated *via* contraction-producing P_2 purinoceptors.

Key words: Apamin — Nitric oxide — ATP — Ileum motor activity

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Introduction

Vladimirova and Shuba (1978) were the first to show that apamin, a polypeptide from bee venom, specifically blocked the transmission of non-adrenergic inhibition, and reported the hyperpolarizing action of exogenous adenosine 5'-triphosphate (ATP) in smooth muscles of the gastrointestinal tract. The action of apamin was determined as reversibly blocking of the smooth muscle ATP receptors (Shuba and Vladimirova 1980) or of the calcium-activated potassium channels (Maas and Den Hertog 1979). Apamin was proposed to provide a pharmacological test in the screening of endogenous substances that might be transmitters of the enteric nerves (Costa et al. 1986) and to enable to identify distinct non-adrenergic non-cholinergic (NANC) mechanisms (Maggi and Giuliani 1993) involved in physiological regulation and pathophysiological processes (Zagorodnyuk et al. 1989). Apamin is an often used agent in current studies aiming to define the role and contribution of the NANC transmitters ATP and nitric oxide to the motor activity of gastrointestinal smooth muscles. There are observations showing that inhibition induced by ATP and analogues in the rat gastric fundus (Lefebvre et al. 1991), small and large intestine of guinea pig (Costa et al. 1986), guinea pig colon (Maggi and Guiliani 1993; Zagorodnyuk and Maggi 1994, 1998); human intestine (Zagorodnyuk et al. 1989) and porcine ileum (Fernandez et al. 1998) utilize apamin-sensitive mechanism(s). The effects of ATP and analogues mediated *via* postjunctional purinoceptors producing contraction (Zagorodnyuk et al. 1989; Zagorodnyuk and Maggi 1998) were described as apamin-sensitive in the guinea pig ileum (Radomirov and Venkova 1988; Ivancheva et al. 2000) and as apamin-resistant in the guinea pig colon (Zagorodnyuk and Maggi 1998). The modulatory action of apamin on the nitric oxide-mediated inhibitory effects is not clear. Neurally-released nitric oxide caused apamin-resistant hyperpolarization or depolarization and was involved in the generation of apamin-resistant inhibitory junction potentials in the guinea pig colon (Zagorodnyuk and Maggi 1994; Watson et al. 1996) and rat caecum (Serio et al. 1996). On the contrary, in the hamster ileum, nitric oxide or a related compound may be the transmitter underlying the apamin-sensitive inhibitory junction potentials (Matsuyama et al. 1999). The nitrergic nerve activation as well as exogenous nitric oxide could cause relaxation in the rat duodenum through apamin-sensitive mechanism (Martins et al. 1995).

In this study isolated guinea pig ileum was used as the experimental model since both excitatory and inhibitory NANC innervation are largely present in this organ (Bauer and Kuriyama 1982; Bauer 1993). Recently, we observed that apamin applied at a high concentration (5 μmol/l) inhibited the ATP-dependent contractile components of the electrically-elicited NANC motor responses of the longitudinal muscle layer and surprisingly, completely eliminated the nitric oxide-mediated relaxation component of these responses (Ivancheva et al. 2000). In view of the above, it appeared of interest to examine, in NANC experimental conditions, the involvement of nitric oxide and ATP in both spontaneous and electrically-induced motor activity with special interest in the apamin-sensitive mechanisms in their action.

Materials and Methods

Animals and preparations

Male guinea pigs (250–300 g) given food and water *ad lib* but starved for 12–14 hours before the experiments were used. The animals were sacrificed by a blow on the neck and were exsanguinated by severing the carotid arteries. The ileum, 18–20 cm proximal to the ileocaecal sphincter, was removed and washed out with Krebs solution at room temperature. In order to retain the myenteric plexus intact 20 mm long segments were cut out.

Mechanographic techniques

The segments were mounted along the longitudinal axis in 10 ml organ baths containing Krebs solution, aerated with 95% O_2 and 5% CO_2 at 36.5°C, pH 7.2. The preparations were initially loaded with a tension equivalent to 10 mN and were allowed to equilibrate for about 60 min. The motor activity of the longitudinal muscle layer was registered by means of a strain gauge (M 1000 B, Microtechna, Czech Republic) connected to a recording device (TZ 4620, Laboratorní přístroje, Czech Republic).

Electrical stimulation

To elicit neurogenic motor responses of the longitudinal muscle layer electrical field stimulation (EFS, square electrical pulses of 0.8 ms duration, 40 V, pulses at relatively low and relatively high frequency of 2 and 20 Hz, 20 seconds stimulus train duration; stimulator ST 02, Experimetria, Hungary) was applied at 5-min intervals by a pair wire platinum electrodes (0.45 mm thick) diametrically opposed on the organ bath walls parallel to the preparation.

The response to EFS was considered a complex response consisting of more than one component. The components which revealed themselves during the EFS were examined.

Solutions and drugs

The composition of the Krebs solution was (mmol/l): 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 drugs used were: phentolamine (CIBA), propranolol hydrochloride (Fluka), atropine sulphate (Merck), apamin, sodium nitroprusside (SNP), adenosine 5-triphosphate (ATP), N-G-nitro-L-arginine (L-NNA), L-arginine, D-arginine (all from Sigma), and tetrodotoxin (TTX, Sankyo). Drug concentrations are presented as final bath concentrations. Drugs were applied in a small volume that did not exceed 1% of the total bath volume and did not affect the pH of the bath solution.

Evaluation of results and statistical analysis

Changes in the NANC spontaneous and electrically-elicited motor activity of the longitudinal muscle layer were examined. The lowest points of the amplitude of the spontaneous phasic contractions 2 min before the application of drugs or electrical

stimulation were considered the baseline for measuring of the responses. Others were expressed as force in mN and in some cases, comparisons were made expressed in percentages. The data are presented as means ± S.E.M. and were analyzed using Student's paired and unpaired t-test. Differences were considered significant at $p < 0.05$. Concentration-response curves for the effects of SNP (0.1–100 μmol/l) in the absence and in the presence of apamin (0.1 μmol/l or 1 μmol/l) were constructed. The points of all curves were expressed as percentages of the maximum effect of SNP (100%) at a concentration of 100 μmol/l in the absence of apamin. The EC_{50} values (the effective concentrations producing 50% of the maximum effect) for SNP before and after apamin pretreatment and their 95% confidence limits were calculated by the points of the concentration-response curves for the effects of SNP using computer programs (Tallarida and Murray 1981).

Results

Spontaneous motor activity

In the presence of phentolamine (5 μmol/l), propranolol (5 μmol/l) and atropine (3 μmol/l) the longitudinal muscle layer of the isolated guinea pig ileum showed spontaneous NANC motor activity characterized by rhythmic phasic low-amplitude (0.2–0.5 mN) contractions without significant changes in tissue tone.

Apamin at a concentration of 0.1 μmol/l enhanced the tone. When added at a concentration of 1 μmol/l it evoked short-lasting fast contraction with an amplitude of 2.6 ± 0.3 mN ($n = 6$) which was followed by an increase in tone with superimposed phasic contractions of higher amplitudes. The changes in the spontaneous activity progressively declined to the baseline within 15 min. The effects of apamin were not influenced by 15-min pretreatment with TTX (0.1 μmol/l) (Fig. 1A, upper trace).

SNP, an exogenous donor of nitric oxide, applied cumulatively at concentrations from 0.1 μmol/l to 100 μmol/l, evoked concentration-dependent relaxation with a magnitude of 3.1 ± 0.4 mN (Fig. 1A, lower trace), the EC_{50} value being 0.39 ± 0.12 μmol/l ($n = 8$). Apamin added at concentrations of 0.1 μmol/l or 1 μmol/l 20 min before the application of SNP, concentration-dependently strongly decreased the SNP effects and shifted the concentration-response curves for SNP to the right (Fig. 1B). The EC_{50} value for SNP in the presence of apamin at a concentration of 0.1 μmol/l was increased to 59.34 ± 36.53 μmol/l ($n = 4$, $p < 0.05$). On the background of apamin at a concentration of 1 μmol/l the maximum effect of SNP was suppressed more than 50%. In this case, the value of EC_{50} for SNP (225.0 ± 161.3 μmol/l) was considered hypothetical.

ATP, applied for 1 min at a single concentration of 1 μmol/l or 50 μmol/l with washing out of the preparations after each application evoked concentration-dependent contractions. The amplitude of the response to ATP at a concentration of 50 μmol/l was 5.6 ± 0.4 mN ($n = 10$). Apamin at a concentration of 1 μmol/l, but not at that of 0.1 μmol/l ($n = 4$), significantly decreased these contractions

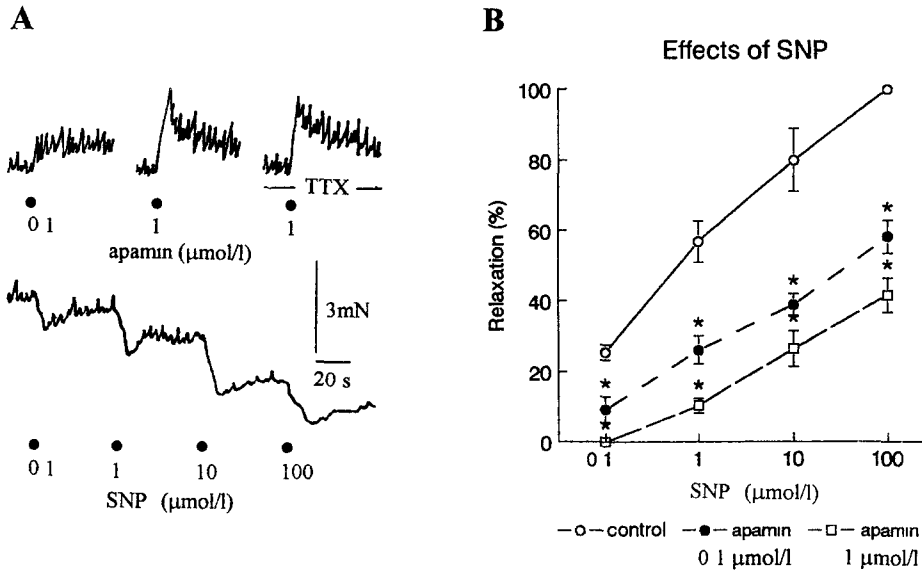

Figure 1. Longitudinal muscle layer of the guinea pig ileum (**A**) Segments of typical tracing showing the effects on the spontaneous NANC motor activity of single applications of apamin in the absence and in the presence of TTX (0 1 μmol/l) (upper trace) and of cumulatively applied SNP (lower trace) (**B**) Concentration-response curves to cumulatively applied SNP in the absence (control) and in the presence of apamin expressed as percentages of the maximum control response to SNP, 100 μmol/l, in the absence of apamin The values are means ± S E M obtained from 4 to 8 experiments Significance of differences *vs* control – $\star p < 0\ 05$

by 48.2 ± 8.3% ($n = 6$, $p < 0.05$) (Fig. 2A, upper trace; Fig. 2B). The contractile responses to ATP (50 μmol/l) were manifested in the presence of TTX (0.1 μmol/l) (not shown).

ATP (50 μmol/l)-induced contractions occurred in the presence of SNP applied at a concentration of 100 μmol/l. SNP (100 μmol/l) provoked relaxation on the background of ATP (50 μmol/l) (Fig. 2A, lower trace).

L-NNA, an inhibitor of nitric oxide synthesis, at concentrations of 0.1 mmol/l or 0.5 mmol/l did not considerably change the spontaneous motor activity. In 4 out of 12 preparations, a slight increase of the tone was observed without changing the phasic contractions. L-arginine (0.5 mmol/l), a substrate for nitric oxide synthesis and D-arginine (0.5 mmol/l), applied to L-NNA-pretreated preparations had no effect on the spontaneous activity.

Electrically-elicited motor responses

As previously described (Ivancheva and Radomirov 1996; Ivancheva et al. 1997), electrical stimulation elicited biphasic NANC motor responses of the longitudinal muscle layer.

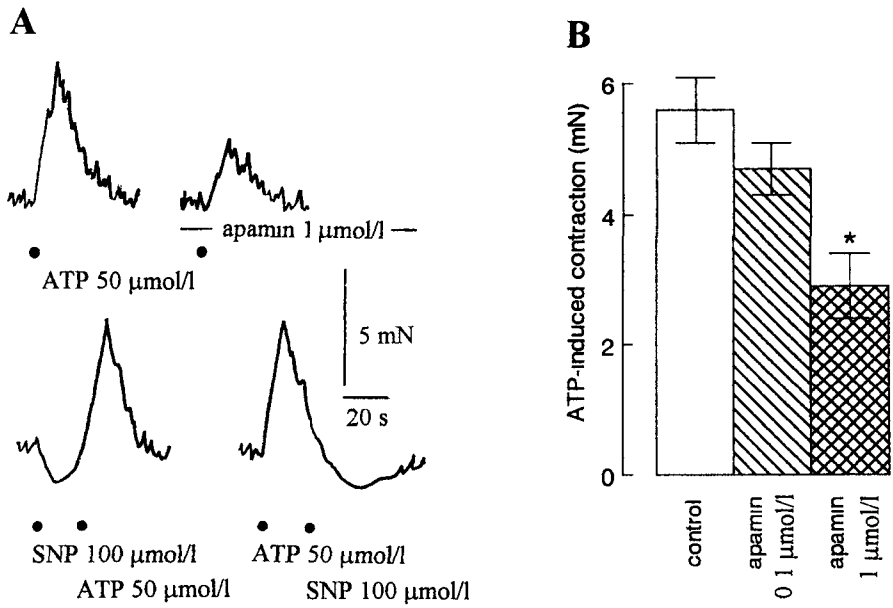

Figure 2. Longitudinal muscle layer of the guinea pig ileum (**A**) Segments of typical tracing showing the effects on the spontaneous NANC motor activity of single applications of ATP in the absence and in the presence of apamin (upper trace) and of single applications of ATP in the presence of SNP or SNP in the presence of ATP (lower trace) (**B**) ATP (50 μmol/l)-induced contractions in the absence (control) and in the presence of apamin The values are means ± S E M obtained from 4 to 10 experiments Significance of differences *vs* control – $\star p < 0\ 05$

The responses during 20 s EFS (0 8 ms, 40 V) applied at frequencies of 2 Hz or 20 Hz consisted of a relaxation, followed by a fast twitch-like contraction and a sustained tonic contraction with superimposed phasic contractions (Fig 3A) Thus, the NANC electrically-elicited responses were considered to consist of a relaxation phase and a phase of contractions The relaxation phase of the NANC responses to EFS occurred immediately after the application of the stimulation and was not longer than 5 s The phase of contractions lasted until the electrical stimulation was switched off The amplitudes of the components of the responses to 20 Hz electrical stimulation were significantly higher as compared to those elicited by EFS at a frequency of 2 Hz (Fig 3B) The amplitude of the relaxation and contractions and the tone of the preparations was not considerably changed during a stimulation period of 75–90 min in control experiments No significant differences were observed between the control responses of the different experimental programs

No electrically-elicited responses were observed in the presence of TTX (0 1 μmol/l) (not shown)

L-NNA (0 5 mmol/l) strongly affected the relaxation phase of the responses to EFS Ten to fifteen min after the addition of L-NNA to the bath, the relaxation

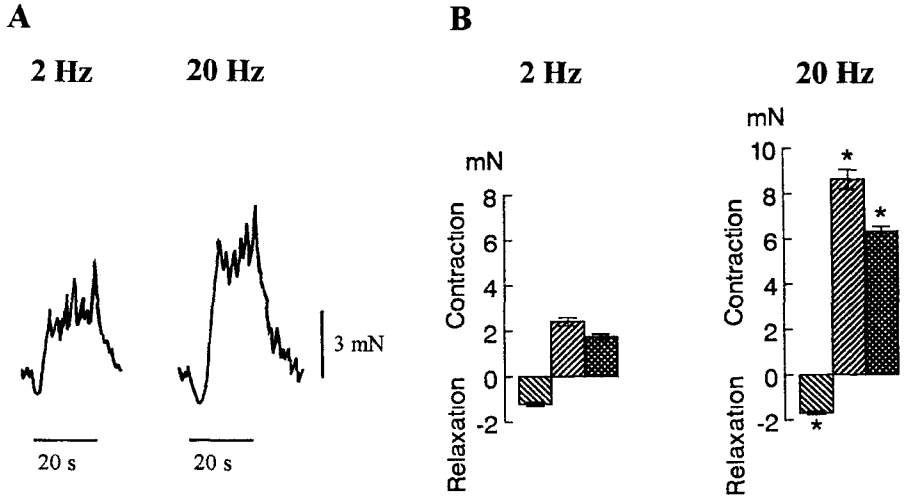

Figure 3. Longitudinal muscle layer of the guinea pig ileum (**A**) Segments of typical tracing showing the NANC electrically (0 8 ms, 40 V, 20 s, 2 or 20 Hz)-elicited motor responses (**B**) Phases of relaxation (▭) and contractions, twitch-like (▨) and tonic (▩) of the electrically-elicited motor responses The values are means ± S E M obtained from 12 experiments Significance of differences *vs* the response to 2-Hz EFS - $*p < 0\ 05$

phase of the responses to 2 Hz or 20 Hz EFS was abolished. The twitch and tonic contractions increased in the presence of L-NNA (0.5 mmol/l) (Fig. 4A,B). There was no significant difference in the efficiency of the L-NNA treatment on the phase of contractions of the responses to EFS applied at frequencies of 2 Hz or 20 Hz. L-arginine (0.5 mmol/l) but not D-arginine (0.5 mmol/l) gradually restored the electrically-elicited responses to the initial level within 30 min in preparations pretreated with L-NNA (0.5 mmol/l). The relaxation phases of the responses to 2 Hz and 20 Hz EFS were 91.5 ± 7.8% and 96.1 ± 6.4% of the respective relaxation of the control responses The twitch and tonic contractions reversed to the control values, too (90.1 ± 8.9% and 93.7 ± 8.4% in the responses to 2 Hz EFS ($n = 6$) and 94.2 ± 8.9% and 101.2 ± 6.4% in the responses to EFS at a frequency of 20 Hz ($n = 7$).

Apamin at concentrations of 0.1 μmol/l or 1 μmol/l completely eliminated the relaxation phase of the responses to 2 Hz or 20 Hz EFS after a 20-min pretreatment of the preparations (Fig. 4A,B). The effects of apamin on the phase of contractions depended on the concentration of the agent and on the frequency of the electrical stimulation. The twitch-like contractions of the responses to 2 Hz EFS increased while the tonic contractions of these responses significantly decreased by apamin at both concentrations used, 0.1 μmol/l or 1 μmol/l (Fig. 4A). Apamin at the concentrations used did not affect the twitch-like contractions of the responses induced by 20 Hz EFS and significantly reduced the tonic contractions of these responses when applied at the higher concentration of 1 μmol/l (Fig. 4B).

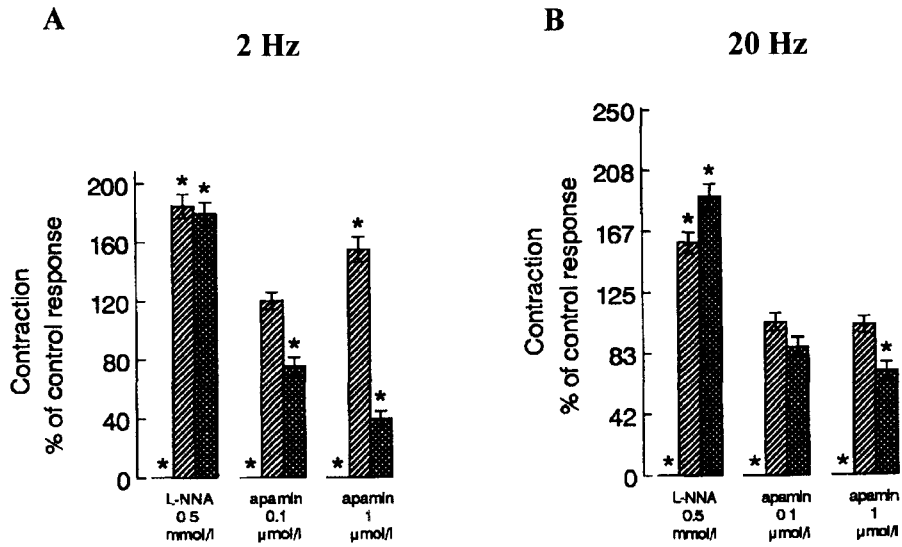

Figure 4. Longitudinal muscle layer of the guinea pig ileum Effects of L-NNA and apamin on the phases of relaxation (—) and contractions, twitch-like (▨) and tonic (▩) of the electrically-elicited at frequencies of stimulation of (**A**) 2 or (**B**) 20 Hz NANC motor responses The values are means ± S E M obtained from 6 to 8 experiments Significance of differences *vs* control – $\star p < 0\ 05$

Discussion

SNP, a nitric oxide donor, used in this study provoked a relaxation. The relaxation phase of the electrically-elicited responses to both low and high frequency of stimulation was abolished by L-NNA and was restored by L-arginine. These findings suggest the nitrergic nature of the drug- or electrically-induced relaxation and correspond with the data that nitric oxide is involved in the inhibitory NANC transmission in a number of tissues throughout the gastrointestinal tract of several mammals (Bult et al. 1990; Shuttleworth et al. 1991; Bauer 1993; Tanobe et al. 1994; Rand and Li 1995) including the guinea pig small intestine (Osthaus and Galligan 1992; Williams and Parsons 1995; Ivancheva et al. 1997, 1998). Applied ATP evoked contractions, thus confirming observations that in some intestinal preparations ATP and analogues could have excitatory action (Moody and Burnstock 1982; Manzini et al. 1986; Radomirov and Venkova 1988; Zagorodnyuk and Maggi 1998; Ivancheva et al. 2000).

SNP-induced relaxation was concentration-dependent and the EC_{50} value (0.39 ± 0.12 µmol/l) was very close to the EC_{50} value (0.29 µmol/l) obtained for the SNP relaxant effect in guinea pig taenia caeci (Shuttleworth et al. 1999). Apamin (0.1 µmol/l or 1 µmol/l) concentration-dependently reduced the SNP-induced re-

laxation, shifted the concentration-response curve for SNP to the right and completely eliminated the nitrergic by nature relaxation phase of the electrically-elicited motor responses, thus suggesting that in the present study the relaxation mediated either by exogenous or by neurally-released nitric oxide was apamin-sensitive Our data are in agreement with more recent observations indicating that nitrergic effects in the rat duodenum (Martins et al 1995), guinea pig taenia caeci (Shuttleworth et al 1999), hamster ileum (Matsuyama et al 1999) and rat proximal colon (Mule et al 1999) are sensitive to apamin (1 nmol/l – 1 μmol/l) In other intestinal preparations such as the whole preparation of the circular muscle of the guinea pig ileum (Bauer 1993) and guinea pig colon (Maggi and Giuliani 1993, Zagorodnyuk and Maggi 1994) and rat caecum (Serio et al 1996) the nitrergic relaxation was found to be apamin (0 1–0 3 μmol/l)-resistant showing that the sensitivity of the nitrergic effects to apamin is probably dependent on the animal species and on the concentration of apamin

The mechanism(s) underlying the sensitivity of nitric oxide-mediated motor events to apamin is a matter of current debate Nitric oxide is known to act *via* stimulation of the soluble guanylate cyclase and accumulation of cyclic GMP, thus inducing smooth muscle relaxation (Waldman and Murad 1987, Kanada et al 1992) Martins et al (1995) tested the influence of apamin on nitrergic effects in the rat proximal duodenum and proposed that the nitrergic nerve activation as well as exogenous nitric oxide caused relaxation by an apamin-sensitive and cyclic GMP-independent mechanism More recent observations showed that the apamin-sensitive action of nitric oxide appears to be mediated *via* cyclic GMP and may involve activation of calcium-dependent potassium channels (Matsuyama et al 1999, Shutleworth et al 1999) The cyclic GMP production system and opening of apamin-sensitive calcium-dependent potassium channels appear to work sequentially in transducing the nitric oxide signal (Mule et al 1999) The calcium-activated potassium channels in the vascular smooth muscle (Bolotina et al 1994, Lee et al 1994) and in the guinea pig proximal and distal colon could be directly modulated by nitric oxide indicating some antagonistic interactions between nitric oxide and apamin at the level of these channels (Watson et al 1996) Since the present experiments demonstrated that apamin prevented the relaxation induced either by exogenous (SNP) or by endogenous nitric oxide it could be suggested that in the longitudinal muscle layer of the guinea pig ileum nitric oxide utilizes apamin-sensitive calcium-activated potassium channels to realize an inhibitory action

Applied ATP evoked TTX-resistant contractions suggesting that postjunctional purinoceptors were involved It has been proposed that in the intestinal smooth muscle cells two subtypes P_2 purinoceptors, relaxation-mediating (Johnson et al 1996) and contraction-mediating (Kennedy and Humphrey 1994) provided the ATP action Zagorodnyuk and Maggi (1998) concluded that at least three types of P_2 purinoceptors are present in the smooth muscle of the guinea pig colon two types inhibitory apamin-sensitive receptors and an excitatory contraction-producing suramin-sensitive receptor Recently, we have found that apamin applied at a high concentration of 5 μmol/l exerted strong inhibitory effects on the

ATP-mediated, suramin-sensitive contractile components of drug- or electrically-induced motor responses in the guinea pig ileum (Ivancheva et al. 2000). The present experiments showed that apamin at a concentration of 1 μmol/l decreased the ATP-induced contractions and reduced the tonic component of the phase of contractions in the electrically-elicited motor responses. The agent was less effective when applied at a smaller concentration of 0.1 μmol/l. A comparison of our present and the previous findings gives us ground to believe that in the longitudinal muscle layer of the guinea pig ileum exogenous or neurally-released ATP could participate in the contractile events *via* subtype of the contraction-mediating P_2 purinoceptors which are suramin-dependent and are sensitive to relatively high ($\geq$ 1 μmol/l) concentrations of apamin.

The ATP-induced contractile effect occurred in the presence of SNP, and SNP-provoked relaxation revealed on the background of ATP indicating that nitric oxide and ATP utilize independent apamin-sensitive mechanisms and act as functional antagonists with respect to the NANC ileal motor activity (Ivancheva et al. 2000).

Apamin stimulated the spontaneous motor activity as well as it increased the twitch-like contractions of the responses to 2 Hz EFS. We have no explanations for this matter. Maggi and Giuliani (1993) observed apamin-induced increase in the guinea pig colon tone and considered that it cannot be decided whether the excitatory effect of apamin is due to the removal of an inhibitory neural influence or to a direct action on the smooth muscle cells. As early as in 1980, Shuba and Vladimirova suggested that many other effects associated with the increase of potassium permeability would be expected to be blocked or changed by apamin

In conclusion, the present results confirm the functional role of nitric oxide and ATP in the modulation of the spontaneous and electrically-elicited motor activity of the longitudinal muscle layer of the guinea pig ileum, and suggest that the apamin-sensitive inhibitory effect of nitric oxide could be realized *via* calcium-activated potassium channels and that the apamin-sensitive excitatory effect of ATP is mediated *via* contraction-producing P_2 purinoceptors.

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