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Tachykinin NK_1 but not NK_2 receptors mediate non-cholinergic excitatory junction potentials in the circular muscle of guinea-pig colon

*Vladimir Zagorodnyuk, Paolo Santicioli & [1]Carlo Alberto Maggi

Pharmacology Department, A. Menarini Pharmaceuticals, Via Sette Santi 3, Florence, Italy and *Department Neuro-muscular Physiology, Bogomoletz Institute of Physiology, Bogomoletz Str. 4, Kiev, Ukraine

1 The effect of tachykinin NK_1 and NK_2 receptor antagonists on noncholinergic excitatory junction potentials (e.j.ps) evoked by electric field stimulation (EFS) in the circular muscle of the guinea-pig proximal colon was investigated by means of a sucrose-gap technique.

2 In the presence of 1 μM atropine, submaximal EFS (10 Hz, 20–30 V, 0.5 ms pulse width, 1 s train duration) evoked an inhibitory junction potential (i.j.p.) followed by e.j.p. with superimposed action potentials (APs) and contraction. Addition of either N^G-nitro-L-arginine (L-NOARG, 0.1 mM) or apamin (0.1 μM) inhibited the evoked i.j.p. and the combined administration of the two agents almost abolished it. In the presence of both L-NOARG and apamin, an atropine-resistant e.j.p. was the only electrical response evoked by EFS in 50% of cases and a small i.j.p. (10% of original amplitude) followed by e.j.p. was evident in the remainder.

3 In the presence of L-NOARG and apamin, the tachykinin NK_1 receptor antagonists, (±)-CP 96,345 and GR 82,334 (10 nM–3 μM) concentration-dependently inhibited the atropine-resistant e.j.p. and accompanying contraction evoked by EFS. EC_{50} values were: 0.77 μM (e.j.p. inhibition) and 0.22 μM (inhibition of contraction) for (±)-CP 96,345; 0.61 μM (e.j.p. inhibition) and 0.20 μM (inhibition of contraction) for GR 82,334. The tachykinin NK_2 receptor antagonists, MEN 10,376 (up to 3 μM) and SR 48,968 (up to 1 μM) had no effect on the atropine-resistant e.j.p. MEN 10,376 (3 μM) but not SR 48,968 produced a slight inhibition of the evoked contraction.

4 (±)-CP 96,345 (3 μM) and GR 82,334 (3 μM) markedly reduced (81 and 89% inhibition, respectively) the atropine-resistant e.j.p. in the absence of L-NOARG and apamin, without affecting the i.j.p. MEN 10,376 (3 μM) and SR 48,968 (1 μM) had no significant effect on noncholinergic i.j.p. and e.j.p. evoked in the absence of apamin and L-NOARG.

5 The electrical and mechanical responses to the NK_1 receptor agonist [Sar^9]substance P (SP) sulfone were blocked by (±)-CP 96,345 (3 μM) or GR 82,334 (3 μM) which, at the same concentration, failed to affect the responses to the NK_2 receptor agonist [βAla^8] neurokinin A (NKA) (4-10). In contrast, MEN 10,376 (3 μM) or SR 48,968 (1 μM) blocked the response to [βAla^8]NKA(4-10) without affecting the response to [Sar^9]SP sulfone.

6 In the presence of L-NOARG and apamin, and in the absence of atropine, EFS of low pulse width (0.02–0.03 ms, other parameters as above) produced cholinergic e.j.ps and contraction which were unaffected by GR 82,334 (3 μM). (±)-CP 96,345 (3 μM) produced 24% reduction in the area of the atropine-sensitive e.j.p. without affecting the peak amplitude of e.j.p. or contraction.

7 These findings demonstrate that the noncholinergic e.j.ps and accompanying contraction of the circular muscle of the guinea-pig colon are produced through activation of intramural tachykininergic nerves and that the resultant smooth muscle response is almost entirely mediated through NK_1 receptors.

Keywords: Tachykinins; tachykinin receptors; guinea-pig proximal colon; NK_1 receptor; tachykinin receptor antagonists; noncholinergic excitatory junction potentials

Introduction

Anatomical, neurochemical and pharmacological evidence implies a major role for tachykinins (TKs) as excitatory neuromuscular transmitters in the mammalian gut (Franco *et al.*, 1979; Bartho *et al.*, 1982; Costa *et al.*, 1985; Bartho & Holzer, 1985 for review). Substance P (SP) and neurokinin A (NKA) are synthesized by enteric neurones projecting to the circular and longitudinal muscle layers of the mammalian gut (Costa *et al.*, 1987; Sternini *et al.*, 1989; Too *et al.*, 1989; Shuttleworth *et al.*, 1991) and the release of endogenous tachykinins in response to depolarizing and physiological stimuli (peristalsis) has been demonstrated (e.g. Donnerer *et al.*, 1984; Theodorsson *et al.*, 1991). SP and NKA are powerful spasmogens in the mammalian gut: the direct contractile activity of tachykinins on smooth muscle cells is mediated, in many instances, through NK_1 (SP-preferring) and NK_2 (NKA-preferring) receptors (e.g. Maggi *et al.*, 1990; 1992; Giuliani *et al.*, 1993). Since both SP and NKA are synthesized by enteric neurones through the expression of the preprotachykinin I gene (Sternini *et al.*, 1989), the concomitant release of the two mediators during nerve activity in the gut, and the simultaneous presence of NK_1 and NK_2 receptors on target smooth muscle cells represents a putative example of tachykininergic co-transmission. This raises the question of the relative contribution of the two mediators to the final response and the mechanisms governing this event (e.g. Bartho *et al.*, 1992).

Non-cholinergic excitatory junction potentials (e.j.ps) have been recorded in the circular (Shuba & Vladimirova, 1980; Bywater & Taylor, 1983; 1986; Crist *et al.*, 1991) and longitudinal (Bauer & Kuriyama, 1982) muscle of the guinea-pig gut. It has been proposed that atropine-resistant e.j.ps in the guinea-pig ileum are caused by the release of SP (Bauer & Kuriyama, 1982; Niel *et al.*, 1983; Crist *et al.*, 1991). Evidence for this was obtained in two ways: (i) by studying

[1] Author for correspondence.

the effect of SP receptor desensitization on the atropine-resistant e.j.ps; (ii) by using non-selective tachykinins antagonists such as [D-Arg[1],D-Pro[2],D-Trp[7,9],Leu[11]]SP or spantide. The use of spantide and other TK receptor antagonists of first generation (Maggi et al., 1993 for review) poses important limits to the final demonstration of a neurotransmitter role for TKs, for the following reasons: (1) spantide and its congeners possess, at certain concentrations, local anaesthetic activity and inhibit the action of mediators (for e.g. bombesin) unrelated to TKs; (2) owing to their low potency, spantide and its congeners do not discriminate between NK_1 and NK_2 receptors (e.g. Buck & Shatzer, 1988).

Previously, we showed that both NK_1 and NK_2 receptors are present in the circular muscle of the guinea-pig colon to mediate the direct contractile response to TKs (Giuliani et al., 1993). The aim of this study was to investigate the effect of novel potent antagonists selective for the NK_1 receptor, (±)-CP 96,345 (Snider et al., 1991) and GR 82,334 (Hagan et al., 1991) or for the NK_2 receptor MEN 10,376 (Maggi et al., 1991) and SR 48,968 (Emonds-Alt et al., 1992), on the atropine-resistant e.j.p. and following contraction produced by electrical field stimulation (EFS) in the circular muscle of the guinea-pig proximal colon. To study noncholinergic e.j.ps in detail it would be necessary to block inhibitory junction potentials (i.j.ps) because EFS excites all nerve fibres within the smooth muscle strip. It appears possible that more than one transmitter determines nonadrenergic noncholinergic relaxation in the gut (Costa et al., 1986; Manzini et al., 1986). As shown in a previous functional study (Maggi & Giuliani, 1993), the combined administration of apamin and N^G-nitro-L-arginine (L-NOARG) was most effective in inhibiting the mechanical nonadrenergic noncholinergic relaxation in the circular muscle of the guinea-pig proximal colon. From this, the effect of selective NK_1 and NK_2 receptor antagonists was studied in the presence of apamin and L-NOARG.

Methods

Male albino guinea-pigs (250–300 g) were stunned and bled. A ring of proximal colon (1–2 cm from the caecum-colonic junction) was excised and placed in oxygenated (96% O_2 and 4% CO_2) Krebs solution of the following composition ($mmol^{-1}$): NaCl 119, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.5, KCl 4.7, $CaCl_2$ 2.5 and glucose 11. The pH of the solutions was 7.4. The ring was opened and pinned flat in a Petri dish and mucosa-free circular muscle strips were dissected. Strips approximately 0.5–0.8 mm wide and 10 mm long were cut in parallel to the circular muscle of the guinea-pig proximal colon. The strips were superfused with oxygenated Krebs solution at a rate of 1 ml min^{-1}. The temperature of Krebs solution was kept constant at 35 ± 0.5°C.

A single sucrose-gap, modified as described in details by Artemenko et al. (1982) and Hoyle (1987) was used to investigate changes in membrane potential and mechanical activity in response to electrical field stimulation. The modifications were made to a rubber membrane type of single sucrose gap apparatus to facilitate the recording of isometric tension in smooth muscle strips and enable the simultaneous recording of electrotonic potentials and junction potentials. Junction potentials were evoked by electrical field stimulation (EFS) of intramural nerves. Unless otherwise stated, atropine (1 μM) was present in the Krebs solution from the beginning of the experiments.

In a preliminary study it was found that submaximal parameters of stimulation were required to demonstrate an atropine-resistant excitatory junction potential (e.j.p.) in response to EFS (see results). For this purpose trains of pulses (pulse width 0.5 ms) were delivered for 1 s at a frequency of 10 Hz at submaximal voltage (20–30 V). In these conditions an inhibitory junction potential (i.j.p.) and e.j.p. were evoked by EFS. The effect of TK receptor antagonists was investigated in the presence of L-NOARG (0.1 mM) and apamin (0.1 μM) to eliminate or inhibit (see results) i.j.ps evoked by EFS. In some experiments, cholinergic e.j.ps were evoked in the presence of L-NOARG and apamin, but in the absence of atropine by using low pulse width (0.02–0.03 ms) EFS (other parameters as above).

GR 82,334, MEN 10,376 and SR 48,968 were applied to the circular muscle for 15 min while (±)-CP 96,345 was applied for at least 20 min. In some experiments, the electrical and mechanical responses produced by the NK_1 receptor selective agonist [Sar[9]]SP sulfone and the NK_2 receptor selective agonist [βAla[8]]NKA(4-10) were recorded in the absence and presence of the various TK receptor antagonists. For each agonist a concentration of 0.3 μM was applied for 10 s through the superfusion system: this concentration was chosen from preliminary experiments showing that it produces a reproducible contractile response of comparable size to [Sar[9]]SP sulfone and [βAla[8]]NKA(4-10). These experiments were performed in the presence of 1 μM atropine.

In preliminary experiments phentolamine (3.1 μM) and propranolol (3.4 μM) did not significantly affect the i.j.ps produced by EFS. For this reason adrenoceptor blockers were not routinely used in the experiments.

For i.j.p. and e.j.p., the following parameters were evaluated: latency, amplitude, duration, time to peak ($t1$) and time of recovery from peak to baseline ($t2$). To evaluate the effect of TK receptor antagonists on e.j.p. the area of depolarization was calculated by use of a MiniMop apparatus (Kontron, Germany).

Statistical analysis

All data in the text are mean ± standard error of the mean (s.e.mean). Statistical analysis was performed by means of Student's t test for paired or unpaired data, or by means of analysis of variance, when applicable.

Drugs

Drugs used were: atropine HCl (Serva, Heidelberg, Germany), phentolamine mesylate (Ciba-Geigy), propranolol HCl, N^G-nitro-L-arginine (L-NOARG) and apamin (Sigma).

MEN 10,376 or [Tyr[5],D-Trp[6,8,9],Lys[10]]NKA(4-10); [βAla[8]]-NKA(4-10) and (±) CP 96,345 ((±)-*cis*-2-(diphenylmethyl)-*N*-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2.]octan-3-amine) were synthesized at Chemistry Department, A. Menarini Pharmaceuticals, Florence, Italy. [Sar[9]]SP sulfone was from Peninsula. GR 82,334 or [D-Pro[9][Spiro-γ-lactam]Leu[10],Trp[11]] physalaemin(1-11) from Neosystem, Strasbourg, France. SR 48,968 ((S)-N-methyl-*N*[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl) butyl] benzamide) was a kind gift of Drs Emonds-Alt & Le Fur (Sanofi, Montpellier, France).

Results

General

In the presence of atropine (Figure 1) a single pulse of EFS (0.5 ms, 20–60 V) evoked i.j.p. but not e.j.p. or contraction. A train of stimuli at a frequency of 10 Hz (1 s duration) evoked i.j.p., an atropine-resistant e.j.p. and contraction when using submaximal voltage (20–30 V) (Figure 1). Supramaximal (60 V) train stimulation caused i.j.p., hyperpolarization (after the first nonadrenergic i.j.p.) and relaxation but no e.j.p. or contraction. After this stimulation the e.j.p. and contraction evoked by submaximal train stimulation were depressed: 10–15 min were required for recovery of the original response to submaximal EFS (Figure 1). Because of these results all junction potentials were evoked by using submaximal parameters of EFS.

In the presence of atropine (1 μM), EFS (10 Hz, 20–30 V, 0.5 ms for 1 s) induced i.j.ps followed by noncholinergic e.j.ps

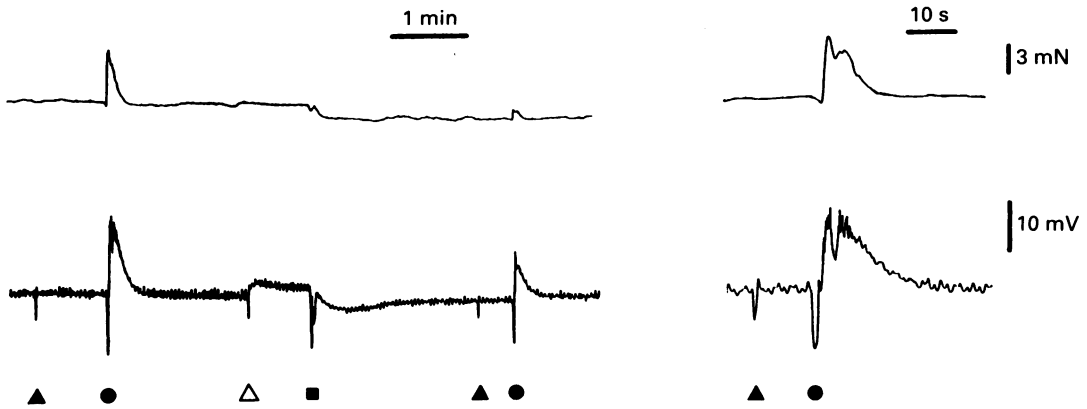

Figure 1 Electrical (lower tracing) and mechanical (upper tracing) response of the circular muscle of the guinea-pig proximal colon to EFS in the presence of atropine (1 μM). Triangles mark the response to single pulse EFS (▲, 20 V; △, 60 V, pulse width was 0.5 ms in both cases). Submaximal (20 V, ●) but not supramaximal (60 V, ■) train EFS (10 Hz for 1 s, 0.5 ms pulse width) evoked an e.j.p. and contraction. After application of a supramaximal (■) train EFS, e.j.p. and contraction produced by submaximal (●) train EFS were depressed and recovered 15 min later (expanded time scale at the right).

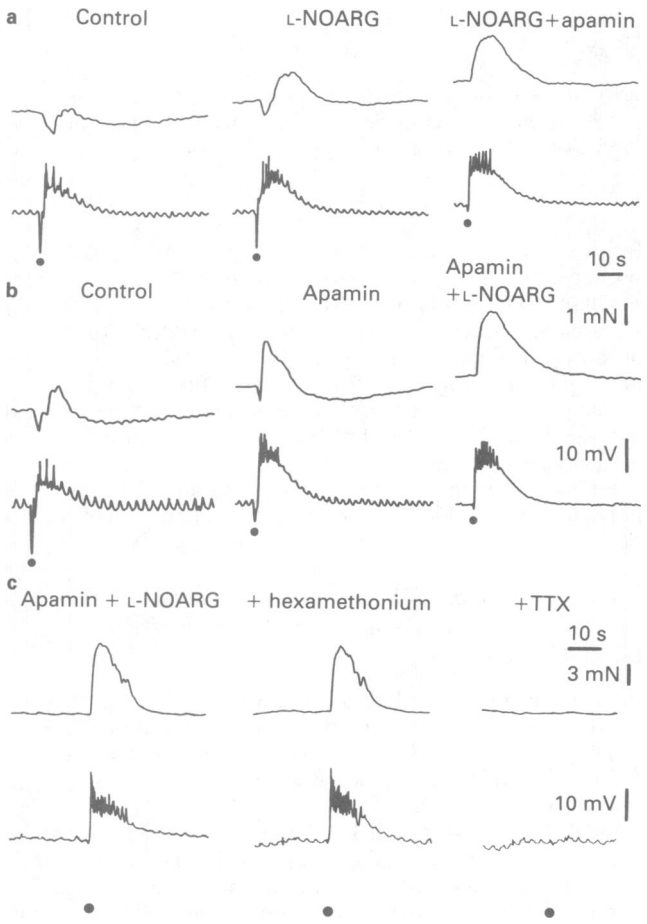

Figure 2 Typical tracings showing the effects of N^G-nitro-L-arginine (L-NOARG) (a) and apamin (b) on junction potentials and changes in tension evoked by EFS (10 Hz, 1 s, 20 V, 0.5 ms, applied at ●) in the circular muscle from guinea-pig proximal colon. Experiments performed in the presence of atropine (1 μM). The effect of L-NOARG (0.1 mM) and apamin (0.1 μM) are shown after 15 min; effect of combined administration of apamin and L-NOARG after 30 min of action. (c) Shows the effect of hexamethonium and tetrodotoxin (TTX) on e.j.p. and contraction of the circular muscle of the guinea-pig colon produced by EFS (10 Hz, 1 s, 20 V, 0.5 ms, applied at ●) in the presence of apamin and L-NOARG (applied 30 min beforehand); the effect of hexamethonium (10 μM) is shown after 20 min and the effect of TTX (0.3 μM) after 15 min of action. Top tracing is muscle tension, lower tracing is change in membrane potential.

(rebound excitation, off-response) and action potentials (APs) (Figure 2a). The mechanical response was dependent on the tone of the smooth muscle strip; when tone was high (Figure 2a) the response was triphasic: (i) a primary relaxation, (ii) a rebound contraction and (iii) a secondary relaxation.

Effects of L-NOARG and apamin on i.j.p.

L-NOARG (0.1 mM) produced an increase in tone of the muscle strips with maximum at 5–10 min from its administration, followed by a decline to baseline. Resting membrane potential and the latency of i.j.ps were not significantly affected by L-NOARG ($n = 5$). The amplitude of i.j.ps was slightly (12%) but significantly ($P < 0.05$, $n = 6$) reduced by L-NOARG. L-NOARG also reduced the duration (37%, $P < 0.01$, $n = 6$) and time of decay of i.j.ps ($t2$, 38%, $P < 0.05$, $n = 6$) (Table 1 and Figure 2a).

Apamin (0.1 μM) caused a small depolarization of cell membrane by 1.96 ± 0.3 mV ($n = 5$) followed by an increase in tone of the muscle strip, which declined (after 15–30 min) to baseline. Apamin markedly reduced the amplitude (56% inhibition, $P < 0.01$, $n = 6$) and increased both latency (51%, $P < 0.01$, $n = 5$) and time to peak ($t1$, 31%, $P < 0.01$, $n = 6$) of i.j.ps (Table 1 and Figure 2b).

The combined application of apamin (0.1 μM) and L-NOARG (0.1 mM) caused depolarization of cell membrane by 1.8 ± 0.3 mV ($n = 17$) followed by an increase of tone of muscle strip. The maximum depolarization and increase of tone was achieved in 10–15 min and with time (30–45 min) both declined to basal values. The i.j.p. was almost abolished by the combined administration of apamin and L-NOARG. After application of apamin and L-NOARG, alone or in combination, a clear enhancement/unmasking of EFS-evoked e.j.p./APs and accompanying contraction was evident (Figure 2). Owing to the preceding i.j.p. and relaxation, these effects were not quantitated nor it is possible to decide whether this is due to removal of inhibition or may also involve prejunctional enhancement of noncholinergic excitation.

In the presence of apamin and L-NOARG, submaximal EFS evoked only noncholinergic e.j.ps in 50% of cases; in the remainder EFS evoked a small nonadrenergic i.j.p. (10% of control) followed by non-cholinergic e.j.p. (Figures 2 and 3). If present, the amplitude and total duration of i.j.ps (were 1.3 ± 0.3 mV ($n = 17$) and 703 ± 70 ms ($n = 7$), respectively. The electrophysiological parameters of non-cholinergic e.j.p. evoked in the presence of apamin and L-NOARG are summarized in Table 2.

The non-cholinergic e.j.p. and APs evoked in the presence of L-NOARG and apamin were not affected by hex-

Table 1 Effect of apamin (0.1 μM) or N^G-nitro-L-arginine (0.1 mM, L-NOARG) on parameters of the inhibitory junction potential (i.j.p.) evoked by electrical field stimulation (10 Hz, 20–30 V, 0.5 ms pulse width for 1 s) in the circular muscle of the guinea-pig proximal colon

	Latency (ms)	*Amplitude* (mV)	*Duration* (ms)	t*1* (ms)	t*2* (ms)
Control	140 ± 6.3 ($n = 5$)	13.8 ± 0.8 ($n = 6$)	1913 ± 130 ($n = 6$)	613 ± 26 ($n = 6$)	1300 ± 122 ($n = 12$)
+ Apamin	212 ± 12** ($n = 5$)	6.1 ± 0.8** ($n = 6$)	1813 ± 123 ($n = 6$)	806 ± 54** ($n = 6$)	940 ± 96 ($n = 6$)
Control	148 ± 4.8 ($n = 7$)	13.8 ± 0.8 ($n = 6$)	2260 ± 97 ($n = 6$)	573 ± 59 ($n = 6$)	1660 ± 93 ($n = 6$)
+ L-NOARG	153 ± 8.4 ($n = 7$)	12.1 ± 0.7* ($n = 6$)	1520 ± 217** ($n = 6$)	486 ± 24 ($n = 6$)	1033 ± 216* ($n = 6$)

Atropine (1 μM) was present throughout the experiment.
*$P < 0.05$; **$P < 0.01$.

Table 2 Electrophysiological parameters of the atropine-resistant and atropine-sensitive excitatory junction potential (e.j.p.) evoked by electrical field stimulation in the circular muscle of the guinea-pig proximal colon

	Latency (ms)	*Amplitude* (mV)	*Duration* (s)	t*1* (s)	t*2* (s)
Atropine-resistant e.j.p.	736 ± 31 ($n = 24$)	12 ± 0.6 ($n = 70$)	31 ± 0.8 ($n = 43$)	3.1 ± 0.3 ($n = 40$)	27.5 ± 0.9 ($n = 40$)
Atropine-sensitive e.j.p.	147 ± 6.7 ($n = 3$)	9.5 ± 0.6 ($n = 9$)	11.3 ± 0.8 ($n = 9$)	1.2 ± 0.1 ($n = 9$)	10.1 ± 0.8 ($n = 9$)

Parameters of EFS were 10 Hz, 20–30 V for 1 s, pulse width was 0.5 ms for atropine-resistant e.j.p. and 0.02–0.03 ms for atropine-sensitive e.j.p. Atropine-resistant e.j.p. was recorded in the presence of atropine (1 μM), apamin (0.1 μM) and N^G-nitro-L-arginine (L-NOARG, 0.1 mM), atropine-sensitive e.j.p. was recorded in the presence of apamin (0.1 μM) and L-NOARG (0.1 mM).

amethonium (10 μM, $n = 3$) but were blocked by tetrodotoxin (TTX, 0.3 μM, $n = 3$) (Figure 2c).

Effects of selective NK_1 and NK_2 receptor antagonists on noncholinergic e.j.ps and contractions evoked by EFS in the presence of L-NOARG and apamin

GR 82,334 (up to 3 μM), MEN 10,376 (up to 3 μM) and SR 48,968 (up to 1 μM) had no agonist effect on mechanical or electrical activity of the circular muscle. (±)-CP 96,345 (3 μM) caused a small but significant depolarization of the cell membrane by 1.0 ± 0.4 mV ($n = 7$, $P < 0.05$).

The non-peptide NK_1 receptor antagonist, (±)-CP 96,345 concentration-dependently (Figures 3 and 4) inhibited the non-cholinergic e.j.p. and contractions, EC_{50} (95% c.l. in parentheses) being 0.77 μM (0.31–1.91 μM) and 0.22 μM (0.17–0.29 μM), respectively.

The peptide NK_1 receptor antagonist GR 82,334 concentration-dependently inhibited the non-cholinergic e.j.p. and contractions (Figures 3 and 4), EC_{50} being 0.61 μM (0.43–0.87 μM) and 0.20 μM (0.09–0.44 μM), respectively. Typical tracings showing the inhibitory effect of NK_1 receptor antagonists on the non-cholinergic e.j.p. and contraction are shown in Figure 3. The effect of GR 82,334 (3 μM) developed faster (more than 50% block occurred within 3–5 min from application) than that of (±)-CP 96,345 (more than 50% block occurred at 10–15 min after application). Data on the concentration-dependent inhibition of e.j.p. and contraction are shown in Figure 4.

The non-peptide NK_2 receptor antagonist SR 48,968 (up to 1 μM) and the peptide NK_2 receptor antagonist MEN 10,376 (up to 3 μM) had no marked effects on non-cholinergic e.j.p. and contractions evoked by EFS (Figures 3 and 4). At the highest concentration tested, MEN 10,376 (3 μM) and SR 48,968 (1 μM) caused a small but significant (against control responses of the same strips) inhibition of non-cholinergic contractions of 32 ± 5% ($n = 6$, $P < 0.01$) and 13 ± 5% ($n = 11$, $P < 0.05$), respectively (Figure 4).

In control experiments, a small spontaneous decay in amplitude of non-cholinergic contractions during 20 min perfusion with Krebs solution was observed. The amplitude of spontaneous decay of contraction was 16 ± 4% ($n = 6$, $P < 0.05$). The inhibitory effect of MEN 10,376 (3 μM) was significantly larger than that of spontaneous decay (measured on different preparations), while the effect of SR 48,968 was not significantly different from spontaneous decay.

The amplitude of non-cholinergic e.j.ps evoked by EFS did not significantly change (1 ± 4%, $n = 6$, NS) during 20 min perfusion with Krebs solution.

Summarizing this section the non-cholinergic e.j.ps were not affected by NK_2 receptor antagonists and a small reduction of contraction was only evident for 3 μM MEN 10,376.

Effects of NK_1 and NK_2 receptor antagonists on the response to EFS in the absence of apamin and L-NOARG

In atropine-containing Krebs solution, GR 82,334 (3 μM) and (±)-CP 96,345 (3 μM) markedly reduced the non-cholinergic e.j.ps by 89 ± 2% ($n = 6$, $P < 0.001$) and 81 ± 4% ($n = 5$, $P < 0.001$) while the amplitude of non-adrenergic i.j.ps was unaffected (Figure 5).

SR 48,968 (1 μM) and MEN 10,376 (3 μM) had no marked effect on e.j.ps or i.j.ps (Figure 5). SR 48,968 (1 μM) slightly reduced the amplitude of non-adrenergic i.j.ps by 8 ± 2% ($n = 6$, $P < 0.01$) while leaving non-cholinergic e.j.ps unaffected. MEN 10,376 (3 μM) caused a small reduction in the amplitude of both e.j.ps and i.j.ps by 17 ± 4 ($n = 7$, $P < 0.01$) and by 8 ± 2% ($n = 9$, $P < 0.01$).

None of these antagonist displayed any significant agonist effect on contractile and electrical activity of the circular muscle under these conditions.

Effect of selective NK_1 and NK_2 receptor antagonists on electrical and mechanical response to [Sar^9]SP sulfone and [βAla^8]NKA(4-10)

Application of the NK_1 receptor selective agonist, [Sar^9]SP sulfone (0.3 μM for 10 s) and of the NK_2 receptor selective agonist, [βAla^8]NKA(4-10) (0.3 μM for 10 s) both produced

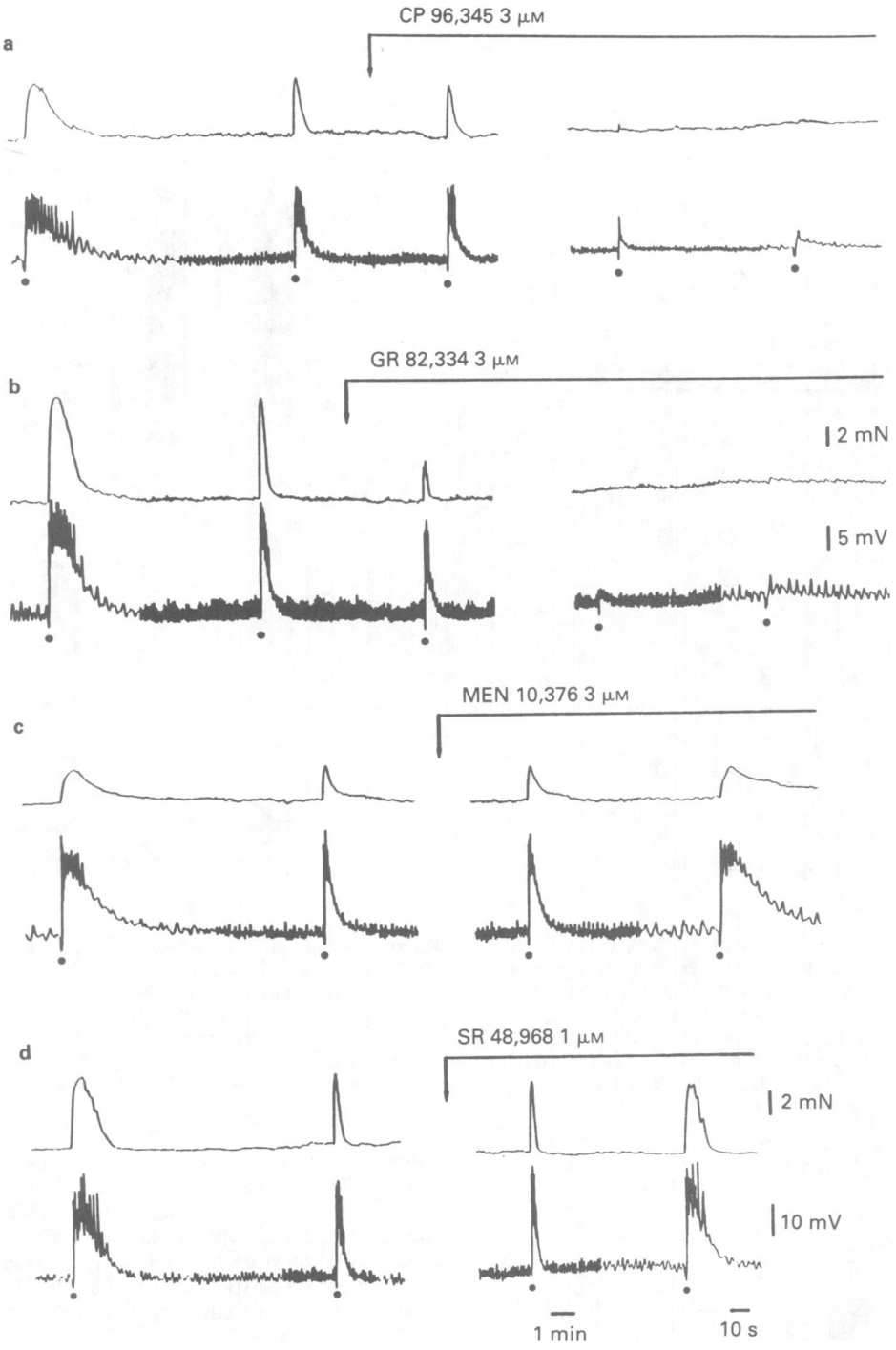

Figure 3 Typical tracings illustrating the effects of (±)-CP 96,345 (a), GR 82,334 (b), MEN 10,376 (c) and SR 48,968 (d) on the non-cholinergic e.j.ps and contractions in the circular muscle from guinea-pig proximal colon in the presence of atropine (1 μM), apamin (0.1 μM) and N^G-nitro-L-arginine (L-NOARG, 0.1 mM). The effect of (±)-CP 96,345 (3 μM) is shown after 23 min of application. The effects of GR 82,334 (3 μM) MEN 10,376 (3 μM) and SR 48,968 (1 μM) are shown at 15 min after application. Upper tracing is muscle tension, lower tracing is change in membrane potential.

depolarization of the membrane by 10.2 ± 0.9 ($n = 17$) and 4.6 ± 0.4 mV ($n = 24$), respectively, and accompanying contraction of the circular muscle of the colon (range 2–5 mN).

The NK_1 receptor antagonist, GR 82,334 (3 μM) inhibited the amplitude of [Sar^9]SP sulfone (0.3 μM)-induced contraction and depolarization by 80 and 75%, respectively ($P < 0.05$). (±)-CP 96,345 (3 μM) inhibited [Sar^9]SP sulfone-induced contraction and depolarization by 95 and 97%, respectively ($P < 0.05$, Figure 6). Neither GR 82,334 nor (±)-CP 96,345 inhibited depolarization and contraction observed in response to [βAla^8]NKA(4-10) (Figure 6).

The NK_2 receptor antagonist, MEN 10,376 (3 μM) inhibited the amplitude of the [βAla^8]NKA(4-10)-induced contraction and depolarization by 75 and 80%, respectively ($P < 0.05$). SR 48,968 (1 μM) inhibited the amplitude of [βAla^8]NKA(4-10)-induced contractions and depolarization by 90 and 88%, respectively ($P < 0.05$, Figure 6). The responses to [Sar^9]SP sulfone were not significantly affected by MEN 10,376 or SR 48,968 (Figure 6).

Effects of NK_1 receptor antagonists on cholinergic e.j.ps and contractions evoked by EFS

A frequency of stimulation of 10 Hz for 1 s, 20–30 V and pulse width of 0.02–0.03 ms was selected to obtain consistent cholinergic e.j.ps followed by APs and contractions in the

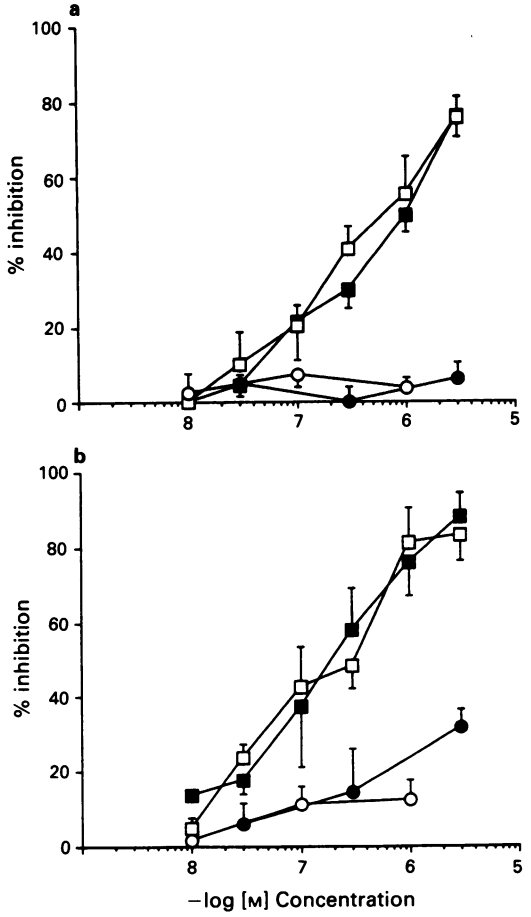

Figure 4 Concentration-dependent inhibitory effect of (±)-CP 96,345 (■), GR 82,334 (□), MEN 10,376 (●) and SR 48,968 (○) on non-cholinergic e.j.ps (a) and following contractions (b) in circular muscle of the guinea-pig colon in the presence of atropine (1 μM), apamin (0.1 μM) and N^G-nitro-L-arginine (0.1 mM). The effect of antagonists on area of depolarization of e.j.p. and maximum amplitude of contraction were calculated. Each value is the mean ± s.e.mean of 4–12 experiments.

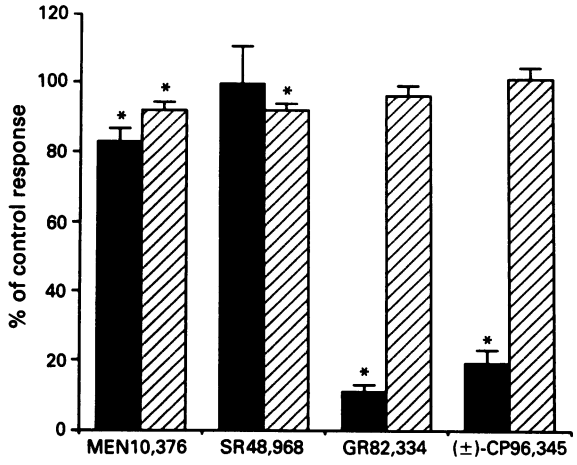

Figure 5 Effect of MEN 10,376 (3 μM), SR 48,968 (1 μM), GR 82,334 (3 μM) and (±)-CP 96,345 (3 μM) on atropine (1 μM)-resistant i.j.p. (hatched columns) and e.j.p. (solid columns) evoked by submaximal train EFS in the circular muscle of the guinea-pig colon in the absence of apamin and N^G-nitro-L-arginine. Each value is mean ± s.e.mean of 5–9 experiments. *Significantly different from controls: $P<0.05$.

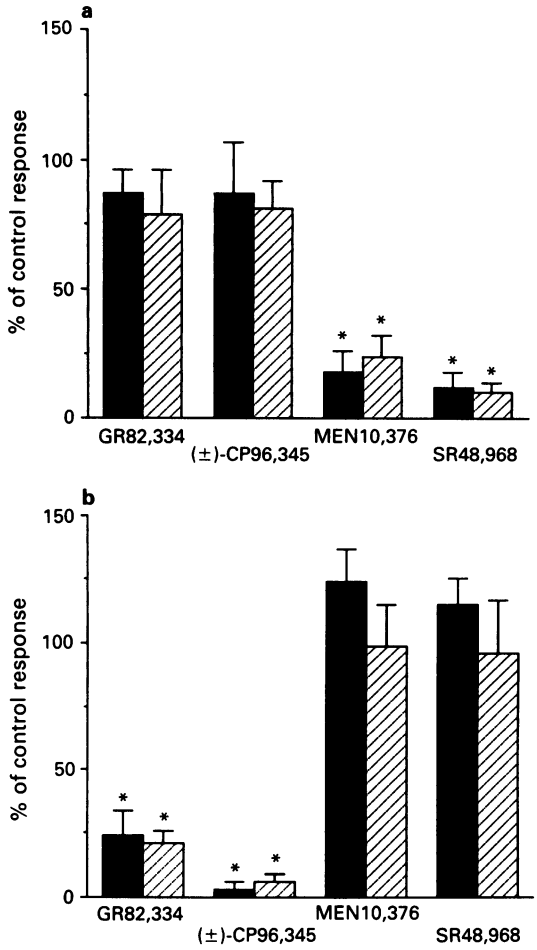

Figure 6 Effect of (±)-CP 96,345 (3 μM), GR 82,334 (3 μM), MEN 10,376 (3 μM) and SR 48,968 (1 μM) on the amplitude of depolarization (solid columns) and contraction (hatched columns) induced by [βAla^8]NKA(4-10) (a) and [Sar^9]SP sulfone (b) in circular muscle strips from guinea-pig proximal colon. Experiments performed in the presence of atropine (1 μM). Each value is the mean ± s.e.mean of 3–6 experiments. *Significantly different from control: $P<0.05$.

presence of apamin (0.1 μM) and L-NOARG (0.1 mM) (Figure 7a,c). The electrophysiological characteristics of cholinergic e.j.ps are summarized in Table 2. The electrical and mechanical activity evoked by EFS under these conditions was abolished by 1 μM atropine (Figure 7).

GR 82,334 (3 μM) and (±)-CP 96,345 (3 μM) did not produce any agonist effect on contractile or electrical activity of the smooth muscle.

GR 82,334 (3 μM) had no effect either on cholinergic e.j.ps or contractions (Figure 7a and b). (±)-CP 96,345 (3 μM) slightly reduced by 24 ± 5% the cholinergic e.j.ps (calculated as area of depolarization), but did not affect the peak amplitude of e.j.ps or contractions evoked by EFS (Figure 7c and d).

Discussion

Recent studies on the role of TKs as enteric excitatory transmitters have taken advantage of novel receptor antagonists of both peptide (second generation) and non-peptide (third generation) nature (Maggi *et al.*, 1993 for review) which are more potent than spantide and also possess remarkable selectivity for either NK_1 or NK_2 receptors. By use of these novel ligands, evidence has been obtained that the NK_1 receptor is the main mediator of atropine-resistant neuromuscular transmission in the longitudinal muscle of the guinea-pig ileum (Taylor & Kilpatrick, 1992), while the NK_2

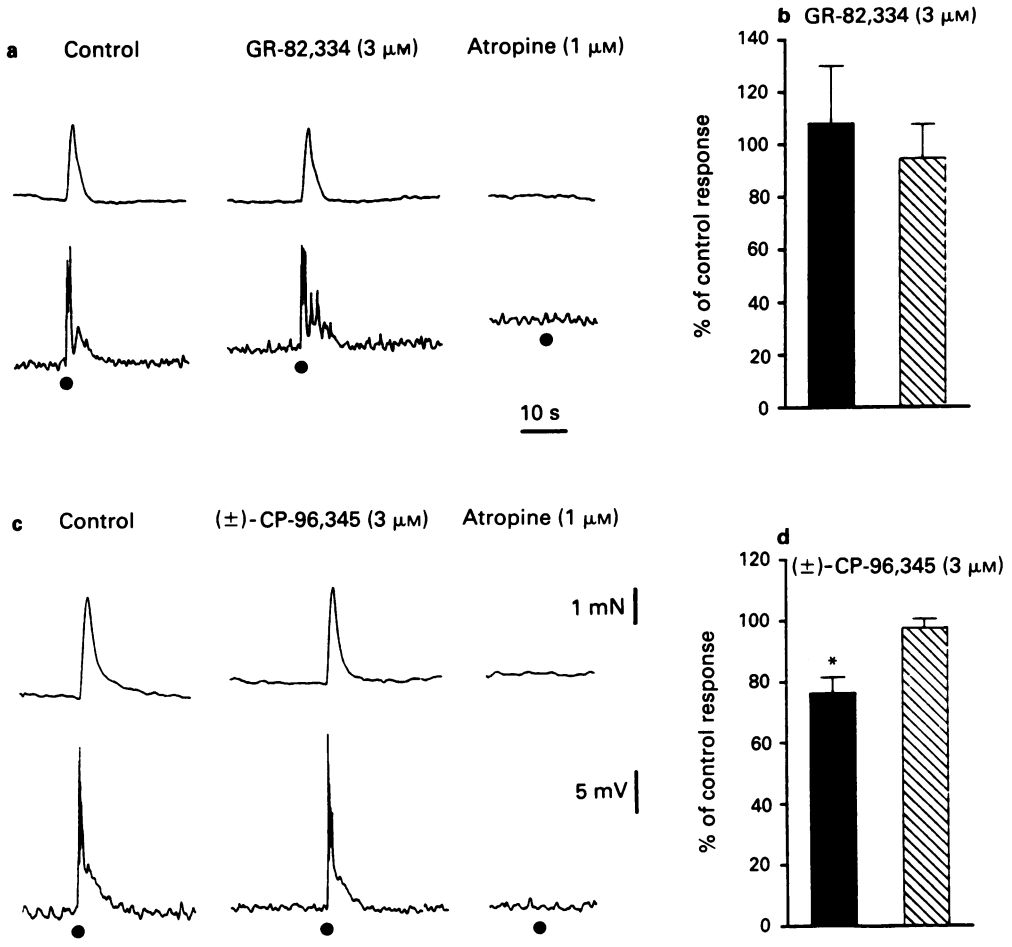

Figure 7 Effects of GR 82,334 (a and b) and (±)-CP 96,345 (c and d) on the atropine-sensitive e.j.ps and contractions evoked by train EFS (10 Hz, 20 V for 1 s, pulse width 0.02 ms) in the presence of apamin (0.1 μM) and N^G-nitro-L-arginine (0.1 mM). In (a) and (c) the effect of GR 82,334 (3 μM) and (±)-CP 96,345 (3 μM) are shown at 15 and 25 min from application, respectively. Upper tracing is muscle tension, lower tracing is change in membrane potential. The area of depolarization of cholinergic e.j.ps (solid columns) and amplitude of contraction (hatched columns) were calculated and are shown in (b) and (d), respectively. Each value is mean ± s.e.mean of 5 experiments. *Significantly different from control: $P<0.05$.

receptor predominates in the circular muscle of the guinea-pig and human ileum (Bartho *et al.*, 1992; Maggi *et al.*, 1992; Holzer *et al.*, 1993).

Because a previous functional study (Giuliani *et al.*, 1993) indicated that functional NK_1 and NK_2 receptors mediate contraction of the circular muscle of the guinea-pig proximal colon, we have used selective NK_1 and NK_2 receptor antagonists to gain information as to whether TK receptors are involved in the generation of noncholinergic e.j.ps in this preparation. GR 82,334 (Hagan *et al.*, 1991) and CP 96,345 (Snider *et al.*, 1991) have been characterized as highly selective NK_1 receptor antagonists, while MEN 10,376 (Maggi *et al.*, 1991) and SR 48,968 (Emonds-Alt *et al.*, 1992) are highly selective NK_2 receptor antagonists. For each one of these ligands, evidence for effective blockade of the respective preferred receptor and selectivity was established by using the selective NK_1 receptor agonist, [Sar^9]SP sulfone (Dion *et al.*, 1987) and the NK_2 receptor selective agonist [βAla^8]NKA(4-10) (Rovero *et al.*, 1989).

The present results indicate that NK_1 but not NK_2 receptor antagonists concentration-dependently inhibit the non-cholinergic e.j.ps and accompanying contraction in response to EFS. The NK_1 receptor antagonists (±)-CP 96,345 (Snider *et al.*, 1991) and GR 82,334 (Hagan *et al.*, 1991) inhibited the atropine-resistant e.j.ps both in the presence and absence of apamin and L-NOARG. This provides strong evidence for predominant involvement of NK_1 receptors in non-cholinergic excitatory neurotransmission to the circular muscle of the guinea-pig proximal colon, where this response is evident either as primary contraction (in the presence of atropine, apamin and L-NOARG) or as a 'rebound' contraction (in the presence of atropine alone).

The EC_{50}s of CP 96,345 and GR 82,334 in inhibiting the atropine resistant e.j.p. and contraction produced by EFS are higher than one would expect on the basis of the affinities of these ligands for the NK_1 receptor in this preparation: thus, when tested against [Sar^9]SP sulfone in the circular muscle of the colon, both (±)-CP 96,345 and GR 82,334 act as competitive antagonists with pK_B values of 8.41 and 7.49, respectively (Maggi, unpublished data). On the other hand, the EC_{50} values of (±)-CP 96,345 and GR 82,334 in blocking the noncholinergic contraction were 0.22 and 0.20 μM, respectively. The reason why higher concentrations of NK_1 receptors antagonists are needed to block endogenous TKs than those which are sufficient to inhibit effectively the action of exogenous TKs is unclear: this may involve a difference in the accessibility of antagonist to NK_1 receptors stimulated by the endogenous agonist vs those stimulated by exogenous agonists and/or a high local concentration of the agonist released during nerve activity. Nevertheless, the EC_{50} of GR 82,334 for inhibiting the atropine-resistant contraction in guinea-pig colon is in good agreement with the EC_{50}, of this antagonist in blocking the atropine-resistant contraction in the longitudinal muscle of the ileum (Taylor & Kilpatrick, 1992; EC_{50} 0.56 μM) vs a pK_B value of 7.64 at the NK_1 receptor in the same preparation (Hagan *et al.*, 1991).

The present results show that the time course of inhibition by GR 82,334 is approximately three times faster than for (±)-CP 96,345. Furthermore, unlike (±)-CP 96,345, the inhibitory effect of GR 82,334 on noncholinergic e.j.p. can be rapidly reversed by washing (unpublished data) The explanation for the difference in time course of action of the two antagonists is not known.

Because of the relatively high concentrations of NK_1 receptor antagonists required to block the atropine-resistant e.j.p., it was important to assess their selectivity. The highest concentration tested of both antagonists is selective at the postjunctional level because the excitatory responses to NK_2 receptor stimulation were unchanged. The present findings also indicate that 3 μM GR 82,334 does not exert nonspecific effects at prejunctional level because neither the nonadrenergic i.j.p. nor the atropine-sensitive e.j.p and contraction were modified by this antagonist. Likewise, 3 μM (±)-CP 96,345 did not inhibit the nonadrenergic i.j.p. and peak amplitude of the atropine-sensitive e.j.p. or contraction: only a minor reduction of the atropine-sensitive e.j.p. was observed, if measured as total area of depolarization. This is probably a nonspecific effect of (±)-CP 96,345 which, at high concentrations, can inhibit voltage-dependent calcium channels (e.g. Schmidt *et al.*, 1992). Although we cannot exclude the possibility that nonspecific effects of (±)-CP 96,345 may have contributed to inhibition of the atropine-resistant e.j.p. and contraction, the extent of the inhibitory effect on the area of the atropine-sensitive e.j.p. is certainly minor as compared to the almost total suppression of atropine-resistant e.j.p. We conclude that the major part of the inhibitory effect of this nonpeptide antagonist on the atropine-resistant e.j.p. and accompanying contraction is due to blockade of the action of endogenous TKs at NK_1 receptors.

At 3 μM, MEN 10,376 produced a minor inhibitory effect on the atropine-resistant mechanical response to EFS which is statistically significant as compared to the spontaneous decay observed in control strips. Since this concentration of MEN 10,376 does not affect the electrical and mechanical response to selective NK_1 receptor stimulation, this may indicate a minor contribution of NK_2 receptors to noncholinergic excitation of the circular muscle. However, SR 48,968 which is more potent than MEN 10,376 in blocking NK_2 receptors in the colon (Giuliani *et al.*, 1993), did not significantly affect the atropine-resistant e.j.p.

After intravenous administration to anaesthetized guinea-pigs, both NK_1 (CP 96,345) and NK_2 (MEN 10,376 and SR 48,968) receptor antagonists produce long-lasting inhibition of atropine-resistant but hexamethonium-sensitive phasic pressure waves produced by radial stretch (balloon distension) in the guinea-pig proximal colon (Giuliani *et al.*, 1993). On the basis of the present findings, it appears that the activity of CP 96,345 could involve blockade on neuromuscular excitatory transmission from enteric tachykininergic nerves to the circular muscle. The activity of NK_2 receptor antagonists in the *in vivo* model (Giuliani *et al.*, 1993) remains unexplained. Since the present study was performed using only one frequency of stimulation to evoke the noncholinergic e.j.p. and accompanying contraction, we cannot exclude the possibility that enteric tachykininergic nerves were activated by balloon distension *in vivo* to a sufficient extent to produce NK_2 receptor stimulation.

In the major part of the present experiments, the effect of NK_1 and NK_2 receptor antagonists was investigated in the presence of apamin and L-NOARG to block the non-adrenergic i.j.ps. Non-adrenergic i.j.ps have a multiple nature in the guinea-pig colon (Vladimirova & Shuba, 1984). Both apamin and L-NOARG effectively but partially inhibited the nonadrenergic i.j.p. and a residual small i.j.p. was observed in about 50% of cases in the presence of both drugs. The identity of the mediators of i.j.ps has not been conclusively demonstrated: for the first, apamin-sensitive, phase of i.j.p., adenosine triphosphate (ATP) was suggested as a likely mediator (Vladimirova & Shuba, 1984). The second, apamin-resistant, phase of i.j.ps was inhibited by the nitric oxide (NO)-synthase blocker, L-NOARG. Thus NO could be responsible for part of the apamin-resistant i.j.ps in the circular muscle of the guinea-pig proximal colon.

In conclusion, the present findings indicate a major role for SP and NK_1 receptors in mediating non-cholinergic e.j.ps and contractions evoked by EFS in the circular muscle of the guinea-pig proximal colon. Since NK_2 receptors mediate tachykininergic neuromuscular transmission to the circular muscle of the guinea-pig ileum (Bartho *et al.*, 1992; Holzer *et al.*, 1993), the use of receptor selective antagonists reveals a remarkable regional specialization in the TK receptor types which mediate neuromuscular transmission to endogenous TKs in the gut.

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