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Evidence that tachykinin NK_1 and NK_2 receptors mediate non-adrenergic non-cholinergic excitation and contraction in the circular muscle of guinea-pig duodenum

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1 In the presence of atropine (1 μM), guanethidine (3 μM), indomethacin (3 μM), apamin (0.1 μM) and L-nitroarginine (L-NOARG, 30 μM), electrical field simulation (EFS) produced a nonadrenergic, non-cholinergic (NANC) excitatory junctional potential (e.j.p.), action potentials and contraction of the circular muscle of the guinea-pig proximal duodenum, recorded by the single sucrose gap technique.

2 The selective tachykinin (TK) NK_1 receptor antagonist, GR 82,334 (30 nM–3 μM) produced a concentration-dependent inhibition of the EFS-evoked NANC e.j.p. and contraction. Similarly, the selective NK_2 receptor antagonists, MEN 10,627 (30 nM–3 μM) and GR 94,800 (100 nM–10 μM), both produced a concentration-dependent inhibition of the EFS-evoked NANC e.j.p. and contraction. GR 82,334 inhibited the electrical and mechanical NANC responses to EFS in an almost parallel manner, while MEN 10,627 and GR 94,800 were more effective in inhibiting the mechanical than the electrical response to EFS.

3 Activation of the NK_1 or NK_2 receptor by the selective agonists, [Sar^9]substance P (SP) sulphone and [βAla^8]neurokinin A (NKA) (4-10), respectively (0.3 μM each), produced depolarization, action potentials and contractions. GR 82,334 selectively inhibited the responses to [Sar^9]SP sulphone, without affecting the responses to [βAla^8]NKA (4-10). MEN 10,627 and GR 94,800 inhibited or abolished the responses to [βAla^8]NKA (4-10), without affecting the responses to [Sar^9]SP sulphone.

4 Nifedipine (1 μM) abolished the action potentials and contraction produced either by EFS or by the TK receptor agonists [Sar^9]SP sulphone or [βAla^8]NKA (4-10).

5 In the presence of nifedipine, the NANC e.j.p. produced by EFS was biphasic: in the majority of strips tested (21 out of 29) an early fast phase of depolarization was followed by a second slow component. The combined administration of GR 82,334 and GR 94,800 (3 μM each) reduced both components, the slow phase being inhibited to a greater extent than the fast phase.

6 The P_2 purinoreceptor antagonist, suramin (100 μM) reduced the fast phase of the e.j.p. produced by EFS in the presence of nifedipine, without affecting the slow phase. The combined administration of suramin, GR 82,334 and GR 94,800 produced a nearly complete blockade of the e.j.p. produced by EFS in the presence of nifedipine.

7 When tested in the absence of apamin and L-NOARG, EFS induced a NANC inhibitory junction potential (i.j.p.) followed by an e.j.p., and the selective P_{2Y} receptor agonist, adenosine-5'-O-(2-thiodiphosphate) (ADPβS, 10 μM), produced membrane hyperpolarization. After addition of apamin and L-NOARG, the i.j.p. was blocked, and EFS produced a pure NANC e.j.p.; ADPβS produced depolarization, action potentials and contraction.

8 Suramin (100 μM) blocked the depolarization, action potentials and contractions produced by ADPβS in the presence of apamin and L-NOARG, without affecting the responses produced by the NK_1 receptor agonist, [Sar^9]SP sulphone.

9 We conclude that NK_1 and NK_2 receptors cooperate in producing NANC excitation and contraction of the circular muscle in the guinea-pig proximal duodenum. Activation of either TK receptor produces membrane depolarization and both receptors contribute to generate action potentials which are essential for producing muscle contraction, via nifedipine-sensitive calcium channels. It appears that endogenous ATP chiefly acts as an inhibitory transmitter but, after blockade of NANC inhibitory mechanism(s), ATP may act as a fast signalling excitatory transmitter.

Keywords: Guinea-pig duodenum; NANC; excitatory junction potentials; tachykinins; tachykinin receptors; ATP

Introduction

Anatomical, neurochemical and pharmacological evidence indicates a major role for peptides of the tachykinin (TK) family as mediators of NANC excitatory neurotransmission to the smooth muscle of the mammalian intestine (Bartho & Holzer, 1985, for review). Two TKs, substance P (SP) and neurokinin A (NKA), via the expression of the preprotachykinin I gene, are produced by some neuronal elements in the myenteric plexus (Deacon *et al.*, 1987; Sternini *et al.*, 1989) which have the adequate anatomical projections to act as effector motoneurones to the circular muscle of the intestine (Brookes *et al.*, 1991). The status of TKs as enteric excitatory transmitters is further documented by the notion that SP- and NKA-like immunoreactive material(s) are released in the gut following neuronal depolarization (Holzer, 1984; Theodorsson *et al.*, 1991; Broad *et al.*, 1992) and by the powerful, receptor-mediated, contractile activity exerted by exogenous TKs in various regions of the mammalian

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intestine. Three types of TK receptors have been isolated and cloned, which are termed NK_1, NK_2 and NK_3, respectively (Maggi *et al.*, 1993, for review): in the circular muscle of the guinea-pig small and large intestine, both NK_1 and NK_2 receptors mediate the direct smooth muscle contraction to TKs, while NK_3 receptors, apparently located on neuronal elements, indirectly affect motility by stimulating the release of other mediators (Maggi *et al.*, 1990; 1994a).

From the above, it appears that TKergic excitatory neurotransmission to the circular muscle of the intestine could involve two mediators (SP and NKA) and two receptors (NK_1 and NK_2). In previous electrophysiological experiments, non-cholinergic excitatory junction potentials (e.j.ps) have been recorded in the longitudinal (Bauer & Kuriyama, 1982) and circular muscle (Niel *et al.*, 1983; Bywater & Taylor, 1986; Crist *et al.*, 1991) of the guinea-pig ileum. In some of these studies, a biphasic noncholinergic e.j.p. has been described, and a differential degree of inhibition of the two phases of e.j.p. was observed with SP antagonists such as [D-Arg[1],D-Pro[2],D-Trp[7,9],Leu[11]]SP (RPWWL-SP, Niel *et al.*, 1983; Bywater & Taylor, 1991) and spantide (Crist *et al.*, 1991). The residual phase of e.j.p. appears to be mediated by an unknown transmitter (Bywater & Taylor, 1986; Crist *et al.*, 1991). Because of their low potency and selectivity, and presence of nonspecific effects (e.g. local anaesthetic action), ligands like RPWWL-SP or spantide, have a limited value for assessing the transmitter role of TKs; furthermore, these ligands are essentially poor instruments to discern a differential contribution of NK_1 and NK_2 receptors in TKergic co-transmission (Buck & Shatzer, 1988; Maggi *et al.*, 1993, for review).

Thanks to the recent availability of antagonists that selectively block the NK_1 (SP-preferring) or NK_2 (NKA-preferring) receptors (Maggi *et al.*, 1993) it has become possible to obtain information about the relative contribution of various TKs and different TK receptors in excitatory NANC responses (Bartho *et al.*, 1992; Zagorodnyuk *et al.*, 1993; Holzer *et al.*, 1993; Holzer & Maggi, 1994; Maggi *et al.*, 1994b). Both NK_1 and NK_2 receptors appear to play a relevant role in NANC contraction evoked by electrical nerve stimulation or intraluminal balloon distension in the circular muscle of the guinea-pig ileum (Bartho *et al.*, 1992; Maggi *et al.*, 1994b), while mainly NK_1 receptors mediate the NANC e.j.p. in the circular muscle of the guinea-pig proximal colon (Zagorodnyuk *et al.*, 1993).

The aim of this study was to assess the relative contribution of NK_1 and NK_2 receptors to NANC excitatory neurotransmission in the circular muscle of the guinea-pig duodenum, by studying the effect of potent and selective receptor antagonists, GR 82,334 (Hagan *et al.*, 1991) for NK_1 receptors, GR 94,800 (McElroy *et al.*, 1992) and MEN 10,627 (Patacchini *et al.*, 1994; Maggi *et al.*, 1994c) for NK_2 receptors. From these studies we found evidence that only part of the NANC e.j.p. produced by EFS in the circular muscle of guinea-pig duodenum can be accounted for by the release of endogenous TKs. In the smooth muscle of guinea-pig stomach and caecum, it has been suggested that apamin unmasks a fast purinergic NANC e.j.p. (Shuba & Vladimirova, 1980). To address the possible role of adenosine-triphosphate (ATP) in the NANC e.j.p. in guinea-pig duodenum, we investigated the effect of the general P_2 purinoceptor antagonist, suramin (Dunn & Blakeley, 1988; Hoyle *et al.*, 1990) and of the selective P_{2Y} receptor agonist, adenosine-5'-O-(2-thiodiphosphate) (ADPβS) (Bertrand *et al.*, 1991).

Methods

A modified single sucrose-gap (Artemenko *et al.*, 1982; Hoyle, 1987) was used to investigate simultaneously changes in membrane potential and contractile activity of the smooth muscle to EFS, as described in detail previously (Zagorodnyuk *et al.*, 1993; 1994). Circular muscle strips of proximal duodenum (1–3 cm from the pyloric sphincter), approximately 0.5–0.8 mm wide and 10 mm long were superfused, at a rate of 1 ml min^{-1}, with oxygenated Krebs solution (35 ± 0.5°C) of the following composition (mM): NaCl 119, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.5, KCl 4.7, $CaCl_2$ 2.5 and glucose 11.

Junction potentials were evoked by submaximal electrical field stimulation (EFS): train of stimuli (30–40 V, 0.15–0.2 ms pulse width) were delivered at a frequency of 32 Hz for 1 s every 3–4 min. In preliminary experiments, we found that the electrical and mechanical responses produced with these parameters of EFS are reproducible for the experimental period (1–2 h) of this study. The Krebs solution routinely contained atropine (1 μM) and guanethidine (3 μM) to block the effect of excitatory cholinergic nerves and adrenergic inhibitory nerves, respectively. Indomethacin (3 μM) was used to exclude the possible involvement of prostanoids. Unless otherwise stated, N^{ω}-nitro-L-arginine (L-NOARG, 30 μM) and apamin (0.1 μM) were used to block NANC inhibitory junction potentials (i.j.ps) evoked by EFS (He & Goyal, 1993; Zagorodnyuk *et al.*, 1993).

In a first series of experiments, performed in the presence of atropine (1 μM), guanethidine (3 μM), indomethacin (3 μM), apamin (0.1 μM) and L-NOARG (30 μM), NANC e.j.ps were produced by train EFS at 3–4 min intervals and the effect of tachykinin receptor antagonists (GR 82,334, MEN 10,627 and GR 94,800) on the electrical and mechanical responses produced by EFS was determined. The effect of antagonists was evaluated as % inhibition of the area of electrical and mechanical responses produced by EFS.

In parallel experiments, the selective NK_1 or NK_2 receptor agonists, [Sar[9]]SP sulphone or [βAla[8]]NKA(4-10), respectively (cf. Zagorodnyuk *et al.*, 1993; 1994), were applied to the circular smooth muscle in the absence or presence of tachykinin receptor antagonists. In preliminary experiments, we found that a concentration of 0.3 μM (applied in superfusion for 20 s) of each agonist determined a reproducible electrical and contractile responses when elicited at 15 min intervals.

In a second series of experiments, performed in the presence of atropine (1 μM), guanethidine (3 μM), indomethacin (3 μM), apamin (0.1 μM) and L-NOARG (30 μM), the role of nifedipine-sensitive calcium channels was determined by studying the effect of 1 μM nifedipine (superfused for at least 30 min) on the NANC responses produced by EFS and on the electrical and mechanical responses to tachykinin receptor agonists, [Sar[9]]SP sulphone and [βAla[8]]NKA(4-10). From these experiments, it was found that a biphasic NANC e.j.p. is produced by EFS in the presence of nifedipine: the effect of GR 82,334 and GR 94,800, alone or in combination, on the nifedipine-resistant NANC e.j.p. was also investigated. Since a fraction of this NANC e.j.p. was still present after the combined administration of the TK receptor antagonists, the hypothesis was advanced that ATP may also act as excitatory transmitter in these experimental conditions. To check this point we investigated the effect of the P_2 purinoceptor antagonist, suramin (100 μM, applied in superfusion for 20–30 min) on the NANC e.j.p.

In a final series of experiments we studied the effect of the selective P_{2Y} receptor agonist adenosine-5'-O-(2-thiodiphosphate) (ADPβS) (10 μM) on the electrical and mechanical activity of the guinea-pig duodenum: ADPβS was applied in superfusion for 30 s at 15 min intervals and its effects were investigated in the absence and presence of apamin and L-NOARG.

Statistical analysis

All data in the text are 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 followed by Dunnett's test, if applicable. A P level <0.05 was considered as statistically significant.

Drugs

Drugs used were: atropine HCl (Serva), guanethidine sulphate (ICF), GR 82,334 or [D-Pro^9,(spiro-γ-lactam) Leu^{10}, Trp^{11}] physalaemin(1-11) (Neosystem), N$^{\omega}$-nitro-L-arginine (L-NOARG), apamin, indomethacin, ADPβS and nifedipine (Sigma). MEN 10,627 or cyclo(Met-Asp-Trp-Phe-Dap-Leu) cyclo(2β-5β) and GR 94,800 or PhCO-Ala-Ala-D.Trp-Phe-D.Pro-Pro-NleNH$_2$ were synthesized at the Chemistry Department of Menarini Pharmaceuticals. Suramin was a kind gift of Prof. M. Costa, Dept. of Human Physiology, Flinders University SA, Bedford Park, Australia.

Results

General

In the presence of atropine (1 μM), guanethidine (3 μM), indomethacin (3 μM), apamin (0.1 μM) and L-NOARG (30 μM), EFS (30 V, 0.15–0.2 ms pulse width, 32 Hz for 1 s) evoked a NANC e.j.p. (mean amplitude of depolarization 10.5 ± 0.7 mV, $n = 42$, latency 426 ± 33 ms, $n = 24$) with superimposed action potentials (mean amplitude 23.8 ± 1.1 mV, $n = 42$) and contraction (5.8 ± 0.4 mN, $n = 42$) (Figures 1, 2 and 3). The mean duration of the NANC e.j.p. was 23.8 ± 3.5 s ($n = 32$). The parameters used to produce a NANC e.j.p. are about two times larger than the threshold EFS stimulation required to produce an e.j.p. followed by action potentials and contraction. Under the

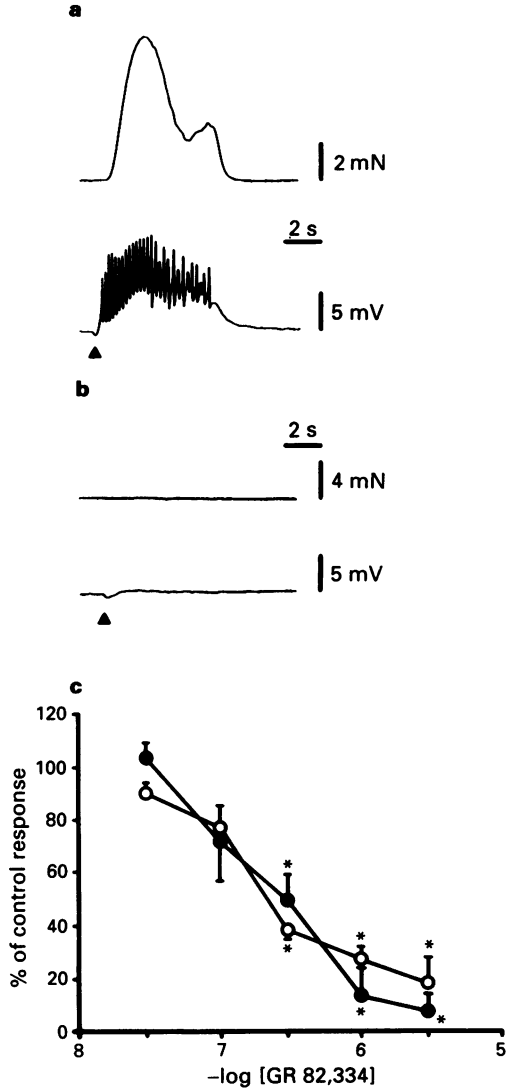

Figure 1 Effect of the tachykinin NK$_1$ receptor antagonist GR 82,334 on the NANC e.j.p. and contraction produced by EFS in the circular muscle of the guinea-pig duodenum. (a and b) Control responses to EFS (32 Hz for 1 s, a), applied at arrowhead and effect of GR 82,334 (3 μM, b) on the responses to EFS in the same preparation. In both panels, upper tracing is muscle tension, lower tracing show changes in membrane potential. (c) Concentration-dependent inhibition of the response to EFS by GR 82,334. The effect of GR 82,334 on the area of depolarization of the NANC e.j.p. (○) and of the area of NANC contraction (●) produced by EFS is expressed as % of the control responses obtained in the absence of the antagonist. Each value is the mean ± s.e.mean of 3–5 experiments. *Significantly different from control, $P<0.05$.

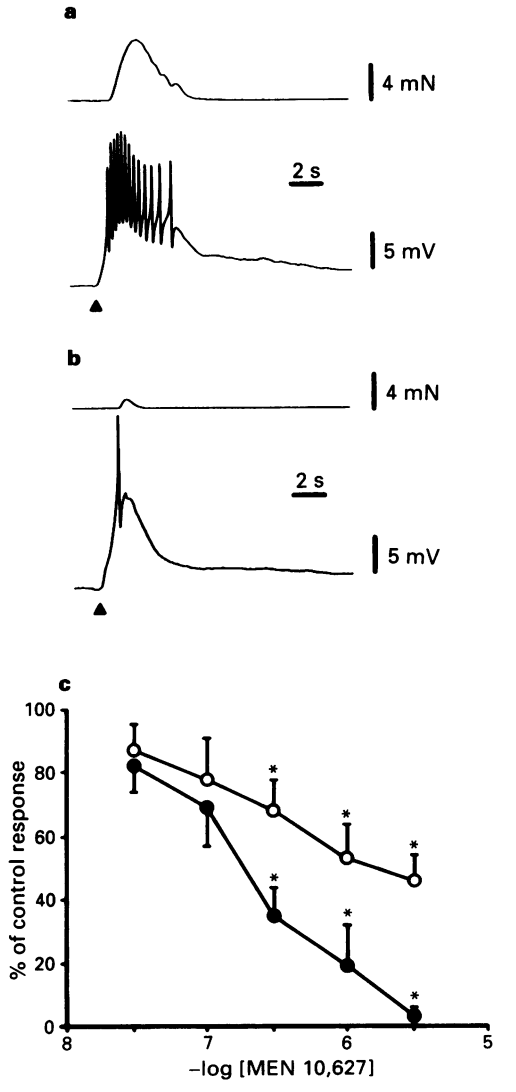

Figure 2 Effect of tachykinin NK$_2$ receptor antagonist MEN 10,627 on the NANC e.j.p. and contraction produced by EFS in the circular muscle of the guinea-pig duodenum. (a and b) Control response to EFS (32 Hz for 1 s, a), applied at arrowhead and effect of MEN 10,627 (3 μM, b) on the responses to EFS in the same preparation. In both panels, upper tracing is muscle tension, lower tracing shows changes in membrane potential. (c) Concentration-dependent inhibition of the response to EFS by MEN 10,627. The effect of MEN 10,627 on the area of depolarization of the NANC e.j.p. (○) and of the area of NANC contraction (●) produced by EFS is expressed as % of the control responses obtained in the absence of the antagonist. Each value is the mean ± s.e.mean of 5–7 experiments. *Significantly different from control, $P<0.05$.

present experimental conditions, the development of contractile responses to EFS (e.g. Figures 1a, 2a and 3a) or exogenous agonists (e.g. Figures 4a, 5a, 8b and 9a) was strictly associated with the presence of action potentials.

Effects of NK_1 and NK_2 receptor antagonists on the NANC e.j.p. and contractions produced by EFS

The NK_1 receptor antagonist, GR 82,334 (30 nM–3 μM), concentration-dependently inhibited the NANC e.j.p. and contractions, EC_{50} values (95% c.l. in parentheses) being 0.23 μM (0.09–0.5 μM) and 0.30 μM (0.19–0.46 μM), respectively. The maximal inhibition of depolarization and contraction produced by GR 82,334 averaged 82 ± 10% ($n = 5$) and 93 ± 7% ($n = 5$), respectively, and the two concentration-response curves were almost superimposable (Figure 1).

The NK_2 receptor antagonist, MEN 10,627 (30 nM–3 μM) concentration-dependently inhibited the NANC e.j.p. and contraction, EC_{50} values (95% c.l. in parentheses) being 0.15 μM (0.11–0.21 μM) and 0.18 μM (0.11–0.29 μM), respectively. As shown in Figure 2, the maximal inhibitory effect of MEN 10,627 on contractility (97 ± 3% inhibition at 3 μM, $n = 7$) was larger than its inhibitory effect on depolarization (54 ± 8% inhibition, $n = 7$). The NK_2 receptor antagonist, GR 94,800 (100 nM–10 μM) concentration-dependently reduced the NANC e.j.p. and contraction, EC_{50} values (95% c.l. in parentheses) being 0.45 μM (0.32–0.63 μM) and 0.20 μM (0.05–0.75 μM), respectively. Also GR 94,800 was more effective in inhibiting contractility (79 ± 8% inhibition, $n = 4$) than depolarization (46 ± 8% inhibition, $n = 4$) (Figure 3). Neither GR 82,334 (up to 3 μM) nor MEN 10,627 (up to 3 μM) or GR 94,800 (up to 10 μM) had any agonist effect on the electrical or contractile activity.

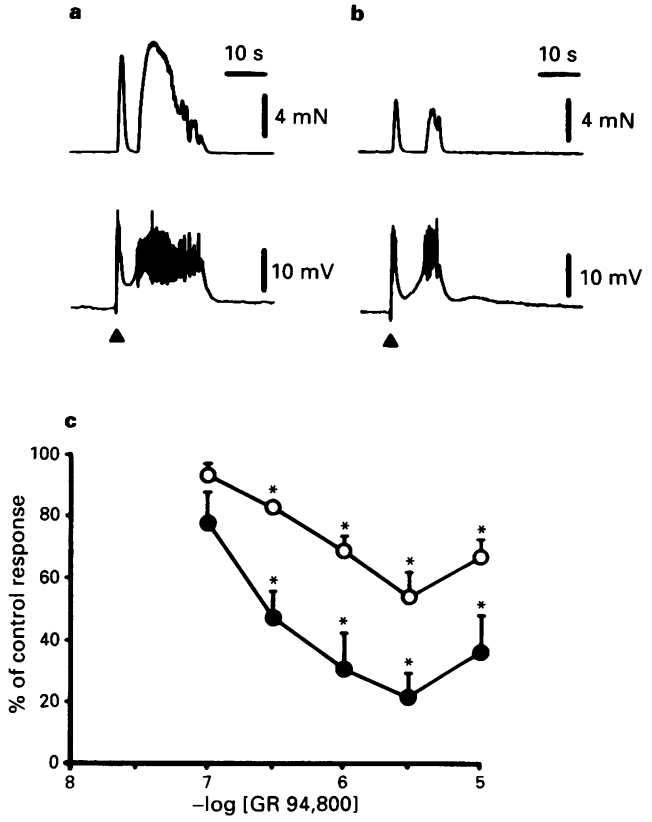

Figure 3 Effect of the tachykinin NK_2 receptor antagonist GR 94,800 on the NANC e.j.p. and contraction produced by EFS in the circular muscle of the guinea-pig duodenum. (a and b) Control response to EFS (32 Hz for 1 s, a), applied at arrowhead and effect of GR 94,800 (3 μM, b) on the responses to EFS in the same preparation. In both panels, upper tracing is muscle tension, lower tracing shows changes in membrane potential. (c) Concentration-dependent inhibition of the response to EFS by GR 94,800. The effect of GR 94,800 on the area of depolarization of the NANC e.j.p. (○) and of the area of NANC contraction (●) produced by EFS is expressed as % of the control responses obtained in the absence of the antagonist. Each value is the mean ± s.e.mean of 4–5 experiments. *Significantly different from control, $P < 0.05$.

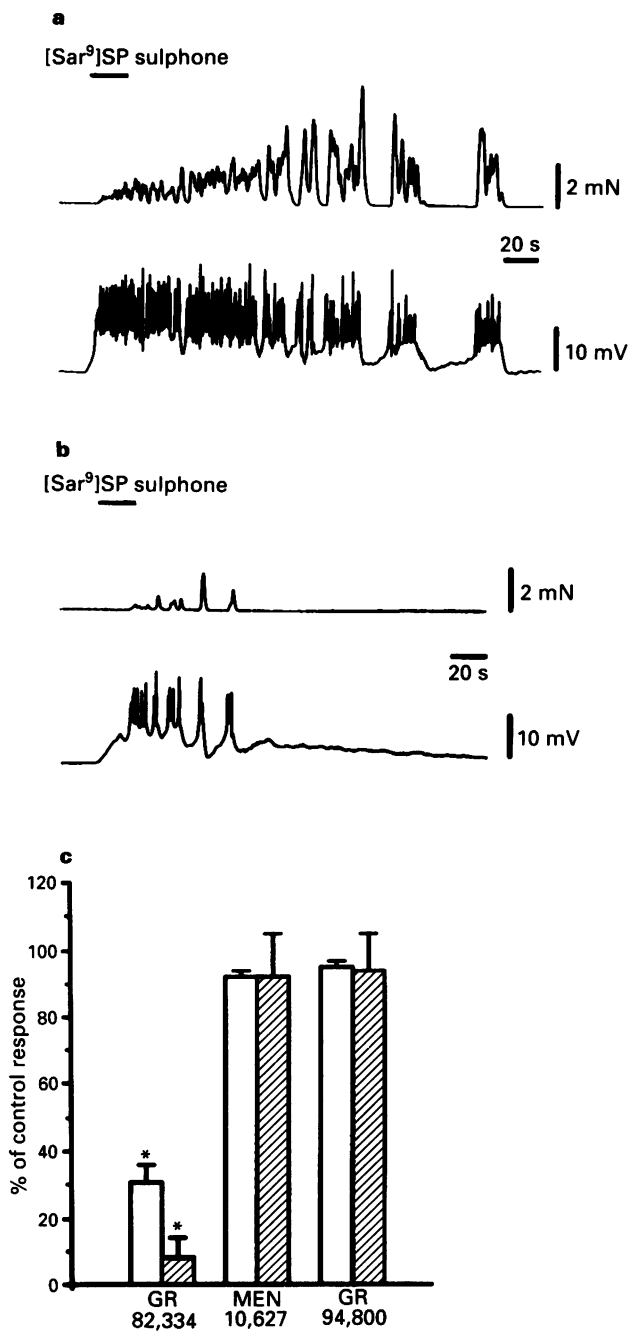

Figure 4 Effect of the tachykinin NK_1 receptor antagonist, GR 82,334 and of the NK_2 receptor antagonists, MEN 10,627 and GR 94,800 on the response induced by the selective tachykinin NK_1 receptor agonist [Sar⁹]SP sulphone. (a) Control response to [Sar⁹]SP sulphone (period of application of the agonist is indicated by the horizontal bar); (b) response to [Sar⁹]SP sulphone in the presence of GR 82, 334 (3 μM); in both panels, upper tracing is muscle tension, lower tracing shows changes in membrane potential. (c) Effect of GR 82,334 (3 μM), MEN 10,627 (3 μM) and GR 94,800 (3 μM) on the area of depolarization (open columns) and area of contraction (hatched columns) induced by [Sar⁹]SP sulphone (0.3 μM for 20 s). The responses to the agonist obtained in the presence of the antagonist are expressed as % of the control response. Each value is the mean ± s.e.mean of 4–5 experiments. *Significantly different from control, $P < 0.05$.

Effect of tachykinin receptor antagonists on the electrical and mechanical responses to selective NK_1 and NK_2 receptor agonists

In the presence of atropine (1 μM), guanethidine (3 μM), indomethacin (3 μM), apamin (0.1 μM) and L-NOARG (30 μM), application of the NK_1 receptor selective agonist, [Sar^9]SP sulphone or of the NK_2 receptor selective agonist, [βAla^8]NKA (4-10) (0.3 μM for 20 s in each case, $n = 8-11$) produced membrane depolarization followed by slow waves with superimposed action potentials and contraction (Figures 4 and 5).

The amplitude of depolarization evoked by the agonists averaged 13.8 ± 0.8 mV ($n = 10$) and 12.1 ± 0.9 mV ($n = 9$) for [Sar^9]SP sulphone and [βAla^8]NKA (4-10), respectively; the amplitude of action potentials averaged 27.5 ± 1.4 and 26 ± 1.9 mV, respectively ($n = 11$ and 8, respectively); the amplitude of evoked contraction averaged 6.2 ± 0.95 and 6.5 ± 0.64 mN, respectively ($n = 11$ and 8, respectively).

GR 82,334 (3 μM) inhibited the [Sar^9]SP sulphone-induced depolarization and contraction by 69 ± 5 and 92 ± 6%, ($n = 4$), respectively, while it did not affect the response to [βAla^8]NKA (4-10) (Figures 4 and 5). MEN 10,627 (1 μM) almost completely abolished the [βAla^8]NKA (4-10)-induced depolarization and contraction (93 ± 4 and 99.8 ± 0.2% inhibition, $n = 3$, respectively). The responses induced by [Sar^9]SP sulphone were not significantly changed by MEN 10,627 (3 μM) (Figures 4 and 5). GR 94,800 (3 μM) completely blocked the [βAla^8]NKA (4-10)-induced depolarization and contraction while the responses evoked by [Sar^9]SP sulphone were not significantly affected (Figures 4 and 5).

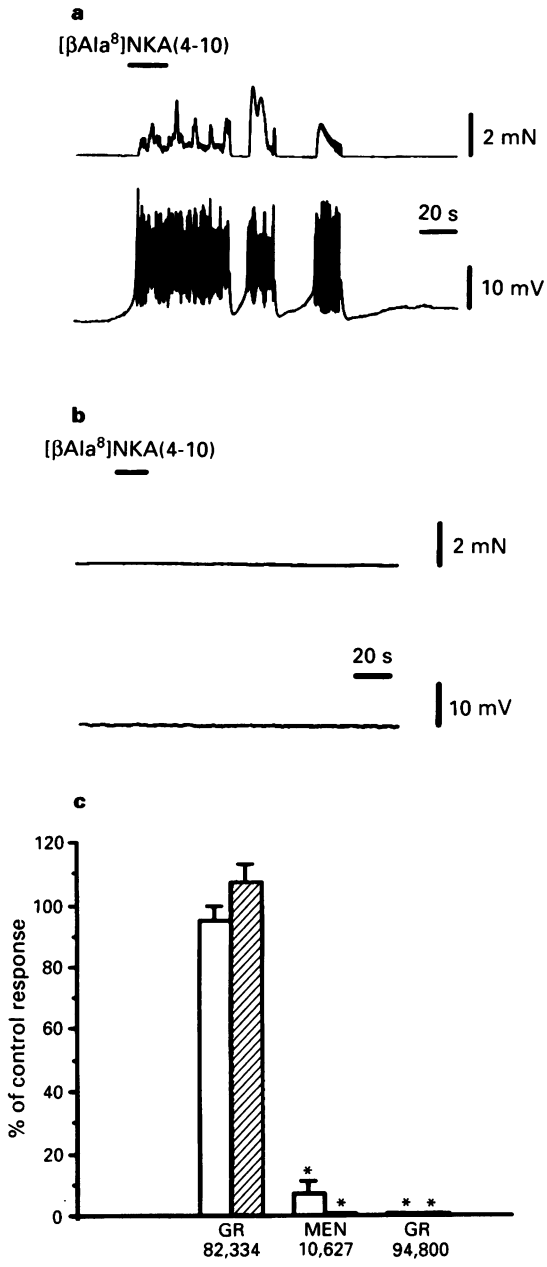

Figure 5 Effect of the tachykinin NK_1 receptor antagonist, GR 82,334 and of the NK_2 receptor antagonist, MEN 10,627 and GR 94,800 on the response induced by the selective tachykinin NK_2 receptor agonist [βAla^8]NKA(4-10). (a) Control response to [βAla^8]NKA(4-10) (period of application of the agonist is indicated by the horizontal bar); (b) response to [βAla^8]NKA(4-10) in the presence of GR 94,800 (3 μM); in both panels, upper tracing is muscle tension, lower tracing shows changes in membrane potential. (c) Effect of GR 82,334 (3 μM), MEN 10,627 (1 μM) and GR 94,800 (3 μM) on the area of depolarization (open columns) and area of contraction (hatched columns) induced by [βAla^8]NKA(4-10) (0.3 μM for 20 s). The responses to the agonist obtained in the presence of the antagonist are expressed as % of the control response. Each value is the mean ± s.e.mean of 3–4 experiments. *Significantly different from control, $P < 0.05$.

Effect of nifedipine on the NANC e.j.p. and responses produced by [Sar^9]SP sulphone or [βAla^8]NKA (4-10)

Nifedipine (1 μM for 30 min) blocked the action potentials superimposed on the EFS-evoked NANC e.j.p. and totally abolished contraction (Figure 6). Nifedipine also reduced the EFS-evoked NANC e.j.p.: the maximal amplitude of depolarization was 15.2 ± 3 mV and 4.7 ± 0.6 mV (70% inhibition, $n = 9$) in the absence and presence of nifedipine, respectively. In most cases (21 out of 29 strips) the NANC e.j.p. evoked by EFS (32 Hz for 1 s, 40 V, 0.2 ms) in the presence of nifedipine was biphasic, a first fast phase of e.j.p. being followed by a second slow component (Figure 6). In 8 out of 29 preparations, the e.j.p. was slow and monophasic. The total duration of the e.j.p. (measured as the duration from the onset of e.j.p. to the 90% of recovery from maximum) averaged 84 ± 9 s ($n = 13$).

Nifedipine totally abolished the action potentials and contraction evoked by [Sar^9]SP sulphone or [βAla^8]NKA(4-10) (0.3 μM and $n = 3$ for each agonist). The depolarization produced by [Sar^9]SP sulphone was inhibited by 72 ± 5% ($n = 3$) while that induced by [βAla^8]NKA(4-10) was reduced at a lesser extent (38 ± 6% inhibition, $n = 3$).

Effect of tachykinin receptor antagonists on the NANC e.j.p. in the presence of nifedipine

In the presence of nifedipine, GR 82,334 (3 μM) reduced the area of the EFS-evoked e.j.p. by 65 ± 7% ($n = 5$); GR 82,334 decreased to a similar extent the peak amplitude of the fast and slow phases of e.j.p. (63 ± 7 and 61 ± 4% inhibition, respectively, $n = 4$ and 5) (Figure 6).

GR 94,800 (3 μM) inhibited the area of the EFS-evoked e.j.p. by 48 ± 5% ($n = 3$) (Figure 6); GR 94,800 decreased to a similar extent the amplitude of the fast and slow phases of the e.j.p. (43 ± 5 and 50 ± 4% inhibition, respectively, $n = 3$).

The combined administration of GR 82,334 (3 μM) and GR 94,800 (3 μM) produced a further inhibition of the area of the EFS-evoked e.j.p. in the presence of nifedipine: only 15 ± 2% ($n = 9$) of the control response remained after 20 min of combined application of the NK_1 and NK_2 receptor antagonists (Figure 6). The combined administration of GR 82,334 and GR 94,800 decreased the peak amplitude of first and second phase of the e.j.p. by 45 ± 12 and 82 ± 4% ($n = 6$ and 7), i.e. the second slow phase of the e.j.p. was preferentially inhibited as compared to the first one.

Effect of suramin on the NANC e.j.p. in the presence of nifedipine

From the above experiments, it appears that a consistent part of the biphasic NANC e.j.p. evoked by EFS in the presence of nifedipine, and especially its fast component, is resistant to the combined blockade of NK_1 and NK_2 receptors by GR 82,334 and GR 94,800. We speculated that adenosine-triphosphate (ATP) maybe involved in this residual response. A NANC e.j.p. was evoked by train EFS (32 Hz for 1 s, 40 V, 0.2 ms pulse width, $n = 10$; Figure 7) and, in some strips ($n = 5$), also by single pulse EFS (30 V, 0.2 ms pulse width). Either suramin (100 μM for 25–30 min) was administered first and the effect of GR 82,334 plus GR 94,800 (3 μM each for 15 min) was investigated on the suramin-resistant response ($n = 6$) *or* GR 82,334 plus GR 94,800 were administered first and the effect of suramin was investigated on the response resistant to the TK receptor antagonists ($n = 4$).

The combined administration of GR 82,334 (3 μM) and GR 94,800 (3 μM) decreased the peak amplitude of the fast phase of e.j.p. in response to train EFS (37 ± 13% inhibition, $n = 4$) and remarkably reduced the area of the second slow phase (73 ± 8% inhibition, $n = 4$; Figure 7). The application of suramin (100 μM) in the presence of GR 82,334 and GR 94,800 produced a further marked inhibition of the peak amplitude of first fast phase of e.j.p. (87 ± 3% inhibition, $n = 4$) while leaving unaffected the residual area of the slow phase of e.j.p. (6 ± 8% increase, $n = 4$, Figure 7).

When applied first, suramin (100 μM) inhibited the peak amplitude of the first fast phase of the e.j.p. in response to train EFS in 4 out of 6 strips by 91 ± 2% ($n = 4$) without affecting the area of the slow phase of the e.j.p. (2 ± 15% increase, $n = 4$) (Figure 7). The combined administration of GR 82,334 and GR 94,800 (3 μM each for 15 min) in the presence of suramin (100 μM) did not affect significantly the peak amplitude of the first residual phase of e.j.p. (3 ± 9% increase, $n = 4$) but remarkably reduced the area of second slow phase of the e.j.p. (83 ± 4% inhibition, $n = 4$). It is also worth noting that suramin significantly increased (2.2 times) the latency of e.j.p.-evoked by train EFS: the latency averaged 268 ± 57 and 590 ± 83 ms ($n = 7$, $P < 0.05$) before and after suramin (100 μM), respectively.

In 2 strips, in which the e.j.p. was also biphasic in response to train EFS, suramin (100 μM) slightly increased (9%) or decreased (16%) the peak amplitude of the first phase and increased the area (35 and 22%) of the second slow phase of the e.j.p. In both cases, the e.j.p. had a large latency (590 and 400 ms, not modified by suramin) and was markedly reduced by the combined administration of GR 82,334 and GR 94,800 (82 and 91% inhibition, respectively) in the presence of suramin.

In the presence of nifedipine, single pulse EFS (30 V, 0.15 ms) evoked a fast NANC e.j.p. with an amplitude of

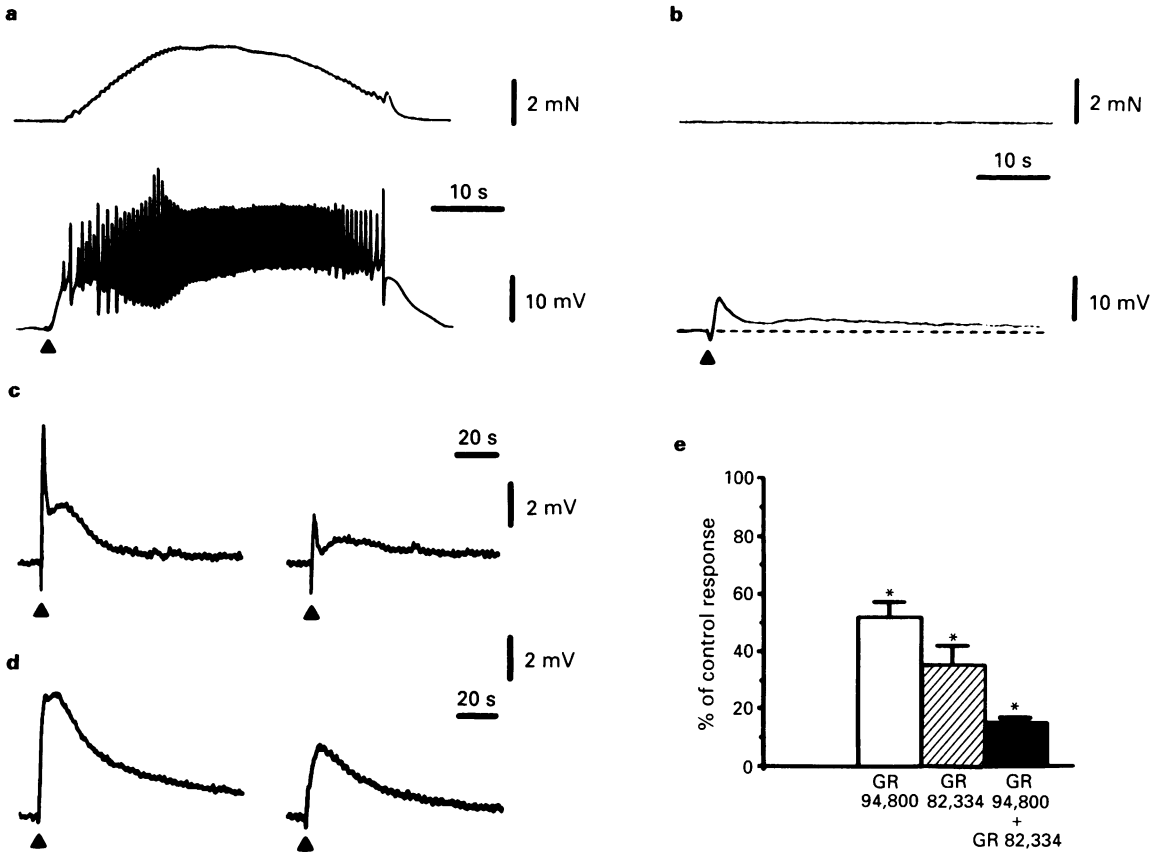

Figure 6 Effect of nifedipine on the NANC e.j.p. produced by EFS (32 Hz for 1 s, applied at arrowheads) and effect of the tachykinin NK_1 receptor antagonist, GR 82,334 and NK_2 receptor antagonist, GR 94,800 on the e.j.p. produced by EFS in the presence of nifedipine. (a and b) Electrical and mechanical responses to train EFS (32 Hz for 1 s, applied to arrowhead) in the absence (a) and presence (b) of nifedipine (1 μM); in both panels, upper tracing is muscle tension, lower tracing shows changes in membrane potential. (c) Effect of GR 82,334 (3 μM) on the nifedipine-resistant e.j.p. to train EFS (32 Hz for 1 s, applied at arrowhead); the control response to EFS is shown on the left, that obtained in the presence of GR 82,334 on the right. (d) Effect of GR 94,800 (3 μM) on the nifedipine-resistant e.j.p. to train EFS (32 Hz for 1 s, applied at arrowhead); the control response to EFS is shown on the left, that obtained in the presence of GR 94,800 on the right. (e) Effect of GR 94,800 (3 μM, open columns), GR 82,334 (3 μM, hatched columns), and combined administration of the two antagonists (solid columns) on the area of the nifedipine-resistant e.j.p. to train EFS. The area of e.j.p. obtained in the presence of antagonists is expressed as % of the control response. Each value is the mean ± s.e.mean of 3–9 experiments. *Significantly different from control response, $P < 0.05$.

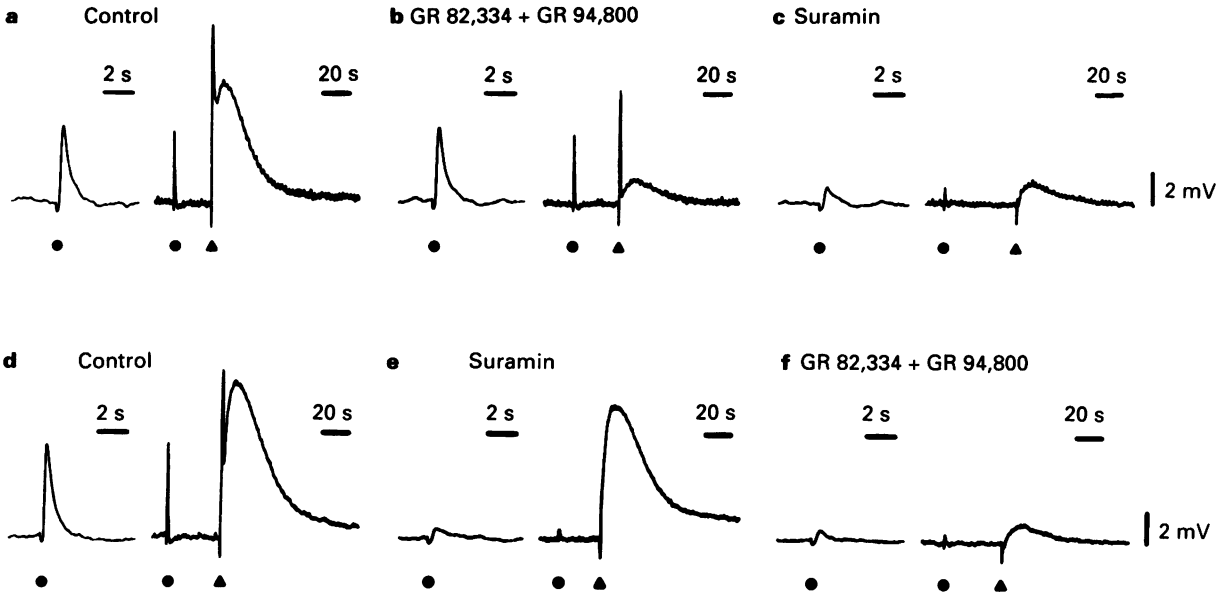

Figure 7 Effect of suramin (100 μM) and of the combined administration of tachykinin receptor antagonist GR 82,334 and GR 94,800 (3 μM each) on the e.j.p. produced by EFS in the presence of nifedipine. In each panel, dots indicate the application of single pulse EFS (30 V, 0.15 ms) and arrowheads indicate the application of train EFS (32 Hz for 1 s, 40 V, 0.25 ms). In the left part of each panel the e.j.p. produced by single pulse EFS is also shown on a magnified time scale. (a) Control responses to EFS; (b) the effect of combined administration of GR 82,334 and GR 94,800; (c) the effect of suramin in the presence of GR 82,334 and GR 94,800. (d) Control responses to EFS; (e) the effect of suramin; (f) the effect of GR 82,334 and GR 94,800 in the presence of suramin.

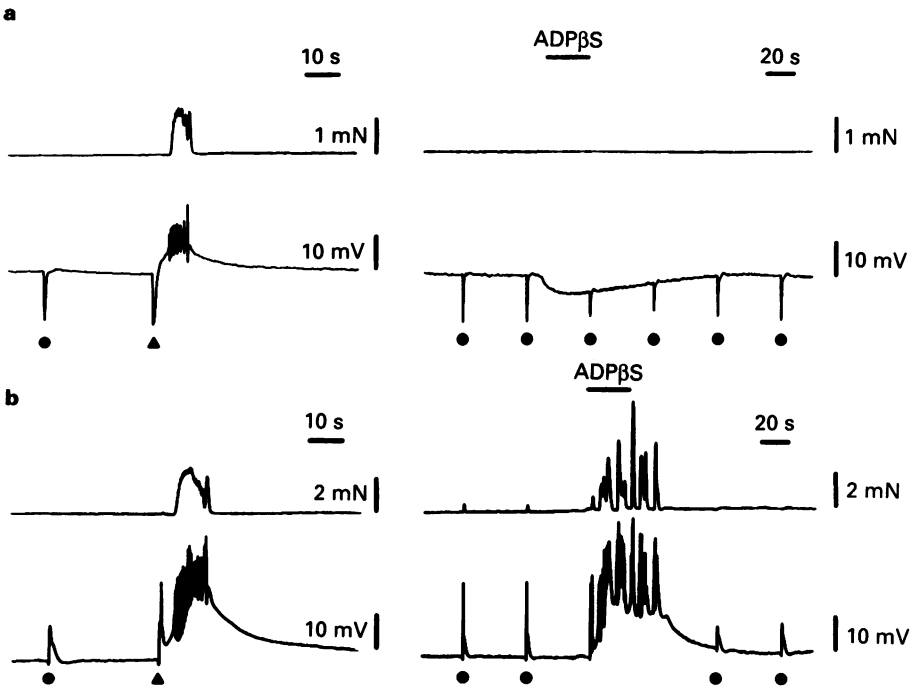

Figure 8 Effect of apamin and N^{ω}-nitro-L-arginine (L-NOARG) on the electrical and mechanical responses to EFS, junction potential and on the electrical and mechanical responses induced by ADPβS (10 μM for 30 s) in the circular muscle of the guinea-pig duodenum. Single pulse (30 V, 0.15 ms) and train EFS (32 Hz for 1 s, 40 V, 0.25 ms) were applied at dots and arrowheads, respectively. In right panels, the period of application of ADPβS is shown by horizontal bars. In each panel upper tracing shows mechanical activity and lower tracing shows changes in membrane potential. (a) Control responses to EFS and ADPβS; (b) responses obtained after 30 min combined administration of apamin (0.3 μM) and L-NOARG (30 μM). Note that, in the absence of apamin and L-NOARG (a, Krebs solution containing atropine, guanethidine and indomethacin) single pulse EFS produced a pure NANC inhibitory junction potential (i.j.p.) and train EFS produced a NANC i.j.p. followed by a NANC e.j.p. and contraction. After addition of apamin and L-NOARG, both single pulse and train EFS evoked a pure NANC e.j.p.; the e.j.p. and contraction were enhanced by administration of apamin and L-NOARG. In the absence of apamin and L-NOARG (a, Krebs solution containing atropine, guanethidine and indomethacin) the P_{2Y} receptor agonist ADPβS produced hyperpolarization of the membrane; this response was converted to depolarization with action potentials and contraction in the presence of apamin and L-NOARG (b).

4.4 ± 1.1 mV; the latency and duration of this e.j.p. were 195 ± 4 ms and 2.1 ± 0.3 s, respectively ($n = 8$). In the same strips, the amplitude of the fast component of the biphasic e.j.p. in response to train EFS averaged 6.5 ± 1.2 mV ($n = 8$). All the strips which showed a fast e.j.p. in response to single pulse EFS ($n = 8$) also showed a biphasic response to train EFS. The combined administration of GR 82,334 and GR 94,800 (3 μM each for 15 min) did not affect significantly (2 ± 4% increase, $n = 3$) the amplitude of the e.j.p. evoked by single pulse EFS. The application of suramin (100 μM for 30 min) in the presence of GR 82,334 and GR 94,800 inhibited the e.j.p. by 84 ± 6% ($n = 3$) (Figure 7). When applied first, suramin inhibited the amplitude of the e.j.p. evoked by single pulse EFS by 90 ± 3% ($n = 5$) while the combined administration of GR 82,334 and GR 94,800 did not affect the residual e.j.p. in the presence of suramin (Figure 7, $n = 5$).

Effect of suramin on the responses induced by ADP βS and [Sar⁹]SP sulphone in the absence of nifedipine

From the above, it would appear that, under certain circumstances, endogenous ATP may act via suramin-sensitive receptors as an excitatory transmitter. We then studied the effect of the selective P_{2Y} receptor agonist, ADPβS before and after blockade of NANC inhibitory transmission.

In the presence of atropine (1 μM), guanethidine (3 μM) and indomethacin (3 μM) single pulse or train (32 Hz for 1 s) EFS evoked an inhibitory junction potential (i.j.p., 16.1 ± 1.2 and 17.8 ± 1.3 mV, $n = 5$, respectively) followed by a NANC e.j.p., action potentials and contraction. The application of ADPβS (10 μM for 30 s) produced a hyperpolarization (7.8 ± 1.3 mV, $n = 5$) of the circular muscle of the guinea-pig duodenum (Figure 8). The combined administration of apamin (0.3 μM) and L-NOARG (30 μM) blocked the EFS-evoked i.j.p. and disclosed a pure NANC e.j.p. in response to train EFS in all cases tested. In 3 out of 5 strips a fast e.j.p. was also evoked by single pulse of EFS (Figure 8).

In the presence of apamin and L-NOARG, the application of ADPβS (10 μM for 30 s) induced a depolarization (7.3 ± 1.1 mV, $n = 9$), appearance of slow waves with action potentials (27.4 ± 2.2 mV, $n = 9$) and contraction (3.7 ± 0.5 mN, $n = 9$) of smooth muscle (Figures 8 and 9). The responses induced by [Sap⁹]SP sulphone (0.3 μM for 10 s) were comparable to those produced by ADPβS: depolarization (7.5 ± 1.3 mV, $n = 3$), action potentials (24.7 ± 4.3 mV, $n = 3$), contraction (2.8 ± 0.8 mN, $n = 3$, Figure 8).

After incubation with suramin (100 μM for 20–30 min), the depolarization and contraction induced by ADPβS were markedly reduced (72 ± 6 and 98 ± 8% inhibition, respectively, $n = 4$) while the responses to [Sar⁹]SP sulphone were not significantly affected (Figure 9).

Discussion

Tachykinin NK_1 and NK_2 receptors mediate NANC excitatory neurotransmission in the circular muscle of guinea-pig duodenum

The natural TKs synthesized by myenteric neurones, SP and NKA, act as full agonists at NK_1 and NK_2 receptors although with different potencies (Maggi *et al.*, 1993); they also act as full agonists at NK_3 receptors, producing neuronal excitation and release of other mediators, including acetylcholine, TKs and NO (e.g. Maggi *et al.*, 1990; 1994e). Therefore natural TKs are not suitable tools for establishing the relative role of NK_1 and NK_2 receptors in neuromuscular transmission; this can be precisely assessed by using receptor-selective agonists and antagonists. The interpretation of the findings presented in this study is based on the assumption that, at the concentrations used, GR 82,334 selectively blocked NK_1 receptors while MEN 10,627 and GR 94,800 selectively blocked NK_2 receptors. The assumption is based on the known pharmacological properties of these ligands (Hagan *et al.*, 1991; McElroy *et al.*, 1992; Patacchini *et al.*, 1994; Maggi *et al.*, 1994c) and has been further checked here by studying their action toward the responses produced by the receptor selective agonists, [Sar⁹]SP sulphone for NK_1 receptors and [βAla⁸]NKA(4-10) for NK_2 receptors (Maggi *et al.*, 1993 for review).

The present results demonstrate that NK_1 and NK_2 receptors are both involved in mediating a NANC e.j.p. and contraction in the circular muscle of the guinea-pig duodenum. This conclusion is supported by the following: (i) GR 82,334 *or* GR 94,800 and MEN 10,627 produced a

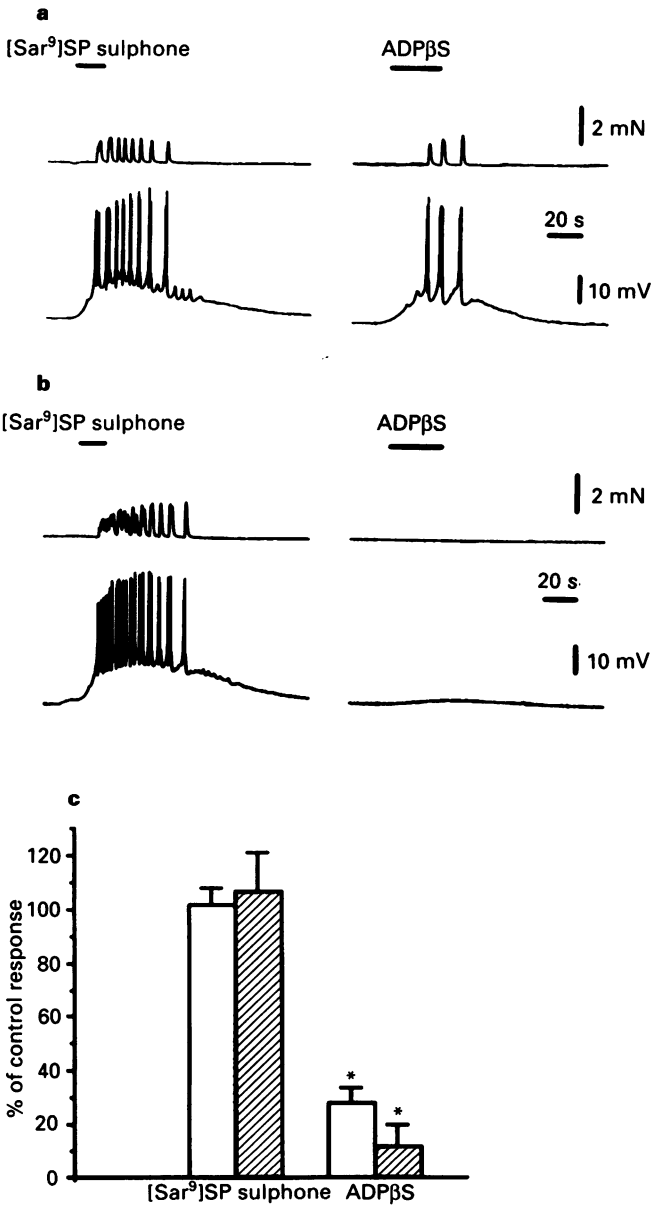

Figure 9 Effect of suramin (100 μM for 20 min) on the responses induced by ADPβS (10 μM for 30 s) and [Sar⁹]SP sulphone (0.3 μM for 10 s) in the circular muscle of the guinea-pig duodenum. (a) Control responses to ADPβS and [Sar⁹]SP sulphone; (b) responses obtained in the presence of suramin. Horizontal bars indicate the period of application of the agonist. In both panels, upper tracing shows muscle tension and lower tracing shows changes in membrane potential. (c) Effect of suramin (100 μM) on the area of depolarization (open columns) and area of contraction (hatched columns) induced by [Sar⁹]SP sulphone and ADPβS. Each value is the mean ± s.e.mean of 3–4 experiments. *Significantly different from control, $P < 0.05$.

concentration-dependent inhibition of the NANC e.j.p. and contraction evoked by EFS; (ii) GR 82,334 and GR 94,800 exerted an additive inhibitory effect on the area of the NANC e.j.p. evoked by EFS in the presence of nifedipine; (iii) GR 82,334, inhibited depolarization and contraction produced by [Sar^9]SP sulphone, without affecting that produced by [βAla^8]NKA(4-10); (iv) MEN 10,627 and GR 94,800 blocked depolarization and contraction produced by [βAla^8]NKA(4-10) but not that produced by [Sar^9]SP sulphone.

Nifedipine abolished the action potentials and totally suppressed the contraction produced by selective NK_1 and NK_2 receptor agonists in the circular muscle of the guinea-pig duodenum, similar to its effect in the guinea-pig ileum (Maggi *et al.*, 1994a). Therefore, L-type calcium channels appear essential for NANC excitation-contraction coupling throughout the guinea-pig small intestine. The experiments using receptor selective agonists demonstrate that, for a certain degree of receptor stimulation, activation of NK_1 *or* NK_2 receptor alone, via depolarization and triggering of action potentials, is sufficient to produce contraction of the guinea-pig duodenum. This does not necessarily reflect the situation occurring when endogenous TKs are released to produce the NANC e.j.p. Indeed, the inhibitory effect of GR 82,334 toward depolarization and contractility in response to EFS is almost parallel while, for both NK_2 receptor antagonists tested, contractility was preferentially inhibited. Moreover, blockade of either NK_1 or NK_2 receptor alone produced a very large inhibition of NANC contraction. We speculate that, at the relative amounts released from intramural nerves during EFS, the action exerted by the endogenous ligand(s) for NK_1 receptor (i.e. SP) could be relatively more efficient than that of the endogenous ligand(s) for NK_2 receptors (i.e. NKA) in producing depolarization. However, the activation of NK_2 receptor may contribute an amplifying mechanism, reinforcing the depolarization produced by NK_1 receptors and facilitating the attainment of threshold for action potentials and contraction. This may provide an explanation for the ability of NK_2 receptor antagonists to produce a large suppression of EFS-evoked NANC contraction while leaving a large proportion of e.j.p. unaffected (e.g. Figure 2a and 2b).

The inhibitory effect produced by NK_1 or NK_2 receptor antagonists was quite variable from one strip to another, yet examples have been found (Figure 1a and b) in which blockade of NK_1 receptor only was sufficient to produce an almost full blockade of both electrical and mechanical responses to EFS. This variability may involve differences in relative amounts of endogenous ligands for NK_1 and NK_2 receptors released from intramural nerves and/or differences in the expression of NK_1 and NK_2 receptors on smooth muscle. A further element of variability arises from the contribution of a third excitatory transmitter (see below) to the fast NANC e.j.p. evoked in the presence of L-NOARG and apamin, since this component showed quite a large variation in strips from different animals.

The relative contribution of the two TK receptors in the overall e.j.p. produced by endogenous TKs is more clearly appreciated in the presence of nifedipine which eliminated the action potentials: in the presence of nifedipine, the NK_1 and NK_2 receptor antagonists were about equieffective (65 and 48% inhibition of the area of e.j.p. by GR 82,334 and GR 94,800, respectively) in reducing the e.j.p. and their combined administration produced an additive inhibitory effect on the area of evoked depolarization (Figure 6e). Nifedipine apparently reduced in a preferential manner the relative contribution of NK_1 receptors to the NANC e.j.p., in agreement with the larger inhibitory effect of nifedipine toward the depolarization induced by the NK_1 vs. NK_2 receptor agonist.

Overall, the present findings support the view that both NK_1 and NK_2 receptors are junctionally activated during NANC e.j.p. in guinea-pig duodenum, yet the clear additivity was evident only after blockade of calcium channels. The observed differential profile of antagonism produced by GR 82,334 and MEN 10,627 *or* GR 94,800 on the electrical and mechanical components of NANC excitatory transmission may be explained by a co-operation of the signal generated by NK_1 and NK_2 receptors in attaining threshold for firing action potentials. Further studies are needed to verify this hypothesis. In any case, the pattern observed in the circular muscle of the guinea-pig duodenum differs markedly from that found in the proximal colon, where the response initiated by activation of NK_1 and NK_2 receptors shows a remarkable specialization leading to NANC excitatory responses with distinct and separate time courses and onset/offset kinetics of contraction (Zagorodnyuk *et al.*, 1993; 1994; Maggi *et al.*, 1994d).

Biphasic NANC e.j.p. in the presence of nifedipine and role of ATP as excitatory transmitter

In most strips tested in the presence of apamin, L-NOARG and nifedipine, train EFS produced a biphasic e.j.p. which involves several mediators; in some strips a fast NANC e.j.p. was also produced by single pulse EFS. These findings are strongly reminiscent of the results obtained in the circular muscle of the guinea-pig ileum, in which a biphasic e.j.p. to nerve stimulation was likewise observed (Niel *et al.*, 1983; Bywater & Taylor, 1986; Crist *et al.*, 1991). Our findings indicate that, in addition to TKs, another excitatory transmitter, possibly ATP (see below), mediates the fast NANC e.j.p. in the guinea-pig duodenum, while the slow e.j.p. is mediated almost exclusively by TKs. Various studies have implicated ATP as a mediator of the NANC i.j.p in the circular muscle of the guinea-pig intestine (e.g. Crist *et al.*, 1991; Zagorodnyuk & Maggi, 1994). In the guinea-pig duodenum, the i.j.p. evoked by EFS was totally eliminated by apamin and L-NOARG: although we did not investigate the effect of these two drugs separately, it appears conceivable that, in analogy with circular muscle of the ileum (cf. He & Goyal, 1993), apamin alone may be sufficient to block the inhibitory action of ATP. The ability of apamin to convert the hyperpolarizing effect of ATP into a depolarization was reported previously in guinea-pig intestine (Shuba & Vladimirova, 1980). The application of apamin and L-NOARG totally eliminated the NANC i.j.p. and, in parallel, converted the hyperpolarization induced by ADPβS into a depolarization. We speculate that after blockade of apamin-sensitive K channels, a depolarizing effect of endogenous ATP was unmasked, involving changes of permeability for some ions. For instance, receptor-gated, calcium permeable, nonselective cation channels are activated by ATP in vascular smooth muscle (Benham & Tsien, 1987).

The evidence for an involvement of ATP in the fast NANC e.j.p. can be summarized as follows: (i) suramin selectively reduced the fast phase of NANC e.j.p.; (ii) when tested in presence of apamin and L-NOARG, ADPβS produced depolarization and contraction; (iii) suramin selectively suppressed the electrical and mechanical responses to ADPβS without affecting that to [Sar^9]SP sulphone. Previously, we suggested that, in addition to acetylcholine and TKs, a third excitatory mediator could be involved in the ascending excitatory reflex to the circular muscle of the guinea-pig ileum, especially in the presence of apamin and L-NOARG (Maggi *et al.*, 1994b). The present findings raise the possibility that, under certain circumstances, ATP may act as excitatory mediator participating in some form of atropine-resistant neuromuscular transmission. On the other hand, the need to block NANC inhibitory mechanisms to demonstrate an ATP-mediated NANC e.j.p. casts some doubt on the physiological relevance of this mechanism.

Interestingly, the fast e.j.p. evoked by single pulse EFS in the presence of apamin and L-NOARG was inhibited by suramin while it was unaffected by the combined administration of GR 82,334 and GR 94,800; on the other hand, a

partial inhibitory effect of the TK receptor antagonists was evident on the fast e.j.p. evoked by train EFS. Therefore the fast e.j.p. to single pulse EFS may represent a pure ATP-mediated event, possibly reflecting the need of more intense stimulation for release of TKs.

In conclusion, both NK_1 and NK_2 receptors mediate NANC e.j.p. 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. In the presence of apamin, L-NOARG and nifedipine, the e.j.p. evoked by a train of EFS is biphasic: the fast component involves tachykinins and another excitatory transmitter, putatively ATP; the second slow phase of e.j.p. is TK-mediated and involves both NK_1 and NK_2 receptors.

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