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Tachykinin NK_1 and NK_2 receptor antagonists and atropine-resistant ascending excitatory reflex to the circular muscle of the guinea-pig ileum

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1 The aim of this study was to investigate the effect of various antagonists, selective for the tachykinin NK_1 or NK_2 receptor, on the atropine-resistant ascending excitatory reflex (AER) to the circular muscle of the guinea-pig ileum elicited by radial stretch (balloon distension) or electrical field stimulation.

2 Submaximal and maximal atropine- (1 μM) resistant AER elicited by balloon distension averaged about 40–50% and 70–90% of maximal circular spasm to 80 mM KCl, respectively. The NK_1 receptor antagonist, (±)-CP 96,345 (1 μM) inhibited both maximal and submaximal AER. FK 888 (1–3 μM) inhibited submaximal AER only. RP 67,580 (1 μM) was ineffective. The NK_2 receptor antagonist, GR 94,800, inhibited both maximal and submaximal AER at all concentrations tested (0.1–3.0 μM), while SR 48,968 was effective only at 1.0 μM. The NK_2 receptor antagonists, MEN 10,376 and MEN 10,573 inhibited both submaximal and maximal AER at 10 and 1.0 μM, respectively.

3 In other experiments, an NK_1 receptor antagonist, (±)-CP 96,345 or FK 888 (1.0 μM in each case) was administered first and the effect of GR 94,800 (1.0 μM) on the residual AER response was determined; or GR 94,800 was administered first and the effect of (±)-CP 96,345 or FK 888 was determined. The results of these experiments indicated an additive effect produced by the combined treatment with NK_1 and NK_2 receptor antagonists.

4 Electrical field stimulation (10 Hz for 0.5 s, 10–20 V, 0.15–0.3 ms pulse width) with electrodes placed at 1.4–1.8 cm anal to the recording site, produced ascending contractions which were almost abolished by 10 μM hexamethonium (electrically-evoked AER). In the presence of apamin (0.1 μM) and N^G-nitro-L-arginine (30 μM) these contractions were reproducible over 10 consecutive stimulation cycles. GR 94,800 (1 μM) and FK 888 (1 μM) both produced a partial inhibition of the electrically-evoked AER and their combined administration produced an inhibitory effect which was larger than that induced by each antagonist alone.

5 FK 888 (1–3 μM), GR 94,800 (1–3 μM), MEN 10,573 (1 μM) and MEN 10,376 (10 μM) did not significantly affect the atropine-sensitive twitch contractions produced by electrical field stimulation of the guinea-pig ileum longitudinal muscle-myenteric plexus preparation, which were abolished by 10–30 μM procaine, 1 μM tetrodotoxin or 1 μM atropine. (±)-CP 96,345 (1 μM) and SR 48,968 (1 μM) produced 12% and 27% inhibition of cholinergic twitches in the longitudinal muscle of the ileum, respectively.

6 We conclude that both NK_1 and NK_2 receptors mediate the atropine-resistant AER to the circular muscle of the ileum. NK_2 receptor activation plays a more important role than NK_1 receptor activation in the AER evoked by radial stretch. Since a consistent fraction of the distension- and electrically-evoked atropine-resistant AER persists in the presence of combined NK_1 and NK_2 receptor blockade, the existence of a third excitatory transmitter to the circular muscle of the ileum, in addition to acetylcholine and tachykinins, is suggested.

Keywords: Tachykinins; guinea-pig ileum; circular muscle; tachykinin receptors; ascending excitatory reflex; enteric nervous system

Introduction

Tachykinins are powerful smooth muscle spasmogens in the guinea-pig ileum and are thought to play a major role as excitatory transmitters to both circular and longitudinal muscle layers (Bartho *et al.*, 1982; Costa *et al.*, 1985; Holzer, 1989; Bartho & Holzer, 1985 for review). A subpopulation of enteric neurones in the myenteric plexus expresses tachykinin-like immunoreactivity: these elements express the preprotachykinin I gene, which encodes the sequence of both substance P and neurokinin A (Sternini *et al.*, 1989) and some of them have appropriate projections for them to be considered the effector motoneurones for reflexly-evoked contraction of the circular muscle (Brookes *et al.*, 1991). Accordingly, both substance P- and neurokinin A-like immunoreactivity have been detected in enteric neurones by immunocytochemistry (e.g. Schmidt *et al.*, 1991; Shuttleworth *et al.*, 1991) and release of both peptides from the mammalian gut has been documented in response to depolarizing stimuli (Theodorsson *et al.*, 1991; Schmidt *et al.*, 1992b). Three main types of tachykinin receptors (NK_1, NK_2 and NK_3) are known to mediate the spasmogenic activity of peptides of this family (Maggi *et al.*, 1993a). In the circular muscle of the guinea-pig ileum, NK_1 and NK_2 receptors mediate the direct spasmogenic effect of tachykinins on muscle cells, while the NK_3 receptor-mediated response is totally indirect and involves the release of endogenous acetylcholine and tachykinins (Maggi *et al.*, 1990; 1994a; Bartho *et al.*, 1992). Since both NK_1 (substance P-preferring) and NK_2 (neurokinin A-preferring) receptors mediate the direct contraction of circular muscle of the ileum, the question arises as to the relative contribution of endogenous tachykinins in mediating atropine-resistant excitation to the circular muscle.

Earlier studies on the role of tachykinins as mediators of

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the atropine-resistant contractility in the guinea-pig ileum have used first generation peptide antagonists, the best known of which is spantide; these compounds suffer from a number of drawbacks, including low potency and lack of selectivity (see Maggi *et al.*, 1993a for recent review). Especially disturbing is the poor ability of these compounds to discriminate between NK_1 and NK_2 receptors which does not enable verification of the relative contribution of different tachykinins/different receptors to the overall physiological response.

Recently, the use of the NK_2 receptor-selective antagonist, MEN 10,376 ([Tyr^5,D-$Trp^{6,8,9}$,Lys^{10}]NKA(4–10)) (Maggi *et al.*, 1991) vs. the NK_1 receptor selective antagonists, GR 71,251 or GR 82,334 (Hagan *et al.*, 1991) has revealed an important role of NK_2 receptors in mediating the ascending excitatory reflex (AER) produced by radial stretch (balloon distension) in the guinea-pig ileum (Bartho *et al.*, 1992; Holzer *et al.*, 1993). From these studies, two basic concepts have emerged: (i) the NK_2 receptor prevails over the NK_1 receptor in mediating the atropine-resistant contraction of the circular muscle of the ileum, suggesting an important role for neurokinin A in mediating neuromuscular transmission at this level (Bartho *et al.*, 1992) and (ii) tachykinin release and NK_2 receptor activation during the AER is not restricted to high degree of stimulation but also occurs in the absence of atropine and when using a submaximal degree of stimulation (Holzer *et al.*, 1993).

In the accompanying paper (Maggi *et al.*, 1994b) we have determined the affinities of a panel of tachykinin receptor antagonists for NK_1 and NK_2 receptor-induced contraction in the circular muscle of the ileum. From this analysis, it appeared that the affinity of MEN 10,376 for NK_2 receptor in the circular muscle of the ileum is lower than that expected on the basis of its potency at NK_2 receptors in other guinea-pig smooth muscles. In particular, MEN 10,376 was found to possess very similar affinity for the NK_2 receptor and the putative novel type of septide-sensitive receptor described by Petitet *et al.* (1992) in the guinea-pig ileum. On this basis it appeared of interest to re-examine the activity of various NK_1 and NK_2 receptor antagonists on the atropine-resistant AER.

Methods

Male albino guinea-pigs weighing 250–350 g were stunned and bled. A 10–15 cm long piece of terminal ileum was excised and placed in warmed (37°C) and oxygenated (96% O_2 and 4% CO_2, pH 7.4) Krebs solution 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. The ileum was placed in a thermostated bath (7 ml) and arranged for isotonic (load 5 mN) recording of circular muscle mechanical activity in response to radial stretch produced by distension of a balloon placed at 1–1.2 cm anal to the recording site: the circular muscle contraction evoked in this way is totally hexamethonium- and tetrodotoxin-sensitive (ascending excitatory reflex, AER), as described previously (Holzer, 1989; Bartho *et al.*, 1992). All experiments were performed in the presence of 1 μM atropine which was added to the Krebs solution from the beginning of the experiment.

After a 45 min equilibration time, the intraluminal balloon located anally to the recording site was manually inflated by a syringe with an amount of saline to evoke the AER. In each preparation, maximal AER response was evoked with 0.2–0.3 ml saline; submaximal AER was obtained by distension with 0.1–0.2 ml saline (see Results).

In each preparation, submaximal and maximal AERs were elicited, 5 min apart from each other, at 20 min intervals, until two reproducible responses to both submaximal and maximal stimulation were evoked. At this time the stated concentration of tachykinin receptor antagonists was added to the bath and their effect on submaximal and maximal AER determined 15 min later. This contact time was the same as that used in experiments aiming to determine the affinities of the various antagonists used in this study for NK_1 and NK_2 receptors in the circular muscle of the ileum (Maggi *et al.*, 1994b). Control experiments showed that submaximal and maximal AER could be elicited for at least seven consecutive stimulation cycles with minimal (10–15% variation) changes in the amplitude of the evoked response.

Control experiments were also performed in which the vehicle dimethylsulphoxide (DMSO) used to dissolve some of the antagonists was added to the preparation after having recorded control responses to submaximal and maximal AER. These experiments ($n = 4$, DMSO 1% final concentration) failed to show any significant effect of the vehicle.

Antagonists tested were: (±)-CP 96,345 (Snider *et al.*, 1991), RP 67,580 (Garret *et al.*, 1991) and FK 888 (Fujii *et al.*, 1992) as NK_1 receptor selective antagonists; MEN 10,376 (Maggi *et al.*, 1991), MEN 10,573 (Quartara *et al.*, 1992), SR 48,968 (Emonds-Alt *et al.*, 1992) and GR 94,800 (McElroy *et al.*, 1992) as NK_2 receptor antagonists.

At the end of the experiment, KCl (80 mM) was added to the bath. This produced a circular muscle contraction corresponding to total occlusion of the ileal lumen. The response to 80 mM KCl was used as internal standard and the amplitude of AER responses were expressed as a % of the response to KCl.

In a separate set of experiments, the atropine-resistant AER was evoked by electrical field stimulation performed by means of a pair of wire platinum electrodes placed in parallel at 1.4–1.8 cm anally to the site of recording of circular muscle activity, using an approach similar to that described by Allescher *et al.* (1992) for evoking the AER in the rat isolated ileum. The electrodes were connected to a GRASS S88 stimulator: trains of pulses were delivered at a frequency of 10 Hz for 0.5 s every 5 min. Pulse width was 0.1–0.3 ms and voltage was 10–20 V. In each preparation, pulse width and voltage were adjusted to produce maximal circular muscle contraction at the recording site oral to the point of stimulation. This averaged 40–70% of the maximal response to 80 mM KCl. Preliminary experiments (and see results) showed that electrical stimulation with these parameters provided ascending circular muscle contractions which were largely or totally hexamethonium-sensitive. All experiments were performed in the presence of 1 μM atropine.

In a first series of experiments, the electrically-evoked atropine-resistant AER obtained in untreated preparations with ten cycles of stimulation at 5 min intervals was compared to that obtained in the presence of apamin (0.1 μM) and N^G-nitro-L-arginine (L-NOARG 30 μM). This was done because preliminary experiments showed a spontaneous fading of the response in untreated preparation while the addition of apamin and L-NOARG improved reproducibility of the response.

In a second series of experiments, in the presence of apamin and L-NOARG, the effect of GR 94,800 (1 μM) or FK 888 (1 μM) alone and in combination was investigated. In these experiments, after having recorded 3–5 control responses at 5 min intervals the drug was added to the bath and its effect recorded over the next 3–4 stimulation cycles. At the end of each experiment, hexamethonium (10 μM) was added to the bath to check the reflex origin of the evoked contractions. All contractile responses were expressed as % of the response to 80 mM KCl.

In other experiments, performed in the absence of atropine, the effects of NK_1 and NK_2 receptor antagonists on twitch contractions of the longitudinal muscle-myenteric plexus preparation produced by electrical field stimulation (0.1 Hz, 60 V, 0.4 ms pulse width) were determined to assess whether the various drugs may possess nonspecific effects on contractility/local anaesthetic activity. After a 90 min equilibration time, the preparations were electrically stimulated until twitch height reached a steady state. At this stage, the stated concentration of tachykinin receptor antagonists was

added to the bath and its effects recorded for at least 15 min Addition of the vehicle (0.1% DMSO, final concentration in the bath) produced a slight (< 10%) and transient enhancement of twitches. In each preparation atropine (1 μM), tetrodotoxin (1 μM) or procaine (10–30 μM) were tested as positive controls.

Data evaluation and statistical analysis

All values in the text, table and figures are mean ± s.e.mean. Statistical analysis was performed by means of the Student's *t* test for paired data or by means of ANOVA, when applicable.

Drugs

Drugs used were: procaine HCl (Sigma) hexamethonium bromide and atropine HCl (Serva) tetrodotoxin (Sankyo).

RP 67,580 ((3α**R**, 7α**R**)-7,7-diphenyl-2-[1-imino-2-(2-methoxyphenyl)ethyl] perhydroisoindol-4-one) was a kind gift of Dr C. Garret, Rhone Poulenc, Vitry, France; SR 48,968 ((S)-N-methyl-*N* [4- (4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl) butyl]benzamide) of Dr X. Emonds-Alt, Sanofi Recherche, Montpellier, France; GR 94,800 (PhCO-Ala-Ala-D-Trp-Phe-D-Pro-Pro-NleNH_2) was a kind gift from Dr R.M. Hagan, GGR, Ware England. (±)-CP 96,345 ((2S,3S)-*cis*-2-(diphenylmethyl)-*N*-[(2-methoxyphenyl)-methyl] -1-azabicyclo [2.2.2]octan-3-amine, MEN 10,376 (Tyr^5,D-$Trp^{6,8,9}$,Lys^{10}] NKA(4–10)), MEN 10,573 (cyclo(LeuΨ[CH_2NH]Asp(OBzl)-Gln-Trp-Phe-βAla) and FK 888 ((2-(N-Me)indolil)-CO-Hyp-Nal-NMeBzl) were synthesized in the Chemistry Department of Menarini Pharmaceuticals.

Results

Ascending enteric reflex by balloon distension

The atropine- (1 μM) resistant ascending enteric reflex (AER) was investigated on 102 preparations. A submaximal AER was elicited at volumes of balloon distension between 0.1–0.2 ml (mean volume 0.13 ± 0.03 ml, $n = 102$) which ranged between 20 and 85% of maximal circular spasm produced by 80 mM KCl (Table 1). Maximal AER was evoked at volumes of 0.2–0.3 ml (mean volume 0.23 ± 0.06 ml, $n = 102$) which ranged between 55 and 100% of maximal circular spasm to KCl.

The effect of various NK_1 and NK_2 receptor antagonists on the submaximal and maximal atropine-resistant AER is shown in Table 1. (±)-CP 96,345 was ineffective at 0.1 μM while it produced 66% and 46% inhibition of submaximal and maximal AER at 1 μM, respectively. FK 888 was ineffective at 0.1 μM and inhibited (50%) the submaximal AER only at 1 μM. A higher concentration (3 μM) of FK 888 was not more effective than 1 μM (Table 1). RP 67,580 was ineffective at 1.0 μM.

The nonpeptide NK_2 receptor antagonist, SR 48,968, inhibited the submaximal and maximal AER (by 63% and 51% respectively) at 1.0 μM while it was ineffective at 0.1 μM. MEN 10,376 was ineffective at 1 μM while at 10 μM it inhibited submaximal and maximal AER by 89% and 60%; MEN 10,573 was ineffective at 0.1 μM, while at 1.0 μM it inhibited submaximal and maximal AER by 75% and 43%, respectively (Table 1).

GR 94,800 inhibited submaximal and maximal AER at both concentrations tested: submaximal AER was inhibited by 48% and 73% at 0.1 and 1.0 μM, respectively; maximal AER was inhibited by 23% and 43% at 0.1 and 1.0 μM, respectively (Table 1). A higher concentration of GR 94,800 (3 μM) was not more effective than 1 μM (Table 1).

Table 1 Effect of various tachykinin receptor antagonists on the atropine (1.0 μM)-resistant ascending enteric reflex evoked by submaximal or maximal balloon distension in the guinea-pig ileum

Antagonist		*Concentration*	*Response to distension* (% of response to KCl) Submaximal	% inh.	Maximal	% inh.
(±)-CP 96,345	($n = 11$)	Control	47 ± 8		88 ± 4	
		0.1 μM	41 ± 8	–	76 ± 5	–
		1.0 μM	16 ± 6*	66%	48 ± 8 *	46%
FK 888	($n = 9$)	Control	48 ± 2		67 ± 3	
		0.1 μM	36 ± 9	–	60 ± 4	–
		1 μM	24 ± 10*	50%	55 ± 6	–
	($n = 4$)	Control	52 ± 6		72 ± 4	
		3 μM	24 ± 6	54%	62 ± 6	–
RP 67,580	($n = 7$)	Control	44 ± 10		71 ± 11	
		1.0 μM	36 ± 12	–	58 ± 9	–
GR 94,800	($n = 21$)	Control	44 ± 6		75 ± 3	
		0.1 μM	23 ± 5*	48%	58 ± 3*	23%
		1.0 μM	12 ± 4*	73%	43 ± 5*	43%
	($n = 4$)	Control	59 ± 6		82 ± 5	
		3.0 μM	18 ± 4*	70%	44 ± 5*	47%
SR 48,968	($n = 11$)	Control	58 ± 8		89 ± 3	
		0.1 μM	41 ± 9	–	70 ± 6	–
		1.0 μM	22 ± 11*	63%	44 ± 15*	51%
MEN 10,573	($n = 15$)	Control	71 ± 6		83 ± 4	
		0.1 μM	69 ± 9	–	80 ± 7	–
		1.0 μM	18 ± 8*	75%	48 ± 6*	43%
MEN 10,376	($n = 6$)	Control	52 ± 7		90 ± 4	
		1.0 μM	42 ± 66	–	79 ± 6	–
		10 μM	6 ± 6*	89%	36 ± 4*	60%

The atropine-resistant AER was evoked in each preparation by submaximal and maximal balloon distension. The amplitude of evoked responses is quantified as % of the control response to KCl. After having established control responses to submaximal balloon distension, the effect of tachykinin antagonists was investigated. For those concentrations of antagonists which produced a statistically significant reduction of evoked AER, the % inhibition values of control response are also presented.
*Significantly different from control value, $P < 0.05$. Inhibitory effects which reached statistical significance are also indicated as % inhibition of control values.

The effect of GR 94,800 (0.1–1.0 μM), administered in the presence of 1 μM (±)-CP 96,345 or FK 888, is shown in Figure 1. In the presence of 1 μM (±)-CP 96,345, GR 94,800 abolished the submaximal AER and inhibited the residual maximal AER by 62% and 85% at 0.1 and 1.0 μM, respectively. In the presence of 1 μM FK 888, GR 94,800 inhibited the submaximal AER by 50% and 79% at 0.1 and 1.0 μM, respectively and the residual maximal AER by 46% and 60% at 0.1 and 1.0 μM, respectively.

The effect of (±)-CP 96,345 (0.1–1.0 μM) or FK 888 (0.1–1.0 μM) in the presence of 1 μM GR 94,800 is shown in Figure 2. In the presence of GR 94,800, (±)-CP 96,345 abolished the residual submaximal AER at 1.0 μM; the maximal AER was further reduced by 36% and 67% at 0.1 and 1.0 μM (±)-CP 96,345, respectively.

In the presence of GR 94,800 (1.0 μM), FK 888 had no further inhibitory effect on either submaximal or maximal AER at 0.1 μM. At 1.0 μM, FK 888 abolished the submaximal AER and inhibited maximal AER by 34% (Figure 2).

Ascending enteric reflex by electrical field stimulation

Electrical field stimulation (10 Hz for 0.5 s, 10–20 V, 0.15–0.3 ms pulse width) evoked atropine (1 μM)-resistant circular muscle contraction at the oral recording site which was placed at 1.6 ± 0.2 cm from the stimulation site ($n = 32$, range 1.4–1.8 cm).

In a first series of experiments, the reproducibility of the evoked response was assessed by delivering trains of stimuli at 5 min intervals and this showed a significant decay (Figure 3). This decay was equally evident when the interstimulus interval was of 10 min (not shown). In the presence of apamin (0.1 μM) and L-NOARG (30 μM) added 45 min before the stimulation, the evoked contraction did not show a significant decay over ten consecutive stimulation cycles, 5 min apart from each other (Figure 3).

In the presence of apamin and L-NOARG, the amplitude of oral contraction evoked by electrical field stimulation averaged 59 ± 5% of maximal circular muscle response to KCl ($n = 18$) and this response was largely >85% inhibited by 1 μM, hexamethonium, indicating its reflex origin. In 13 out of 18 cases tested, including the example shown in Figure 4a, hexamethonium completely abolished the evoked response.

In a second series of experiments, the effect of GR 94,800 (1 μM) and FK 888 (1 μM) on the electrically-evoked AER was determined (Figure 4b, and Figure 5). GR 94,800 produced 36% inhibition of the electrically-evoked AER and, in its presence, FK 888 produced a further 35% reduction of the residual response ($n = 8$, Figure 5). When added first, FK 888 produced 29% inhibition of the electrically evoked AER and, in its presence, GR 94,800 produced 45% inhibition of the residual response ($n = 8$, Figure 5). In both series of experiments, the residual response in the presence of GR 94,800 and FK 888 was almost abolished by 10 μM hexamethonium (Figures 4 and 5).

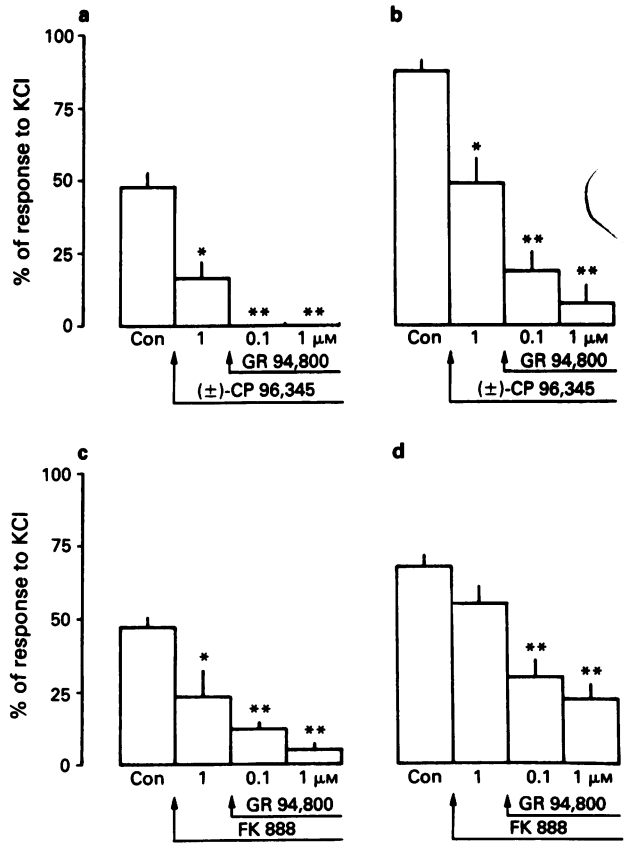

Figure 1 Effect of tachykinin receptor antagonists on the atropine-resistant AER evoked by submaximal (a,c) or maximal (b,d) balloon distension in the guinea-pig ileum. In the experiments shown in (a) and (b), (±)-CP 96,345 was administered first and, in its presence, GR 94,800 was investigated. In (c) and (d), FK 888 was added first and, in its presence, the effect of GR 94,800 was investigated. Each value is mean ± s.e.mean of at least six determinations. *Significantly different from control (con) response: $P < 0.05$. **Significantly different from the response obtained in the presence of (±)-CP 96,345 (a,b) or FK 888 (c,d), $P < 0.05$.

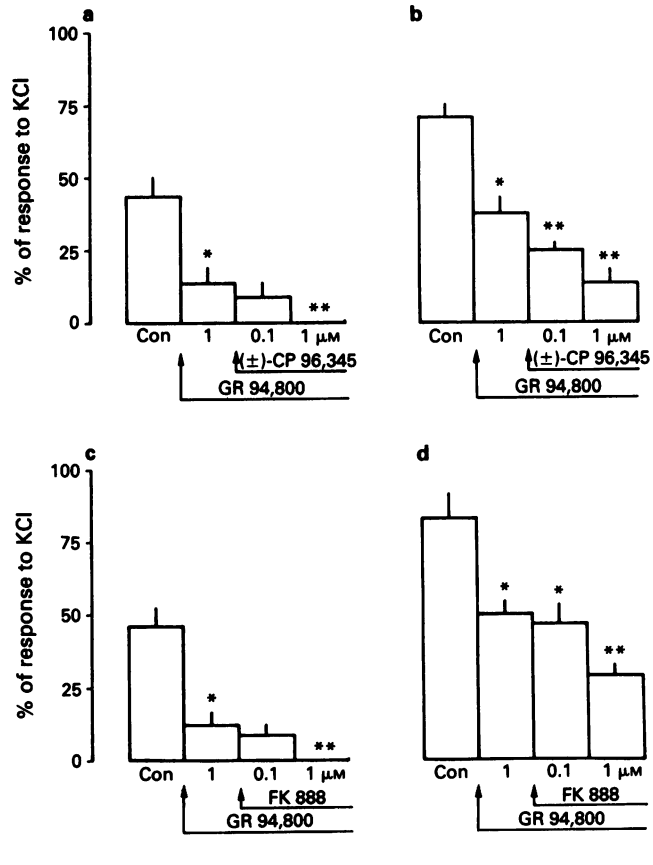

Figure 2 Effect of tachykinin receptor antagonists on the atropine-resistant AER evoked by submaximal (a,c) or maximal (b,d) balloon distension in the guinea-pig ileum. In the experiments shown in (a) and (b), GR 94,800 was administered first and, in its presence, (±)-CP 96,345 was investigated. In (c) and (d), GR 94,800 was added first and, in its presence, the effect of FK 888 was investigated. Each value is mean ± s.e.mean of at least six determinations. *Significantly different from control (Con) response $P < 0.05$. **Significantly different from the response obtained in the presence of GR 94,800, $P < 0.05$.

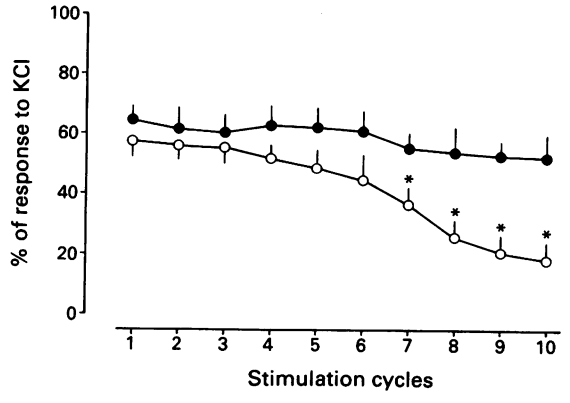

Figure 3 Effect of apamin (0.1 μM) and N^G-nitro-L-arginine (L-NOARG, 30 μM) on time course of the electrically-evoked AER induced by 10 consecutive cycles of stimulation: (○) control; (●) apamin plus L-NOARG. Each value is mean ± s.e.mean of six experiments.

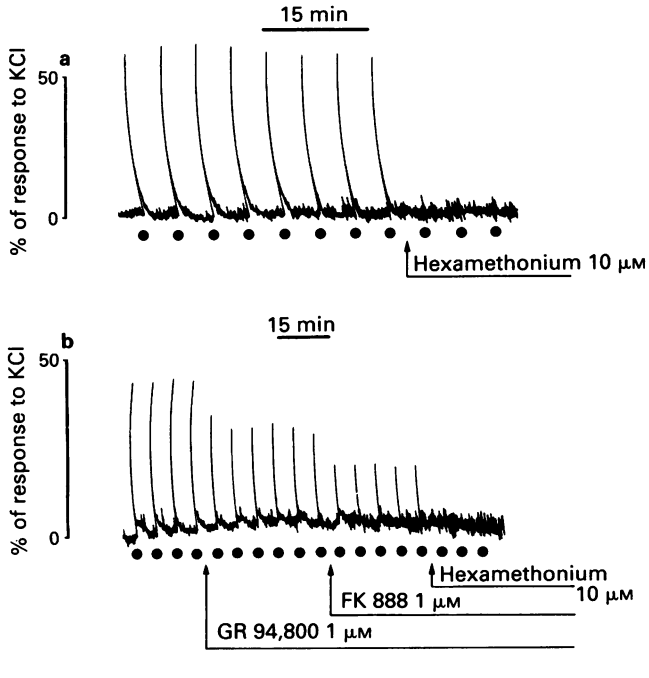

Figure 4 Electrically-evoked atropine-resistant AER in the guinea-pig ileum in the presence of apamin (0.1 μM) and N^G-nitro-L-arginine (L-NOARG, 30 μM): effect of hexamethonium (a); effect of GR 94,800, FK 888 and hexamethonium (b).

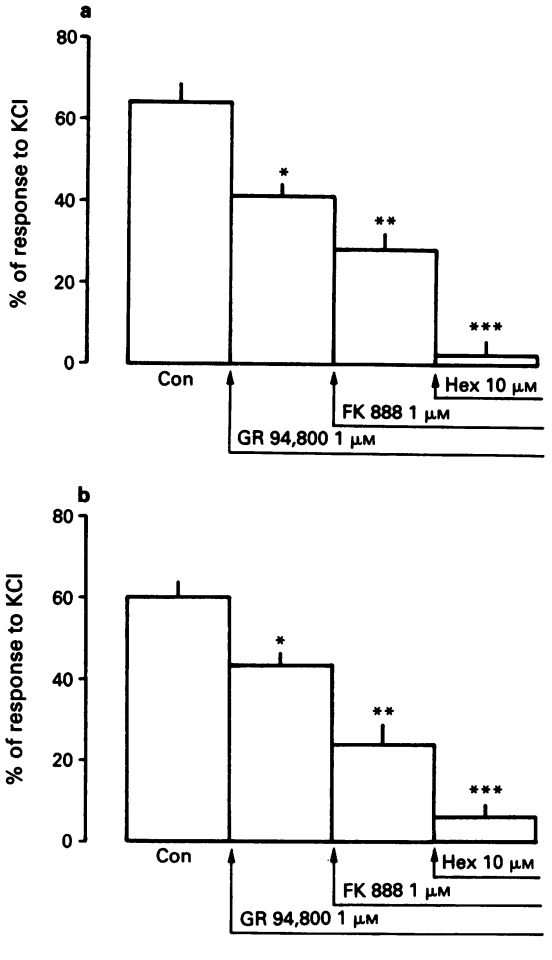

Figure 5 Effect of GR 94,800 and FK 888 on the atropine-resistant AER evoked by electrical stimulation in the guinea-pig ileum in the presence of apamin (0.1 μM) and N^G-nitro-L-arginine (L-NOARG, 30 μM). In experiments shown in (a), GR 94,800 was added first and, in its presence, the effect of FK 888 was determined. In the experiments shown in (b), the effect of FK 888 was determined first and, in its presence, the effect of GR 94,800 was determined. At the end of the experiments hexamethonium (Hex) was added to check the reflex nature of the evoked response. Each value is mean ± s.e.mean of at least six experiments. *Significantly different from control (Con) $P<0.05$; **Significantly different from the value recorded in the presence of GR 94,800 (a) or FK 888 (b), $P<0.05$; ***Significantly different from the value recorded in the presence of FK 888 and GR 94,800, $P<0.05$.

Effect of tachykinin receptor antagonists on twitch response of the longitudinal muscle to electrical field stimulation

FK 888 (1–3 μM), GR 94,800 (1–3 μM), MEN 10,573 (1 μM) and MEN 10,376 (10 μM) ($n = 4$ for each antagonist) had no significant inhibitory effect on the amplitude of twitch contractions of the guinea-pig ileum longitudinal muscle-myenteric plexus preparation evoked by electrical field stimulation (0.4 ms pulse width, maximal voltage delivered at a frequency of 0.1 Hz). (±)-CP 96,345 (1 μM) and SR 48,968 (1 μM) produced 12 ± 3% and 29 ± 7% inhibition of twitches ($n = 4$ for each antagonist). The evoked contractions were suppressed by atropine (1 μM), tetrodotoxin (1 μM) or procaine (10–30 μM, $n = 4$).

Discussion

Three different inputs have prompted us to re-investigate the role of NK_1 and NK_2 receptors in the AER of the guinea-pig ileum; first, the availability of novel and more potent antagonists for both receptors, especially the nonpeptide antagonists CP 96,345 and SR 48,968 and the peptide antagonists FK 888 and GR 94,800. Second, Suzuki & Gomi (1992) reported that CP 96,345, at a concentration of 1 μM, inhibits or suppresses the atropine-resistant peristalsis of the guinea-pig isolated ileum. Although this concentration of CP 96,345 is near to those producing nonspecific depressant effects on the contractility of the guinea-pig ileum (Lecci *et al.*, 1991; Legat *et al.*, 1992), these observations were interpreted as evidence of a major role for NK_1 receptors (substance P preferring) in mediating intestinal peristalsis. Third, the proposal for the existence of a novel, septide-sensitive receptor in the guinea-pig ileum (Petitet *et al.*, 1992; Maggi *et al.*, 1993b) further complicated the interpretation of results, because the activity of the various antagonists at the putative novel septide-sensitive receptor was unknown.

Both the distension- and the electrically-evoked atropine-resistant circular muscle contraction elicited with the present set up are abolished by hexamethonium, indicating that at least one nicotinic synapse is involved in their genesis. The

simplest arrangement to account for the AER to the circular muscle of the ileum requires the existence of a sensory neurone which detects radial stretch to distension, an interneurone and an effector neurone projecting to circular muscle. Tonini & Costa (1990) provided evidence that acetylcholine, through nicotinic and muscarinic receptors, plays a role at each one of the three transmission sites of this polysynaptic reflex. Tachykinins, via NK_1 and NK_2 receptors, are thought to play a role as final effectors of the atropine-resistant neuromuscular transmission to the circular muscle and, through a different receptor (putatively NK_3), could also play a role in hexamethonium-resistant neuro-neuronal communication (Bartho *et al.*, 1989; 1992; Holzer, 1989; Tonini & Costa, 1990; Holzer *et al.*, 1993). At the present time, there is no evidence to show that NK_1 and/or NK_2 receptors play a role in neuro-neuronal communication in enteric neural pathways, although such a possibility cannot be ruled out.

As a preliminary step to this study, we assessed the affinities of the various antagonists for NK_1 and NK_2 receptors mediating contraction of the circular muscle of the guinea-pig ileum (Maggi *et al.*, 1994b). This gave us information on the potency and selectivity of the various ligands used here to block the effect of endogenous tachykinins.

Interpretation of the present results is critically dependent upon: (a) the selectivity and specificity of tachykinin receptor antagonists in blocking NK_1 and NK_2 and, (b) the assumption that maximal inhibitory effects were produced by individual antagonists used in experiments in which the combined effects of an NK_1 and an NK_2 antagonist were investigated. With regard to the first point, it appears that because of the combination of their potency, selectivity for only one tachykinin receptor and lack of nonspecific effects, FK 888 and GR 94,800 are the most interesting tools for producing a selective blockade of NK_1 and NK_2 receptors, respectively, in the circular muscle of the guinea-pig ileum. The interpretation of results obtained with other antagonists is more difficult either because of nonspecific depressant effects on ileal contractility at certain concentrations (e.g. CP 96,345 and SR 48,968 which depress cholinergic twitches of the longitudinal muscle) or because of a more limited ability to discriminate between NK_1 and NK_2 receptors. Notwithstanding, the results obtained here with several antagonists which belong to different chemical classes are in general agreement with the basic idea that tachykinins are the main transmitters responsible for atropine-resistant excitatory reflex in the ileum. The actions of FK 888 and GR 94,800 were assessed in further detail by studying the effect of their combined administration and by using a different stimulus (electrical stimulation) to evoke the AER: the assumption that 1 μM concentration of these antagonists produced maximally effective inhibitory effects was verified by showing that at 3 μM they did not produce a larger inhibitory effect on the AER.

Tachykinin NK_1 receptors in the AER to the circular muscle of the guinea-pig ileum

The present findings disclose a small but sizeable role of NK_1 receptors in mediating the atropine-resistant AER, especially when a submaximally effective stimulus is used to produce radial stretch of the ileal wall. CP 96,345 and FK 888 both inhibited the submaximal AER at 1 μM. The nonpeptide antagonist, RP 67,580, which is ineffective at NK_1 receptors of the ileum up to 1 μM (higher concentrations produce nonspecific reduction of contractile responses to various agonists) (Maggi *et al.*, 1994b), was ineffective toward the submaximal or maximal AER. At the highest concentrations used in this study, both (±)-CP 96,345 and FK 888 were ineffective toward circular muscle contraction induced by the NK_2 receptor agonist, [βAla^8]neurokinin A(4–10) (Maggi *et al.*, 1994b): this excludes a nonspecific depressant effect on smooth muscle contractility in the observed AER depression.

(±)-CP 96,345 was more effective than FK 888 in inhibiting the distension-evoked AER, since the maximal response to distension was also inhibited by the nonpeptide antagonist. Since CP 96,345 is more potent than FK 888 in blocking NK_1 receptors in the circular muscle of the ileum (Maggi *et al.*, 1994b) this may indicate a larger contribution of NK_1 receptors to the AER than that detected through the inhibitory action of FK 888. On the other hand, it is doubtful if the greater effectiveness of (±)-CP 96,345 can be explained in terms of NK_1 receptor blockade only: (±)-CP 96,345 interacts, at μM concentrations, with both calcium and sodium channels (Schmidt *et al.*, 1992a; Caeser *et al.*, 1993). Since the activation of the AER involves neuro-neuronal communication in the myenteric plexus, we cannot exclude the possibility that 1.0 μM (±)-CP 96,345 produced some nonspecific depressant effect on neuronal excitability, partly responsible for the observed AER depression. A recent study (Tamura *et al.*, 1993) showed that CP 96,345 exerts a nonselective local anaesthetic effect on the excitability of myenteric neurones in the guinea-pig ileum, the lowest effective concentration being 10 μM. The highest concentration of (±)-CP 96,345 tested in this study, 1 μM produces a slight but significant inhibition of cholinergic twitches in the longitudinal muscle of the ileum (cf. Legat *et al.*, 1992).

Since the contribution of NK_1 receptors to the atropine-resistant AER is relatively minor as compared to that exerted by NK_2 receptors, it is not easy to dissect out the relative contribution of the putative 'septide-sensitive' receptor, as opposed to the 'classical' NK_1 receptor to the AER. (±)-CP 96,345 possesses a very high affinity for the 'septide-sensitive' receptor in the ileum (pK_B 9.24), its dissociation constant measured against septide as agonist being about 1000 times lower than the highest concentration (1 μM) tested here toward the atropine-resistant AER. Because both (±)-CP 96,345 and FK 888 are more potent toward septide than toward a 'classical' NK_1 receptor agonist (Maggi *et al.*, 1994b) our conclusion about a sizeable, albeit minor, NK_1 receptor-mediated component in the atropine-resistant AER applies equally well to the 'septide-sensitive' and 'classical' NK_1 receptor.

In the present study we also used a technique, developed by Allescher *et al.* (1992) for studying the electrically-evoked AER in the rat ileum. The evoked response can be assumed to at least partly overlap with that evoked by balloon distension. The inhibitory effects of FK 888 in this model confirm the results obtained with the balloon distension model, and further imply a certain degree of activation of NK_1 receptors in the atropine-resistant AER.

Tachykinin NK_2 receptors in the AER to the circular muscle of the guinea-pig ileum

Our results add further weight to the conclusion (Bartho *et al.*, 1992; Holzer *et al.*, 1993) that NK_2 receptors, putatively activated by endogenous neurokinin A, play a dominant role in mediating the atropine-resistant AER: the selective NK_2 receptor antagonists, GR 94,800 and SR 48,968 inhibited the atropine-resistant AER with relative potency (GR 94,800 > SR 48,968) consistent with their relative potency (pK_B 8.85 and 8.09 for GR 94,800 and SR 48,968, respectively) in antagonizing NK_2 receptor-mediated contraction in the circular muscle of the ileum (Maggi *et al.*, 1994b). AER inhibition produced by SR 48,968 at 1.0 μM is not likely to involve blockade of NK_2 receptors only for two reasons: (i) 1.0 μM SR 48,968 reduces (by 20–30%) the maximal contractile response to NK_1 receptor stimulation in the circular muscle of the ileum (Maggi *et al.*, 1994b); (ii) 1.0 μM SR 48,968 reduces to a similar extent cholinergic twitches of the longitudinal muscle, indicating some nonspecific effect.

The involvement of NK_2 receptors in the atropine-resistant AER is further supported by experiments with MEN 10,573 and MEN 10,376, although the affinity of these ligands for NK_2 receptors in the circular muscle of the ileum is lower than that of GR 94,800 and SR 48,968: the cyclic pseudopeptide, MEN 10,573 is slightly more potent (pK_B 7.18) than

MEN 10,376 (pK_B 6.44) at NK_2 receptors in the circular muscle of the ileum (Maggi *et al.*, 1994b) and is likewise more potent than MEN 10,376 in inhibiting the submaximal and maximal AER evoked by balloon distension. Since MEN 10,376 possesses comparable affinities at NK_2 and 'septide-sensitive' receptor in the circular muscle of the ileum (Maggi *et al.*, 1994b) it is quite conceivable that its inhibitory effect on AER involves the blockade of more than one tachykinin receptor.

Relative contribution of NK_1 and NK_2 receptors to the atropine-resistant AER

The present results indicate that the relative contribution of NK_2 receptors to the distension-evoked AER is greater than that of NK_1 receptors. Of the various antagonists used in this study, FK 888 and GR 94,800 are, because of their potency and selectivity (Maggi *et al.*, 1994b), and lack of nonspecific effects, the most suitable tools to dissect the relative contribution of different tachykinin receptors in the overall physiological response. FK 888 at concentrations (1.0–3.0 μM) which are about 30–300 times higher than its dissociation constant for NK_1 receptors in the circular muscle of the ileum, produced 50% reduction of submaximal atropine-resistant AER without affecting the maximal response to balloon distension. GR 94,800 at a concentration (0.1 μM) which is about 100 times higher than its dissociation constant for NK_2 receptors, produced 50% reduction of the submaximal AER and 23% reduction of the maximal AER induced by balloon distension. Higher (1.0–3.0 μM) concentrations of GR 94,800 produced further depression of maximal AER to balloon distension. These results indicate that the submaximal atropine-resistant AER produced by balloon distension is mediated by both NK_1 and NK_2 receptors while the maximal AER is not significantly blunted by blockade of NK_1 receptors only. This interpretation is supported by the results of experiments in which the combined administration of GR 94,800 and FK 888 or of GR 94,800 and CP 96,345 abolished the submaximal AER induced by balloon distension. In these experiments, the addition of FK 888 in the presence of GR 94,800 produced some inhibition of the distension-induced maximal AER indicating that, after occlusion of NK_2 receptors, a contribution of NK_1 receptors to the maximal AER can be demonstrated. Overall, the results of these experiments indicate an additive effect of the degree of blockade produced by NK_1 and NK_2 receptor antagonists.

This conclusion is further supported by the additive inhibitory effect produced by FK 888 and GR 94,800 on the electrically-evoked atropine-resistant AER. In these experiments, the degree of blockade of FK 888 was somewhat larger than that expected on the basis of its effectiveness toward the distension-induced AER. However, since apamin and L-NOARG were used to improve the reproducibility of the electrically-evoked response, the results of the two experiments are not strictly comparable.

A third excitatory mediator of the atropine-resistant AER?

The atropine-resistant AER evoked by submaximal distension of an intraluminal balloon is practically abolished by combined NK_1 and NK_2 receptor blockade. On the other hand, a consistent fraction of the maximal response produced by balloon distension is still evident in the presence of NK_1 and NK_2 receptor antagonists at concentrations which are at least 100 times higher than their dissociation constant from the respective receptors. Likewise, about 30–40% of the overall atropine-resistant AER evoked by electrical stimulation was resistant to the combined administration of FK 888 and GR 94,800. It appears unlikely that the residual component of the atropine-resistant AER, still observed in the presence of both FK 888 and GR 94,800 may involve NK_3 receptors, since the contractile response to NK_3 receptor agonists in the circular muscle of the ileum is totally, indirect, being mediated through the release of both acetylcholine and endogenous tachykinins (Maggi *et al.*, 1990; 1994a). The present results raise the possibility that, in addition to acetylcholine and tachykinins, a third excitatory transmitter to the circular muscle of the ileum exists. This hypothesis is indirectly supported by electrophysiological evidence that two distinct types of atropine-resistant excitatory junction potential can be evoked in circular muscle cells of the guinea-pig ileum, and only one of them is sensitive to tachykinin receptor antagonists (Bywater & Taylor, 1986; Crist *et al.*, 1991).

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