British Journal of Pharmacology (1996) 118, 1262-1268

Evidence that tachykinin NK_2 receptors modulate resting tone in the rat isolated small intestine

[1]Carlo Alberto Maggi & Sandro Giuliani

Pharmacology Department, Menarini Ricerche, Florence, Italy

1 In the progress of experiments aimed at evaluating the role of tachykinins as enteric nonadrenergic noncholinergic (NANC) transmitters, we noted that certain tachykinin receptor antagonists produce a relaxation of circular muscle strips in the rat small intestine. This study aimed to assess the nature of this response and to determine the receptor type involved. The majority of the experiments were performed in capsaicin- (10 μM for 15 min) pretreated mucosa-free circular muscle strips from the rat small intestine, in the presence of atropine (1 μM), guanethidine (3 μM) and indomethacin (10 μM).

2 Under isometric recording of mechanical activity, the tachykinin NK_1 receptor antagonist SR 140,333 (0.1 μM) had no effect on resting tone or spontaneous activity in duodenal or ileal circular muscle strips. The NK_2 receptor antagonists, MEN 10,627 (0.1 μM) and GR 94,800 (0.1 μM) produced, after a delay of 10-15 min, a relaxation which averaged 61 ± 3 and $57\pm6\%$ ($n=6$ and 4, respectively) of the maximal response (E_{max}) to isoprenaline (1 μM). The effect of maximal concentrations of MEN 10,627 and GR 94,800 when applied together was non-additive. The relaxant effect of MEN 10,627 (0.1 μM) was similar in the absence and presence of apamin (0.3 μM) and L-nitroarginine (100 μM).

3 Under isotonic recording of mechanical activity, MEN 10,627 (10 nM-1 μM) produced a concentration- and time-related relaxation of duodenal strips. The maximal relaxation averaged 72 ± 4 and $69\pm4\%$ ($n=5$ each) of E_{max} to isoprenaline (1 μM) and was achieved 15-20 or 20-30 min after application of 1.0 or 0.1 μM MEN 10,627, respectively.

4 Duodenal strips were relaxed by other NK_2 receptor selective antagonists (values in parentheses are % of E_{max} to isoprenaline at the given concentration of antagonist): GR 94,800 ($69\pm3\%$ at 1 μM, $n=4$), SR 48,968 ($60\pm3\%$ at 1 μM, $n=4$) and MDL 29,913 ($66\pm4\%$ at 1 μM, $n=4$). SR 48,965 (1 μM), the inactive enantiomer of SR 48,968, was without effect. The NK_1 receptor selective antagonists, SR 140,333 (0.1 μM), FK 888 (10 μM) RP 67,580 (1 μM) and GR 82,334 (10 μM) were also without effect ($n=4-5$).

5 A cocktail of peptidase inhibitors, thiorphan, bestatin and captopril (1 μM each) had no significant effect on tone or spontaneous activity of duodenal strips. In the presence of peptidase inhibitors, MEN 10,627 (1 μM) produced a relaxation of duodenal strips ($72\pm6\%$ of E_{max} to isoprenaline, $n=5$), whilst GR 82,334 (10 μM, $n=6$) had no significant effect.

6 The relaxant response to MEN 10,627 was preserved in mucosa-free strips not pre-exposed to capsaicin. Tetrodotoxin (1 μM), saxitoxin (1 μM), hexamethonium (100 μM) and ω-conotoxin (0.1 μM) had no significant effect on the resting tone of duodenal strips nor did they affect the relaxation to MEN 10,627. L-Nitroarginine (100 μM) increased the tone of the strips but did not affect the response to MEN 10,627. Nifedipine (1 μM) relaxed the strips by $62\pm4\%$ ($n=4$), but in its presence a small relaxant effect to MEN 10,627 ($26\pm5\%$, $n=4$) was still evident.

7 Under isotonic recording of mechanical activity along the longitudinal axis, MEN 10,627 (1 μM) produced a slowly developing relaxation ($39\pm3\%$ of E_{max} to isoprenaline; $n=6$) of whole segments of rat duodenum. When similar experiments were performed on whole segments of rat proximal colon MEN 10,627 had no effect.

8 The present findings document the observation that tachykinin NK_2 receptors contribute to the maintenance of resting tone of the rat isolated small intestine. We found no evidence to suggest that this effect follows the blockade of the contractile effect of spontaneously released endogenous tachykinins. The present findings raise the possibility that constitutively active NK_2 receptors account for the relaxant effect produced by NK_2 receptor antagonists in rat small intestine.

Keywords: Tachykinins; NK_2 receptors; small intestine; circular muscle; spontaneous activity; tachykinin receptor antagonists; substance P; neurokinin A

Introduction

Substance P (SP) and neurokinin A (NKA), two members of the tachykinin family of peptides, fulfil the criteria required for them to be considered as excitatory transmitters to the circular muscle in the mammalian gut. Contraction of the circular muscle induced by SP and NKA is mediated by NK_1 (SP-preferring) and NK_2 (NKA-preferring) receptors (see Barthò & Holzer, 1985; Holzer-Petsche, 1995 for reviews).

A number of potent and selective tachykinin receptor antagonists of both peptide and nonpeptide nature have been developed in recent years (see Maggi *et al.*, 1993 for review): these tools enable precise assessment of the role of endogenous tachykinins as enteric neurotransmitters and of different tachykinin receptors in these events (e.g. Barthò *et al.*, 1992; Zagorodnyuk *et al.*, 1993; 1995; Maggi *et al.*, 1992; 1994b,c,

[1]Author for correspondence at: Pharmacology Department, Menarini Ricerche, Via Sette Santi 3, 50131, Florence, Italy.

d,e,f; Holzer *et al.*, 1993; Holzer & Maggi, 1994). These studies have provided good evidence for the concept that both tachykinins and acetylcholine are the major excitatory transmitters to the circular muscle in the mammalian gut, and have highlighted the existence of a remarkable regional specialization in terms of the role played by different tachykinin receptors.

Recently, we started investigating the effect of various high affinity tachykinin receptor antagonists on nonadrenergic noncholinergic (NANC) neurotransmission in the circular muscle of the rat small intestine. During this study (Maggi & Giuliani, 1995), we noted that certain tachykinin receptor antagonists reduce the resting tone of circular muscle strips from the rat isolated duodenum or ileum. The aim of the present study was to investigate in detail this phenomenon in an attempt to assess whether the relaxation involves a specific blockade of tachykinin receptors and, if so, which receptors are involved. After having established that occupancy of NK_2 receptors by the antagonists is involved in the phenomenon under study, we were interested in determining whether a background release of endogenous tachykinins from neuronal or non-neuronal sources (e.g enterochromaffin cells in the mucosa, Simon *et al.*, 1992) could be involved. With this aim several pharmacological pretreatments, including administration of channel blockers and peptidase inhibitors, were performed to study their possible influence on the relaxation produced by NK_2 receptor occupancy in mucosa-free circular muscle strips from rat duodenum. To understand better the significance of the relaxation produced by NK_2 receptor antagonists the phenomenon was investigated under both isometric and isotonic recording conditions and in both circular muscle strips and whole segments of rat duodenum.

Methods

General

All experiments were performed on intestinal segments obtained from male albino rats of the Wistar strain (340–360 g) killed by stunning and bleeding. A 2 cm segment of small intestine was excised from the proximal duodenum (at about 1 cm from the pyloric sphincter), terminal ileum (at about 5 cm from the ileo-caecal valve) or proximal colon (at about 1 cm from the caecum) and placed in oxygenated Krebs solution (95% O_2 and 5% CO_2, pH 7.4 at 37°C) of the following composition (mmol l^{-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 majority of the experiments was performed in mucosa-free circular muscle strips from rat duodenum and ileum under isometric (protocol A, described below) or isotonic (protocol B) tension recording. A few experiments were performed on whole segments of rat duodenum or proximal colon (protocol C). For experiments with circular muscle strips, indomethacin (10 μM) was added to the Krebs solution, to increase the spontaneous activity of the preparations (see Maggi *et al.*, 1994c). All experiments commenced after an equilibration period of 90 min during which time the bath solution was renewed every 15 min. Isoprenaline (1 μM), a relaxant, was used in all experiments as an internal standard.

Mucosa-free circular muscle strips from rat duodenum or ileum were prepared as follows: the intestinal segment was opened along the mesenteric border and pinned flat in a Petri dish; the mucosa was removed by gentle scraping. A mucosa free strip (about 2 mm wide) was then excised along the circular axis and mounted in a 5 ml organ bath.

Experimental protocols

Unless stated otherwise, only one concentration of one antagonist was investigated in each preparation. For experiments performed in muscle strips, as described under Protocols A and B (see below) multiple strips (up to 4) were usually prepared from each duodenal segment: one strip served as control for time-related changes in tone/spontaneous activity or for studying the effect of the vehicle and the others received one concentration of the stated tachykinin receptor antagonist.

Protocol A These experiments were performed in mucosa-free circular muscle strips from rat duodenum or ileum. Mechanical activity was recorded with an isometric force transducer connected to a Basile 7050A polygraph. A load of 10 mN was initially applied to the strips which were then allowed to relax; after 30 min equilibration with intervening washout periods, the resting tension was adjusted to 5 mN. All experiments were performed in preparations pre-exposed to 10 μM capsaicin for 15 min, in order to prevent the release of transmitters from peripheral endings of sensory nerves (Maggi & Meli, 1988 for review). In all experiments, the Krebs solution contained atropine (1 μM), guanethidine (3 μM) and indomethacin (10 μM). Under these conditions, we studied the effect of tachykinin NK_1 receptor antagonist SR 140,333 (0.1 μM) (Emonds-Alt *et al.*, 1993) and of the tachykinin NK_2 receptor antagonist MEN 10,627 (Maggi *et al.*, 1994a) and GR 94,800 (McElroy *et al.*, 1992) (0.1 μM each) for 30 min. The effect of MEN 10,627 was also investigated in the presence of N^{ω}-nitro-L-arginine (L-NOARG; 100 μM) and apamin (0.3 μM), which were applied to the bath 30 min before the administration of MEN 10627. The concentrations of antagonists were selected on the basis of their known affinities and selectivities for tachykinin receptors; for SR 140,333 and MEN 10,627, previous experiments have shown that a concentration of 0.1 μM of these antagonists effectively and selectively occludes NK_1 and NK_2 receptors, respectively, in circular muscle strips of rat duodenum (Maggi & Giuliani, 1995).

Protocol B These experiments were performed in mucosa-free circular muscle strips from rat duodenum; the mechanical activity was recorded by isotonic force transducers under a load of 2 mN.

One series of experiments was performed in capsaicin-pretreated strips and in the presence of atropine (1 μM), guanethidine (3 μM) and indomethacin (10 μM) to study the effect of various concentrations of NK_1 and NK_2 receptor antagonists on mechanical activity. The NK_1 receptor antagonists tested were SR 140,333 (1 μM), RP 67,580 (1 μM) (Garret *et al.*, 1991), FK 888 (10 μM) (Fujii *et al.*, 1992) and GR 82,334 (10 μM) (Hagan *et al.*, 1991); the NK_2 receptor antagonists tested were MEN 10,627 (10 nM–1 μM), GR 94,800 (0.1–1 μM), MDL 29,913 (1 μM) (Van Giersbergen *et al.*, 1991), the non-peptide antagonist SR 48,968 (0.1–1 μM) and its inactive enantiomer, SR 48,965 (1 μM) (Emonds-Alt *et al.*, 1992).

In a second series of experiments, the effect of various pretreatments on the relaxant response to 1.0 μM MEN 10,627 was determined in strips not pre-exposed to capsaicin but exposed to a cocktail of peptidase inhibitors (thiorphan, bestatin and captopril, 1 μM each), 100 μM hexamethonium, 1 μM tetrodotoxin, 1 μM saxitoxin, 1 μM nifedipine, 100 μM L-NOARG or 0.1 μM ω-conotoxin fraction GVIA (CTX). All inhibitors or channel blockers were applied for 30 min before administration of MEN 10,627.

Protocol C These experiments were performed on whole segments of rat duodenum and proximal colon (1 cm long). The segments were suspended under a resting load of 5 mN for isotonic recording of mechanical activity in Krebs solution containing atropine (1 μM) and guanethidine (3 μM). After 60 min equilibration period, the effect of MEN 10,627 (1 μM) was recorded on resting tone and spontaneous activity and compared with the effect of 1 μM isoprenaline.

Data evaluation and statistical analysis

All data in text and figures are mean ± s.e.mean. In the text and figures all relaxant responses are expressed as % of the maximal relaxant response (E_{max}) induced by 1 μM isoprenaline.

Statistical analysis was performed by means by Student's t test or by means of analysis of variance, when applicable. A P level <0.05 was considered statistically significant.

Drugs

Drugs used were: capsaicin, N^{ω}-nitro-L-arginine (L-NOARG), apamin, isoprenaline, nifedipine, indomethacin, guanethidine and captopril (Sigma, St Louis, MO, U.S.A.), thiorphan (Bachem, Bubendorf, Switzerland), tetrodotoxin (Sankyo, Tokyo, Japan), saxitoxin (Calbiochem, San Diego, CA, U.S.A.), atropine HCl (Serva, Heidelberg, Germany), GR 82,334 ([D-Pro^9,(spiro-γ-lactam)Leu^{10},Trp^{11}] physalaemin(1–11); Neosystem, Strasbourg, France), bestatin and ω-conotoxin fraction GVIA (Peninsula, Belmont, CA, U.S.A.). MDL 29,913 (cyclo[Leu-Ψ(CH_2NCH_3)Leu-Gln-Trp-Phe-Gly]) was provided by Dr S.H. Buck, Marion Merrell Dow, Cincinnati, OH, U.S.A. RP 67,580 ((3αR,7αR)-7,7-diphenyl-2-[1-imino-2-(2-methoxyphenyl)ethyl]perhydroisoindol-4-one) was a kind gift of Dr C. Garret, Rhone Poulenc Rorer, Vitry, France. SR 48,968 ((S)-N-methyl-N[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl]benzamide), its inactive enantiomer, SR 48,965 and SR 140,333 ((S)1-{2-[3-(3,4-dichloropheynl)-1-3(3-isopropoxyphenylacetyl)piperidin-n-3-yl]ethyl}4-phenyl-1-azoniabicyclo[2,2,2]octane chloride) were a kind gift of Dr X. Emonds-Alt, Sanofi Research, Montpellier, France. MEN 10,627 (cyclo(Met-Asp-Trp-Phe-Dap-Leu)cyclo(2β-5β)), GR 94,800 (PhCO-Ala-Ala-DTrp-Phe-DPro-Pro-NleNH_2) and FK 888 ((2-(N-Me)indolil)-CO-Hyp-Nal-NMeBzl) were synthesized at the Chemistry Department of Menarini Ricerche, Florence, Italy.

Nifedipine (1 mM) and capsaicin (10 μM) were dissolved in ethanol and diluted in Krebs solution: the final concentration of the vehicle in the bath (0.1%) had no effect on tone or

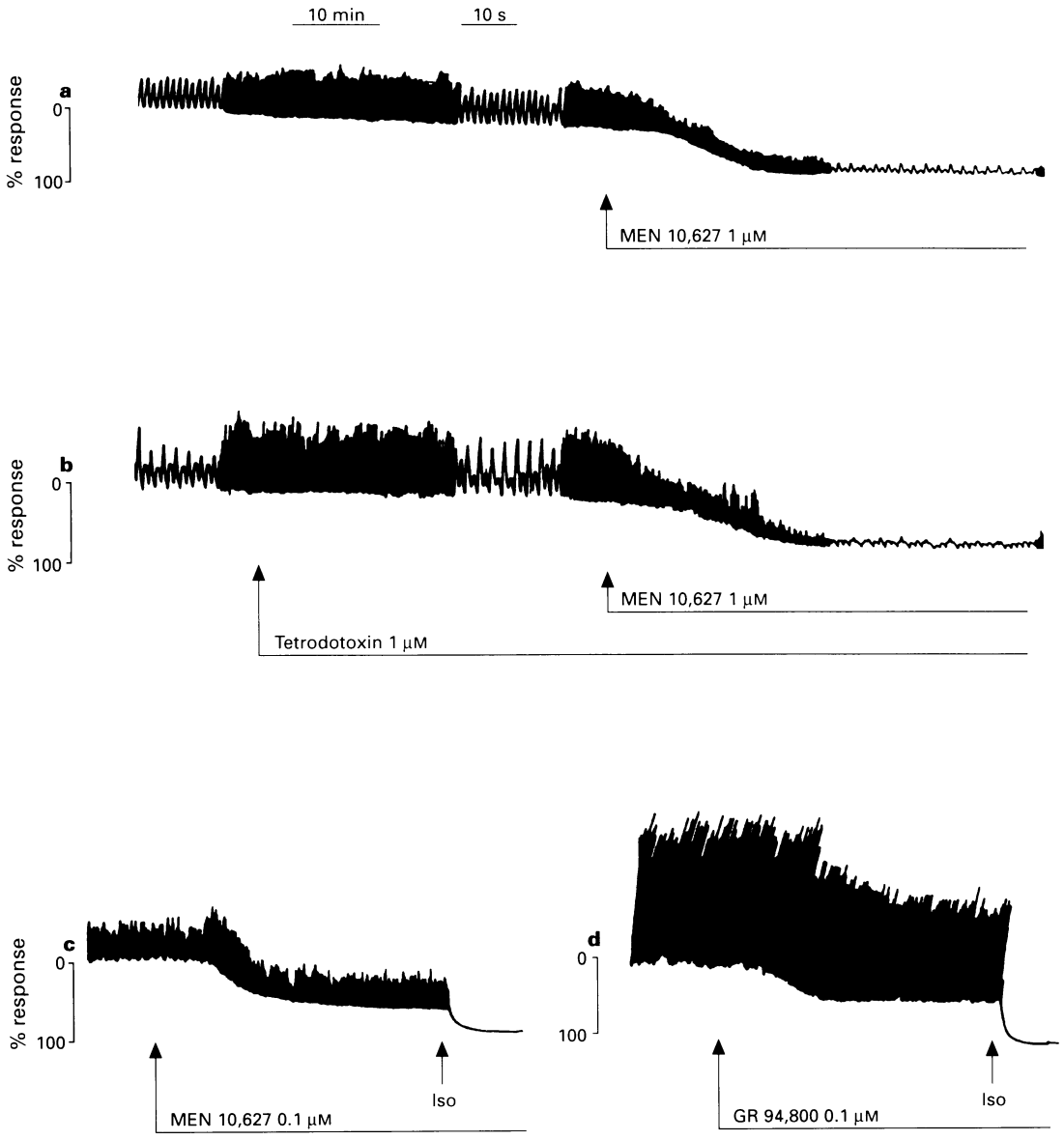

Figure 1 Relaxant activity of tachykinin NK_2 receptor antagonists on mucosa-free circular muscle strips from rat isolated duodenum. Mechanical activity was recorded isotonically in the presence of atropine (1 μM), guanethidine (3 μM) and indomethacin (10 μM) in capsaicin-pretreated preparations. In (a–d), vertical bars represent the % relaxant response to 1 μM isoprenaline (Iso). (a and b) Relaxant effect of MEN 10,627 1 μM in the absence (a) and presence (b) of 1 μM tetrodotoxin. Note that in these strips MEN 10,627 in addition to producing relaxation, also reduced the amplitude of spontaneous contractions without affecting their frequency. Tetrodotoxin had no effect on resting tone or the relaxation to MEN 10,627. (c and d) Relaxant effect of MEN 10,627 (0.1 μM) and GR 94,800 (0.1 μM); note that relaxation was not accompanied by any change in the amplitude of spontaneous contractions. Similar results were obtained in at least 4 other preparations.

spontaneous activity of the strips. MEN 10,627 (1 mM) and MDL 29,913 (1 mM) were dissolved in DMSO and diluted in Krebs solution: the final concentration of the vehicle in the bath (0.1%) had no effect on tone or spontaneous activity of the strips. Indomethacin (0.1 M) was dissolved in DMSO and diluted in Krebs solution to give a final concentration of 1 μM. All other chemicals were dissolved in bidistilled water or saline and diluted in Krebs solution.

Result

Isometric recording of mechanical activity

In the presence of atropine (1 μM), guanethidine (3 μM) and indomethacin (10 μM), and after *in vitro* capsaicin pretreatment, mucosa-free circular muscle strips excised from the rat proximal duodenum and ileum displayed an irregular spontaneous phasic mechanical activity at a frequency of 32±1.2 and 23±1.5 cycles min^{-1} ($P<0.05$) and with an amplitude of 4.61±0.8 and 5.64±0.9 mN ($n=15$ and 16), respectively. Isoprenaline (1 μM) promptly relaxed duodenal and ileal strips by 1.97±0.18 and 1.21±0.06 mN, respectively ($P<0.05$, $n=10-20$).

The NK_1 receptor antagonist, SR 140,333 (0.1 μM for 60 min) did not significantly affect resting tone or spontaneous activity of duodenal or ileal strips (both $n=6$). The NK_2 receptor antagonist, MEN 10,627 (0.1 μM) consistently relaxed all duodenal strips tested (61±3% of E_{max} to isoprenaline, $n=6$); the effect of MEN 10,627 ensued after a delay of about 10–15 min and developed fully about 30 min following application. A 10 fold higher concentration (1.0 μM) of MEN 10,627 produced a similar relaxant effect (62±3%, $n=4$) but the effect ensued within 5 min from bath application.

A relaxant effect of MEN 10,627 (0.1 μM) was also evident in 5 out of 7 ileal strips tested, approaching only about 25% of the E_{max} to isoprenaline. For this reason, further experiments were performed in duodenal strips only.

To assess whether activation of NANC relaxant mechanisms could be involved in the relaxant action of MEN 10,627, its effect was investigated in the presence of L-NOARG (100 μM) and apamin (0.3 μM). MEN 10,627 (0.1 μM) relaxed duodenal strips by 63±3% ($n=4$). Application of L-NOARG and apamin did not increase the tone of strips but increased the amplitude and regularity of spontaneous contractions.

In a further series of experiments, we studied the effect of another high affinity and selective NK_2 receptor antagonist, GR 94,800 (McElroy *et al.*, 1992). These experiments were performed on paired duodenal strips from the same animal, one of which received MEN 10,627 (0.1 μM) followed, 30 min later, by GR 94,800 (0.1 μM); in the other strip the antagonists were added in the reverse order. When applied first, GR 94,800 produced a delayed relaxation of the strips, similar to that observed with MEN 10,627: the effect of GR 94,800 averaged 57±6% ($n=4$) and the subsequent addition of MEN 10,627 had no further relaxant effect. When added first, MEN 10,627 relaxed the strips by 56±2% ($n=4$) and the subsequent addition of GR 94,800 had no further effect on tone.

Isotonic recording of mechanical activity

Effect of tachykinin receptor antagonists In the presence of atropine (1 μM), guanethidine (3 μM) and indomethacin (10 μM) and after *in vitro* capsaicin pretreatment, application of MEN 10,627 (1 nM–1 μM) produced a concentration- and time-related relaxation of duodenal strips (Figures 1 and 2). The amplitude of spontaneous contractions was either unaffected (Figure 1c) or even increased during the relaxation produced by MEN 10,627 while in other strips the amplitude was reduced (Figure 1a) but never totally suppressed.

The relaxant effect of MEN 10,627 was reproduced by other NK_2 receptor selective antagonists (Figures 1 and 2): in particular, the linear peptide antagonist, GR 94,800 (0.1–1 μM), the cyclic pseudopeptide antagonist MDL 29,913 (1 μM) and the nonpeptide antagonist, SR 48,968 (0.1–1 μM) were all effective in producing a slowly developing relaxation of duodenal strips (Figure 2). The maximal effect produced by these antagonists was 69±3% for GR 94,800 at 1 μM ($n=4$), 66±4% for MDL 29,913 at 1 μM ($n=4$) and 60±3% for SR 48,968 at 1 μM ($n=4$) (Figure 2). The inactive enantiomer, SR 48,965 (1 μM) was without effect (Figure 2c).

In sharp contrast, the NK_1 receptor selective antagonists, SR 140,333 (1 μM, $n=5$, Figure 2), RP 67,580 (1 μM, $n=4$, not shown), FK 888 (10 μM, $n=4$, not shown) and GR 82,334 (10 μM, $n=4$, Figure 2) failed to induce a significant relaxation over a 30 min observation period.

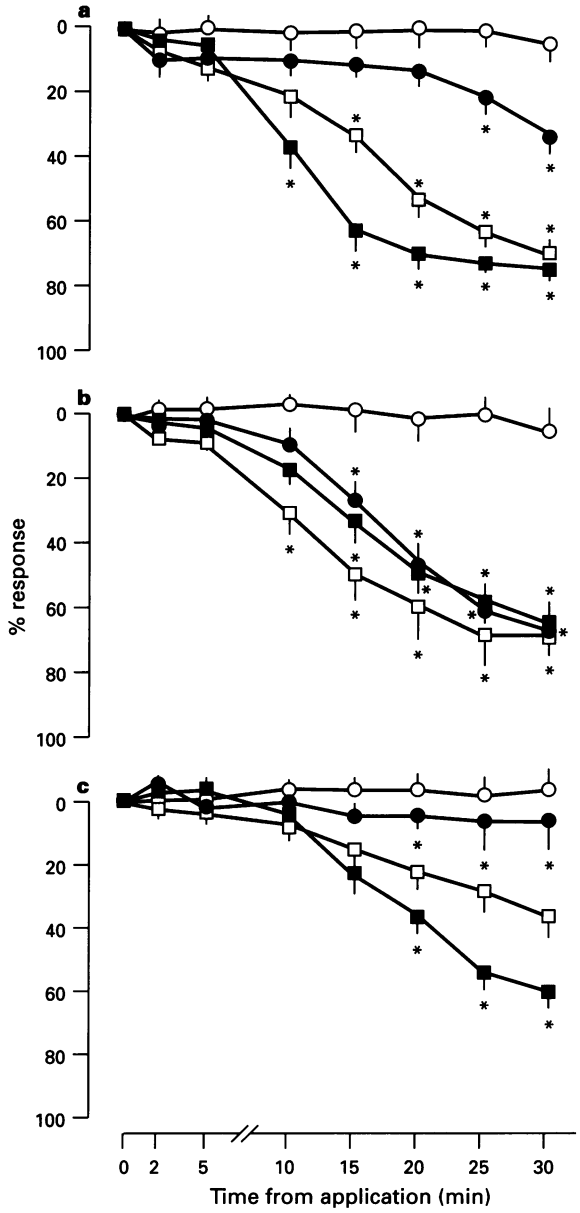

Figure 2 Effect of various tachykinin NK_1 and NK_2 receptor antagonists on resting tone of circular muscle strips from rat duodenum. Mechanical activity was recorded isotonically in the presence of atropine (1 μM), guanethidine (3 μM) and indomethacin (10 μM) in capsaicin-pretreated preparations. Vertical axes represent the % relaxant response to 1 μM isoprenaline. (a) Vehicle (1% DMSO, ○, $n=5$), MEN 10627, 0.01 μM (●, $n=5$), MEN 10627, 0.1 μM (□, $n=5$) and MEN 10627, 1 μM (■, $n=5$). (b) GR 82334, 10 μM (○, $n=4$), GR 94800, 0.1 μM (●, $n=4$), MDL 29913, 1 μM (■, $n=4$) and GR 94800 1 μM (□, $n=4$). (c) SR 140333, 1 μM (○, $n=5$), SR 48965, 1 μM (●, $n=4$), SR 48968 0.1 μM (□, $n=4$) and SR 48968 1 μM (■, $n=4$). Each value is mean±s.e.mean. *Significantly different from control, $P<0.05$.

Factors influencing the relaxant response to NK_2 receptor blockade Application of a cocktail of peptidase inhibitors, thiorphan, bestatin and captopril (10 μM each, contact time 60 min, $n=11$) had no consistent effect on tone or spontaneous activity of duodenal strips. In the presence of peptidase inhibitors, MEN 10,627 (1 μM) produced a relaxation of duodenal strips, averaging $72\pm6\%$ ($n=5$) of the E_{max} to isoprenaline, while GR 82,334 (10 μM) had a small, not statistically significant, relaxant effect ($20\pm6\%$, NS, $n=6$).

Pretreatment with tetrodotoxin (1 μM, $n=8$), saxitoxin (1 μM, $n=4$), ω-conotoxin (0.1 μM, $n=8$) or hexamethonium (100 μM, $n=6$) did not significantly affect the tone or spontaneous activity of the strips, while L-NOARG (100 μM, $n=7$) produced a prompt increase in tone and frequency of contractions which reached a steady state in about 20 min. In the presence of L-NOARG, tetrodotoxin, saxitoxin, ω-conotoxin or hexamethonium, MEN 10627 (1 μM) produced a relaxation similar, in both amplitude (Figure 3) and time course, to that observed in controls.

Nifedipine (1 μM) suppressed the spontaneous activity of the strips and produced a relaxation averaging $62\pm4\%$ of the E_{max} to isoprenaline ($n=4$). In the presence of nifedipine, bath application of MEN 10,627 produced a further relaxation of the strips, averaging $26\pm5\%$ ($n=4$, $P<0.05$ vs. controls).

One possibility is that the relaxant effect of MEN 10,627 and other NK_2 receptor antagonists results from the previous application of capsaicin, yielding a massive release of endogenous tachykinins, which remain to occupy NK_2 receptors despite the washouts applied during the equilibration period. To test this hypothesis, the effect of MEN 10,627 (1 μM) was also studied in the presence of atropine, guanethidine and indomethacin in strips not subjected to capsaicin pretreatment: MEN 10,627 (1 μM) produced a relaxation ($59\pm7\%$, $n=5$) not significantly different ($P>0.05$) from that produced in capsaicin-pretreated duodenal strips.

Effect of MEN 10,627 on whole segments of rat duodenum and proximal colon

Whole segments of rat duodenum or proximal colon displayed a resting tone and spontaneous activity (Figure 4): bath application of 1 μM isoprenaline produced a relaxation in both cases ($n=4-6$; Figure 4). MEN 10,627 (1 μM) produced a slowly developing relaxation of whole segments of rat duodenum (Figure 4), similar to the effect observed in mucosa-free circular muscle strips. The maximal relaxant effect of MEN 10,627 averaged $39\pm3\%$ and reached a steady state in 23 ± 2 min, without inhibiting the amplitude of spontaneous contractions ($n=6$). In sharp contrast, no relaxant effect was produced in whole segments of proximal colon ($n=4$; Figure 4).

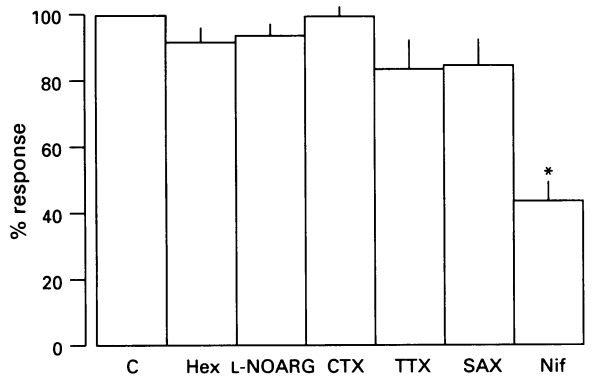

Figure 3 Effect of hexamethonium (Hex, 100 μM), L-nitroarginine (L-NOARG, 100 μM), ω-conotoxin (CTX, 0.1 μM), tetrodotoxin (TTX, 1 μM), saxitoxin (SAX, 1 μM) and nifedipine (Nif, 1 μM) on the amplitude of relaxation induced by 1 μM MEN 10,627 in mucosa-free circular muscle strips from rat duodenum. Mechanical activity was recorded isotonically in the presence of atropine (1 μM), guanethidine (3 μM) and indomethacin (10 μM) in capsaicin-pretreated preparations. The relaxant effect produced by MEN 10,627 in the presence of various drugs is normalized to % of the relaxation induced in control strips (C). Each value is mean$\pm$s.e.mean of 4–8 experiments. *Significantly different from the effect of MEN 10,627 in controls, $P<0.05$.

Discussion

Tachykinin NK_2 receptors contribute to maintenance of resting tone in rat small intestine

The present results indicate that tachykinin NK_2 receptor antagonists produce relaxation of the circular muscle of rat isolated small intestine, especially the rat duodenum. This effect is shared by several, chemically-unrelated, peptide-based NK_2 receptor antagonists (MEN 10, 627, MDL 29,913 and GR 94,800) and by the nonpeptide NK_2 receptor antagonist, SR 48,968, but not by its inactive enantiomer, SR 48,965. All these antagonists possess nanomolar affinity for NK_2 receptors expressed in the rat gastrointestinal tract (Mussap & Burcher, 1993; Maggi *et al.*, 1994a). The need to use micromolar concentrations of these antagonists to produce the maximal effect within a reasonable experimental time is not in itself an argument against the involvement of NK_2 receptors, since the relaxant effect of all the NK_2 receptor antagonists tested is clearly time-dependent. The reason(s) underlying the pronounced time-dependency of the action of the NK_2 receptor antagonists is unclear when considering that, for many of these ligands shorter incubation times are sufficient to reach equilibrium in competition experiments with exogenous tachykinins (e.g. Maggi *et al.*, 1994a for MEN 10,627). This point will be discussed further below.

An important observation is that the maximal relaxation produced by NK_2 receptor antagonists did not exceed 60–70% of the E_{max} to isoprenaline: this same maximal effect was produced by MEN 10,627, GR 94,800 and MDL 29,913 at

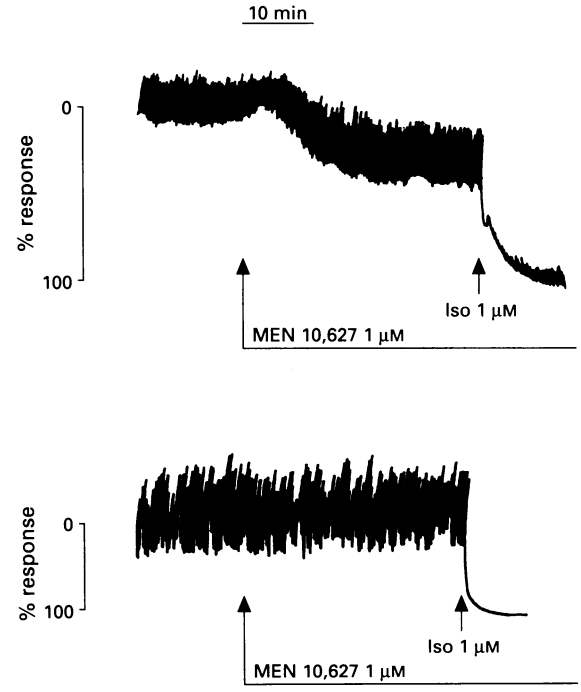

Figure 4 Tracings showing the relaxant effect of MEN 10627 (1 μM) on a whole segment of rat duodenum (a) and lack of effect on resting tone on a whole segment of rat proximal colon (b). Vertical scales represent the % relaxant response to 1 μM isoprenaline (Iso). The figure is representative of 4 similar experiments.

concentrations of 0.1, 0.1 and 1.0 μM, respectively; moreover, the relaxation produced by maximally effective concentrations of MEN 10,627 and GR 94,800, albeit lower than the E_{max} to isoprenaline, was non-additive, indicating a common mechanism of action.

In marked contrast to the effect of NK_2 receptor antagonists, no significant relaxation was observed after application of micromolar concentrations of several peptide and nonpeptide NK_1 receptor antagonists (GR 82,334, SR 140,333, FK 888, RP 67,580); this is despite the nanomolar or subnanomolar affinities that these compounds, especially RP67,580 (Garret *et al.*, 1991) and SR 140,333 (Edmonds-Alt *et al.*, 1993), possess for rat NK_1 receptors. Moreover, SR 140,333 (0.1 μM) produced a partial blockade of the NANC contractile response to electrical field stimulation in the circular muscle of rat duodenum and selectively inhibited the contractile response to the NK_1 receptor selective agonist, [Sar^9] substance P sulphone (Maggi & Giuliani, 1995).

In view of the above observations, we conclude that the relaxation produced by NK_2 receptor antagonists is a specific effect linked to occupancy of NK_2 receptors. For experiments aimed to assess the mechanism underlying this effect, we used 1 μM MEN 10,627 as an effective concentration to produce a maximal relaxant effect in a suitable experimental time. The choice was based on: (a) the affinity and selectivity of this ligand for rat NK_2 receptors (Maggie *et al.*, 1994a); (b) the knowledge that, up to 1 μM, MEN 10,627 is inactive as an antagonist at rat NK_1 or NK_3 receptors; (c) MEN 10,627 does not exhibit any significant binding affinity for other transmitter or peptide receptors or for sodium, calcium and potassium ion channels (Maggi *et al.*, 1994a) and, (d) no relaxant or inhibitory effect has been observed following application of 1 μM MEN 10,627 in various isolated smooth muscle preparations, including the rat isolated portal vein, the rabbit pulmonary artery, the hamster trachea, the guinea-pig renal pelvis and the longitudinal muscle of guinea-pig ileum (Maggi *et al.*, 1994a). Moreover, as shown here, MEN 10,627 is devoid of any relaxant effect on the longitudinal muscle of the rat isolated proximal colon, while producing a clear relaxation when applied to the rat duodenum. Altogether, these observations provide a firm basis for the exclusion of nonspecific effects (unrelated to NK_2 receptor occupancy) in the relaxant action of MEN 10,627.

Nature of the relaxation produced by NK_2 receptor occupancy in the rat small intestine

The present data enabled us to exclude a number of factors as potential contributory mechanisms involved in the relaxation produced by occupancy of tachykinin NK_2 receptors. In particular we can exclude the involvement of: (a) tachykinin release from primary afferent nerves (experiments in capsaicin-pretreated strips); (b) tachykinin release from neuroendocrine cells in the mucosa (cf. Simon *et al.*, 1992) (experiments in mucosa-free strips); (c) tachykinin release produced by mechanical trauma of dissection (experiments on whole duodenal segments); (d) induced release of inhibitory mediators (experiments with guanethidine, L-NOARG and apamin and experiments with tetrodotoxin).

In previous studies we established that the contraction produced by stimulating NK_2 receptors differentially utilizes nifedipine-sensitive calcium channels in different regions of the intestine (Maggi *et al.*, 1994e; Zagorodnyuk *et al.*, 1995; Maggi & Giuliani, 1995). The present findings showed that the relaxant effect of MEN 10,627 was still evident in the presence of 1 μM nifedipine, indicating that a nifedipine-resistant mechanism of contraction activated by NK_2 receptors in rat duodenum (cf. Maggi & Giuliani, 1995) is involved in the maintenance of resting tone of this preparation.

Since tachykinins (SP and NKA) act as NANC excitatory transmitters in the circular muscle of rat duodenum by activating NK_1 and NK_2 receptors (Burcher *et al.*, 1986; Ekblad *et al.*, 1987; Sternini *et al.*, 1989; Maggi & Giuliani, 1995), the relaxant response produced by NK_2 receptor antagonists may imply the existence of a background release of tachykinins from intramural nerves, to provide a basal peptidergic input which regulates resting tone of the small intestine. However, a number of observations do not support this hypothesis. Firstly, the relaxant effect of MEN 10,627 was not significantly modified by blockers of neuronal conduction, tetrodotoxin and saxitoxin, or by the blocker of neuronal N-type Ca channels, ω-conotoxin. These results contradict a basal release of tachykinins from enteric neurones as a contributory mechanism to the maintenance of resting tone in the circular muscle of rat duodenum, unless the release process is such that it does not require propagated action potential and does not involve Ca influx via N-type Ca channels. In particular, the possibility of a 'leak' release of tachykinins from nerve fibres/neurones cannot be totally excluded on the basis of these findings.

On the other hand a 'neuronal' explanation remains unlikely when considering that both NK_1 and NK_2 receptor antagonists are needed to block the NANC contraction to electrical field stimulation in the circular muscle of rat duodenum (Maggi & Giuliani, 1995) whilst, contrary to NK_2 receptor antagonists, NK_1 receptor antagonists do not affect the resting tone of the preparation. Thus, the failure of NK_1 receptor antagonists to affect resting tone argues against the existence of a basal or 'leak' release of tachykinins from a neuronal source. It may be argued that the ligand(s) for the NK_1 receptor (putatively substance P) is degraded by peptidases at a faster rate than the ligand for the NK_2 receptor (putatively NKA); this, for the relatively small amounts of endogenous tachykinins 'leaking' from nerve terminals, could explain the lack of activation of NK_1 receptors. If this hypothesis were correct, then the addition of peptidase inhibitors should increase the resting tone of the strips and unmask a relaxant effect of NK_1 receptor antagonists. However, a cocktail of peptidase inhibitors, at concentrations which enhance the NANC contraction of circular muscle of rat duodenum to nerve stimulation (data not shown), did not affect the resting tone of the strips or reveal a relaxant effect of the NK_1 receptor antagonist, GR 82,334.

Overall, the present results indicate that occupancy of tachykinin NK_2 receptors by receptor selective antagonists produces a specific relaxant effect in the rat small intestine which is especially evident in the circular muscle of the rat duodenum. However, no evidence has been obtained to support the idea that a basal release of tachykinins from intrinsic nerve could be responsible for this effect.

As an alternative explanation, there is the possibility that a fraction of NK_2 receptors in the rat small intestine is constitutively active in the absence of an endogenous agonist (cf. Lefkowitz *et al.*, 1993; Kenakin, 1995; Milligan *et al.*, 1995 for reviews): this mechanism, which has been repeatedly demonstrated to occur in transfected cell systems, but also *in vivo* in transgenic mice (Bond *et al.*, 1995), may account for a non-neural, yet receptor-mediated contribution to the resting tone of the rat small intestine. According to this model, the relaxant activity of MEN 10,627 and the other antagonists could be viewed as an example of inverse agonism (Milligan *et al.*, 1995). Almost by definition, the demonstration of such an effect would be possible in conditions in which neural influences are totally eliminated from the system (as happens in transfected cells); the lack of relaxant action of NK_1 receptor antagonists would then not be a 'discrepancy' as it is if a 'neural' explanation is examined. When considering inverse agonism as a possible explanation for the present findings, the need to use relatively high concentration of the ligands to produce an effect and the existence of a pronounced time-dependency to reach equilibrium may reflect the existence of differences between the agonist-bound and the constitutively active conformations of the receptor (see Kenakin, 1995 for discussion on this point). Clearly the hypothesis that constitutively active NK_2 receptors exist in the rat small intestine requires further experimental evaluation.

References

BARTHÓ, L. & HOLZER, P. (1985). Search for a physiological role of substance P in gastrointestinal motility. *Neuroscience*, **16,** 1–32.

BARTHÓ, L., SANTICIOLI, P., PATACCHINI, R. & MAGGI, C.A. (1992). Tachykininergic transmission to the circular muscle of the guinea-pig ileum: evidence for the involvement of NK_2 receptors. *Br. J. Pharmacol.*, **105,** 805–810.

BOND, R.A., LEFF, P., JOHNSON, T.D., MILANO, C.A., ROCKMAN, H.A., MCMINN, T.R., APPARSUNDARAM, S., HYEK, M.F., KENAKIN, T.P., ALLEN, L.F. & LEFKOWITZ, R.J. (1995). Physiological effects of inverse agonists in transgenic mice with myocardial overexpression of the β2-adrenoceptor. *Nature*, **374,** 272–276.

BURCHER, E., BUCK, S.H., LOVENBERG, W. & O'DONOHUE, T.L. (1986). Characterization and autoradiographic localization of multiple tachykinin binding sites in gastrointestinal tract and bladder. *J. Pharmacol. Exp. Ther.*, **236,** 819–831.

EKBLAD, E., WINTHER, C., EKMAN, R., HÅKANSON, R. & SUNDLER, F. (1987). Projections of peptide-containing neurons in rat small intestine. *Neuroscience*, **20,** 169–188.

EMONDS-ALT, X., DOUTREMEPUICH, J.D., HEAULME, M., NELIAT, G., SANTUCCI, V., STEINBERG, R., VILAIN, P., BICHON, D., DUCOUX, J.P., PROIETTO, V., VAN BROECK, D., SOUBRIE, P., LE FUR, G. & BRELIERE, J.C. (1993). In vitro and in vivo biological activities of SR 140,333 a novel potent nonpeptide tachykinin NK_1 receptor antagonist. *Eur. J. Pharmacol.*, **250,** 403–413.

EMONDS-ALT, X., VILAIN, P., PROIETTO, V., VAN BROECK, D., ADVENIER, C., NALINE, E., NELIAT, G., LE FUR, G. & BRELIER, J.C. (1992). A potent and selective non-peptide antagonist of the neurokinin A (NK_2) receptor. *Life Sci.*, **50,** 101–106.

FUJII, T., MURAI, M., MORIMOTO, H., MAEDA, Y., YAMAOKA, M., HAGIWARA, D., MIYAKE, H., IKARI, N. & MATSUO, M. (1992). Pharmacological profile of a high affinity dipeptide NK_1 receptor antagonist FK 888. *Br. J. Pharmacol.*, **107,** 785–789.

GARRET, C., CARRUETTE, A., FARDIN, V., MOUSSAOUI, S., PEYRONEL, J.F., BLANCHARD, J.C. & LADURON, P.M. (1991). Pharmacological properties of a potent and selective nonpeptide substance P antagonist. *Proc. Natl. Acad. Sci. U.S.A.*, **88,** 10208–10211.

HAGAN, R.M., IRELAND, S.J., BAILEY, F., MCBRIDE, C., JORDAN, C.A. & WARD, P. (1991). A spirolactam conformationally-constrained analogue of physalaemin which is a peptidase resistant, selective NK_1 receptor agonist. *Br. J. Pharmacol.*, **102,** 168P.

HOLZER, P. & MAGGI, C.A. (1994). Synergistic role of muscarinic acetylcholine and tachykinin NK_2 receptors in intestinal peristalsis. *Naunyn Schmiedeberg's Arch. Pharmacol.*, **349,** 194–201.

HOLZER, P., SCHLUET, W. & MAGGI, C.A. (1993). Ascending enteric reflex contraction: roles of acetylcholine and tachykinins in relation to distension and propagation of excitation. *J. Pharmacol. Exp. Ther.*, **264,** 391–396.

HOLZER-PETSCHE, U. (1995). Tachykinin receptors in gastrointestinal motility. *Regul. Peptides*, **57,** 19–42.

KENAKIN, T. (1995). Pharmacological proteus? *Trends Pharmacol. Sci.*, **16,** 256–258.

LEFKOWITZ, R.J., COTECCHIA, S., SAMAMA, P. & COSTA, T. (1993). Constitutive activity of receptors coupled with guanine nucleotide regulatory proteins. *Trends Pharmacol. Sci.*, **14,** 303–308.

MAGGI, C.A., ASTOLFI, M., GIULIANI, S., GOSO, C., MANZINI, S., MEINI, S., PATACCHINI, R., PAVONE, V., PEDONE, C., QUARTARA, L., RENSETTI, A.R. & GIACHETTI, A. (1994a). MEN 10,627, a novel polycyclic peptide antagonist at tachykinin NK_2 receptor. *J. Pharmacol. Exp. Ther.*, **271,** 1489–1500.

MAGGI, C.A. & GIULIANI, S. (1995). Role of tachykinins as excitatory mediators of NANC contraction in the circular muscle of rat small intestine. *J. Autonom. Pharmacol.*, **15,** 335–350.

MAGGI, C.A., GIULIANI, S., PATACCHINI, R., SANTICIOLI, P., THEODORSSON, E., BARBANTI, G., TURINI, D. & GIACHETTI, A. (1992). Tachykinin antagonists inhibit nerve-mediated contractions in the circular muscle of the human ileum: involvement of NK_2 receptors. *Gastroenterology*, **102,** 88–96.

MAGGI, C.A., HOLZER, P. & GIULIANI, S. (1994b). Effect of ω-conotoxin on cholinergic and tachykininergic excitatory neurotransmission to the circular muscle of the guinea-pig colon. *Naunyn Schmiedeberg's Arch. Pharmacol.*, **350,** 529–536.

MAGGI, C.A.. & MELI, A. (1988). The sensory-efferent function of capsaicin-sensitive sensory neurons. *Gen. Pharmacol.*, **19,** 1–43.

MAGGI, C.A. PATACCHINI, R., BARTHÓ, L., HOLZER, P. & SANTICIOLI, P. (1994c). Tachykinin NK_1 and NK_2 receptor antagonists and atropine-resistant ascending excitatory reflex to the circular muscle of the guinea-pig ileum. *Br. J. Pharmacol.*, **112,** 161–168.

MAGGI, C.A., PATACCHINI, R., MEINI, S. & GIULIANI, A. (1994d). Effect of longitudinal muscle myenteric plexus removal and indomethacin on the response to tachykinin NK_2 and NK_3 receptor agonists in the circular muscle of the guinea-pig ileum. *J. Autonom. Pharmacol.*, **14,** 49–60.

MAGGI, C.A., PATACCHINI, R., MEINI, S., QUARTARA, L., SISTO, A., POTIER, E., GIULIANI, A. & GIACHETTI, A. (1994e). Comparison of tachykinin NK_1 and NK_2 receptors of the guinea-pig ileum and proximal colon. *Br. J. Pharmacol.*, **112,** 150–160.

MAGGI, C.A., PATACCHINI, R., ROVERO, P. & GIACHETTI, A. (1993). Tachykinin receptors and tachykinin receptor antagonists. *J. Autonom. Pharmacol.*, **13,** 23–93.

MAGGI, C.A., ZAGORODNYUK, V. & GIULIANI, S. (1994f). Specialization of tachykinin NK_1 and NK_2 receptors in producing fast and slow atropine-resistant neurotransmission to the circular muscle of the guinea-pig colon. *Neuroscience*, **63,** 1137–1152.

MCELROY, A.B., CLEGG, S.P., DEAL, M.J., EWAN, G.B., HAGAN, R.M., IRELAND, S.J., JORDAN, C.C., PORTER, B., ROSS, B.C., WARD, P. & WHITTINGTON, A.R. (1992). Highly potent and selective heptapeptide antagonists of the neurokinin NK-2 receptor. *J. Med. Chem.*, **35,** 2582–2591.

MILLIGAN, G., BOND, R.A. & LEE, M. (1995). Inverse agonism: pharmacological curiosity or potential therapeutic strategy? *Trends Pharmacol. Sci.*, **16,** 10–13.

MUSSAP, C.J. & BURCHER, E. (1993). Characterization and autoradiographic localization of tachykinin receptors in rat gastric fundus. *J. Pharmacol. Exp. Ther.*, **266,** 1043–1053.

SIMON, C., PORTALIER, P., CHAMOIN, M.C. & TERNAUX, J.P. (1992). Substance P-like immunoreactivity release from enterochromaffin cells of rat caecum mucosa. Inhibition by serotonin and calcium-free medium. *Neurochem. Int.*, **4,** 529–536.

STERNINI, C., ANDERSON, K., FRANTZ, G., KRAUSE, J.E. & BRECHA, N. (1989). Expression of substance P/neurokinin A encoding preprotachykinin messenger ribonucleic acids in the rat enteric nervous system. *Gastroenterology*, **97,** 348–356.

VAN GIERSBERGEN, P.L.M., SHATZER, S.A., HARBESON, S.L., ROUISSI, N., NANTEL, F. & BUCK, S.H. (1991). Multiple NK_2 receptor subtypes are suggested by physiological and biochemical studies with neurokinin A (NKA) analogues and antagonists. *Ann. New York Acad. Sci.*, **632,** 483–484.

ZAGORODNYUK, V., SANTICIOLI, P. & MAGGI, C.A. (1993). Tachykinin NK_1 but not NK_2 receptors mediate noncholinergic excitatory junction potential in the circular muscle of guinea-pig colon. *Br. J. Pharmacol.*, **110,** 795–803.

ZAGORODNYUK, V., SANTICIOLI, P., MAGGI, C.A. & GIACHETTI, A. (1995). Evidence that tachykinin NK_1 and NK_2 receptors mediate NANC excitation and contraction in the circular muscle of guinea-pig duodenum. *Br. J. Pharmacol.*, **115,** 237–246.

(Received December 20, 1995
Revised March 6, 1996
Accepted March 15, 1996)