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Tachykinin receptors in the circular muscle of the guinea-pig ileum

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1 We have studied the mechanical response of circular strips of the guinea-pig ileum to tachykinins and characterized the receptors involved by means of receptor-selective agonists.

2 The strips responded to both substance P (SP) and neurokinin A (NKA), as well as to [Pro^9]-SP sulphone (selective NK_1-receptor agonist), [βAla^8]-NKA(4–10) (selective NK_2-receptor agonist) and [MePhe^7]-neurokinin B (selective NK_3-receptor agonist). The ED_{50}s of the various peptides (calculated as the concentration of agonist which produced 50% of the response to 10 μM carbachol) were similar, in the range of 40–200 nM, i.e. no clearcut rank order of potency was evident.

3 The response to a submaximal (10 nM) concentration of SP or NKA was unaffected in the presence of peptidase inhibitors (thiorphan, captopril and bestatin, 1 μM each).

4 The response to the NK_1-agonist was totally atropine-resistant, but was reduced (about 30% inhibition) by tetrodotoxin. The response to the NK_3-receptor agonist was halved by atropine and abolished by tetrodotoxin. The response to the NK_2-agonist was unaffected by either atropine or tetrodotoxin.

5 The response to the selective NK_2-agonist was unchanged after desensitization of NK_1- or NK_3-receptors.

6 The response to the NK_2-selective agonist was strongly inhibited by [Tyr^5, D-Trp^{6,8,9}, Arg^{10}]-NKA(4–10) (MEN 10,207) a selective NK_2-receptor antagonist which did not modify the response to the NK_1-selective agonist.

7 Our findings indicate that all the three known types of tachykinin receptors mediate the contractile response of the circular muscle of the guinea-pig ileum to peptides of this family. The response to activation of NK_3-receptors is totally neurogenic and partially mediated by endogenous acetylcholine, the response to activation of NK_1-receptors is partly neurogenic and largely myogenic and the response to activation of NK_2-receptors is totally myogenic.

Introduction

Ample evidence, based on pharmacological, physiological, anatomical and neurochemical findings indicates that tachykinins (TKs) play a physiological role as excitatory transmitters in the guinea-pig ileum (Franco *et al.*, 1979; Costa *et al.*, 1981; 1985; Donnerer *et al.*, 1984; Smith & Furness, 1988; Llewellyn-Smith *et al.*, 1988; 1989; Bartho' *et al.*, 1989; Holzer, 1989; see also Bartho' & Holzer 1985 for review). In the guinea-pig intestine, substance P (SP) and other TKs, such as neurokinin A (NKA) (Too *et al.*, 1989) are stored in intrinsic neurones thought to play a role as final effectors of the atropine-resistant peristalsis (Bartho' & Holzer, 1985 for review).

The contractile response of the guinea-pig ileum to TKs is mediated by specific receptors (Lee *et al.*, 1982). In the longitudinal muscle, at least two TK receptors are present: one receptor (NK_1) mediates the direct response of muscle cells while the other (NK_3) activates intramural effector neurones which in turn release acetylcholine and possibly endogenous TKs (Kilbinger *et al.* 1986; Laufer *et al.*, 1986; 1988; Guard & Watson, 1987). Some studies have also suggested that a third type of TK receptor (NK_2) may mediate the contractile response of the guinea-pig ileal longitudinal muscle (Jacoby *et al.*, 1986; Dion *et al.*, 1987) while other investigations have excluded this possibility (Laufer *et al.*, 1988).

Also in the circular muscle of the guinea-pig ileum TKs are potent spasmogens (Holzer *et al.*, 1980; Costa *et al.*, 1985). However, the type of TK receptors mediating their contractile response at this level has not been determined. Complex mechanisms are likely to be involved, because the contractile response to SP has both a direct and an indirect component (Holzer *et al.*, 1980; Costa *et al.*, 1985).

The aim of this study was to characterize the TK receptors mediating the contractile response of the circular muscle of the guinea-pig ileum to these peptides. With this aim, the effect of SP and NKA as well as of receptor-selective synthetic TK agonists were investigated.

Methods

Male albino guinea-pigs (250–300 g) were stunned and bled. A segment of the ileum was excised and placed in oxygenated (96% O_2 and 4% CO_2) Krebs solution. The ileum was opened along the mesenteric border and pinned flat on a Petri dish. A small strip (< 3 mm wide) was then dissected along the circular axis as described by Costa *et al.* (1985) except that no attempt was made to remove the mucosal layer, in order to avoid any possible damage to the inner circular muscle. The strips were transferred to a 5 ml bath for isotonic recording (load 5 mN) of mechanical activity via a Basile 7050 Unirecord. In some experiments, the strips were electrically stimulated by means of two wire platinum electrodes placed at the top and bottom of the organ bath, connected to a Grass S11 stimulator.

All experiments started after a 90 min equilibration period. The contractile response to carbachol (10 μM) was assessed at 15–20 min intervals until reproducible responses were obtained. The response to carbachol was used as an internal standard to express contractile responses to peptides or field stimulation. Non-cumulative concentration-response curves to peptides were constructed at 15–20 min intervals with washing between doses. Some experiments were performed in presence of atropine (3 μM, contact time 15 min) or tetrodotoxin (1 μM, contact time 15 min). In these experiments the response to a 1 μM concentration of the three receptor-selective agonists was determined at 30 min intervals until reproducible responses were obtained before testing the effect of atropine or tetrodotoxin. In some experiments the response to a submaximal concentration of SP or NKA (10 nM) was determined before and after application of a mixture of peptidase inhibitors

(thiorphan, captopril and bestatin to block endopeptidase 24.11, angiotensin converting enzyme and aminopeptidases, respectively, 1 μM for each inhibitor).

In other experiments the response to a submaximal concentration of the peptides was studied before and after application (contact time 15 min) of [Tyr^5, D-Trp^{6,8,9}, Arg^{10}]-neurokinin A (4–10) (MEN 10,207) a newly developed selective NK_2-tachykinin receptor antagonist (Rovero *et al.*, 1990; Maggi *et al.*, 1990b). Attempts were made to construct cumulative concentration-response curves to the peptide in order to study the effect of the antagonist on the full concentration-response curves but marked desensitization was found in all cases tested ($n = 4$ from 4 animals).

All data in the text and figures are mean ± s.e.mean of 5–8 determinations. Statistical analysis was made by Student's t test for paired or unpaired data or by means of analysis of variance, when applicable. A P level < 0.05 was considered statistically significant.

EC_{50}s of SP, NKA and of the receptor-selective synthetic agonists were calculated, using linear regression analysis and the least square method, as the concentration of each peptide which produced 50% of the response to 10 μM carbachol.

Drugs used were: atropine HCl (Serva), tetrodotoxin (Sankyo), substance P and neurokinin A and bestatin (Peninsula), thiorphan (Sigma), captopril (Squibb). [Pro^9]-SP sulphone was a kind gift of Prof. D. Regoli, Department of Pharmacology, University of Sherbrooke, Canada. [β Ala^8]-NKA(4–10), [MePhe^7]-neurokinin B and [Tyr^5, D-Trp^{6,8,9}, Arg^{10}]-NKA(4–10) (MEN 10,207) were synthesized by Dr P. Rovero, Department of Chemistry, Menarini Pharmaceuticals by a conventional solid phase method. All peptides were dissolved in saline and kept frozen until use.

Results

About 50% of the circular strips showed an irregular, low-amplitude phasic activity (15% of the response to carbachol) which often waned and re-appeared during the course of the experiment. Electrical field stimulation at 20 Hz for 5 s (0.5 ms pulse width, 60 V) evoked phasic contractions which ranged between 72 and 94% of the response to carbachol. These responses were reduced by atropine (46 ± 7% inhibition, $n = 8$) and abolished by tetrodotoxin (1 μM). Atropine (3 μM) fully inhibited the contraction to 10 μM carbachol ($n = 4$).

Both SP and NKA produced a concentration (1 nM–1 μM)-dependent contraction of the circular muscle of the guinea-pig ileum (Figures 1 and 2). The curves to these two peptides were virtually superimposable. At 1 μM, the peak contractile response to SP and NKA averaged 86 ± 3 and 95 ± 2% of that to carbachol, respectively ($n = 5$ for each peptide). EC_{50}s and 95% confidence limits (in parentheses) were as follows: SP 42 nM (29–79), NKA 103 nM (62–129).

A consistent, concentration-dependent contractile response (Figures 1 and 2) was also obtained with each one of the three receptor selective agonists ($n = 6$–8). The responses at 1 μM averaged 61 ± 4, 68 ± 3 and 88 ± 4% of that to carbachol for [Pro^9]-SP sulphone ($n = 9$), [βAla^8]-NKA(4–10) ($n = 6$) and [MePhe^7]-neurokinin B ($n = 7$), respectively. EC_{50}s and 95% confidence limits (in parentheses) were as follows: [Pro^9]-SP sulphone 209 nM (115–402); [βAla^8]-NKA(4–10) 199 nM (152–279); [MePhe^7]-neurokinin B 68 nM (50–91).

The question was raised whether activity of TKs might have been underestimated because of local metabolism by tissue peptidases. To address this point the response to a submaximal concentration of SP and NKA (10 nM) was determined in the absence and the presence of a mixture of peptidase inhibitors (thiorphan, captopril and bestatin, 1 μM each, 15 min beforehand). The mixture of peptidase inhibitors did not change the response to carbachol ($n = 4$) nor affected the response to added peptides. In fact the responses to 10 nM SP averaged 18 ± 4 and 20 ± 5% and that to 10 nM NKA 25 ± 4 and 23 ± 3% of the response to carbachol before and after addition of the peptidase inhibitors, respectively ($n = 4$ for each peptide, NS).

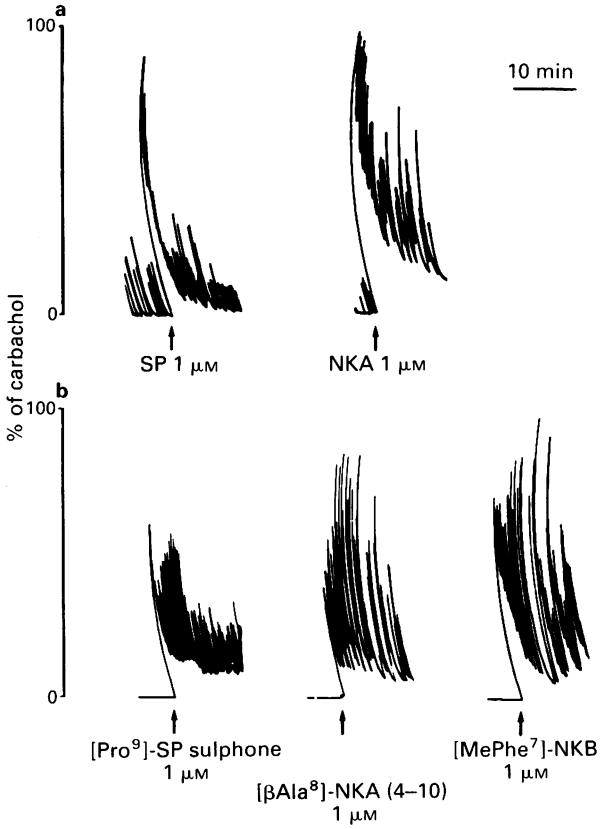

Figure 1 Typical tracings showing the contractile response of the circular muscle of the guinea-pig ileum to (a) 1 μM substance P (SP), neurokinin A (NKA) and (b) the receptor-selective agonists, [Pro^9]-SP sulphone (NK_1-receptor), [βAla^8]-NKA(4–10) (NK_2-receptor) and [MePhe^7]-neurokinin B (NKB). Vertical bars indicate the maximal contractile response to carbachol (10 μM).

Figure 3 shows the effect of atropine (3 μM) and tetrodotoxin (1 μM) on the contractile responses produced by the synthetic receptor-selective agonists. The response to [Pro^9]-SP sulphone was not significantly changed by atropine, although in individual experiments either an enhancement or a reduction was observed ($n = 11$). Conversely, tetrodotoxin produced a statistically significant reduction (about 30%) of the response to the NK_1-receptor agonist ($n = 8$, Figure 3). The response to [MePhe^7]-neurokinin B was significantly reduced (about 50%) in the presence of atropine and was vir-

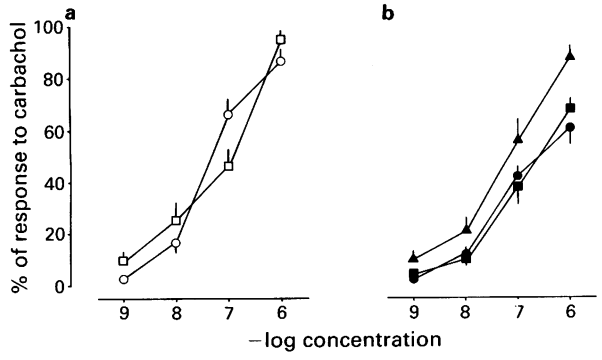

Figure 2 Concentration-response curves showing the contractile effect of (a) substance P (SP, ○, $n = 5$), neurokinin A (□, $n = 5$) and (b) the receptor-selective agonists [Pro^9]-SP sulphone (●, NK_1-receptor, $n = 8$), [βAla^8]-NKA(4–10) (■, NK_2-receptor, $n = 7$) and [MePhe^7]-neurokinin B (▲, NK_3-receptor, $n = 6$). Each point is mean with s.e.mean shown by vertical lines.

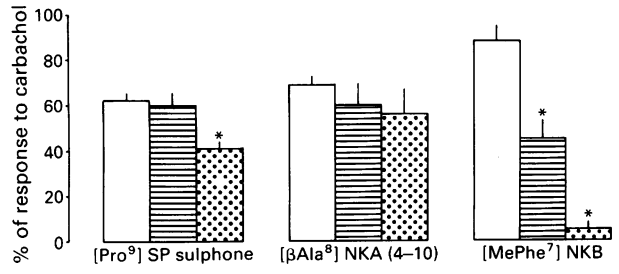

Figure 3 Effect of atropine (3 μM horizontally lined columns) or tetrodotoxin (1 μM, stippled columns) on the contractile response of the circular muscle of the guinea-pig ileum to 1 μM application of [Pro^9]-SP sulphone (NK_1-receptor, $n = 8$–11), [βAla^8]-NKA(4–10) (NK_2-receptor, $n = 6$) and [MePhe^7]-neurokinin B (NK_3-receptor, $n = 6$). Control: open columns. Significantly different from the control response, $^*P < 0.05$.

tually abolished by tetrodotoxin ($n = 6$ each). By contrast the response to [βAla^8]-NKA(4–10) was unaffected by either atropine or tetrodotoxin ($n = 6$ each, Figure 3).

Cross-desensitization experiments were also performed in order to assess whether NK_2-receptors might contribute to the contractile response to TKs. As shown in Figure 4, a second application of either [Pro^9]-SP sulphone or [MePhe^7]-neurokinin B (1 μM for each peptide) failed to reproduce the contractile response observed at the first challenge, indicating desensitization of NK_1- and NK_3-receptors, respectively. In the presence of each of the two desensitizing peptides, 1 μM [βAla^8]-NKA(4–10) produced a contractile response not different from the control, indicating the lack of cross-desensitization. Similar results were obtained for each desensitizing peptide in 4 preparations from different animals.

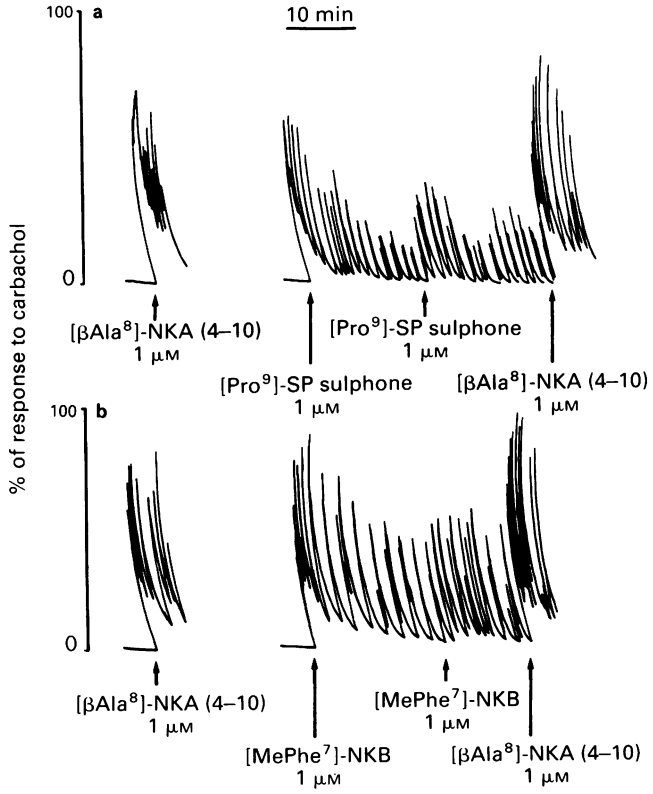

Figure 4 Typical tracings showing the lack of cross desensitization between the response to [Pro^9]-SP sulphone (NK_1-receptor selective) and [βAla^8]-NKA(4–10) (NK_2-receptor selective) (a) and [MePhe^7]-neurokinin B (NKB, NK_3-receptor selective) and [βAla^8]-NKA (4–10) (b). In each panel the control response to the NK_2-receptor agonist, obtained 30 min before application of the first dose of the NK_1- or NK_3-receptor selective agonist is shown. Experiments were performed on paired strips from the same animal.

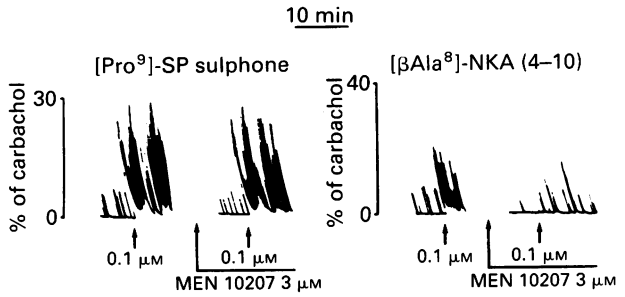

Figure 5 Typical tracings showing the effect of MEN 10,207 ([Tyr^5, D-Trp^{6,8,9}, Arg^{10}]-neurokinin A (4–10)) on the contractile response produced by a submaximal concentration (0.1 μM) of the NK_1-receptor selective agonist, [Pro^9]-SP sulphone or the NK_2-receptor selective agonist [βAla^8]-NKA(4–10).

Data in Figures 5 and 6 show the effect of MEN 10,207 (3 μM, contact time 15 min), a newly developed NK_2-receptor selective antagonist (Rovero *et al.*, 1990; Maggi *et al.*, 1990b) on the responses produced by a submaximal concentration (0.1 μM) of [Pro^9]-SP sulphone or [βAla^8]-NKA(4–10). MEN 10,207 did not affect the response to the NK_1-selective agonist, while strongly reduced the contraction produced by the NK_2-selective agonist ($n = 5$ in each case).

Discussion

This study confirms previous reports describing the powerful contractile effects exerted by TKs on the circular muscle of the guinea-pig ileum (Holzer *et al.*, 1980; Costa *et al.*, 1985). As compared to the earlier study of Costa *et al.* (1985) we noted a lower sensitivity of the circular strip to SP, a finding possibly related to the presence, in our preparation, of the mucosal layer which was not removed in order to prevent any possible damage to the inner circular muscle. To be noted is that Bauer & Kuriyama (1982) observed a similar low sensitivity to SP in mucosa-free preparations of the circular muscle of the guinea-pig ileum.

Recently, evidence has been obtained indicating a role for certain tissue peptidases in degrading TKs in the periphery (Turner, 1987; Devillier *et al.*, 1988; Patacchini *et al.*, 1989).

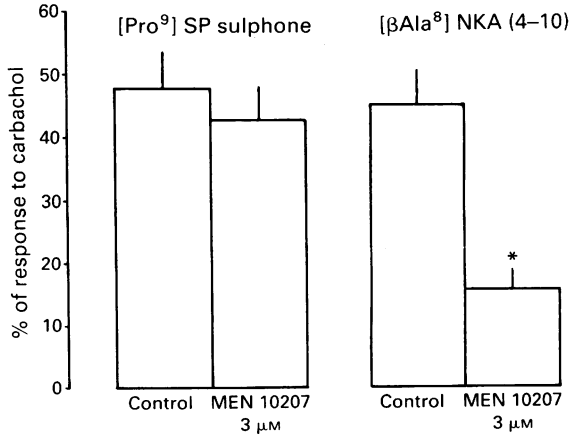

Figure 6 Effect of MEN 10,207 ([Tyr^5, D-Trp^{6,8,9}, Arg^{10}]-neurokinin A (4–10)) on the contractile response produced by application of a submaximal concentration (0.1 μM) of the NK_1-receptor selective agonist [Pro^9]-SP sulphone or the NK_2-receptor selective agonist [βAla^8]-NKA(4–10). Each column is mean of 5 experiments; s.e.mean shown by vertical lines. Statistically different from the control response $^*P < 0.05$.

The present findings do not provide evidence for a significant degradation of TKs by either endopeptidase 24.11 (thiorphan as inhibitor), angiotensin converting enzyme (captopril as inhibitor) or aminopeptidases (bestatin as inhibitor) in the guinea-pig ileum. However, the possibility cannot be ruled out that other peptidases are important for TK degradation at this level.

The contractile response of the circular strip of the ileum to TKs application is likely to involve activation of the specific TK receptors detected on circular muscle cells by autoradiography (Burcher *et al.*, 1986). However the possibility that the contractile responses to TKs and synthetic peptides might have been influenced by other mediators released from the mucosal layer cannot be ruled out.

The results obtained with the receptor-selective synthetic agonists are of interest because they delineate differentiated functions and discrete localizations of the TK receptor subtypes in the guinea-pig ileum. It is evident that the conclusions which can be drawn from the present experiments are critically dependent by the specificity of the selective agonists and antagonists used to identify TK receptors. The available evidence, obtained in selected, monoreceptorial bioassays for NK_1-, NK_2- and NK_3-receptors, indicates that [Pro^9]-SP sulphone is as potent as SP at NK_1-sites while being virtually inactive at NK_2- or NK_3-receptors (Drapeau *et al.*, 1987). For both the NK_2- ([βAla^8]-NKA(4–10)) and NK_3-([$MePhe^7$]-neurokinin B) agonists used in this study, the available evidence indicates that they possess similar or stronger affinity as compared to natural TKs at the respective preferred receptor while their activity at NK_1/NK_3 sites for the NK_2-agonist or at NK_1/NK_2-sites for the NK_3-agonist is markedly reduced, by about two orders of magnitude (Drapeau *et al.*, 1987; Rovero *et al.*, 1988). To improve the possibility of discriminating between the responses produced by the synthetic receptor-selective agonists we also investigated the effect of atropine and tetrodotoxin.

The response to [$MePhe^7$]-neurokinin B, a selective NK_3-receptor agonist (Drapeau *et al.*, 1987; Dion *et al.*, 1987) was totally neurogenic, being abolished by tetrodotoxin. A consistent fraction of this response was also atropine-resistant indicating that endogenous acetylcholine is not the only mediator involved. In fact the possibility that the endogenous TKs account for the atropine-resistant responses to the NK_3-agonist (cf. Guard & Watson, 1989) and to electrical field stimulation (Costa *et al.*, 1985 and present findings) deserves consideration.

The response to [Pro^9]-SP sulphone, a selective NK_1-receptor agonist (Drapeau *et al.*, 1987; Dion *et al.*, 1987) was partially neurogenic as indicated by the effect of tetrodotoxin. Thus both a direct and indirect components might participate in circular muscle contraction produced by NK_1-receptors activation. The lack of a significant inhibitory action by atropine indicates that the cholinergic contribution to the NK_1-mediated response was quantitatively very small, if any. The ability of NK_1-receptors to activate neurogenic mechanisms affecting motility was also shown in canine small intestine (Fox & Daniel, 1986; Fox *et al.*, 1986). In view of the extremely high selectivity of [Pro^9]-SP sulphone for NK_1- vs. NK_2-/NK_3-receptors, the possibility of a cross-talk of this peptide with the neurogenic response activated by the NK_3-agonist seems very unlikely.

[βAla^8]-NKA(4–10) is a newly developed NK_2-receptor selective agonist (Rovero *et al.*, 1989). The use of this peptide has enabled us to obtain evidence that NK_2-receptors mediate the contractile response to TKs in both the longitudinal and circular muscle of the human ileum (Maggi *et al.*, 1989; 1990a). The possibility that [βAla^8]-NKA(4–10) was acting on NK_1- or NK_3-receptors in the circular muscle of the ileum can be excluded because: (a) the response mediated by NK_3-receptor activation is totally indirect while the response to [βAla^8]-NKA(4–10) is atropine- and tetrodotoxin-resistant and (b) the action of [βAla^8]-NKA(4–10) is not modified after desensitization of NK_1- or NK_3-receptors achieved by repeated exposure to [Pro^9]-SP sulphone or [$MePhe^7$]-neurokinin B.

Further differentiation of the response to [Pro^9]-SP sulphone and [βAla^8]-NKA(4–10) comes from the use of MEN 10,207, a newly developed highly selective NK_2-receptor antagonist (Rovero *et al.*, 1990; Maggi *et al.*, 1990b).

In conclusion, NK_1-, NK_2- and NK_3-receptors appear to mediate, with different mechanisms, the contractile response of the circular muscle of the guinea-pig ileum to TKs. In this respect, a marked species-related difference exists in comparison to the results of similar experiments performed on the circular muscle of the human ileum. In this latter tissue we found that the contractile response is entirely sustained by activation of NK_1- and NK_2-receptors (Maggi *et al.*, 1990a).

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