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# Tachykinin receptors in the guinea-pig isolated oesophagus: a complex system

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**1** The tachykinin receptors mediating contraction of isolated longitudinal strips of the guinea-pig oesophageal body were characterized with substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) as well as the analogues, [Sar$^9$,Met(O$_2$)$^{11}$]SP, [Nle$^{10}$]NKA(4–10) and [MePhe$^7$]NKB, selective for NK$_1$, NK$_2$ and NK$_3$, receptors, respectively. Experiments were performed both in the absence and presence of a cocktail of peptidase inhibitors, captopril (1 $\mu$M), thiorphan (1 $\mu$M) and amastatin (20 $\mu$M), in order to determine whether membrane bound proteases are important in the metabolism of tachykinins in this preparation.

**2** All agonists produced concentration-dependent contractile effects. The presence of the peptidase inhibitors shifted the concentration-response curves of SP, [Nle$^{10}$]NKA(4–10) and [MePhe$^7$]NKB significantly leftwards and the concentration-response curve of NKB was shifted significantly rightwards. However, the EC$_{50}$ values were significantly different only for [Nle$^{10}$]NKA(4–10) and NKB.

**3** In the presence of the peptidase inhibitors, the EC$_{50}$ values of the selective agonists, [MePhe$^7$]NKB (0.6 nM) and [Nle$^{10}$]NKA(4–10) (66 nM) indicated the presence of both tachykinin NK$_3$ and NK$_2$ receptors. [MePhe$^7$]NKB produced less than 50% of the maximal response obtained with the other agonists. Since [Sar$^9$,Met(O$_2$)$^{11}$]SP produced a small response in the nanomolar concentration range in about 30% of the preparations tested, it is possible that some NK$_1$ receptors were also present.

**4** Assuming competitive antagonism, the NK$_2$-selective antagonist SR 48,968 (30 nM) gave apparent p$K_B$ values of 8.13 and 8.65 for [Nle$^{10}$]NKA(4–10) in the absence and presence of peptidase inhibitors, respectively, supporting the presence of NK$_2$ receptors.

**5** The NK$_3$-selective antagonist SR 142,801 (0.1 $\mu$M), suppressed responses to low (0.1–10 nM) concentrations of [MePhe$^7$]NKB. These contractile responses to [MePhe$^7$]NKB were also abolished by atropine (0.6 $\mu$M) suggesting that this response was mediated via cholinergic nerves.

**6** It is concluded that the guinea-pig oesophagus is a complex system which has both NK$_2$ and NK$_3$ receptors and possibly some NK$_1$ receptors as well.

**Keywords:** Guinea-pig oesophagus; tachykinin receptors; peptidase inhibitors; [Nle$^{10}$]NKA(4–10); [MePhe$^7$]NKB; [Sar$^9$,Met(O$_2$)$^{11}$]SP; SR 48,968; SR 142,801 endogenous tachykinins

## Introduction

Vagal nerve stimulation of the guinea-pig isolated oesophagus has been shown to induce a triphasic tetrodotoxin-sensitive contractile response (Kerr *et al*., 1995). The first response involved activation of cholinergic motor neurones innervating striated muscle while the second response was due to stimulation of preganglionic parasympathetic neurones innervating smooth muscle. The third response involved the smooth muscle as well, via retrograde stimulation of afferent neurones. This latter response was of interest since, although it was resistant to ganglion blocking drugs, it was atropine sensitive. This response, which was selectively abolished by capsaicin, may be mediated by the action of a substance P-like neuropeptide released from sensory nerve endings which subsequently activates cholinergic neurones. Tachykinin-like immunoreactivity has been detected in sensory nerves of the guinea-pig oesophagus by radioimmunoassay and immunohistochemistry (Hua *et al*., 1985).

Neurokinin receptor characterization has not been fully determined in the guinea-pig oesophagus. Kamikawa and Shimo (1984) studied the contractile responses to substance P (SP) and two non-mammalian tachykinins, eledoisin and physalaemin, in the muscularis mucosae-submucous plexus preparation of the guinea-pig oesophagus, but they did not have the advantage of using the receptor selective neurokinin analogues which are currently available. Also, their determinations were not carried out in the presence of peptidase inhibitors.

The aim of this study was to characterize the tachykinin receptors in the body of the guinea-pig oesophagus. The endogenous tachykinins, substance P, neurokinin A (NKA) and neurokinin B (NKB), act preferentially but not exclusively on NK$_1$, NK$_2$ and NK$_3$ receptors, respectively (Maggi *et al*., 1993). The order of agonist potencies was determined with the endogenous agonists as well as the agonists, [Sar$^9$,Met(O$_2$)$^{11}$]SP, [Nle$^{10}$]NKA(4–10), and [MePhe$^7$]NKB, selective for NK$_1$, NK$_2$ and NK$_3$ receptors, respectively (Drapeau *et al*., 1987; Regoli *et al*., 1988). The effects of the selective NK$_1$ receptor antagonist SR 140,333 (Emonds-Alt *et al*., 1993), the selective NK$_2$ receptor antagonist SR 48,968 (Emonds-Alt *et al*., 1992) and the selective NK$_3$ receptor antagonist SR 142,801 (Emonds-Alt *et al*., 1995), all non-peptide antagonists, were also determined. Since responses to tachykinins are markedly affected by peptidase inhibitors (Stephens-Smith *et al*., 1988) and differential rates of degradation of the neuropeptides by endogenous peptidases may affect the rank order of potency (Sekizawa *et al*., 1987; Shore & Drazen, 1989), the study was performed both in the absence and presence of several peptidase inhibitors.

Functions of tachykinins in the oesophagus are of interest as they may have clinical relevance in the pathophysiology of gastro-oesophageal reflux. The release of tachykinins from sensory nerves in the oesophagus may occur during reflux since protons stimulate peptide release from capsaicin-sensitive

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nerves (Bevan & Gepetti, 1994). This may serve as a protective mechanism whereby the oesophagus is cleared of refluxed material as studies have shown that the rate of primary and secondary oesophageal contractions is directly proportional to a low oesophageal pH (Corazziari *et al*., 1978; Dent *et al*., 1980). Also, gastro-oesophageal reflux has been implicated in Sudden Infant Death Syndrome in some infants (Leape *et al*., 1977).

Further, it is of interest that studies of antagonist affinities have shown that there is a pharmacological homology between guinea-pig and human tachykinin receptors in the case of $NK_1$, $NK_2$ and $NK_3$ receptor types (Maggi *et al*., 1993; Emonds-Alt *et al*., 1995).

## Methods

Male Dunkin Hartley guinea-pigs were stunned and bled. The oesophagus was opened by cutting along its ventral length and a 2.5 cm length was cut proximal from the level of the diaphragm. The strip was pinned out, muscularis mucosa side upwards, on a wax slab submerged in Krebs-Henseleit solution and slit in half longitudinally with a scalpel blade. The 2.5 cm long strips were each set up in a 3 ml siliconized tissue bath (Sigmacote; Sigma, St Louis, MO, U.S.A.) containing modified Krebs-Henseleit solution (2.5 ml) of the following composition (mM): NaCl 116, KCl 5.4, $MgSO_4.7H_2O$ 0.6, $NaH_2PO_4.2H_2O$ 1.2, $NaHCO_3$ 25, glucose 11.1 and $CaCl_2$ 2.5, gassed with 95% $O_2$, 5% $CO_2$. The bath temperature was maintained at 30°C to reduce spontaneous activity. A tension of 1.5 g wt. was applied to the tissue which was then equilibrated for 1 h, washing every 15 min. Isometric responses were recorded from a Grass FT.03 transducer connected to a MacLab/2e unit and a Macintosh SE computer. Responses to a maximal dose of KCl (100 mM), added to the bath for 20 s, were recorded every 15 min until reproducible (usually only two doses). After washing every 10 min over a 30 min period, a single maximum concentration of agonist was applied for 3 min. Then, after washing every 10 min for 45 min, a cumulative log concentration-response curve of the test agonist was obtained, each concentration being added when the effect of the preceding one had reached a steady state (*ca* 3 min). These curves were used to estimate the $EC_{50}$ and $E_{max}$ values. The values obtained from the maximal concentrations applied singly and cumulatively were compared as a check for tachyphylaxis. In order to determine the apparent $pK_B$ values of the selective antagonists, the cumulative concentration-response curves of the selective agonists were repeated after 1 h equilibration with their respective selective antagonists, SR 140,333 (0.1 $\mu$M), SR 48,968 (30 nM) and SR 142,801 (0.1 $\mu$M), or after 1 h equilibration with antagonist vehicle which served as antagonist vehicle-time controls.

The peptidase inhibitors, amastatin (20 $\mu$M), thiorphan (1 $\mu$M) and captopril (1 $\mu$M) were used to inhibit aminopeptidases, endopeptidase-24.11 and angiotensin converting enzyme, respectively (Turner *et al*., 1985). In experiments performed in the presence of peptidase inhibitors, cumulative concentration-response curves of the agonists were carried out and repeated after 1 h of equilibration with the $NK_2$ receptor antagonist SR 48,968 (30 nM), in order to determine the apparent $pK_B$ value of the selective $NK_2$ receptor antagonist against [$Nle^{10}$]NKA(4–10) and the extent to which each of the other five agonists were acting on $NK_2$ receptors. Any shifts in the concentration-response curves were corrected for shifts in appropriate vehicle-time control curves when calculating apparent $pK_B$ values. Also, in the presence of peptidase inhibitors, concentration-response curves to [$MePhe^7$]NKB were performed after 1 h equilibration with either the selective $NK_3$ receptor antagonist, SR 142,801 (0.1 $\mu$M) or antagonist vehicle only. Concentration-response curves of [$MePhe^7$]NKB were also performed after pre-incubation with atropine (0.6 $\mu$M).

As preliminary experiments showed that both captopril and thiorphan caused contractions *per se* (*ca* 25% of KCl, peaking around 7 min), they were added together to the tissue bath for 10 min, then washed out. When tension had returned to baseline, they were added in the same manner until no response resulted (usually after three additions). After washout, captopril, thiorphan and amastatin were then added together, 30 min before each concentration-response curve, since the maximum inhibition of aminopeptidases by amastatin has been shown to require a 30 min equilibration period (Rich *et al*., 1984).

### *Statistics and data evaluation*

Log concentration-response curves were normalized by plotting responses as a percentage of the response to KCl (100 mM) by use of the programme PRISM, version 1.0 (GraphPad Software, San Diego CA). Significance of differences between curves in the absence and presence of peptidase inhibitors were calculated by two-way ANOVA. Maximal effects of agonists ($E_{max}$) were calculated as percentages of the responses to KCl. Student's *t* test for paired data was used to determine whether there was any desensitization to the effects of the tachykinins and for unpaired data to ascertain whether the $EC_{50}$ and $E_{max}$ values were significantly different in the presence of peptidase inhibitors. As maxima were not the same for all concentration-response curves, order of potencies were compared in two ways; by using the $EC_{50}$ value, the concentration of agonist producing 50% of its maximal response, and also be determining the $EC_{10\%KCl}$ value, the molar concentration of agonist that produced a response equal to 10% of the internal standard.

Apparent $pK_B$ estimates for antagonists were determined from individual concentration-ratios by use of the Schild equation (Arunlakshana & Schild, 1959) for competitive inhibition at equilibrium, $pK_B = \log_{10}(DR-1) - \log_{10}[A]$, where DR is the concentration-ratio and [A] the antagonist concentration.

### *Drugs and solutions*

SP, NKA, NKB, [$Sar^9$,$Met(O_2)^{11}$]SP, [$Nle^{10}$]NKA(4–10), [$MePhe^7$]NKB, amastatin, and (±)-thiorphan were purchased from Auspep (Melbourne, Australia). Captopril was purchased from Research Biochemicals Incorporated (Natick, MA, U.S.A.) and atropine from Sigma (St. Louis, MO, U.S.A.). SP and [$Sar^9$,$Met(O_2)^{11}$]SP were dissolved in distilled water as stock solutions of 2.5 mM. NKA and [$Nle^{10}$]NKA(4–10) were dissolved in distilled water and 0.1 M ammonia solution, respectively, to form stock solutions of 0.25 mM. NKB and [$MePhe^7$]NKB were dissolved in 0.01 M HCl and 0.02 M acetic acid, respectively, to form stock solutions of 2.5 mM. Further dilutions of peptides were made in normal saline, separated into aliquots, and stored frozen. Stock solutions of (±)-thiorphan (2.5 mM) in 5% ethanol and amastatin (1.25 mM) in 0.01 M HCl were made in aliquots and stored frozen. The stock solution of captopril (2.5 mM) in saline, was stored refrigerated. All subsequent dilutions were made in normal saline. Stock solutions (2.5 mM) of SR 140,333 ((**S**)1-{2-[3-{3,4,-dichlorophenyl)-1-{3-isopropoxyphenylacetyl)piperidin-3yl]ethyl}-4-phenyl-1-azoniabicyclo[2.2.2]octane chloride), SR 48,968 ((**S**)-N-methyl-N[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl) butyl]benzamide) and SR 142,801 ((**S**)-(N)-(1-(3-(1-benzoyl-3-(3,4-dichloro- phenyl)-piperidin-3-yl)propyl)-4-phenylpiperidin-4-yl)-N-methylacetamide) (Sanofi Recherche, Montpellier, France) were made in absolute alcohol and stored refrigerated.

## Results

### *Effect of peptidase inhibitors on the natural and selective tachykinin agonists*

All the agonists produced concentration-dependent contractile responses; the concentration-response curves are shown in

Figure 1. There were no significant differences ($P>0.05$) between the $E_{max}$ values obtained after the addition of a single maximal concentration of agonist and the cumulative addition of agonists up to the same concentration.

The $pD_2$ values in the absence and presence of peptidase inhibitors are shown in Table 1. The presence of peptidase inhibitors did not affect the $E_{max}$ values. The peptidase inhibitors significantly ($P<0.0001$) potentiated the concentration-response curve to SP; this was more pronounced with lower concentrations of the agonist (Figure 1a). However, the change in $EC_{50}$ values (dose-ratio 0.1) was not significant ($P>0.05$). The $NK_1$-selective analogue [$Sar^9$,$Met(O_2)^{11}$]SP was not affected by the peptidase inhibitors (Figure 1b). In three out of eight strips, in the absence of peptidase inhibitors, and in 1 of 4 strips in the presence of peptidase inhibitors, the response to the $NK_1$-selective analogue appeared biphasic, as shown in Figure 2a.

While the presence of the peptidase inhibitors had no significant effect on the concentration-response curves of NKA (Figure 1c), they significantly ($P<0.0001$) potentiated the response to the $NK_2$-selective agonist, [$Nle^{10}$]NKA(4–10), $EC_{50}$ dose ratio = 0.12, (Figure 1d). Responses to [$Nle^{10}$]NKA(4–10) above 3 $\mu$M could not be determined, since the vehicle for this agonist, ammonia (0.1 M) solution, caused an increase in baseline tension, when added at an equivalent dilution for final bath concentrations of $>3$ $\mu$M [$Nle^{10}$]NKA(4–10).

The concentration-response curve of NKB was significantly inhibited ($P=0.005$) by the peptidase inhibitors, $EC_{50}$ dose ratio = 8.5, (Figure 1e), whereas the concentration-response curve of the $NK_3$-selective agonist, [$MePhe^7$]NKB, which was clearly biphasic, was significantly enhanced, ($P<0.0001$) for the total curve (Figure 2b) and ($P=0.005$) for the lower concentration range of 0.1–30 nM (Figure 1f). However, the change in $EC_{50}$ values (dose ratio = 0.23) was not significant. Since the vehicle for the NKB solutions, hydrochloric acid (0.01 M), produced a contraction of the oesophagus when added at the same dilution as the NKB solutions for final bath

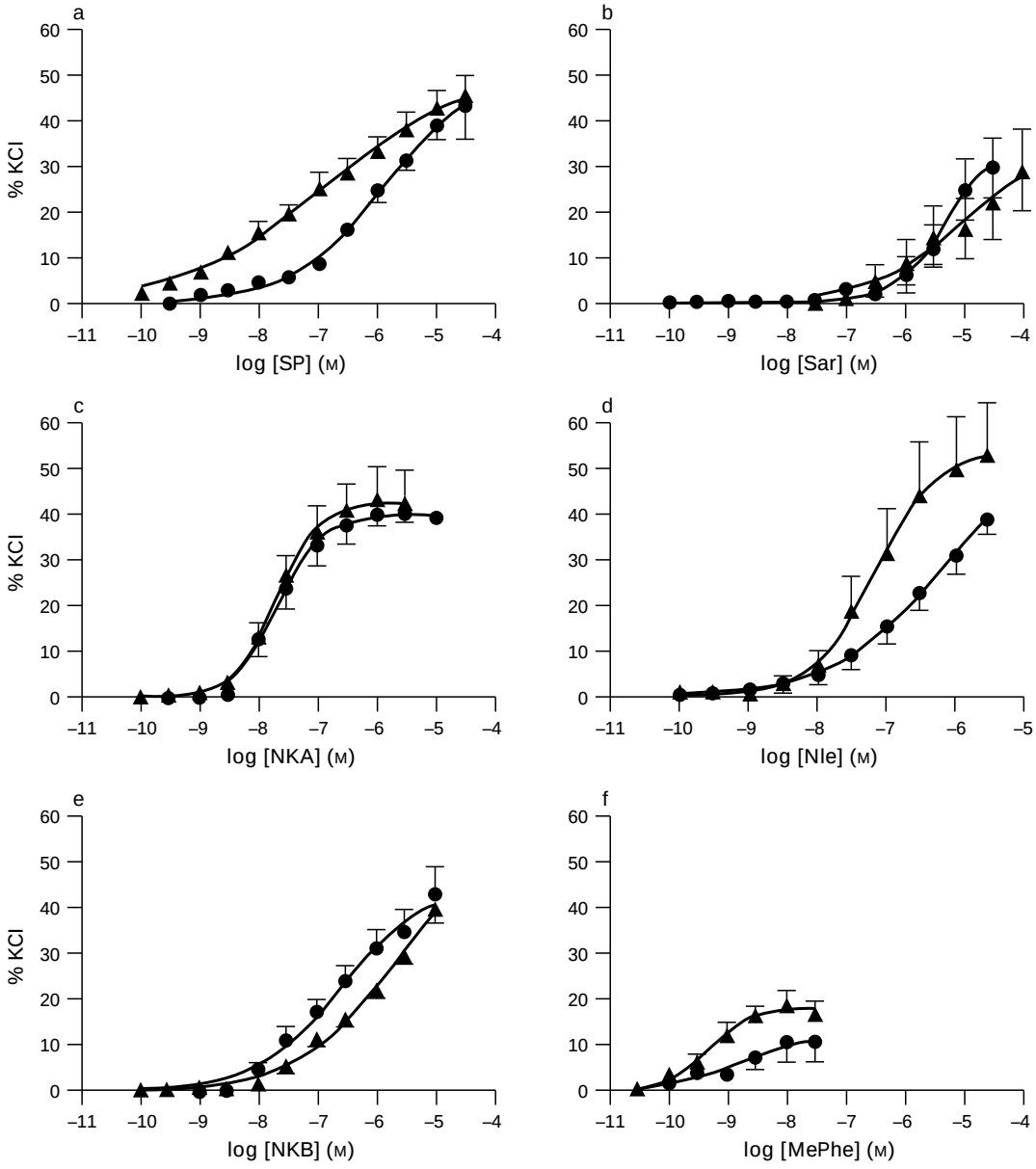


**Figure 1** Concentration-response curves for (a) SP, (b) [$Sar^9$,$Met(O_2)^{11}$]SP (Sar), (c) NKA, (d) [$Nle^{10}$]NKA(4–10) (Nle), (e) NKB and (f) [$MePhe^7$]NKB in the absence (●) and in the presence (▲) of peptidase inhibitors. Each point is the mean of 4–7 experiments; vertical lines show s.e.mean. Error bars which are not shown lie within the dimensions of the symbol. The plots show non-linear regressions through the data points. In the case of [$MePhe^7$]NKB only the data for concentrations $\leqslant$30 nM are shown, since the concentration-response relationship for the whole concentration range studied appeared biphasic as shown in Figure 2b.

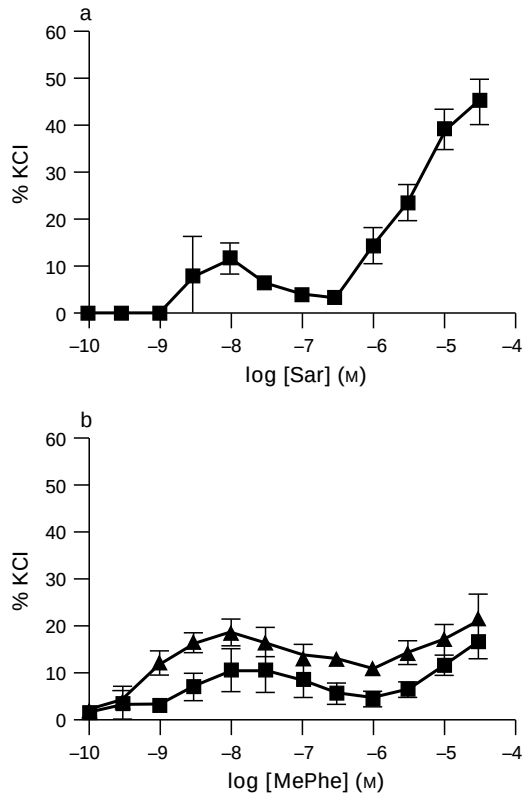


**Figure 2** (a) Concentration-response curve of [Sar$^9$,Met(O$_2$)$^{11}$]SP (Sar) showing a biphasic response present in three out of eight strips. (b) Concentration-response curves of [MePhe$^7$]NKB (MePhe) in the absence (■) and presence of peptidase inhibitors (▲). Each point is the mean of 3–7 experiments; vertical lines show s.e.mean. Error bars which are not shown lie within the dimensions of the symbol.

**Table 1** Negative log $EC_{50}$ ($pD_2$) values and relative potencies of tachykinins and selective analogues

| *Agonist* | *Control* $pD_2$ | n | *RP ($EC_{50}$)* |
|---|---|---|---|
| SP | 5.87±0.30 | 4 | 1.50 |
| NKA | 7.68±0.03 | 4 | 100 |
| NKB | 6.62±0.17 | 4 | 8.68 |
| [Sar$^9$,Met(O$_2$)$^{11}$]SP | 5.58±0.15 | 4 | 0.80 |
| [Nle$^{10}$]NKA(4–10) | 6.25±0.08† | 5 | 3.76 |
| [Mephe$^7$]NKB | 8.60±0.56 | 4 | 840 |
| | *Peptidase inhibitors* | | |
| SP | 6.85±0.38 | 7 | 14.1 |
| NKA | 7.70±0.02 | 7 | 100 |
| NKB | 5.69±0.29* | 7 | 0.97 |
| [Sar$^9$,Met(O$_2$)$^{11}$]SP | 5.30±0.29‡ | 4 | 0.40 |
| [Nle$^{10}$]NKA(4–10) | 7.17±0.15** | 4 | 29.85 |
| [Mephe$^7$]NKB | 9.24±0.09 | 7 | 3509 |

RP: relative potency, derived from $EC_{50}$ values, expressed as a % of potency of NKA. $pD_2$ value are presented as mean±s.e.mean. *Significantly different from the control, $P=0.0367$. **Significantly different from the control, $P<0.0001$. †Since it was not possible to achieve a maximum response to the curve in the absence of peptidase inhibitors, the $EC_{50}$ value was obtained from the concentration which gave an equi-effective response to the $EC_{50}$ value of the agonist in the presence of peptidase inhibitors (Figure 1d). ‡Since it was not possible to achieve a maximum response to the curve in the presence of peptidase inhibitors, the $EC_{50}$ value was obtained from the concentration which gave an equi-effective response to the $EC_{50}$ value of the agonist in the absence of peptidase inhibitors (Figure 1b).

concentrations of NKB > 10 μM, responses to such concentrations were not determined. Acetic acid (0.02 M), the vehicle for [MePhe$^7$]NKB, had no effect on baseline tension when added to the tissue bath at the same dilution as any of the [MePhe$^7$]NKB solutions used.

### *Relative potency of agonists*

The relative potencies of the tachykinins and selective analogues are shown in Tables 1 and 2. In the case of the natural peptides, relative potencies were compared using data derived from $EC_{50}$ values (Table 1). Thus, the order of potency, in the presence of the peptidase inhibitors, was: NKA > SP > NKB (Table 1) which indicates the presence of a mixed receptor population (Regoli *et al*., 1989; Watson & Girdlestone, 1996). For the purposes of receptor classification, the potency determination should accurately reflect the concentration of agonist available to activate the receptor. Since the activity of NKB was suppressed by the presence of peptidase inhibitors, the relative potency in the case of NKB should utilize the $EC_{50}$ value obtained in the absence of peptidase inhibitors. However, even when taking this factor into consideration, the order remained the same. In the case of the selective peptides, the relative potencies were derived from both $EC_{50}$ and $EC_{10\%KCl}$ values, as the maxima were not the same for the concentration-response curves (Figure 1b, d and f). However, the relative potency values derived from both methods were similar (Tables 1 and 2). These values, which indicate the presence of both $NK_3$ and $NK_2$ receptors, are also shown in Table 3 together with relative potency values obtained in other multi- and mono-receptor preparations.

It should be noted that the small response obtained with low concentrations of [Sar$^9$,Met(O$_2$)$^{11}$]SP in some preparations, which peaked around 10 nM (Figure 2a), was not taken into account when determining the order of potency, since this response could not be consistently produced either in the presence or absence of peptidase inhibitors. However, the occurrence of this response argues for the presence of some $NK_1$ receptors.

### *Effects of antagonists in the absence of peptidase inhibitors*

In the absence of peptidase inhibitors, the selective $NK_2$ receptor antagonist, SR 48,968, (30 nM) shifted the concentration-response curve of the selective $NK_2$ receptor agonist, [Nle$^{10}$]NKA(4–10) in a parallel fashion, with an apparent $pK_B$ value of 8.13±0.27 ($n=5$). However, neither the selective $NK_1$ receptor antagonist, Sr 140,333 (0.1 μM), nor the selective $NK_3$

**Table 2** Negative log $EC_{10\%KCl}$ values and relative potencies of tachykinins and selective analogues

| *Agonist* | *Control* $pEC_{10\%KCl}$ | n | *RP($EC_{10\%KCl}$)* |
|---|---|---|---|
| SP | 7.05±0.09 | 4 | 8.36 |
| NKA | 8.13±0.11 | 4 | 100 |
| NKB | 7.50±0.18 | 4 | 23.28 |
| [Sar$^9$,Met(O$_2$)$^{11}$]SP | 5.85±0.31 | 4 | 0.52 |
| [Nle$^{10}$]NKA(4–10) | 7.40±0.27 | 5 | 18.71 |
| [Mephe$^7$]NKB | 7.92±0.38 | 4 | 61.52 |
| | *Peptidase inhibitors* | | |
| SP | 8.64±0.25 | 7 | 315.5 |
| NKA | 8.14±0.10 | 7 | 100 |
| NKB | 6.98±0.15 | 7 | 6.95 |
| [Sar$^9$,Met(O$_2$)$^{11}$]SP | 5.79±0.53 | 4 | 0.45 |
| [Nle$^{10}$]NKA(4–10) | 7.85±0.27 | 4 | 51.52 |
| [Mephe$^7$]NKB | 9.17±0.17 | 7 | 1084 |

RP: relative potency, derived from $EC_{10\%KCl}$, expressed as a % of the potency of NKA. $pEC_{10\%KCl}$ values are presented as mean±s.e.mean.

**Table 3** Negative log $EC_{50}$ ($pD_2$) values and relative potencies (RP values) of tachykinins and selective analogues

| | *GPO* | | *$NK_3/NK_2$ RD* | | *$NK_2/NK_3$ HUB* | | *$NK_1$ DCA* | | *$NK_2$ RPA* | | *$NK_3$ RPV* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Agonist* | $pD_2$ | RP | $pD_2$ | RP† | $pD_2$ | RP‡ | $pD_2$ | RP‡ | $pD_2$ | RP† | $pD_2$ | RP‡ |
| SP | 6.85 | 14.1 | 6.50 | 2 | 5.57 | 1.48 | 10.00 | 398 | 6.13 | 0.8 | 5.82 | 23.51 |
| NKA | 7.70 | 100 | 8.22 | 100 | 7.40 | 100 | 9.40 | 100 | 8.22 | 100 | 6.45 | 100 |
| NKB | 6.62 | 8.68 | 8.15 | 85 | 7.20 | 63.07 | 8.90 | 31.59 | 7.45 | 17 | 7.68 | 1699 |
| [$Sar^9$,$Met(O_2)^{11}$]SP | 5.30 | 0.40 | 6.67 | 3 | I | I | 10.45 | 1121 | I | I | I | I |
| [$Nle^{10}$]NKA (4–10) | 7.18 | 29.85 | 8.02 | 63 | 7.09 | 48.95 | 7.00 | 0.4 | 7.90 | 48 | I | I |
| [$Mephe^7$]NKB | 9.24 | 3509 | 8.66 | 275 | 6.14 | 5.50 | 7.15 | 0.56 | 5.24 | 0.1 | 8.30 | 7086 |

GPO: guinea-pig oesophagus; RD: rat duodenum; HUB: hamster urinary bladder; DCA: dog carotid artery; RPA: rabbit pulmonary artery; RPV: rat portal vein. RP: relative potency, derived from $EC_{50}$ values, expressed as a percentage of the potency of NKA (see Table 1) †Relative potency derived from $EC_{50}$ values, expressed as a percentage of the potency of NKA (Regoli *et al*., 1989). ‡Relative potency, derived from $EC_{50}$ values, recalculated as a percentage of the potency of NKA (Regoli *et al*., 1989). I-inactive.

receptor antagonist, SR 142,801 (0.1 $\mu$M), affected the concentration-response curves of their respective selective agonists, [$Sar^9$,$Met(O_2)^{11}$]SP and [$MePhe^7$]NKB at high concentrations (0.1–100 $\mu$M). Antagonist vehicle-time controls also showed no significant effect on responses to the three selective agonists. Responses to [$Sar^9$,$Met(O_2)^{11}$]SP and [$MePhe^7$]NKB at low concentrations (0.1 nM–0.1 $\mu$M) were absent or weak. It was subsequently found that responses to these agonists in the low concentration range were not reproducible in the same preparation and it was not possible to test the effect of antagonists on these reponses.

### *Effects of the $NK_2$-selective antagonist, SR 48,968 in the presence of peptidase inhibitors*

The $NK_2$-selective antagonist, SR 48,968 (30 nM) shifted the concentration-response curve of the $NK_2$-selective agonist, [$Nle^{10}$]NKA(4–10), to the right with an apparent $pK_B$ value of $8.65 \pm 0.30$ (Figure 3a), but suppressed the concentration-response curve of NKA (Figure 3b). Antagonist vehicle-time controls showed the responses to NKA to be reproducible.

SR 48,968 also shifted the concentration-effect curve of NKB to the right with an apparent $pK_B$ value of $8.42 \pm 0.32$ ($n = 3$); corrected for the *ca* 2.5 fold rightward shift seen in the antagonist vehicle-time controls. SR 48,968 suppressed the concentration-response curve for high concentrations of [$MePhe^7$]NKB (1–30 $\mu$M).

While the concentration-response curve of SP was also shifted by SR 48,968, an apparent $pK_B$ value for the antagonist could not be determined as time controls to SP showed a *ca* 10 fold rightwards shift of the curve and since these controls were not performed on paired preparations from the same oesophagus, it was not possible to make relevant corrections. The concentration-response curve for high concentrations (micromolar range) of [$Sar^9$,$Met(O_2)^{11}$]SP, was shifted to the right by SR 48,968 with an apparent $pK_B$ value of $8.24 \pm 0.06$ ($n = 4$).

### *Effects of the $NK_3$-selective antagonist, SR 142,801, on responses to [$MePhe^7$]NKB in the presence of peptidase inhibitors*

The first phase (nanomolar range) of the concentration-response curve of [$MePhe^7$]NKB, once performed, could not be repeated ($n = 3$) in the same tissue. However, it was shown, by use of paired tissues, that the concentration-response curve for low concentrations (0.1 nM–10 nM) of [$MePhe^7$]NKB was significantly ($P = 0.0003$) suppressed by the $NK_3$ selective antagonist SR 142,801 (0.1 $\mu$M) ($n = 3$). The peak response (17% of KCl), which occurred at 10 nM, was reduced to 8% of KCl with this concentration of the $NK_3$ selective agonist. Pretreatment with atropine (0.6 $\mu$M) prevented the first phase (0.1 nM–0.1 $\mu$M) of the concentration-response curve of [$MePhe^7$]NKB ($n = 3$).

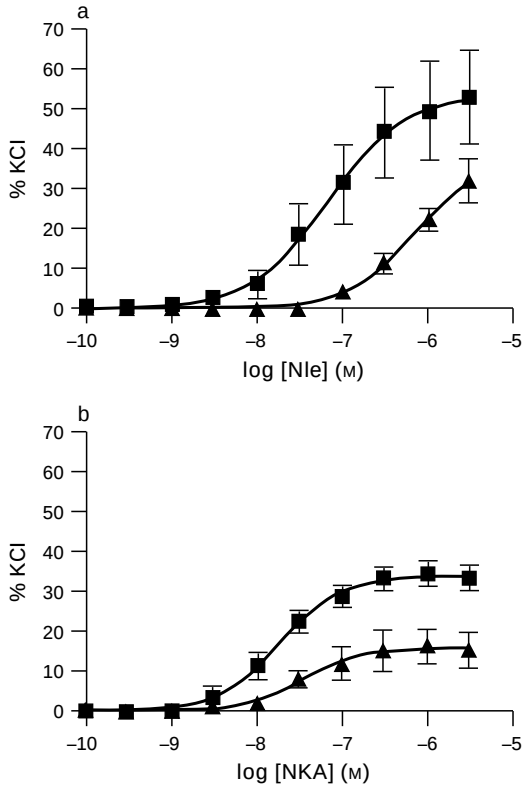


**Figure 3** (a) Concentration-response curves for [$Nle^{10}$]NKA(4–10) alone (■) and in the presence of SR 48,968 (30 nM) (▲). (b) Concentration-response curves for NKA alone (■) and in the presence of SR 48,968 (30 nM) (▲). Each point is the mean of four experiments, performed in the presence of peptidase inhibitors; vertical lines show s.e.mean. Error bars which are not shown lie within the dimensions of the symbol.

## Discussion

### *Effects of peptidase inhibitors*

Since the concentration-response curves of SP, [$Nle^{10}$]NKA(4–10), and [$MePhe^7$]NKB were significantly enhanced by the presence of the peptidase inhibitors, it is apparent that one or more of the peptidases, angiotensin converting enzyme, endopeptidase-24.11, and aminopeptidases is present in the guinea-pig oesophagus. The increased potency of SP could be the result of inhibition of angiotensin converting enzyme which hydrolyses some of the peptide bonds of SP (Skidgel *et al*., 1984). Alternatively, active fragments of SP generated by the enzyme, dipeptidyl aminopeptidase IV would be rapidly in-

activated by aminopeptidase N (Wang *et al*., 1991), in which case inhibition by amastatin would result in potentiated responses to SP. However, thiorphan does not appear to play a role in this potentiation of SP as responses to both NKA and NKB, which are more susceptible to neutral endopeptidase than SP (Nau *et al*., 1986; Sekizawa *et al*., 1987), were not enhanced by thiorphan. The decrease in potency of NKB can be explained by the fact that, in the absence of peptidase inhibitors, peptidase activity yields fragments of NKB which are more potent than NKB itself. It has been shown that while NKB has similar potencies for $NK_2$ ($pD_2 = 7.45$) and $NK_3$ ($pD_2 = 7.68$) receptors, the NKB (4–10) fragment is about 3 times as active as NKB on the rabbit pulmonary artery, an $NK_2$ monoreceptor system, but about 10 times less active than NKB on the rat portal vein, an $NK_3$ monoreceptor system (Drapeau *et al*., 1987). Accordingly, this suppression of activity of NKB by the peptidase inhibitors suggests that $NK_2$ receptors are more important than $NK_3$ receptors when NKB elicits a response in the guinea-pig oesophagus.

[$Nle^{10}$]NKA(4–10) was the only peptide which showed a significant reduction in the $EC_{50}$ value in the presence of the peptidase inhibitors. This was most probably due to the inhibition of aminopeptidase A which cleaves N-terminal glutamyl and aspartyl residues and of aminopeptidase N which hydrolyses many N-terminal amino acids (Turner *et al*., 1985). Giuliani *et al*., (1993) found that the activities of linear peptide derivatives of NKA with an N-terminal aspartyl residue were reduced by an amastatin-sensitive peptidase. The estimated potency of [$\beta Ala^8$]NKA(4–10) was significantly increased from a $pD_2$ value of 6.87 to 7.44 by amastatin (Astolfi *et al*., 1994).

It is surprising that the combination of peptidase inhibitors had no significant effect on responses to the natural peptide NKA in view of the fact that it is a substrate for various peptidases and the termination of action of peptides generally does not involve re-uptake into nerve terminals (Iverson *et al*., 1976; Segawa *et al*., 1977; Maggi, 1991). A possible explanation might be that amastatin blocks two aminopeptidases, one of which produces a more active fragment which is then susceptible to inactivation by the other enzyme. NKA undergoes sequential N-terminal hydrolysis by aminopeptidase-N (Wang *et al*., 1991). NKA fragments (2–10), (3–10) and (4–10) show considerable activity: for example the fragment NKA(4–10) is actually twice as active as the parent compound, NKA, in both the rabbit pulmonary artery, an $NK_2$ monoreceptor system, and in the rat portal vein, an $NK_3$ monoreceptor system (Regoli *et al*., 1994). Subsequently, further aminopeptidase activity produces fragments which are much weaker (Regoli *et al*., 1994). The active NKA(4–10) fragment is susceptible to aminopeptidase-A by virtue of its N-terminal aspartyl residue (Turner *et al*., 1985). Thus, aminopeptidase-A is apparently more important than aminopeptidase-N in the inactivation of NKA and thus, it would be expected that inhibition of aminopeptidase-A only and not both aminopeptidases-A and N would result in an enhanced response to NKA.

### *Receptor characterization*

The results of this study indicate the presence of both $NK_2$ and $NK_3$ receptors in the guinea-pig oesophagus. There is also some evidence suggesting the presence of $NK_1$ receptors. This conclusion is based on agonist potencies and antagonist affinities. The relative potencies of the selective tachykinin agonists clearly indicate the presence of both $NK_2$ and $NK_3$ receptors in the body of the guinea-pig oesophagus, as these values are of a similar relative order of magnitude as values previously obtained in mixed $NK_2/NK_3$ receptor systems (Table 3). Also, the relative potency value of the $NK_2$-selective agonist, [$Nle^{10}$]NKA(4–10), as a percentage of the potency of NKA, in the guinea-pig oesophagus is of a similar order of magnitude as that found in the rabbit pulmonary artery, an $NK_2$ monoreceptor system. Likewise, the potency value of the $NK_3$-selective agonist, [$MePhe^7$]NKB relative to NKA, in the guinea-pig oesophagus is of a similar order of magnitude as that of [$MePhe^7$]NKB in the rat portal vein, an $NK_3$-monoreceptor system. The use of the naturally occurring tachykinins offers less discrimination for receptor characterization because they are relatively non-selective. Nevertheless, the order of potency derived from $EC_{50}$ values, of the natural peptides, NKA > SP > NKB, indicated the presence of a mixed receptor population (Regoli *et al*., 1989; Watson & Girdlestone, 1996). Further, the $pD_2$ values of the three endogenous agonists are consistent with their acting on $NK_2$ and $NK_3$ receptors rather than on $NK_1$ receptors when compared with their respective values in each of the three monoreceptor systems (Table 3).

### *Further evidence for the presence of $NK_2$ receptors*

Evidence for the presence of $NK_2$ receptors is based on results obtained with the selective $NK_2$ receptor agonist, [$Nle^{10}$]NKA(4–10) and the potent non-peptide $NK_2$ receptor selective antagonist, SR 48,968. [$Nle^{10}$]NKA(4–10) produced concentration-dependent contractile responses in the submicromolar range and the apparent $pK_B$ value of 8.65 for the selective $NK_2$ receptor antagonist, SR 48,968 *vs* [$Nle^{10}$]NKA(4–10) was in good agreement with the $pA_2$ value of 8.73 obtained by Zeng and Burcher (1994) for SR 48,968 against the selective $NK_2$-receptor agonist, [$Lys^5$,$MeLeu^9$,$Nle^{10}$]NKA(4–10), in the guinea-pig bronchus.

However, a higher $pA_2$ value of 10.51 has been found for SR 48,968 vs the selective $NK_2$ receptor agonist, [$\beta Ala^8$]NKA(4–10), in the guinea-pig trachea (Advenier *et al*., 1992). There is a fairly wide range of apparent $pA_2$ values for SR 48,968 in the literature. A possible explanation for this might be differences in antagonist incubation times. Advenier *et al*. (1992) showed that the potency of the antagonist was increased by prolonging the contact time ($pA_2 = 7.50$ for 10 min and 8.11 for 120 min in the hamster urinary bladder). However, Zeng and Burcher (1994), found no differences between incubation times of 15 and 150 min in experiments on the guinea-pig bronchus. Our apparent $pK_B$ value of 8.65, which was obtained at 60 min, for SR 48,968 *vs* [$Nle^{10}$]NKA(4–10) is closer to the $pA_2$ value of 8.11 found in the hamster urinary bladder (Advenier *et al*., 1992). It is interesting that this preparation is also a mixed $NK_2/NK_3$ receptor system.

Another reason for the range of apparent $pA_2$ values for SR 48,968 in the literature concerns receptor subtypes. Studies of antagonist affinities have indicated the presence of $NK_2$ receptor 'subtypes' which are species dependent. SR 48,968 exhibits higher affinities for $NK_2$ receptors found in man ($pA_2 = 9.40$ in the bronchus) and guinea-pigs ($pA_2 = 10.51$ in the trachea) than the $NK_2$ receptors in the hamster, where $pA_2$ values of 8.11 and 8.66 have been found in the urinary bladder and trachea, respectively (Advenier *et al*., 1992). Receptors found in man and guinea-pigs have been designated $NK_{2A}$ and those in hamsters $NK_{2B}$ (Maggi *et al*., 1993). Our apparent $pK_B$ value of 8.65 for [$Nle^{10}$]NKA(4–10) is closer to the $pA_2$ value found in the hamster urinary bladder, which has the $NK_{2B}$ receptor 'subtype'. This raises the possibility that $NK_{2B}$ as well as $NK_{2A}$ receptors exist in the guinea-pig. The apparent $pA_2$ value obtained by Advenier *et al*. (1992) in the guinea-pig trachea indicates $NK_{2A}$ receptors and the values obtained by Zeng and Burcher (1994) in the guinea-pig bronchus and by us in the oesophagus suggest $NK_{2B}$ receptors. The presence of both receptor subtypes in the same species has not been demonstrated; this would indicate a true receptor subtype.

In contrast to the apparently competitive nature of the antagonism of SR 48,968 against the $NK_2$-selective agonist, [$Nle^{10}$]NKA(4–10), the antagonism of NKA was clearly non-competitive. Since NKA is not a selective tachykinin agonist and the response to the $NK_3$-selective agonist, [$MePhe^7$]NKB was less than maximal compared to the other agonists, it would seem that NKA acts predominantly on $NK_3$ receptors in the presence of SR 48,968 resulting in the appearance of a non-competitive effect.

With NKB as agonist in place of [Nle$^{10}$]NKA(4–10) a similar apparent p$K_B$ value (8.42) for SR 48,968 was obtained. This indicates that the natural peptide, NKB is primarily activating the $NK_2$ receptors in the guinea-pig oesophagus, which supports our earlier conclusion that peptidase inhibitors prevented the formation of the NKB(4–10) fragment which is more active at $NK_2$ receptors.

The presence of $NK_2$ receptors in the guinea-pig oesophagus have been demonstrated previously by Kamikawa and Shimo (1984). Their findings suggested the presence of $NK_2$ rather than $NK_3$ receptors. Our p$D_2$ value of 5.87 for SP, in the absence of peptidase inhibitors, as they used none, is in good agreement with their value of 5.72.

While responses to [MePhe$^7$]NKB in the high concentration range (>1 $\mu$M) were not affected by the selective $NK_3$ receptor antagonist, SR 142,801, they were inhibited by SR 48,968 (30 nM) indicating that [MePhe$^7$]NKB was also acting on $NK_2$ receptors at these concentrations. Maggi (1995) has also found [MePhe$^7$]NKB to stimulate $NK_2$ receptors in micromolar concentrations.

### *Further evidence for the presence of $NK_3$ receptors*

Evidence for the presence of $NK_3$ receptors is based on responses obtained with the selective $NK_3$ receptor agonist, [MePhe$^7$]NKB and the effect of the selective $NK_3$ receptor antagonist, SR 142,801. [MePhe$^7$]NKB gave a concentration-dependent contractile response over the nanomolar concentration range although the maximum response was only one third of that obtained with [Nle$^{10}$]NKA(4–10). A limited response to $NK_3$ receptor activation has been noted by others, for example Wormser *et al.* (1986) found that the maximal contraction resulting from the stimulation of the $NK_3$ receptor in the guinea-pig ileum was about 20% lower than that resulting from stimulation of the $NK_1$ receptor. In our experiments [MePhe$^7$]NKB did not display the same maximal responses as other agonists. The inability of [MePhe$^7$]NKB to produce the same maxima as the other agonists was not due to desensitization because the application of a single maximum concentration (3 $\mu$M) of this agonist to the preparation still resulted in a similar low response. This could be explained by a small receptor population or a less efficient transduction mechanism for these receptors.

Responses to [MePhe$^7$]NKB over the low concentration range (0.1 nM–0.1 $\mu$M) could not be reproduced in the same preparation. This suggests that $NK_3$ receptor desensitization occurred on repeated administration. Stimulation of the $NK_3$ receptors may also have resulted in desensitization when [MePhe$^7$]NKB was administered cumulatively at sub-micromolar concentrations as the response reached a maximum and then declined. It is of interest that Legat *et al.* (1996) found that $NK_3$ receptor activation contributed to part of the contractile response to SP in the guinea-pig ileum via acetylcholine release, but this effect was only of 1 min in duration.

The selective $NK_3$ antagonist, SR 142,801 (0.1 $\mu$M) inhibited responses to [MePhe$^7$]NKB. An apparent p$K_B$ value could not be determined, as the maximal response to [MePhe$^7$]NKB in the low (nanomolar concentration range) was suppressed by SR 142,801 indicating that it might act non-competitively.

Atropine (0.6 $\mu$M) completely abolished responses to [MePhe$^7$]NKB at low concentrations (0.1–100 nM) which suggested that the $NK_3$ receptors were present on cholinergic neurones. The fact that Kamikawa & Shimo (1984) only found $NK_2$ receptors in the muscularis mucosae-submucous plexus preparation of the guinea-pig oesophagus, in which the myenteric plexus was absent, indicates the likely location of the $NK_3$ receptors in our preparation as being on myenteric neurones. A neuronal location of $NK_3$ receptors on myenteric ganglia of cholinergic neurones in the guinea-pig ileum has been shown previously (Yau & Youther, 1982; Laufer *et al.*, 1985).

### *Evidence for the presence of $NK_1$ receptors*

This study also presents evidence that a small population of $NK_1$ receptors may be present in the guinea-pig oesophagus. This was indicated by the finding that the selective $NK_1$ receptor agonist, [Sar$^9$,Met(O$_2$)$^{11}$]SP, produced a small response (10% of the internal standard) peaking around 10 nM in *ca* 30% of preparations. Responses to high concentrations of [Sar$^9$,Met(O$_2$)$^{11}$]SP (micromolar range) were not affected by the selective $NK_1$ receptor antagonist, SR 140,333 which indicated that [Sar$^9$,Met(O$_2$)$^{11}$]SP had a non-selective action at high concentrations in this tissue. The selective $NK_2$-receptor antagonist, SR 48,968, shifted the responses to the right with an apparent p$K_B$ value of 8.24, which suggested that high concentrations of [Sar$^9$,Met(O$_2$)$^{11}$]SP activated $NK_2$ receptors as this value was similar to that obtained for SR 48,968 with the $NK_2$-selective agonist, [Nle$^{10}$]NKA(4–10).

Further evidence for the presence of $NK_1$ receptors came from the observation that nanomolar concentrations of SP, in the presence of peptidase inhibitors, caused contraction of the strips. Also, the order of potency of the natural peptides, derived from $EC_{10\%KCl}$ values: SP>NKA>NKB, indicates the presence of $NK_1$ receptors (Regoli *et al.*, 1989; Watson & Girdlestone, 1996). Responses to [Sar$^9$,Met(O$_2$)$^{11}$]SP at low concentrations may not be reproducible as one of the time-control curves showed a response at low concentrations of [Sar$^9$,Met(O$_2$)$^{11}$]SP but this response was absent when repeated. Also, as in the case of [MePhe$^7$]NKB, responses to [Sar$^9$,Met(O$_2$)$^{11}$]SP produced less than 50% of the maximum response obtained with the other agonists. Thus, the $NK_1$ receptors showed a similarity to the $NK_3$ receptors in that their activation resulted in a smaller maximal response compared to activation of $NK_2$ receptors. Also both the $NK_1$ and $NK_3$ receptors appeared to exhibit desensitization. Like the $NK_3$ receptors, the $NK_1$ receptors might also be neuronal as Legat *et al.* (1996) have shown the presence of both $NK_1$ and $NK_3$ receptors on cholinergic neurones in the guinea-pig ileum.

In conclusion, these findings indicate the presence of both $NK_2$ and $NK_3$ receptors in the body of the guinea-pig oesophagus. [Nle$^{10}$]NKA(4–10), as well as some of the other peptides investigated, produced concentration-dependent contractile responses by activation of $NK_2$ receptors, presumably located on the smooth muscle. The $NK_3$ receptors, which were activated by [MePhe$^7$]NKB in nanomolar concentrations, appeared to be situated on cholinergic neurones. Some $NK_1$ receptors may be present also. Concentration-response curves to SP, [Nle$^{10}$]NKA(4–10) and [MePhe$^7$]NKB were enhanced by the presence of thiorphan, captopril and amastatin, whereas the response to NKB was inhibited by this combination of peptidase inhibitors.

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