Two affinities for a single antagonist at the neuronal NK₁ tachykinin receptor: evidence from quantitation of receptor endocytosis

*¹Karl M. Jenkinson, ¹Bridget R. Southwell & ¹John B. Furness

¹Department of Anatomy and Cell Biology, University of Melbourne, Parkville, Victoria 3052, Australia

1 In smooth muscle contractility assays, many NK₁ receptor (NK₁r) antagonists inhibit responses to the neurotransmitter, substance P (SP), and its analogue, septide, with markedly different potency, leading to the proposal that there is a septide-preferring receptor related to the NK₁r.

2 We used fluorescence immunohistochemistry and confocal microscopy to visualize agonist-induced NK₁r endocytosis and analyse agonist/antagonist interactions at native NK₁r in neurons of the myenteric plexus of guinea-pig ileum.

3 SP and septide gave sigmoid log concentration-response curves and were equipotent in inducing NK₁r endocytosis.

4 The NK₁r antagonists, CP-99994 (2S,3S)-3-(2-methoxybenzyl)amino-2-phenylpiperidine dihydrochloride and MEN-10581, cyclo(Leu/[CH₂NH]Lys(benzyloxycarbonyl)-Gln-Trp-Phe-βAla) were both more potent in inhibiting endocytosis (50× and 8× greater respectively) against septide than against SP.

5 The results suggest that SP and septide interact differently with the NK₁r, and that a single antagonist can exhibit different affinities at a single NK₁r population, depending on the agonist with which it competes. Thus it may not be necessary to posit a separate septide-preferring tachykinin receptor.

Keywords: Confocal microscopy; CP-99994; MEN-10581; NK₁ receptor; receptor endocytosis; septide; substance P; tachykinin

Abbreviations: FITC, fluorescein isothiocyanate; HBSS, Hanks balanced salt solution; NK₁r, neurokinin 1 receptor; NK₁r-IR, NK₁ receptor immunoreactivity; PBS, sodium phosphate buffered saline; PSS, physiological salt solution; SP, substance P

Introduction

Differences in pharmacological profiles between the natural ligand for the NK₁r, SP, and its analogue, septide, (pGlu⁶,Pro⁹]-SP (6–11), have been revealed in organ bath and receptor-binding studies. Septide contracts a number of smooth muscle preparations, including the guinea-pig ileum, with similar potency to SP (EC₅₀, 1–9 nM), whereas it competes with low affinity for SP binding (Laufer *et al.*, 1988; Petit *et al.*, 1992; Carruette *et al.*, 1992; Pradier *et al.*, 1993; Glowinski, 1995; Nguyen-Le *et al.*, 1996). Many NK₁r antagonists inhibit responses of isolated tissues to septide with markedly greater potency (higher pA₂ values) than they inhibit responses to SP, when both agonists are active at nanomolar concentrations, and this led to the suggestion that septide may interact with a 'septide-preferring' tachykinin receptor, distinct from the NK₁r (Petit *et al.*, 1992; Longmore *et al.*, 1994; Zeng & Burcher, 1994; Patacchini *et al.*, 1995; Glowinski, 1995; Nguyen-Le *et al.*, 1996). Receptor cloning has failed to identify such a receptor (Maggi & Schwartz, 1997). However, in transfected cells that express only the NK₁r, homologous binding data indicate that septide and SP bind to the receptor with similar nanomolar affinity (Hastrup & Schwartz, 1996). In addition to the nanomolar binding of SP and septide, a subnanomolar affinity binding that is selective for SP has been identified (Pradier *et al.*, 1993; Hastrup & Schwartz, 1996).

Maggi & Schwartz (1997) postulated that the pharmacological and binding data can be explained if there are two sites or conformations of a single NK₁r, one (the general tachykinin conformer) at which SP and septide both bind with nanomolar affinity (K_D , 0.5 nM) and one (the SP-preferring conformer) which binds SP at subnanomolar affinity (K_D , 0.05 nM) and septide at relatively low affinity (K_D , 50 nM). If this is true, there may be no separate septide receptor; however, the differential effects of antagonists against nanomolar concentrations of SP and septide remain unexplained.

The aim of the present study was to clarify whether SP and septide activate the same tachykinin receptor and determine whether selective NK₁r antagonists differentially inhibit responses to each agonist when acting at the same immunohistochemically identified receptor. The NK₁r is a G-protein coupled receptor that undergoes endocytosis into cytoplasmic organelles when activated by agonists. We have previously shown that confocal images can provide quantitative data on trafficking of the NK₁r in enteric neurons (Southwell *et al.*, 1998a,b). In this study we have used this technique for pharmacological analysis of activation of immunohistochemically identified receptors on individual cells in intact tissue. We have examined SP- and septide-induced endocytosis of native NK₁r on enteric neurons and investigated the effects of the selective NK₁r antagonists CP-99994 and MEN-10581 on this endocytosis. Both CP-99994 and MEN-10581 have previously been shown to have different potencies when acting against SP and septide in smooth muscle

*Author for correspondence.

E-mail: k.jenkinson@anatomy.unimelb.edu.au

contractility studies (Patacchini *et al.*, 1995; Nguyen-Le *et al.*, 1996).

Methods

Experimental protocol

NK₁r endocytosis in enteric neurons in the guinea-pig ileum was studied using methods that have been characterized previously (Grady *et al.*, 1995; Southwell *et al.*, 1996, 1998a). Guinea-pigs of either gender (250–350 g) were killed by a blow to the head and exsanguination. One cm segments of ileum were isolated and incubated for 1 h at 37°C in modified physiological salt solution (PSS; composition in mM: NaCl 118; KCl 4.8; NaHCO₃ 25; NaH₂PO₄ 1.0; MgSO₄ 1.2, glucose 11.1; CaCl₂ 2.5; containing 3 µM nicardipine and 0.3 µM tetrodotoxin; bubbled with 95% O₂ and 5% CO₂), to allow receptor to locate on the cell surface (Southwell *et al.*, 1996). Tissues were rinsed in ice-cold Hanks balanced salt solution (HBSS; containing 3 µM nicardipine, 1% bovine serum albumin, 50 µM monensin, and the protease inhibitors 4 µg ml⁻¹ chymostatin, 40 µg ml⁻¹ bacitracin and 4 µg ml⁻¹ leupeptin), and incubated for 1 h at 4°C in HBSS in the absence or presence of SP (10 pM–10 µM) or septide (10 pM–10 µM). At 4°C the agonists are able to diffuse into the tissue and bind receptors, but membranes are solidified and so receptor endocytosis cannot occur. Tissues were then rinsed in warm PSS and incubated in PSS containing 50 µM monensin for 20 min at 37°C. Monensin blocks the exchange of H⁺ ions across endosomal membranes (Maxfield, 1982; Pless & Wellner, 1996). One effect of this block is to inhibit the recycling of NK₁r without preventing their endocytosis (Grady *et al.*, 1995; Southwell *et al.*, 1998a). We have previously shown that maximum endocytosis of NK₁r occurs after 20 min warming (Southwell *et al.*, 1996). Endocytosed NK₁r is recycled to the cell surface within 30 min of endocytosis (Southwell *et al.*, 1996; 1998a), and so monensin was used in the present study to prevent NK₁r recycling.

To investigate the effects of the NK₁r antagonists CP-99994, (2S,3S)-3-(2-methoxybenzyl)amino-2-phenylpiperidine dihydrochloride (10 pM–10 nM) or MEN-10581, cyclo (Leuψ[CH₂NH]Lys(benzyloxycarbonyl)-Gln-Trp-Phe-βAla) (10 nM–3 µM) on agonist-induced NK₁r endocytosis, tissue was incubated with antagonist for 1 h at 37°C in PSS without agonist, prior to incubation for 1 h at 4°C in HBSS with antagonist and with or without agonist. The antagonists, but not agonists, were also included in the final 20 min incubation in PSS at 37°C.

At the end of the incubations, tissues were transferred to ice cold PBS (0.9% NaCl in 0.01 M sodium phosphate buffer, pH 7.0) containing 3 µM nicardipine, opened along the mesenteric attachment, stretched and pinned to balsa board and fixed overnight in 2% formaldehyde plus 0.2% picric acid in 0.1 M sodium phosphate buffer, pH 7.0.

Immunohistochemistry

Fixed segments of ileum were dehydrated in dimethylsulphoxide (3 × 10 min) and rehydrated in PBS (3 × 10 min). Wholmount preparations of myenteric plexus were incubated for 1 h in PBS containing 1% Triton X-100 and 10% normal horse serum to block non-specific binding. Gut was incubated overnight at room temperature in hypertonic PBS containing antisera against the rat NK₁r (rabbit polyclonal, code 94168, to the intracellular 15 C-terminal amino acids of the rat NK₁r;

(Grady *et al.*, 1996); diluted 1 : 500) and against synaptophysin (mouse monoclonal, Dako Laboratories, code M776; 1 : 100). This NK₁r antibody binds to the full length NK₁r, but does not bind to the NK₂ or NK₃ tachykinin receptors, or to the C-terminus truncated form of the NK₁r (Grady *et al.*, 1996). Synaptophysin is a synaptic vesicle protein. It is in high concentrations in varicosities that surround nerve cells and was used to indicate the location of nerve cell surfaces. Tissues were washed in PBS (3 × 10 min) then incubated for 2 h at room temperature in PBS containing biotinylated horse anti-mouse antiserum (Vector Laboratories, code H0508; 1 : 100). Preparations were again washed in PBS (3 × 10 min), and incubated for 90 min in PBS containing streptavidin-Texas red complex (Amersham, Australia, batch No 31; 1 : 50) and sheep anti-rabbit antiserum coupled to fluorescein isothiocyanate (FITC) (Wellcome Laboratories, Australia, code K0722; 1 : 200). Tissue was washed in PBS (3 × 10 min) and mounted on microscope slides in buffered glycerol (pH 8.6).

Confocal microscopy

Samples were viewed on a Biorad MRC1024 confocal scanning laser system with a krypton/argon laser, installed on a Zeiss Axioplan 2 microscope. Fluorophores were visualized using a 488 nm excitation filter and 522–535 nm emission filter for FITC and a 568 nm excitation filter and 605–632 nm emission filter for Texas red. Single confocal images (optical section, 0.5 µM), that included the nucleus and a large area of cytoplasm, were taken through the centre of NK₁r-immunoreactive neurons chosen at random. Light emitted as green (NK₁r-IR) or red (synaptophysin) fluorescence was collected simultaneously using a 100 × objective (NA 1.4) and 1.4 zoom. Images of 512 × 512 pixels were obtained and processed using Confocal Assistant, Adobe Photoshop and Corel Draw software.

Fluorescence quantitation

Subcellular distribution of NK₁r-IR was quantified in imaged neurons, using NIH-Image software (<http://rsb.info.nih.gov/nih-image/>) as previously described (Southwell *et al.*, 1998a,b). Nuclear fluorescence was measured and this value was set as a threshold for measuring specific staining. Total cell and cytoplasmic FITC fluorescence were measured by counting the number of pixels with intensity above threshold in the whole cell and then in the cytoplasm only, and the percentage of fluorescence attributable to endocytosed NK₁r-IR was calculated.

Data analysis

Ten cells were imaged per tissue and each experiment was performed 3–4 times on tissue from different animals. Differences between groups were compared by two-way ANOVA followed by Student-Newman-Keuls test using Sigma Stat software. A *P* value < 0.05 was taken as significant. Antagonist activities were quantified as pA₂ values determined by the intercepts on the abscissa of Schild plots (Arunlakshana & Schild, 1959). The values determined are described as apparent pA₂ values as only two data points are used for each calculation.

Drugs

Bacitracin (Calbiochem, Australia); chymostatin (Sigma, U.S.A.); cyclo (Leuψ[CH₂NH]Lys(benzyloxycarbonyl)-Gln-

Trp-Phe- β Ala) (MEN-10581; Menarini, Italy); leupeptin (Auspep, Australia); (2S,3S)-3-(2-methoxybenzyl)amino-2-phenylpiperidine dihydrochloride (CP-99994; Pfizer, U.S.A.); monensin (Sigma, U.S.A.); nicardipine (Sigma, U.S.A.); septide (Auspep, Australia); substance P (SP; Auspep, Australia); tetrodotoxin (Alomone, Israel).

Results

Agonist-induced endocytosis of NK₁r

In tissues not exposed to agonist, $80.9 \pm 1.6\%$ of NK₁r-IR was present as a smooth band on the plasma membrane of a subpopulation of nerve cells in myenteric ganglia and the residual $19.1 \pm 1.6\%$ was located in small aggregations throughout the cytoplasm (Figure 1A). Both SP and septide caused concentration-dependent endocytosis of NK₁r-IR in the nanomolar range (Figure 2). As agonist concentration was increased, the proportion of NK₁r-IR on cell membranes was progressively reduced with a concomitant increase in the number of NK₁r-IR aggregations in the cytoplasm (Figure 1B–E, Figure 2). At concentrations of SP or septide of $1 \mu\text{M}$ or higher, most NK₁r-IR was present in aggregations distributed evenly throughout the cytoplasm of the cell; the continuous band of immunoreactivity was no longer evident on the surfaces of cells (Figure 1C and E). Both agonists produced a maximum receptor endocytosis of approximately 75% at $10 \mu\text{M}$, the highest concentration used, with the remaining 25% being present on, or very near, the cell surface (Figure 2). The effects of SP and septide on the subcellular distribution of NK₁r did not differ either qualitatively (Figure 1) or quantitatively (Figure 2). The two agonists induced NK₁r endocytosis with equivalent potency (EC_{50} : $\text{SP} = 5.6 \pm 1.1 \text{ nM}$;

septide = $8.5 \pm 1.9 \text{ nM}$). Nonlinear regression analysis of agonist concentration-response curves produced Hill coefficients that did not differ between SP (0.43 ± 0.10) and septide (0.45 ± 0.11).

Effects of NK₁r antagonists on receptor endocytosis

We investigated the effects of the NK₁r antagonists, CP-99994 and MEN-10581, on agonist-induced receptor endocytosis. Neither CP-99994 (10 pM – 10 nM) nor MEN-10581 (10 nM – $3 \mu\text{M}$) induced NK₁r endocytosis in myenteric neurons (Figure 4A and B), confirming our previous observations that endocytosis is dependent on the activation of receptor by agonist, but is not induced when antagonists bind to the receptor (Southwell *et al.*, 1996). Both CP-99994 and MEN-10581 markedly inhibited receptor endocytosis induced by either SP or septide (Figure 4C–F), producing rightward shifts in the concentration-response curves to the two agonists (Figure 4). Both antagonists were markedly more potent inhibitors of responses to septide than they were of responses to SP (Figures 3 and 4).

Schild analysis of antagonist activities produced apparent pA_2 values of 10.2 and 11.9 for CP-99994 acting against SP and septide, respectively, and 7.5 and 8.4 for MEN-10581 acting against SP and septide, respectively (Figure 4E and F), indicating that the antagonists were $50 \times$ (CP-99994) and $8 \times$ (MEN-10581) more potent against septide than against SP. Slopes of the Schild plots were close to unity: for CP 99994, -0.96 versus SP and -1.02 versus septide; for MEN-10581, -1.15 versus SP and -0.94 versus septide. These slopes are consistent with agonists and antagonists interacting competitively. Both antagonists have been shown in smooth muscle contractility studies to be competitive antagonists at the NK₁r (Longmore *et al.*, 1994; Zeng & Burcher, 1994; Patacchini *et al.*, 1995; Nguyen-Le *et al.*, 1996). However, in the present study the agonist concentration-response curves became steeper in the presence of MEN-10581, suggesting an allosteric interaction may occur for this antagonist.

Figure 1 Agonist-induced endocytosis of NK₁ receptor immunoreactivity (NK₁r-IR) in myenteric neurons from guinea-pig ileum. Each micrograph is a single $0.5 \mu\text{m}$ thick optical section obtained by confocal microscopy. The dark areas are the cell nuclei. Neurons were incubated with: (A), no agonist; (B), 10 nM substance P (SP); (C), $1 \mu\text{M}$ substance P; (D), 10 nM septide; and (E), $1 \mu\text{M}$ septide. Prior to agonist exposure, NK₁r-IR is at the cell surface (arrows). Both substance P and septide induced a concentration-dependent endocytosis of NK₁ receptors. Scale bar, $20 \mu\text{m}$.

Figure 2 Concentration-response curves for NK₁ receptor endocytosis induced by substance P (SP) and septide in myenteric neurons from guinea-pig ileum. The subcellular distribution of NK₁ receptor immunoreactivity in imaged neurons was measured using quantitative confocal microscopy. Both substance P and septide induced a concentration-dependent endocytosis of NK₁ receptors. The effects of substance P and septide did not differ from each other ($P > 0.05$). Values are means $\pm$ s.e.mean. ($n = 3$; 30 cells, three experiments, ten cells were imaged for each experiment).

Figure 3 Confocal images (single 0.5 μ m optical sections) showing the effects of the NK₁ receptor antagonists CP-99994 and MEN-10581 on endocytosis of NK₁ receptor immunoreactivity (NK₁r IR) induced by substance P (SP) or septide in myenteric neurons from guinea-pig ileum. Neurons were incubated with: (A), 100 nM SP; (B), 100 nM septide; (C), 100 nM SP and 10 nM CP-99994; (D), 100 nM septide and 100 pM CP-99994; (E) 100 nM SP and 3 μ M MEN-10581; and (F), 100 nM septide and 100 nM MEN-10581. Both CP-99994 and MEN-10581 inhibited NK₁ receptor endocytosis induced by SP or septide. Both antagonists were markedly more potent when acting against septide than against SP. Scale bar, 20 μ m.

As NK₃ receptors almost certainly exist on guinea-pig myenteric neurons (Johnson *et al.*, 1998), we tested the possibility that interaction of SP with NK₃ receptors may influence our results. Segments of guinea-pig ileum were incubated with SP in the presence of the NK₃ receptor antagonist SR-142801 to block binding to NK₃ receptor. This did not alter the percentage of NK₁r endocytosed in response to SP ($n=2$, data not shown).

Discussion

The present study investigated ligand-induced endocytosis of NK₁r on myenteric neurons of the guinea-pig ileum and demonstrates that the neurotransmitter, SP, and its artificial analogue, septide, induce endocytosis of the NK₁r with equivalent potency. Endocytosis of the NK₁r resulted from agonist binding to receptors and was not induced by binding of the selective NK₁r antagonists CP-99994 or MEN-10581. Analysis of agonist-induced endocytosis of NK₁r produced sigmoidal log concentration-response curves, equivalent in form to those obtained in conventional pharmacological studies (Figure 2). Moreover, the agonist concentrations producing receptor endocytosis were in the nanomolar range, similar to the concentrations with which these agonists contract smooth muscle preparations (Laufer *et al.*, 1988; Petitet *et al.*, 1992; Hall *et al.*, 1994), including the guinea-pig ileum (Carruette *et al.*, 1992; Nguyen-Le *et al.*, 1996), with which they stimulate inositol phosphate formation in transfected cells (Sagan *et al.*, 1996) and which is observed in homologous binding studies (Hastrup & Schwartz, 1996). As this technique produces agonist concentration-response data

Figure 4 Concentration-response curves for the effects of CP-99994 (A and C) and MEN-10581 (B and D) on endocytosis of NK₁ receptor immunoreactivity (NK₁r IR) induced by substance P (SP) (A and B) or septide (C and D) in myenteric neurons from guinea-pig ileum. Both CP-99994 and MEN-10581 inhibited receptor endocytosis induced by either SP or septide. Both antagonists were more potent inhibitors of NK₁r endocytosis induced by septide than of that induced by SP. Values are means $\pm$ s.e. mean ($n=3-4$; 30 or 40 cells, 3-4 experiments, ten cells were imaged for each experiment). (E and F): Schild plots for the antagonism by CP-99994 (E) and MEN-10581 (F) against septide and SP. Scale on ordinate is log concentration ratio minus 1, where the concentration ratio ($[A']/[A]$) is the ratio of the EC₅₀ concentrations for the agonist in the presence and absence of antagonist.

similar to that obtained by more established methods, we suggest that quantitative confocal microscope analysis of receptor endocytosis provides a useful new method for pharmacological analysis of receptor activation. The current technique has the advantages that the receptor population is visualized by specific antibody probes and the analysis is performed at the single cell level.

The competitive antagonists, CP-99994 and MEN-10581, differentially inhibited endocytosis of the NK₁r induced by nanomolar concentrations of SP or septide. There are two possible explanations for this finding: either the NK₁r antibody recognizes both the NK₁r and the proposed 'septide-sensitive' receptor; or SP and septide bind differently in the nanomolar concentration range to a single NK₁r population. The NK₁r antibody used in this study was raised against the 15 C-terminal amino acids of the rat NK₁r, and binds the full-length NK₁r, but not the NK₂ or NK₃ receptors or the C-terminal

truncated form of the NK₁r (Grady *et al.*, 1996). It is possible that a 'septide-sensitive' receptor exists that differs to a small extent from the NK₁r in a ligand binding domain(s) but not in the C-terminus; however, this seems unlikely as receptor cloning studies have failed to identify such a receptor (Glowinski, 1995; Maggi & Schwartz, 1997). Moreover, homologous binding data from cells transfected with cloned human NK₁r demonstrates that SP and septide bind differently to this receptor (Hastrup & Schwartz, 1996).

The hypothesis that SP and septide bind differently to the NK₁r at nanomolar concentrations is supported by earlier smooth muscle contractility studies, although the earlier studies could not distinguish between there being two receptors or two states for one receptor. The contractility studies demonstrated that NK₁r antagonists are as much as 16 times more potent against septide than against SP when both agonists are used in nanomolar concentrations. Furthermore, septide is about 200 times less effective than SP in displacing (Pro⁹)-SP (an agonist which behaves like SP (Hall *et al.*, 1994; Sagan *et al.*, 1996)). Hastrup & Schwartz (1996) used a homologous binding assay to identify two agonist binding affinities at the NK₁r, subnanomolar binding of SP, but not septide, and nanomolar binding of SP and septide. In view of the present results, we suggest that the NK₁r exhibits three different agonist binding properties: subnanomolar binding of SP, but not septide; nanomolar binding of septide; and nanomolar binding of SP, all of which have been shown to cause receptor activation (Laufer *et al.*, 1988; Petitet *et al.*, 1992; Pradier *et al.*, 1993; Sagan *et al.*, 1996). The last two binding properties are distinguished by the differential potency of antagonists acting against nanomolar concentrations of SP and septide in this study and in previous studies (Petitet *et al.*, 1992; Longmore *et al.*, 1994; Zeng & Burcher, 1994; Patacchini *et al.*, 1995; Nguyen-Le *et al.*, 1996), and by the low potency of septide in displacing nanomolar binding of (Pro⁹)-SP and SP (Petitet *et al.*, 1992; Pradier *et al.*, 1993; Sagan *et al.*, 1996).

Receptor mutagenesis demonstrates that the same amino acid residues are necessary for binding SP and septide to the NK₁r (Huang *et al.*, 1994; 1995). In fact, subnanomolar binding of SP and nanomolar binding of both agonists were completely abolished by either of two point mutations of the receptor (at N85 and N89), demonstrating that all three binding properties of the receptor result from interaction with the same binding site. The observation that subnanomolar SP displaces nanomolar concentrations of septide (Hastrup & Schwartz, 1996) supports this conclusion. However, the relative involvement of other residues (Y92 and N96) in agonist binding varies markedly between SP and septide, suggesting that the two agonists fit differently into this common binding site. Binding domains for small non-peptide antagonists, such as those used in the present work, only partly overlap with the agonist binding region (Huang *et al.*, 1994; 1995). Consequently, the region of overlap between antagonist binding and binding of SP may be different from the overlap between antagonist and septide; this difference in spatial overlap may partly explain differences in pA₂ values for antagonism of septide and SP.

Differences in agonist binding to a shared region may also involve differences in compatibility of the agonists with different conformations of the NK₁r (Huang *et al.*, 1995; Maggi & Schwartz, 1997). This is suggested by experiments on the superior cervical ganglion, where SP and septide have

similar nanomolar potency. At 37°C, the NK₁r antagonist, GR82334, is about 12 times more potent against septide than against SP, whereas at 22°C the difference is only 2 fold (Hawcock *et al.*, 1995). Thus, lower temperature may favour a conformation that increases the interaction of the antagonist with SP binding. Similarly, the effects of stable GTP analogues on SP binding to transfected and native NK₁r is consistent with agonist binding being influenced by receptor conformation (Aharony *et al.*, 1991; Cascieri *et al.*, 1992). In the absence of GTP analogues, SP bound the receptor with single subnanomolar affinity, whereas in their presence the majority of binding was at nanomolar affinity (Aharony *et al.*, 1991; Cascieri *et al.*, 1992).

The present results allow us to extend the proposal of Maggi & Schwartz (1997) that a single site on the NK₁r exhibits two conformations, the subnanomolar SP-preferring conformer and the nanomolar general tachykinin conformer. We suggest that septide and SP fit differently into the general tachykinin conformer. This would explain previous data showing differences in agonist displacement kinetics and antagonist potencies when both agonists are used at nanomolar concentrations. Thus, it is not necessary to posit the existence of the elusive 'septide-preferring' tachykinin receptor.

The pA₂ values calculated for CP-99994 and MEN-10581 in the present study are higher than those previously reported for either antagonist acting at the NK₁r (Longmore *et al.*, 1994; Patacchini *et al.*, 1995; Nguyen-Le *et al.*, 1996). The equilibration period used for antagonists in the present study (60 min) was considerably longer than that used by Patacchini *et al.* (1995) for MEN-10581 (15 min), and by Nguyen-Le *et al.* (1996) for CP-99994 (7–8 min). If the equilibration period used in these previous studies was insufficient to allow the antagonists to reach an equilibrium with the NK₁r prior to addition of agonist, this may help explain why the antagonists appear more potent in the present study. However, Longmore *et al.* (1994) equilibrated CP-99994 for the same time used in the present study, and so this cannot explain why CP-99994 was more potent in this study than in that by Longmore *et al.* (1994).

The concept that the pA₂ value for a particular antagonist acting at a receptor is constant, and is the same against all agonists for the receptor, has been a fundamental tenet of receptor classification (Arunlakshana & Schild, 1959; Bowman & Rand, 1988). In fact, it is often on the basis of different pA₂ values that sub-types of receptor have been identified. The current work suggests that antagonists may exhibit two pA₂ values at the NK₁r. Thus it may not be possible to define the NK₁r by a unique pA₂ value for a given antagonist.

In conclusion, the present study has demonstrated that SP and septide are equipotent agonists at the NK₁r, and that the competitive NK₁r antagonists, CP-99994 and MEN-10581, differentially inhibit receptor endocytosis induced by each agonist. These findings suggest that SP and septide bind differently to the NK₁r in the nanomolar concentration range.

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