Pharmacological analysis of [³H]-senktide binding to NK₃ tachykinin receptors in guinea-pig ileum longitudinal muscle-myenteric plexus and cerebral cortex membranes

*¹Steven Guard, *Stephen P. Watson, †John E. Maggio, †H. Phon Too & ‡Keith J. Watling

*University Department of Pharmacology, South Parks Road, Oxford OX1 3QT; †Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, U.S.A. and ‡Merck Sharp & Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Harlow, Essex CM20 2QR

1 The binding properties and pharmacological specificity of the selective NK₃ tachykinin receptor agonist [³H]-senktide ([³H]-succinyl[Asp⁶,MePhe⁸] substance P (6–11)) have been examined in homogenates of guinea-pig ileum longitudinal muscle-myenteric plexus (LM/MP) and cerebral cortex.

2 Scatchard analysis of saturation binding studies in guinea-pig ileum LM/MP and cerebral cortex membranes indicated that [³H]-senktide bound to a single site with apparent high affinity, $K_D = 2.21 \pm 0.65$ nM; $B_{max} = 13.49 \pm 0.04$ fmol mg⁻¹ protein in ileum and $K_D = 8.52 \pm 0.45$ nM; $B_{max} = 76.3 \pm 1.6$ fmol mg⁻¹ protein in cortex (values are means $\pm$ ranges; $n = 2$).

3 The pharmacological profile for tachykinins and analogues in displacing [³H]-senktide from ileum membranes was: [MePhe⁷] neurokinin B > neurokinin B (NKB) $\approx$ senktide > eledoisin > substance P (SP) > neurokinin A (NKA) > physalaemin > [Sar⁹,Met(O₂)¹¹]SP > [Nle¹⁰]NKA(4–10) = [Glp⁶,L-Pro⁹]SP(6–11) > substance P methyl ester, consistent with [³H]-senktide binding to an NK₃ subtype of tachykinin receptor. A similar rank order of affinity was obtained for these peptides in displacing [³H]-senktide from cortex membranes.

4 Several tachykinin receptor agonists were tested for their ability to displace [³H]-senktide from ileal and cortical NK₃ binding sites and were found to be either weak displacers ($pIC_{50} < 5.00$) or inactive.

5 The binding of [³H]-senktide to cortex membranes was inhibited by GTP ($pIC_{50} = 6.49$) and GTP- γ -S ($pIC_{50} = 6.67$) with ATP being at least three orders of magnitude less potent ($pIC_{50} = 3.55$).

6 These results indicate that both central and peripheral NK₃ receptors share a similar pharmacological specificity and that they may be labelled selectively with the NK₃ receptor agonist [³H]-senktide.

Introduction

Substance P (SP) and the recently discovered mammalian neuropeptides, neurokinin A (NKA) and neurokinin B (NKB), belong to a family of peptides known as tachykinins, the biological actions of which are mediated by multiple receptors (for reviews see: Watson, 1984; 1987; Regoli *et al.*, 1987a; Quirion & Dam, 1988). The current classification of tachykinin receptors, based primarily on relative potencies of agonists in various bioassays and radioligand binding assays, recognizes three subtypes: NK₁, NK₂ and NK₃ (Henry *et al.*, 1987) where SP, NKA and NKB appear to be the preferred agonists respectively, although each peptide can activate all three receptor types. Alternative tachykinin receptor nomenclature and classification has been discussed elsewhere (see Mantyh *et al.*, 1989).

NK₃ receptors were identified as the tachykinin receptor type which mediates the release of acetylcholine from myenteric plexus neurones of the guinea-pig ileum (Laufer *et al.*, 1985). NKB is approximately two orders of magnitude more potent than NKA and SP in activating this neuronal tachykinin receptor in the guinea-pig ileum (Wormser *et al.*, 1986). A similar rank order of potency, indicative of an NK₃ receptor, is observed for tachykinin-induced contraction of the rat portal vein (Mastrangelo *et al.*, 1986; McKnight & Maguire, 1987).

The presence of NK₃ tachykinin binding sites in the mammalian brain was first demonstrated with [¹²⁵I]-Bolton-Hunter conjugated eledoisin ([¹²⁵I]-BHE; Cascieri & Liang, 1984; Beaujouan *et al.*, 1984). In these studies [¹²⁵I]-BHE

labelled a tachykinin binding site which was distinct from that labelled with [¹²⁵I]-BH-substance P ([¹²⁵I]-BHSP), or [³H]-SP, both in terms of pharmacology and distribution. Subsequent radioligand binding studies showed that [¹²⁵I]-BHE and [³H]-NKB labelled the same site in rat brain and exhibited identical pharmacological profiles and distribution (Bergström *et al.*, 1987).

The presence of NK₃ receptors has been further confirmed by the development of a highly selective agonist, senktide (succinyl-[Asp⁶,MePhe⁸]SP(6–11)). This analogue of substance P has been shown to be extremely selective for NK₃ receptors while having little, if any, activity at NK₁ or NK₂ receptors (Wormser *et al.*, 1986). The Bolton Hunter-conjugated analogue of senktide ([¹²⁵I]-BH-[Asp^{5,6},MePhe⁸]SP(6–11)) has been synthesized and shown to label a tachykinin binding site in rat cerebral cortex indicative of an NK₃ receptor (Laufer *et al.*, 1986).

Although the existence of NK₃ receptors is now widely accepted, previous authors have been unable to detect NK₃ binding sites in peripheral tissues using a range of radioligands including [¹²⁵I]-BHE, [¹²⁵I]-BH-NKB, [³H]-NKB, 2-[¹²⁵I]-iodohistidyl-NKB, or [¹²⁵I]-BH-senktide (Burcher *et al.*, 1986; Mantyh *et al.*, 1988; 1989). This may not be surprising, given the low selectivity of many of these ligands for NK₃ receptors in addition to the possibility that the densities of NK₃ sites in peripheral tissues may be quite low. In this study, therefore, we have used [³H]-senktide as a selective radioligand for the NK₃ receptor in an attempt to make a direct comparison of both peripheral (myenteric plexus) and central (cerebral cortex) NK₃ tachykinin receptors. The pharmacological specificity of these sites has been determined with a range of naturally occurring tachykinins and several tachykinin receptor agonists and antagonists shown to be selective

¹ Author for correspondence at present address: Douglas Hospital Research Centre, 6875 Boulevard Lasalle, Verdun, Québec, Canada H4H 1R3.

for the NK₁, NK₂ or NK₃ subtype of receptor. In addition, the affinities of these analogues and naturally occurring tachykinins for [³H]-senktide binding sites have been compared with their biological potencies in the rat portal vein bioassay (Drapeau *et al.*, 1987; McKnight & Maguire, 1987). A preliminary account of this work has been presented to the British Pharmacological Society (Guard *et al.*, 1989).

Methods

Preparation of ileal longitudinal muscle-myenteric plexus membranes

Ileal longitudinal muscle-myenteric plexus membranes (LM/MP) were prepared from Hartley strain guinea-pigs of either sex (300–500 g), essentially as previously described (Watson & Iversen, 1984) with minor modifications. Tissue from several animals was pooled and homogenised in 10 vol of ice-cold 20 mM HEPES buffer (pH 7.4) by polytron at position 7 for 10–15 s. The homogenate was centrifuged at 48,000 *g* for 30 min at 4°C. The pellet was resuspended in 20 mM HEPES buffer (pH 7.4) by polytron at position 7 for 5 s and incubated on ice in the presence of 300 mM KCl and 10 mM EDTA for 30 min with intermittent mixing. The homogenate was then filtered through nylon mesh (250 × 250 µm) and the filtrate centrifuged at 48,000 *g* for 30 min. The final pellet was resuspended in Krebs-HEPES buffer (pH 7.4), composition (mM): NaCl 120, KCl 4.8, CaCl₂ 1, MgSO₄ 1, HEPES 20, to a protein concentration of 2–3 mg ml⁻¹. Membranes were used immediately or stored in aliquots at -20°C. Protein concentration was determined by the method of Bradford (1976) with bovine serum albumin (BSA) as a standard.

Preparation of cerebral cortex membranes

Cerebral cortex membranes were prepared from Hartley strain guinea-pigs of either sex (300–500 g) essentially as described by Laufer *et al.* (1986). Brains were dissected on ice and cerebral cortices placed in ice-cold 10% sucrose (w/v). Pooled tissues were homogenised in 10 vol of 10% sucrose using a teflon glass homogeniser (10–12 strokes, 800 r.p.m.). The homogenate was centrifuged at 1,600 *g* for 10 min and the resulting supernatant was centrifuged at 20,000 *g* for 10 min. The crude synaptosomal pellet was lysed by resuspension (polytron, position 7 for 5 s) in 10 vol of 5 mM HEPES, pH 7.5. This suspension was centrifuged at 30,000 *g* for 30 min. The pellet was resuspended in the same buffer and centrifuged at 30,000 *g* for a further 30 min. The final pellet was resuspended in Krebs-20 mM HEPES buffer, pH 7.4 (composition as described above) to give a protein concentration of 2–3 mg ml⁻¹.

Radioligand binding assays

Binding assays were performed in polypropylene tubes containing, in a final volume of 0.2 ml, assay buffer (Krebs-20 mM HEPES plus 0.1% BSA, pH 7.4) 2 mM MnCl₂, 2 µg ml⁻¹ chymostatin, 4 µg ml⁻¹ leupeptin, 40 µg ml⁻¹ bacitracin, [³H]-senktide (1.3–1.5 nM), membranes (~300 µg protein) and various test substances. After incubation at 20°C for 60 min, the reaction was terminated by the addition of 2 ml ice-cold assay buffer containing 2 mM MnCl₂, 0.05% BSA and 0.01% sodium dodecyl sulphate (SDS). Membranes were then filtered, using a Brandel Cell Harvester, through Whatman GF/C filters (presoaked in a solution of 0.3% polyethylenimine/0.5% Triton X-100 for 1–2 h) and washed 3 times with 3 ml of ice-cold assay buffer containing 2 mM MnCl₂, 0.05% BSA and 0.01% SDS. Non-specific binding was estimated from determinations in the presence of 1 µM non-radioactive senktide. Bound radioactivity was measured by liquid scintillation spectrometry.

Data analysis

The ability of natural and synthetic tachykinins to inhibit the binding of [³H]-senktide was determined using 6–9 test concentrations (0.1 nM–100 µM) assayed in duplicate or triplicate. Data were analysed by nonlinear regression using an iterative curve fitting programme (GraphPAD, ISI Software).

Saturation analyses of [³H]-senktide binding to ileum LM/MP and cerebral cortex membranes were carried out over a concentration range of 0.10–100 nM. At concentrations greater than 2.0 nM, [³H]-senktide was supplemented with non-radioactive peptide. Equilibrium dissociation constants (*K_d*) and maximum number of binding sites (*B_{max}*) were determined by non-linear regression by the above curve fitting programme.

Degradation of [³H]-senktide

[³H]-senktide (1.5 nM) was incubated with ileal or cortical membranes (~300 µg tissue per tube) in assay buffer with or without the peptidase inhibitors bacitracin (40 µg ml⁻¹), leupeptin (4 µg ml⁻¹) and chymostatin (2 µg ml⁻¹) for 60 min at room temperature. Incubations were terminated by the addition of 1 ml 5% acetonitrile-0.1% trifluoroacetic acid followed by centrifugation at 10,000 *g* for 5 min. Approximately 1 nmol of non-radioactive carrier senktide was added to the supernatant and a sample then examined by h.p.l.c. The stability of [³H]-senktide was determined by reverse phase h.p.l.c. on a µBondapak C₁₈ column (3.9 × 300 mm). Parallel incubations were performed with [¹²⁵I]-BHSP, instead of [³H]-senktide, in the presence or absence of peptidase inhibitors as a control for enzyme activity. The peptides were eluted with a linear gradient: 25–41% B over 16 min at 1 ml min⁻¹. In this system, solvent A was 0.01 M trifluoroacetic acid/water and solvent B was 0.01 M trifluoroacetic acid/acetonitrile. Thirty second fractions were collected and radioactivity counted.

Materials

Substance P, eledoisin, neurokinin B and physalaemin were purchased from Cambridge Research Biochemicals, U.K. Neurokinin A and neuropeptide K were obtained from Peninsula Laboratories, U.K. Substance P methyl ester and [D-Pro⁴,D-Trp^{7,9,10}]SP(4–11) were obtained from Bachem Biochemicals, Basel, Switzerland. [Sar⁹,Met(O₂)¹¹]SP, [Nle¹⁰]NKA(4–10) and [MePhe⁷]NKB were purchased from IAF Biochem International, Québec, Canada. Senktide (succinyl-[Asp⁶,MePhe⁸]SP(6–11)), cyclo[Gln,Trp,Phe,Gly,Leu,Met] (L-659,877), Ac-Leu,Met,Gln,Trp, Phe.NH₂(L-659,874) and [Glp⁶,L-Pro⁹]SP(6–11), were synthesized and characterized by Dr B.J. Williams at Merck Sharp and Dohme Research Laboratories, Harlow, U.K. [Gly⁶]NKB(3–10) and [Arg³,D-Ala⁶]NKB(3–10) were a kind gift from Prof. Hashimoto, Kanazawa, Japan. Guanine and adenine nucleotides were obtained from Sigma, Poole, U.K. [³H]-senktide (specific activity 50 Ci mmol⁻¹ labelled at the N-methyl group, supplied in ethanol containing 5 mM β-mercaptoethanol) was a kind gift from New England Nuclear, Boston, U.S.A. [¹²⁵I]-BH-substance P (2,200 Ci mmol⁻¹) was obtained from Amersham International.

Results

Degradation of [³H]-senktide by ileal and cortical membranes

Chromatographic analysis of [³H]-senktide incubated at 20°C with either ileal or cortical membranes, in the absence of peptidase inhibitors, indicated that approximately 92% of the radioactivity eluted in the same position as unlabelled senktide. In the presence of peptidase inhibitors, degradation of [³H]-senktide was almost completely prevented in both ileum and cortex with >98% of the radioactivity co-eluting with standard senktide. This same cocktail of peptidase inhibitors

Figure 1 Association time course of [^3H]-senktide specific binding to (a) guinea-pig ileum longitudinal muscle-myenteric plexus (LM/MP) and (b) cerebral cortex membranes. Membranes ($\sim 300 \mu\text{g}$ protein) were incubated with radioligand (2.5 nM) and incubations terminated at various times by filtration. Non-specific binding (in the presence of $1 \mu\text{M}$ non-radioactive senktide) was determined simultaneously for all the time points. Insets: plots of $\ln(B_{\text{eq}}/B_{\text{eq}} - B_t)$ versus time, where B_{eq} = amount of [^3H]-senktide specifically bound at equilibrium and B_t = amount specifically bound at time t .

also effectively inhibited the metabolism of [^{125}I]-BHSP (not shown).

Kinetic characteristics of [^3H]-senktide binding

The association time courses for the binding of [^3H]-senktide to guinea-pig ileal LM/MP and cerebral cortex membranes are shown in Figure 1a and b respectively. The specific binding of [^3H]-senktide increased with time, reaching equilibrium after 30 min of incubation at 20°C and remained stable for 60 min. As the total binding did not exceed 10% of the amount of [^3H]-senktide added, and there was no degradation of the radioligand detectable by h.p.l.c., the data were linearised according to a pseudo first order rate equation (Weiland & Molinoff, 1981). Plots of $\ln(B_{\text{eq}}/B_{\text{eq}} - B_t)$ versus time are shown (insets to Figure 1a and b) where B_{eq} is the amount of [^3H]-senktide specifically bound at equilibrium and B_t is the amount specifically bound at time t . The apparent association rate constants for ileum and cortex sites were calculated to be 0.24 min^{-1} and 0.15 min^{-1} , respectively. [^3H]-senktide specific binding was reversible as shown by the dissociation of the radioligand following addition of $1 \mu\text{M}$ non-

Figure 2 Dissociation time course of [^3H]-senktide specific binding to (a) guinea-pig ileum LM/MP and (b) cerebral cortex membranes. Membranes were incubated with radioligand for 45 min and senktide was then added to a final concentration of $1 \mu\text{M}$ (at zero time). The reaction mixture was diluted and filtered at various times thereafter. Each time point represents the mean of triplicate determinations (with s.e.mean $< 10\%$) from a single representative experiment which was repeated one further time with similar results. Insets: data plotted as a first order reaction according to the formula $\ln(B_t/B_0) = -k_{-1}t$, where B_0 represents the amount of radioligand specifically bound before dissociation and B_t is the amount bound at various times t . The fast phase of dissociation ($\circ$) was obtained following subtraction of the contribution of the slow phase ($\bullet$).

radioactive senktide (Figure 2a and b). The dissociation curves were curvilinear and were best fitted to a double exponential decay equation using the curve fitting programme Graph-PAD. The insets to Figure 2a and b show the data plotted as a first order reaction according to the formula $\ln(B_t/B_0) = -k_{-1}t$ where B_0 represents the amount of radioligand specifically bound before dissociation and B_t is the amount bound at various times t . The receptor-ligand dissociation comprised two distinct phases. The initial fast phase of dissociation was calculated after subtraction of the contribution of the slow phase and had a dissociation rate (k_{-1}) of 0.48 min^{-1} ($t_{1/2} = 1.26 \text{ min}$) and 0.30 min^{-1} ($t_{1/2} = 0.78 \text{ min}$) in ileum and cortex, respectively. The second phase was much slower: $k'_{-1} = 0.004 \text{ min}^{-1}$ ($t_{1/2} = 54.7 \text{ min}$) and $k'_{-1} = 0.010 \text{ min}^{-1}$ ($t_{1/2} = 64.2 \text{ min}$) in ileum and cortex, respectively. Similar biphasic dissociations have been described for the binding of [^{125}I]-BHE and [^3H]-NKB to NK_3 receptors in rat cerebral cortex membranes (Beaujouan *et al.*, 1984; Bergström *et al.*, 1987). Since the dissociation time courses were biphasic, estimates of the kinetic K_D values have not been calculated.

Figure 3 Saturation analysis of [^3H]-senktide specific binding to (a) guinea-pig cerebral cortex membranes and (b) ileum LM/MP membranes. Membranes ($\sim 300\mu\text{g}$ protein) were incubated with [^3H]-senktide over a concentration range of 0.10–100 nM. At concentrations greater than 2.0 nM [^3H]-senktide was supplemented with non-radioactive peptide. Each time point represents triplicate determinations from a single representative experiment which was repeated one further time with similar results.

Equilibrium binding studies

[^3H]-senktide specific binding represented 70–75% of total binding in ileum LM/MP and 80–85% of total binding in cortex. Non-specific binding to filters amounted to approximately 10% of the total binding and 0.2% of total radioactivity added. Equilibrium binding assays were performed using $\sim 300\mu\text{g}$ protein at room temperature ($20 \pm 2^\circ\text{C}$) for 45 min.

Saturation analysis of [^3H]-senktide binding (0.10–100 nM) in ileum LM/MP and cerebral cortex membranes showed that specific binding was saturable (Figure 3a and b). Scatchard analysis of saturation experiments, performed in parallel to ileum LM/MP and cerebral cortex membranes, showed that the binding of [^3H]-senktide in ileum was to a single site of high affinity, $K_D = 2.21 \pm 0.65\text{ nM}$ and $B_{\text{max}} = 13.49 \pm 0.04\text{ fmol mg}^{-1}$ protein, whereas in cerebral cortex [^3H]-senktide bound to a single site of $K_D = 8.52 \pm 0.45\text{ nM}$ and $B_{\text{max}} = 76.3 \pm 1.6\text{ fmol mg}^{-1}$ protein (means $\pm$ ranges of 2 separate experiments; Figure 4).

Effect of nucleotides

The effect of guanine and adenine nucleotides (10 nM–1 mM) on [^3H]-senktide specific binding to cerebral cortex membranes was examined using guanosine triphosphate (GTP), the non-hydrolysable analogue guanosine 5'-O-(3-thiotriphosphate) (GTP- γ -S), and adenosine triphosphate (ATP). GTP and

Figure 4 Scatchard analysis of the data shown in Figure 3; cortex ($\blacksquare$) and ileum ($\bullet$); see text for mean K_D and B_{max} values.

Table 1 Effect of GTP, GTP- γ -S, and ATP (30 nM–1 mM) on specific binding of [^3H]-senktide (1.5 nM) to guinea-pig cerebral cortex membranes

Nucleotide	% maximal inhibition	pIC_{50}
GTP	82.8 ± 5.8	6.49 ± 0.19
GTP- γ -S	83.1 ± 5.7	6.67 ± 0.09
ATP	57.6 ± 14.9	3.55 ± 0.12

Data are expressed as the percentage maximal inhibition of specific binding (defined by $1\mu\text{M}$ senktide) in the presence of 1 mM nucleotide, and as pIC_{50} values (the negative logarithm of the concentration required to inhibit 50% of the maximal displaceable binding). Values represent the mean $\pm$ s.e. mean of 4 separate determinations.

GTP- γ -S produced a concentration-dependent inhibition of [^3H]-senktide specific binding with similar pIC_{50} values, whereas ATP was approximately three orders of magnitude less potent (Table 1).

Pharmacological specificity of [^3H]-senktide binding

The naturally occurring mammalian tachykinins NKB, NKA and SP all produced a concentration-dependent inhibition of [^3H]-senktide binding in guinea-pig cerebral cortex (Figure 5b) with a rank order of affinity of $\text{NKB} > \text{NKA} \approx \text{SP}$. A similar rank order was observed in ileum (Figure 5a), NKB having between 25 and 80 times greater affinity than SP and NKA. Neuropeptide K displayed approximately one order of magnitude lower affinity than SP and NKA. The non-mammalian tachykinins, physalaemin and eledoisin also displaced [^3H]-senktide binding with eledoisin having approximately 100 times greater affinity than physalaemin. The affinities of these peptides and synthetic analogues for [^3H]-senktide binding sites in cortex and ileum are shown in Table 2. A strong correlation was observed between the affinities of tachykinins and synthetic analogues for myenteric plexus and cortical [^3H]-senktide binding sites ($r = 0.98$, Figure 6). A good correlation was also obtained between biological potencies of these peptides in the rat portal vein bioassay (data from McKnight & Maguire, 1987; Drapeau *et al.*, 1987) and their affinities for cortical ($r = 0.95$) and ileal sites ($r = 0.92$).

Of the synthetic analogues tested which exhibit receptor selectivity, a recently described NK_3 -selective agonist, [MePhe 7]NKB (Drapeau *et al.*, 1987), had approximately 4–5 times greater affinity than senktide itself in both cortex and ileum. The NK_2 receptor-selective analogue [Nle 10]NKA(4–10) (Drapeau *et al.*, 1987) was virtually inactive, as were the

Figure 5 Inhibition of [^3H]-senktide binding to (a) guinea-pig ileum LM/MP and (b) guinea-pig cerebral cortex membranes by neurokinin B ($\circ$), neurokinin A ($\bullet$) and substance P ($\square$). Curves were fitted using an iterative curve-fitting procedure described in the text and the values are the mean percentage inhibition of specific binding from 3–5 separate experiments.

Figure 6 Correlation between pIC_{50} values for inhibition of $[^3H]$ -senktide binding to guinea-pig cerebral cortex and ileum LM/MP membranes by tachykinins and analogues. pIC_{50} values were taken from Table 2. The line was fitted by linear regression and had a slope of 0.92. The correlation coefficient was 0.98. (1) $[MePhe^7]NKB$, (2) neurokinin B, (3) senktide, (4) eldoisin, (5) neurokinin A, (6) substance P, (7) physalaemin, (8) $[Sar^9, Met(O_2)^{11}]SP$, (9) $[Gly^6]NKB(3-10)$.

selective NK_1 agonists substance P methyl ester (Watson *et al.*, 1983), $[Glp^6, L-Pro^9]SP(6-11)$ (Wormser *et al.*, 1986) and $[Sar^9, Met(O_2)^{11}]SP$ (Drapeau *et al.*, 1987).

The affinities of several tachykinin receptor antagonists for NK_3 sites were also determined (Table 2). The non-selective NK_1/NK_2 antagonist $[D-Pro^4, D-Trp^{7,9,10}]SP(4-11)$ (Featherstone *et al.*, 1986) and the recently described selective NK_2 receptor antagonists cyclo-(Gln, Trp, Phe, Gly, Leu, Met) (L-659,877) and Ac-Leu, Met, Gln, Trp, Phe, NH_2 (L-659,874) (McKnight *et al.*, 1988) failed to produce any significant displacement of $[^3H]$ -senktide specific binding at concentrations up to $10 \mu M$. The putative NK_3 antagonists $[Gly^6]NKB(3-10)$ and $[Arg^3, D-Ala^6]NKB(3-10)$ (Hashimoto *et al.*, 1987) were similarly weakly active or inactive respectively.

Discussion

Until recently, some relatively non-selective radioligands have been used to label tachykinin binding sites. For example,

$[^{125}I]$ -BHE not only labels NK_3 sites, but also NK_2 (Lee *et al.*, 1986) and NK_1 sites (Lee *et al.*, 1986; Guard *et al.*, 1988a). Similarly, $[^{125}I]$ -BHNKA labels not only NK_2 sites (Buck *et al.*, 1984; Burcher & Buck, 1986), but also NK_3 (Foster & Tridgett, 1988) and NK_1 sites (Souquet *et al.*, 1987). Furthermore, recent attempts at labelling peripheral NK_3 sites with non-selective ligands such as $[^3H]$ -NKB and 2- $[^{125}I]$ -iodohistidyl-NKB (Mantyh *et al.*, 1988; 1989) have proved unsuccessful. In this study we have used the selective NK_3 receptor agonist $[^3H]$ -senktide to investigate NK_3 receptors in longitudinal muscle-myenteric plexus membranes rather than whole ileum, thereby effectively increasing the density of neuronal NK_3 sites. Thus, we have been able to characterize pharmacologically both peripheral and central NK_3 tachykinin binding sites.

Under the conditions used, $[^3H]$ -senktide bound with high affinity (nM) to both ileum LM/MP and cerebral cortex membranes as determined by Scatchard analysis of saturation data. In competition experiments, the naturally occurring tachykinins displaced $[^3H]$ -senktide binding from ileal and cortical high affinity sites with the following rank order of affinities: NKB > eldoisin > NKA $\geq$ SP > physalaemin $\geq$ NPK. This is in good agreement with the rank order of potencies observed for these peptides in the rat portal vein (Mastrangelo *et al.*, 1986; McKnight & Maguire, 1987) or the neuronal NK_3 receptor bioassay in the guinea-pig ileum (Laufer *et al.*, 1985). Indeed, the affinities of the naturally occurring tachykinins and synthetic analogues for $[^3H]$ -senktide binding sites correlate well with biological data in the literature, suggesting that these are functional receptor sites.

Further evidence that $[^3H]$ -senktide is labelling NK_3 receptors in these tissues comes from the use of receptor-selective agonists. The selective NK_1 agonists substance P methyl ester and $[Glp^6, L-Pro^9]SP(6-11)$ did not displace $[^3H]$ -senktide (1.3–1.5 nM) at concentrations up to $10 \mu M$. $[Sar^9, Met(O_2)^{11}]SP$, another NK_1 selective agonist, was weakly active at $[^3H]$ -senktide sites having approximately 500 times lower affinity than senktide itself. Conversely, and in agreement with these findings, we have previously shown that senktide is inactive at guinea-pig ileum longitudinal smooth muscle NK_1 sites labelled with $[^{125}I]$ -BHSP (Guard *et al.*, 1988a). The selective NK_2 agonist $[Nle^{10}]NKA(4-10)$ was also a very weak displacer of $[^3H]$ -senktide specific binding. In contrast, the NK_3 receptor agonist $[MePhe^7]NKB$ had approximately 5 times greater affinity than senktide, whilst NKB has similar affinity. These observations are consistent

Table 2 Affinities of natural and synthetic tachykinins for $[^3H]$ -senktide binding sites in guinea-pig ileal longitudinal muscle-myenteric plexus and guinea-pig cerebral cortex membranes

Peptide	Ileum		Cortex	
	pIC_{50}	nH	pIC_{50}	nH
Neurokinin B	8.43 (0.05)	0.86 (0.07)	8.35 (0.03)	0.72 (0.03)
$[MePhe^7]NKB$	9.05 (0.05)	1.24 (0.15)	8.71 (0.10)	1.09 (0.25)
Senktide	8.44 (0.03)	1.04 (0.07)	8.06 (0.02)	1.04 (0.04)
Eldoisin	8.11 (0.02)	0.78 (0.02)	7.39 (0.06)	0.77 (0.07)
Neurokinin A	6.53 (0.07)	0.59 (0.03)	6.85 (0.07)	0.59 (0.05)
Substance P	7.01 (0.05)	0.80 (0.06)	6.54 (0.24)	0.62 (0.06)
Physalaemin	6.13 (0.05)	0.72 (0.06)	5.92 (0.02)	0.77 (0.03)
$[Sar^9, Met(O_2)^{11}]SP$	5.68 (0.05)	0.71 (0.06)	5.42 (0.03)	0.80 (0.05)
SP methyl ester	< 5.00		< 5.00	
Neuropeptide K	ND		5.86 (0.007)	0.89 (0.12)
$[Nle^{10}]NKA(4-10)$	5.38 (0.04)	0.92 (0.09)	< 5.00	
$[Glp^6, L-Pro^9]SP(6-11)$	< 5.30		< 5.00	
$[Gly^6]NKB(3-10)$	4.82 (0.03)	0.90 (0.06)	4.74 (0.09)	0.67 (0.10)
$[Arg^3, D-Ala^6]NKB(3-10)$	< 4.00		< 4.00	
PTTT	< 5.00		< 5.00	
L-659,877	< 5.00		< 5.00	
L-659,874	< 5.00		< 5.00	

Data are expressed as pIC_{50} values (the negative logarithm of the molar concentration of peptide required to inhibit 50% of $[^3H]$ -senktide specific binding), nH is the Hill coefficient. Results are the means ($\pm$ s.e. mean) of 2–5 separate experiments performed in duplicate or triplicate; ND, not determined. PTTT: $[D-Pro^4, D-Trp^{7,9,10}]SP(4-11)$; L-659,877 = cyclo(Gln, Trp, Phe, Gly, Leu, Met); L-659,874 = AcLeu, Met, Gln, Trp, Phe, NH_2 .

with the relative potencies obtained for these analogues in the rat portal vein bioassay (Drapeau *et al.*, 1987).

The moderately potent NK₃ antagonists [Gly⁶]NKB(3–10) and [Arg³,D-Ala⁶]NKB(3–10) described recently by Hashimoto *et al.* (1987), were poor displacers of [³H]-senktide binding in both tissues. [Gly⁶]NKB(3–10) has been shown to be a specific, competitive antagonist of NKB-induced contraction of the guinea-pig ileum and rat portal vein (Hashimoto *et al.*, 1987). However, the low pIC₅₀ values obtained for this NKB analogue in displacing [³H]-senktide from cerebral cortex and ileum membranes (<5.00) is in contrast to its reported biological activity as an NK₃ receptor antagonist. The other putative NK₃ antagonist [Arg³,D-Ala⁶]NKB(3–10) failed to inhibit [³H]-senktide binding in either tissue up to 30 µM. This is in contrast to its antagonist activity against NKB in the guinea-pig ileum, but consistent with its lack of antagonist activity in the rat portal vein (Hashimoto *et al.*, 1987). The non-selective NK₁/NK₂ receptor antagonist [D-Pro⁴,D-Trp^{7,9,10}]SP(4–11) and the selective NK₂ antagonists L-659,877 and L-659,874 were unable to displace [³H]-senktide binding from either ileum or cortex membranes at concentrations up to 10 µM, in agreement with the lack of antagonist activity of these analogues in bioassays for NK₃ receptors (Featherstone *et al.*, 1986; McKnight *et al.*, 1988).

The neuronal NK₃ receptor in the guinea-pig ileum mediates not only acetylcholine release from myenteric plexus neurones (Laufer *et al.*, 1986), but also the release of a tachykinin-like substance (Guard & Watson, 1987). We have also recently provided evidence for the coupling of NK₃ receptors in the guinea-pig ileum to the hydrolysis of inositol phospholipids (Guard *et al.*, 1988b). Activation of phospholipase C by NK₃ receptors in this preparation most likely occurs via a GTP regulatory binding protein, since the binding of [³H]-senktide is inhibited by GTP and its analogue GTP-γ-S. More recently, Hanani *et al.* (1988) have demonstrated electrophysiologically that depolarisation of myenteric neurones following application of selective NK₃ analogues is associated with an increase in input resistance which reverses at about –90 mV, indicating that this response is likely to be due to the closure of potassium channels. Senktide is 20–100 times more potent than SP in depolarizing myenteric neurones, consistent with our affinity estimates for

senktide and SP at ileal NK₃ binding sites. Although the neuronal NK₃ receptor has been described as NKB-preferring (Laufer *et al.*, 1985), the origin of NKB which might act upon peripheral NK₃ receptors has not been established. Using specific radio-immunoassays we have been unable to detect NKB in extracts of whole guinea-pig ileum which contain significant concentrations of SP and NKA (Too *et al.*, 1989). In contrast, extracts of guinea-pig CNS tissues contain all three tachykinins (SP, NKA and NKB) in easily quantified amounts. Nonetheless, given that SP and NKA exhibit appreciable affinity for the NK₃ receptor and are apparently co-localised in myenteric neurones (Deacon *et al.*, 1987), it is conceivable that these peptides may be the endogenous ligands for the neuronal NK₃ receptor.

At present very little is known about the functional role of central NK₃ receptors. Stoessl *et al.* (1988a) have recently demonstrated 5-HT-mediated behaviour in mice and rats following both central and peripheral administration of senktide. In addition, and by analogy to the actions of tachykinins in the enteric nervous system, behavioural studies have revealed NK₃ receptor-mediated tachykinin-cholinergic interactions in the CNS (Stoessl *et al.*, 1988b).

In conclusion, we have compared the pharmacological specificities of peripheral and central NK₃ tachykinin binding sites using the selective NK₃ receptor agonist [³H]-senktide. We have shown that there is a great deal of similarity between central and peripheral NK₃ binding sites, as revealed by the good correlation between the affinities of tachykinins and analogues (both agonists and antagonists) for [³H]-senktide binding sites in cortex and ileum. Furthermore, the correlation between biological potencies and the data presented here suggest that NK₃ binding sites represent functional receptors and that both central and peripheral NK₃ receptors can be selectively labelled with the metabolically stable NK₃ receptor ligand [³H]-senktide.

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