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Characterization of the [^{125}I]-neurokinin A binding site in the circular muscle of human colon

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1 Neurokinin A (NKA) is a potent contractile agonist of human colon circular muscle. These responses are mediated predominantly through tachykinin NK_2 receptors. In the present study, the NK_2 receptor radioligand [^{125}I]-NKA has been used to characterize binding sites in this tissue, using tachykinin agonists and antagonists.

2 ^{125}INKA labelled a single, high affinity binding site. Specific binding (95% of total binding) of [^{125}I]-NKA was saturable (K_D 0.47 ± 0.05 nM), of high capacity (B_{max} 2.1 ± 0.1 fmol mg^{-1} wet weight tissue) and reversible (kinetically derived K_D 0.36 ± 0.07 nM).

3 The rank order of agonists competing for the [^{125}I]-NKA binding site was neuropeptide γ (NPγ) ≥ NKA ≥ [Lys^5,MeLeu^9,Nle^{10}]NKA (4–10) (NK_2 agonist) > > substance P (SP) > neurokinin B (NKB) ≥ [Pro^9]SP (NK_1 agonist) > > senktide (NK_3 agonist), indicating binding to an NK_2 site.

4 The nonpeptide selective NK_2 antagonist SR48968 showed higher affinity for the [^{125}I]-NKA site than selective peptide NK_2 antagonists. The rank order of potency for NK_2 antagonists was SR48968 ≥ MEN11420 > GR94800 ≥ MEN10627 > MEN10376 ≥ R396. The NK_1 antagonist SR140333 was a weak competitor.

5 The competition curve for SP could be resolved into two sites. When experiments were repeated in the presence of SR140333 (0.1 μM), the curve for SP became monophasic and showed a significant shift to the right, whereas curves to NKA and NKB were unaffected.

6 In conclusion, binding of the radioligand [^{125}I]-NKA to membranes from circular muscle is predominantly to the NK_2 receptor. There may be a small component of binding to the NK_1 receptor. The NK_2 receptor mediates circular muscle contraction, whereas the role of the NK_1 receptor in circular muscle is unclear.

Keywords: Tachykinins; tachykinin NK_2 receptor; human colon; circular muscle; [^{125}I]-NKA; radioligand binding

Abbreviations: BSA, bovine albumin; [^{125}I]-NKA, (2-[^{125}I]-iodohistidyl^1)-neurokinin A; NKA, neurokinin A; NKB, neurokinin B; NPγ, neuropeptide γ; SP, substance P

Introduction

Neurokinin A (NKA) is one of the five mammalian neuropeptides belonging to the tachykinin family. During evolution, the C-terminal sequence Phe-Xaa-Gly-Leu-Met-NH_2, common to all tachykinins, has been conserved. Tachykinins are distributed throughout the peripheral and central nervous systems and have a diverse range of biological actions. These actions are mediated through three distinct receptor subtypes, NK_1, NK_2 and NK_3, at which the endogenous ligands are substance P (SP), NKA and neurokinin B (NKB), respectively (Mussap *et al*., 1993; Maggi *et al*., 1993). The 21 amino acid tachykinin neuropeptide γ (NPγ), which has a copy of NKA at its C-terminus, also has very high affinity for the NK_2 receptor (Mussap *et al*., 1993).

In the mammalian intestine, tachykinins play a role as non-cholinergic excitatory transmitters that mediate the ascending excitatory reflex and atropine-resistant peristalsis (Bártho & Holzer, 1985; Costa *et al*., 1985) either directly through specific receptors located on smooth muscle cells or indirectly by activating intramural neurons (Bártho & Holzer, 1985; Holzer & Holzer-Petsche, 1997a,b). Tachykinin-like immunoreactivity originates primarily from intrinsic enteric neurons but also the peripheral endings of capsaicin-sensitive afferent neurons (Holzer & Holzer-Petsche, 1997a). In the human large intestine, tachykinin-like immunoreactive motor neurons observed within longitudinal and circular muscle layers (Wattchow *et al*., 1988) are seen to project orally from the myenteric plexus (Wattchow *et al*., 1997), although the majority of neurons in human colon are cholinergic (Porter *et al*., 1996). Furthermore, NKA-like immunoreactivity can be released from human jejunum or colon, by stretch at the anal end of the segment (Grider, 1989) or by depolarizing stimuli, respectively (Maggi *et al*., 1990).

Previous studies in human colon indicate that NK_2 receptors play a dominant role in the excitation of the circular muscle by tachykinins (Giuliani *et al*., 1991; Patacchini *et al*., 1997; Croci *et al*., 1998), and autoradiographic studies have demonstrated binding sites for [^{125}I]-Bolton-Hunter NKA on the circular muscle (Gates *et al*., 1989). To date, no radioligand membrane binding study has been performed with tachykinins in this tissue. This study has characterized the [^{125}I]-NKA binding site in the circular muscle of the human colon. The role of peptidases in the possible metabolism of tachykinins in circular muscle was also observed, using selective peptidase inhibitors.

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Methods

Specimen collection

Human sigmoid colon specimens were obtained from patients undergoing resection surgery for colon carcinoma, and was approved by the Human Ethics Committees of the University of NSW and the St George Hospital. The tissue was obtained from 24 patients (13 female, 11 male) aged 68–86 years, who had not undergone radiation therapy or chemotherapy. Specimens were taken 10–20 cm from the tumour. Immediately after collection, specimens were placed in ice-cold carbogenated Krebs-Henseleit solution and stored overnight at 4°C. The mucosa, submucosa, serosa and taenia coli were removed, using a dissecting microscope. The muscle (described as 'circular muscle' hereafter) left after this procedure also contained a thin layer of longitudinal muscle, with the myenteric plexus and small blood vessels (verified microscopically in initial studies). This muscle was frozen in liquid nitrogen and stored at −70°C. Specimens appeared macroscopically normal without any signs of inflammation. Specimens with disease other than carcinoma were not used in this study.

Radioligand binding studies

(2-[^{125}I]-iodohistidyl^{1})-neurokinin A ([^{125}I]-NKA, specific activity 2000 Ci mol^{-1}) was used to characterize the tachykinin NK_2 receptors in human colon circular muscle. Crude membranes (3% w v^{-1}) were prepared as previously described (Mussap & Burcher, 1990). Membranes were homogenized, centrifuged and finally resuspended in incubation buffer constituted of 50 mM Tris-HCl (pH 7.4, 25°C), 3 mM $MnCl_2$ 0.02 % bovine albumin (BSA) and peptidase inhibitors, and incubated for 60 min at 25°C with 50–70 pM [^{125}I]-NKA. In preliminary studies, the effects of various peptidase inhibitors were tested (Figure 1), and in subsequent studies chymostatin (4 μg ml^{-1}), bestatin (10 μM) and phosphoramidon (10 μM) were used in the incubation buffer. Non-specific binding was defined by 1 μM NKA. The binding was terminated by rapid filtration of membranes through Whatman GF/B glass filter paper (presoaked in 0.5% BSA overnight) using a Brandel cell harvester. Filters were washed (3 × 3 ml) with ice-cold 50 mM Tris buffer containing 3 mM $MnCl_2$ and 0.02% BSA. Filter bound radioligand was quantified using a Wallac Wizard Gamma counter (>78% efficiency).

Kinetic studies (association and dissociation) of 70 pM [^{125}I]-NKA were performed as previously described (Bylund & Yamamura, 1990). For association experiments, crude membranes were incubated with radioligand for 5, 15, 30, 60, 90 and 120 min before filtration. For dissociation experiments, membranes were incubated for 60 min. Dissociation was initiated by addition of NKA (1 μM). Membrane aliquots were then removed at intervals and filtered using a Millipore cell harvester. 'Cold' saturation experiments with 70 pM [^{125}I]-NKA were performed to determine the parameters K_D and B_{max} (Mussap & Burcher, 1990). In competition experiments, a range of natural tachykinins, analogues and antagonists were used at varying concentrations (0.01 nM–100 μM) with 50 pM [^{125}I]-NKA.

All experiments were carried out in duplicate. Four to five independent experiments with tissue from individual patients were performed. Raw binding data were analysed using the computer program PRISM (GraphPad Software Inc.). Data were analysed using single and multiple site models and the F test was used to determine the most appropriate model. $P<0.05$ was considered statistically significant. Data from peptidase inhibition experiments were compared using ANOVA followed by the Bonferroni test. The pIC_{50} values for SP, NKA and NKB were compared in the presence and absence of SR140333 using Student's t-test. Unless otherwise stated, data are expressed as the mean ± s.e.mean.

Materials

[^{125}I]-NKA (50 μCi) was purchased from NEN Life Science Products Inc, Boston, U.S.A., reconstituted in distilled water and stored frozen at −70°C in 100 μl aliquots. NKA, NPγ, NKB, SP, [Pro^{9}]SP, senktide and antagonist GR 94800 (PhCO-Ala-Ala-D-Trp-Phe-D-Pro-Pro-NleNH_2) were obtained from Auspep Pty Ltd, Melbourne, Australia. MEN 10376 ([Tyr^{5},D-Trp^{6,8,9},Lys^{10}] NKA 4–10) and R396 (Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH_2) were obtained from Neosystems, Groupe SNPE, France. [Lys^{5},MeLeu^{9},Nle^{10}]NKA (4–10) was a gift from Dr S. Lavielle (Université Pierre et Marie Curie, Paris, France). MEN10627 (cyclo[(Met-Asp-Trp-Phe-Dap-Leu)cyclo(2β-5β)]) and MEN11420 (cyclo {[Asn(β-D-GlcNAc)-Asp-Trp-Phe-Dap-Leu]cyclo (2β-5β)}) were gifts from Dr C. A. Maggi (Menarini Ricerche S.p.A, Florence, Italy). SR140333[(*S*)-1{2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl)piperin-3-yl]ethyl}-4-phenyl-1-azoniabicyclo[2,2,2]octanechloride] and SR48968 [(*S*)-*N*-methyl-*N*-(4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl) butyl) benzamide] were gifts from Dr X. Emonds-Alt (Sanofi Recherche, Montpellier, France). Stock solutions of tachykinins, analogues and antagonists were prepared in 0.01 M acetic acid with β-mercaptoethanol, or in dimethylsulphoxide, and stored as aliquots at −20°C. The peptidase inhibitors phosphoramidon, chymostatin, bestatin, amastatin, leupeptin, captopril, bacitracin and pepstatin A were purchased from Sigma Chemical Company, Australia. All other reagents were of analytical grade.

Results

Effect of peptidase inhibitors

Initially, the effects of eight peptidase inhibitors on specific binding of [^{125}I]-NKA were observed (Figure 1). Chymostatin (4 μg ml^{-1}) significantly increased specific binding of [^{125}I]-NKA by 92 ± 36% ($n=4$, ANOVA $P<0.05$) compared with control. Phosphoramidon (10–100 μM) and bestatin (10–100 μM) used separately had no effect on specific binding ($n=4$) but a combination of three peptidase inhibitors (phosphoramidon 10 μM, bestatin 10 μM and chymostatin 4 μg ml^{-1}) showed an increase in specific binding (90 ± 37%; $P<0.05$, $n=3$) of [^{125}I]-NKA, and these inhibitors were used in subsequent experiments to protect competitors as well as radioligand.

Kinetic studies

The association of [^{125}I]-NKA to human colon circular muscle membranes reached equilibrium at 1 h and remained stable for at least 2 h (Figure 2A). At equilibrium, specific binding represented 95 ± 0.25% ($n=15$) of total binding. Association curves were consistent with pseudo first-order kinetics, with a rate constant k_{+1} of 0.097 min^{-1} nM^{-1}. The specific binding of [^{125}I]-NKA was reversible, with 85% of radioligand dissociated from the receptor by 1 μM NKA at 90 min (Figure 2B). The dissociation rate constant k_{-1} was 0.035 min^{-1}. The kineti-

cally derived dissociation constant was K_D 0.36±0.07 nM ($n=4$).

Saturation studies

'Cold' saturation experiments performed with [125I]-NKA (70 pM) showed binding to a single, high affinity site, with a K_D value of 0.47±0.05 nM and maximum number of binding sites (B_{max}) 2.1±0.1 fmol mg^{-1} wet weight tissue ($n=5$). Scatchard plots were linear, with Hill coefficients approaching unity (Figure 3).

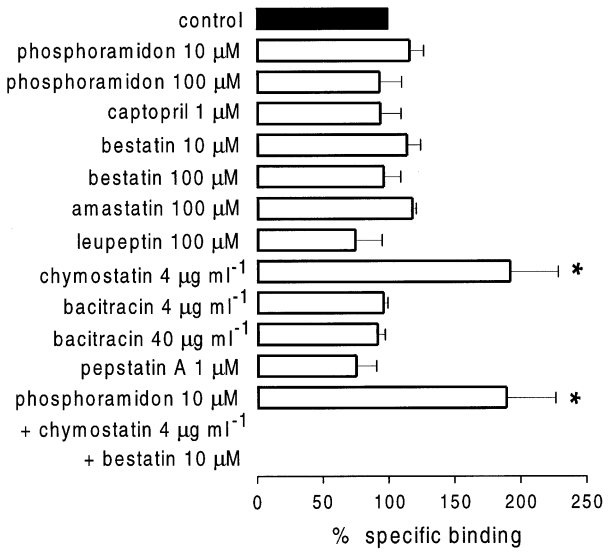

Figure 1 Effect of peptidase inhibitors on specific binding of [125I]-NKA to human colon circular muscle. Data represent specific c.p.m. bound, expressed as a percentage of the control incubated without peptidase inhibitors. Bars are mean±s.e.mean of data from 3–4 individual patients. *Indicates $P<0.05$ (ANOVA).

Competition studies

Pharmacological characterization of the [125I]-NKA binding site was made using a selection of natural and synthetic tachykinin agonists and antagonists. The most potent competitors of [125I]-NKA binding were the NK_2 receptor agonists NPγ, NKA and [Lys^5,MeLeu^9, Nle^{10}]NKA (4–10) and the NK_2 antagonists SR48968 and MEN11420 (K_i values <1 nM, Table 1). The rank order of potency for agonists competing at the [125I]-NKA binding site was NP$\gamma \geqslant$ NKA $\geqslant$ [Lys^5, MeLeu^9, Nle^{10}]NKA (4–10) >> SP > NKB $\geqslant$ [Pro^9]SP >> senktide (Figure 4A). The potency order of antagonists was SR48968 > MEN11420 >> GR94800 > MEN10627 > R396 > SR140333 (Figure 4B). The selective NK_1 antagonist SR140333 displayed 1000 fold lower affinity than SR48968. These data are consistent with binding to the NK_2 receptor, although the potency order NKA > SP > NKB is unusual.

All competitors except SP had high slope factors, indicating binding to one site. However, the slope factor for SP was low, and binding was resolved into two sites of high (pIC_{50} 10.5; 25% of sites) and low affinity (pIC_{50} 7.8; 75% of sites). Since [125I]-NKA has been demonstrated to have affinity for the NK_1 receptor (Geraghty *et al.*, 1992), the competition curves for these three natural tachykinins were repeated in the presence of the NK_1 receptor antagonist SR140333 (0.1 μM). The curve for SP became monophasic and showed a significant 14 fold shift

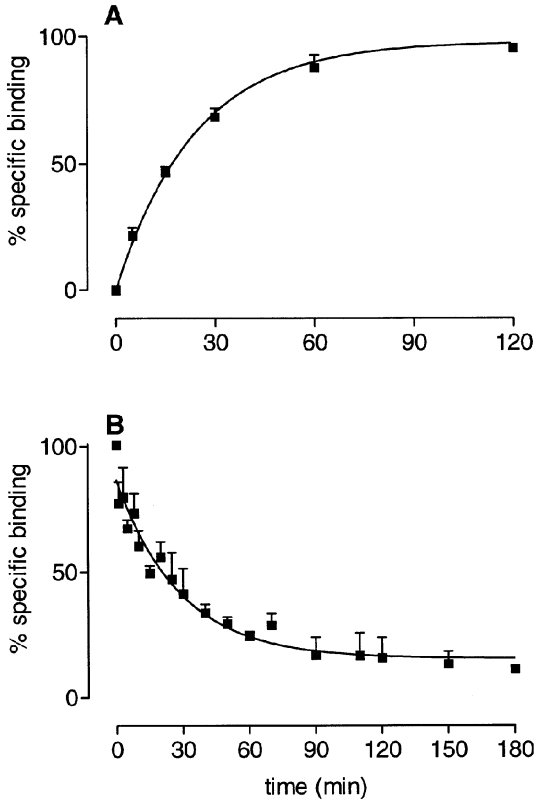

Figure 2 Kinetics of [125I]-NKA (70 pM) specific binding to circular muscle of human colon. Values represent means±s.e.mean. (A) Association time course k_{+1} 0.097 min^{-1} nM^{-1} (B) Dissociation time course (initiated by 1 μM NKA at equilibrium) k_{-1} 0.035 min^{-1}. The calculated kinetic K_D was 0.36±0.07 nM ($n=4$).

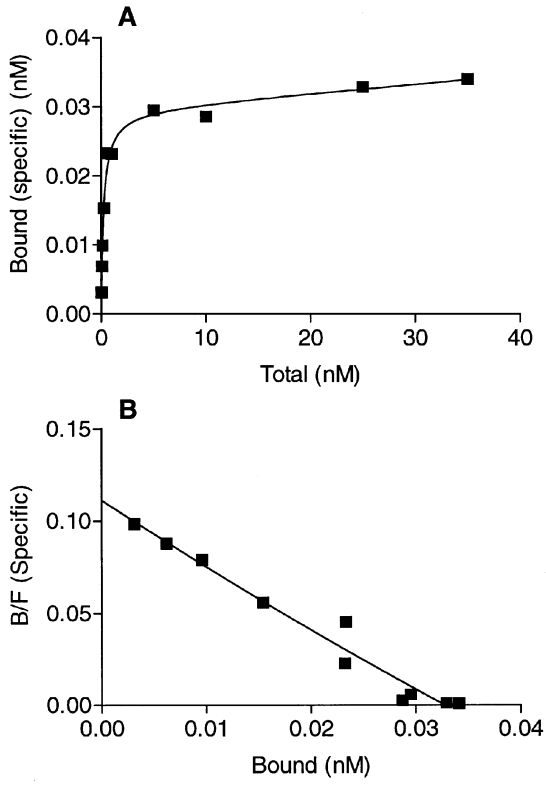

Figure 3 Representative (A) Saturation curve and (B) Scatchard plot calculated from 'cold' saturation experiments with 70 pM [125I]-NKA in circular muscle of human colon. K_D was 0.47±0.05 nM with corresponding B_{max} 2.1±0.1 fmol mg^{-1} wet weight tissue. Four independent experiments were performed in duplicate. Non-specific binding was determined by 1 μM NKA.

Table 1 Binding parameters for tachykinins and analogues as competitors for [^{125}I]-NKA binding in circular muscle membranes

Competitor		K_i (nM) *95% C.L.*	*IC_{50}* (nM)	*pIC_{50} + s.e.mean*	*Slope factor*
Neuropeptide γ		0.26 (0.18–0.36)	0.29	9.5±0.07	0.85
Neurokinin A		0.42 (0.32–0.56)	0.48	9.3±0.06	1.02
[Lys^5,MeLeu^9,Nle^{10}]NKA (4–10)		0.77 (0.56–1.06)	0.87	9.1±0.07	0.87
Substance P	(H 25%)	0.03 (0.003–0.46)	0.03	10.5±0.55	0.58
	(L 75%)	13 (8.1–22)	14	7.8±0.10	
Neurokinin B		60 (40–92)	68	7.2±0.09	0.83
[Pro^9]SP		130 (80.8–212)	150	6.8±0.10	0.76
Senktide		12500 (6300–25000)	14000	4.9±0.14	N.D.
SR48968		0.28 (0.19–0.41)	0.31	9.5±0.08	1.06
MEN11420		0.73 (0.63–0.85)	0.81	9.1±0.03	1.09
GR94800		3.1 (2.4–4.1)	3.5	8.4±0.06	0.85
MEN10627		10 (5.9–18)	11	7.9±0.12	0.76
MEN10376		63 (42–95)	71	7.2±0.09	0.78
R396		99 (52–190)	110	6.9±0.14	0.72
SR140333		330 (240–440)	370	6.4±0.06	0.90

Inhibition constants (K_i) (means with 95% confidence limits), IC_{50}, pIC_{50} (negative log of IC_{50}) (mean±s.e.mean) and slope values were calculated from competition curves by PRISM. K_i values for high (H) and low (L) affinity components are shown for SP which yielded a significant two-site fit ($P<0.05$). Experiments were performed in duplicate with ($n=4-6$) determinations for each competitor. N.D. not determined.

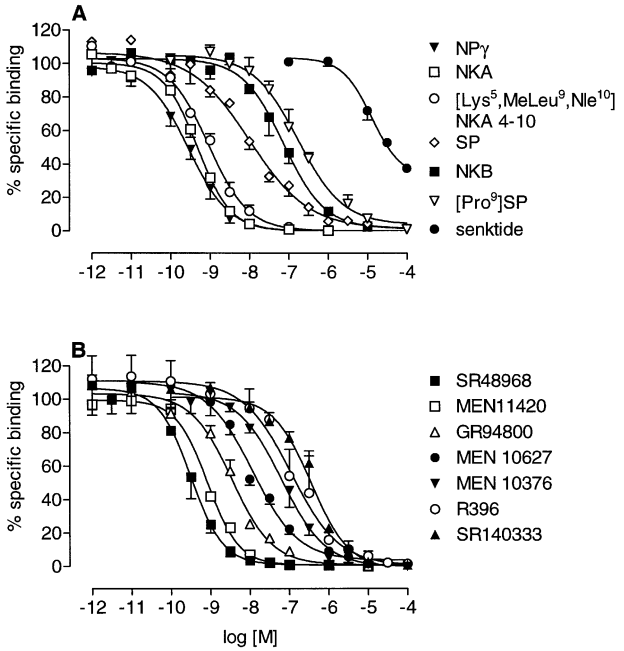

Figure 4 Competition curves of tachykinin and analogues for [^{125}I]-NKA (50 pM) binding to circular muscle of human colon. Curves represent mean values±s.e.mean of 4–5 independent experiments in duplicate. (A) tachykinin agonists (B) tachykinin antagonists.

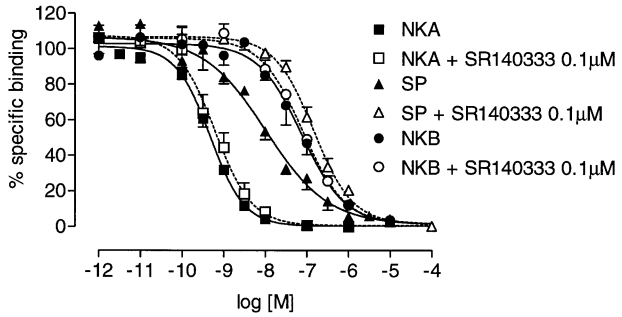

Figure 5 Competition curves to NKA, NKB and SP in the absence (filled symbols, solid line) and in the presence (open symbols, dotted line) of selective NK_1 receptor antagonist SR140333 (0.1 μM). Note the effect on the curve of SP which was steepened (slope 0.58–0.93) and shifted to the right (IC_{50} 10.3–145 nM). Curves represent mean values±s.e.mean of 3–5 independent experiments, performed in duplicate.

to the right (pIC_{50} 6.85; $P<0.0001$, $n=3$, Student's t-test), while the competition profiles of NKA (pIC_{50} 9.21) and NKB (pIC_{50} 7.12) were unaffected by SR140333 (Figure 5). In the presence of SR140333, the potency order became NKA >>NKB>SP, consistent with binding to the NK_2 receptor.

Discussion

Using the technique of radioligand membrane binding, the present study investigated the [^{125}I]-NKA binding site in the circular muscle of human colon. Scatchard analysis of 'cold' saturation data showed binding to a single, saturable, high affinity site, while kinetic studies suggested binding to a

homogenous class of receptors. The K_D value obtained from saturation studies was in good agreement with that from kinetic studies. As shown previously for rat fundus membranes, specific binding of this radioligand was enhanced by chymostatin (Mussap & Burcher, 1990). Binding of [^{125}I]-NKA appeared to be to the NK_2 receptor site, since the most potent competitors were NPγ, NKA, the enzyme resistant, selective NK_2 receptor agonist [Lys^5, MeLeu^9, Nle^{10}]NKA (4–10) and the NK_2 receptor antagonists SR48968, MEN11420 and GR94800. NPγ was a very potent competitor at the circular muscle NK_2 receptor, in support of some of our previous studies where NPγ was a preferred functional agonist and binding competitor at NK_2 receptors in human airways (Burcher *et al*., 1991) and hamster and dog urinary bladder (van Giersbergen *et al*., 1991; Mussap *et al*., 1996).

The few previous studies investigating tachykinin receptors in human colon have shown that the NK_2 receptor is most important in the excitation of the circular muscle. Autoradiographic studies showed localization of [^{125}I]-Bolton-Hunter NKA binding sites on circular muscle only (Gates *et al*., 1989). In support, *in vitro* functional studies using exogenously applied tachykinins demonstrated that NK_2 receptor agonists were more potent contractile agents in human colon circular muscle, than NK_1 and NK_3 receptor agonists (Giuliani *et al*., 1991; Croci *et al*., 1998). These studies indicate that NK_2 receptors are the predominant receptor mediating the spasmogenic activity by tachykinins, through direct activation of receptors located on the smooth muscle cells (Giuliani *et al*., 1991; Croci *et al*., 1998).

Competition studies using [^{125}I]-NKA in circular muscle showed binding affinities for NK_2 agonists to be similar to those seen for other peripheral NK_2 systems; such as human urinary bladder (Zeng *et al*., 1995), rat fundus (Mussap & Burcher, 1990) and rat duodenum (Emonds-Alt *et al*., 1992). Binding in human bladder detrusor muscle (HB) (Zeng *et al*., 1995) and rat duodenum (RD) (Emonds-Alt *et al*., 1992) also show similar binding constants for NK_2 antagonists SR48968 (HB pIC_{50} 8.9; RD pIC_{50} 9.3) and/or GR94800 (HB pIC_{50} 8.2). The rank order of antagonists as competitors in our study is similar to the published values of antagonist potency in functional studies, in human colon (Giuliani *et al*., 1991; Patacchini *et al*., 1997; Croci *et al*., 1998) and human bladder (Zeng *et al*., 1995).

Although [^{125}I]-NKA has been used as a high affinity radioligand at the NK_2 receptor, it is not entirely selective and is able to label NK_1 sites, as shown in guinea-pig airways (Geraghty *et al*., 1992). In the present competition study, we found SP to have greater binding affinity than NKB at the [^{125}I]-NKA binding site, whereas the usual rank order of potency of mammalian tachykinins observed at NK_2 receptors is NKA > NKB >> SP. The slope factor for SP was low and analysis showed binding to two sites. When the competition studies were repeated in the presence of the selective NK_1 receptor antagonist SR140333, the curve for SP showed a significant shift to the right with a new slope factor approaching unity, whereas the curves to NKA and NKB were not affected. In addition, the highly selective NK_1 agonist [Pro^9]SP and antagonist SR140333 showed full displacement of [^{125}I]-NKA from its binding site, although the selective NK_3 agonist senktide was comparably weaker. This suggests that [^{125}I]-NKA may be binding to a proportion of NK_1 sites in addition to NK_2 sites, even though the kinetic and 'cold' saturation studies indicated binding to only one site. 'Hot' saturation studies were not an option due to the expense involved. Whether the additional NK_1 sites were in circular muscle or in the small amount of longitudinal muscle and/or myenteric plexus is not known.

The question of whether functional NK_1 receptors are actually present in human colon circular muscle is interesting. Immunoreactivity for both SP (the putative endogenous ligand at NK_1 receptors) and NKA is found in human colon, and release of SP and NKA, associated with ascending circular muscle contraction, has been reported from human jejunum (Grider, 1989). Autoradiographic studies in normal human colon show NK_1 binding sites over circular muscle (Gates *et al*., 1989), as well as to submucosal and longitudinal muscle blood vessels (Markus *et al*., 1998). Our preliminary studies in circular muscle membranes show a moderate number of high affinity binding sites for [^{125}I]-Bolton-Hunter[Sar^9,Met$(O_2)^{11}$]SP, characterized as NK_1 sites (Markus *et al*., 1998). If NK_1 receptors were present on circular smooth muscle, then SP and NK_1 agonists such as [Sar^9,Met$(O_2)^{11}$]SP would be expected to show high potency in functional studies, but these agents were actually very weak contractile agents (Giuliani *et al*., 1991). Therefore, in spite of autoradiographic and radioligand binding evidence that NK_1 binding sites are present in circular muscle, they do not seem to be coupled to contractile mechanisms. Other NK_1 receptors are found in other regions of the colon and may well be expressed on endothelial cells, macrophages and nerves within the circular muscle.

Our data using the technique of membrane binding support the broad conclusion from earlier studies using functional and autoradiographic techniques, that the receptor involved primarily in the contraction of human colon circular muscle is the NK_2 receptor. The endogenous ligand for this receptor is probably NKA, even though SP is present and able to interact with the NK_2 receptor, but with lower affinity than NKA. The potential exists for NK_2 receptor antagonists to be of therapeutic importance in conditions which incur an increased or exaggerated gut motility such as diarrhoea, and irritable bowel diseases. Whilst there appears to be a population of tachykinin NK_1 binding sites in human colon circular muscle, functional data suggest that these may not be involved in mediating a contractile response. The role of SP in the colon may be more concerned with vascular, neural and inflammatory mechanisms (Mantyh *et al*., 1988) than with smooth muscle contraction.

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