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Identification of both NK_1 and NK_2 receptors in guinea-pig airways

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1 NK_1 and NK_2 receptors have been characterized in guinea-pig lung membrane preparations by use of [^{125}I-Tyr^8]-substance P and [^{125}I]-neurokinin A binding assays in conjunction with tachykinin-receptor selective agonists ([Sar^{9}Met(O$_2$)^{11}]substance P for NK_1 and [βAla^8]neurokinin A (4–10) for NK_2) and antagonists (CP-99,994 for NK_1 and SR48968 for NK_2).

2 The presence of high affinity, G-protein-coupled NK_1 receptors in guinea-pig lung parenchymal membranes has been confirmed. The rank order of affinity for competing tachykinins was as predicted for an NK_1 receptor: substance P = [Sar^{9}Met(O$_2$)^{11}]substance P > substance P-methyl ester = physalaemin > neurokinin A = neurokinin B >> [βAla^8]neurokinin A (4–10). The novel NK_1 antagonist CP-99,994 has a K_i of 0.4 nM at this NK_1 site.

3 In order to characterize [^{125}I]-neurokinin A binding to guinea-pig lung, the number of [^{125}I]-neurokinin A specific binding sites was increased 3–4 fold by purification of the parenchymal membranes over discontinuous sucrose gradients. The rank order of affinity determined for NK_1- and NK_2-receptor agonists and antagonists in competition for these sites showed that the majority (80%) of [^{125}I]-neurokinin A specific binding was also to the NK_1 receptor.

4 Under conditions where the guinea-pig lung parenchymal NK_1 receptor was fully occupied by a saturating concentration of either [Sar^{9}Met(O$_2$)^{11}]substance P (1 μM) or CP-99,994 (2.7 μM), residual [^{125}I]-neurokinin A specific binding was inhibited in a concentration-dependent manner by both [βAla^8]neurokinin A and SR48968. This result shows that the NK_2 receptor is also present in these preparations.

5 Similar studies using guinea-pig tracheal membranes demonstrated that [^{125}I]-neurokinin A specific binding was composed of a NK_1-receptor component (60%), inhibited by both [Sar^{9}Met(O$_2$)^{11}]substance P and CP-99,994, and a significant NK_2-receptor component, inhibited by both [βAla^8]neurokinin A and SR48968.

6 In summary, these data demonstrate that guinea-pig lung parenchyma and guinea-pig trachea express both NK_1 and NK_2 receptors.

Keywords: Tachykinin; substance P; CP-99,994; SR48968; guinea-pig airways; neurogenic inflammation; radioligand binding

Introduction

Substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) form part of the mammalian tachykinin family of biologically active peptides (Maggio, 1988). The wide spectrum of neuronal and non-neuronal physiological events involving tachykinins are receptor-mediated. These peptides all share the conserved carboxyl-terminal sequence (Phe-X-Gly-Leu-Met-NH_2) which is essential for receptor activation, while their diverse amino terminal domains are thought to govern receptor selectivity (Krause *et al.*, 1992). Substance P, NKA and NKB have been shown to interact preferentially with NK_1, NK_2 and NK_3 receptors, respectively, three distinct G-protein-coupled receptor types which have now been cloned and sequenced (Masu *et al.*, 1987; Yokoto *et al.*, 1989; Shigemoto *et al.*, 1990).

The results of *in vitro* and *in vivo* studies show that tachykinins are mediators of bronchoconstriction, plasma extravasation, mucous production and vasodilatation, all characteristic of neurogenic inflammation in the respiratory tract (Barnes *et al.*, 1990). These peptides are released from sensory nerve fibres originating in the airway epithelium in response to a variety of noxious stimuli (Solway & Leff, 1991), producing pronounced spasmogenic effects on respiratory smooth muscle. In addition, SP and NKA are known to modulate immune cell function through their effects on T-lymphocytes, B-lymphocytes, macrophages, mast cells and monocytes (Casale, 1991). It has been proposed, therefore, that the tachykinins may contribute to the pathogenesis of certain respiratory disease states such as bronchial asthma, both by decreasing airway calibre and modulating the cells involved in the inflammatory response.

The precise role played by the individual tachykinins and their receptor types in pulmonary pathophysiology is currently being elucidated. It has been demonstrated that plasma extravasation in guinea-pig upper airways (trachea and main bronchi) is mediated via NK_1 receptors (Murai *et al.*, 1992), whereas in lower airways (secondary bronchi and intraparenchymal airways) plasma extravasation is mediated via both NK_1 and NK_2 receptors (Tousignant *et al.*, 1993a,b). Similarly, bronchoconstriction in guinea- pig airways appears to be mediated via both NK_1 and NK_2 receptor types (Regoli *et al.*, 1987). In support of these functional studies suggesting that guinea-pig respiratory tissues contain multiple tachykinin-receptor types, several groups have characterized NK_1 receptors in guinea-pig lung membranes by radioligand binding techniques (Coats & Gerard, 1989; Aharony *et al.*, 1991; Geraghty *et al.*, 1992). However, as yet, NK_2 receptors have not been identified unambiguously to be present in these preparations.

Delineating the receptor types present in guinea-pig lung is complicated by the non-selectivity of the endogenous tachykinins normally used as probes and, in addition, the extreme susceptibility of these peptides to metabolism by a variety of proteases. These problems have been partially alleviated by the development of metabolically stable and highly selective peptide agonists {[Sar^9,Met(O$_2$)^{11}]-SP (Regoli

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et al., 1988) at the NK_1 site and [β-Ala^8]NKA (4–10) (Rovero et al., 1989) at the NK_2 site} and non-peptide antagonists {CP-99,994 (McLean et al., 1992) at the NK_1 site and SR48968 (Emonds-Alt et al., 1992) at the NK_2 site}. In this paper we have used these selective agonists and antagonists, in conjunction with radioligand binding techniques, to identify both NK_1 and NK_2 receptors in guinea-pig lung parenchyma and trachea.

Methods

Guinea-pig lung parenchymal and tracheal membrane preparation

Guinea pigs (male; 350 g) were killed by cervical dislocation and the lung tissues removed. All subsequent procedures were performed either on ice or at 4°C. The tracheae were removed and processed as described below. The remaining connective tissue, major airways and large blood vessels were then dissected away and the lung tissue (principally parenchyma) was finely minced prior to homogenization in 10 volumes of 10 mM HEPES/KOH, pH 7.4, containing 0.25 M sucrose, 2 mM EDTA, 2 mM phenylmethylsulphonyl fluoride, 10 μg ml^{-1} pepstatin, 10 μg ml^{-1} leupeptin, and 10 μM E-64, using 5 s bursts of a Polytron homogenizer (Brinkman Instruments). The homogenate was then subjected to differential centrifugation at 1,000 *g* for 10 min, at 10,000 *g* for 15 min, and finally at 100,000 *g* for 40 min. The resulting pellets were washed by resuspension in 20 vol of 10 mM HEPES/ KOH, pH 7.4, followed by centrifugation at 150,000 *g* for 40 min. The final membrane pellets were resuspended in 50 mM Tris/HCl, pH 7.4, at a final concentration of 7–10 mg ml^{-1} of membrane protein and stored at − 80°C.

For the [^{125}I]-NKA binding assays, the guinea-pig lung membranes were further purified over discontinuous sucrose gradients. The 100,000 *g* membrane fraction was resuspended in 10 mM HEPES/KOH, pH 7.4, containing 10% (w/v) sucrose, at a final protein concentration of 1–2 mg ml^{-1}. The tissue suspension was then carefully layered over 10 mM HEPES/KOH, pH 7.4, containing 40% (w/v) sucrose in a 3:2 (v/v) ratio. The discontinuous sucrose gradient was centrifuged in a swinging bucket rotor at 83,000 *g* for 60 min. The membrane fraction located at the interface of the sucrose gradient layers was recovered using an automated pump (Buchler Instruments) and then washed by a 10 fold dilution in 10 mM HEPES/KOH, pH 7.4, followed by centrifugation at 150,000 *g* for 40 min. The final membrane pellets were again resuspended in 50 mM Tris/HCl, pH 7.4, at a final concentration of 3–5 mg ml^{-1} of membrane protein and stored at − 80°C.

Guinea-pig tracheal membranes were prepared by homogenization, as described for the parenchymal preparations. The homogenate was initially centrifuged at 1,000 *g* for 15 min, and then at 100,000 *g* for 40 min. The membrane pellets were resuspended at 50 mM Tris/HCl, pH 7.4, at a final concentration of 3–8 mg ml^{-1} of tracheal membrane protein and stored at − 80°C.

[^{125}I]-neurokinin radioreceptor binding assays

[^{125}I]-neurokinin ([^{125}I]-NK) binding assays were routinely performed in 50 mM Tris/HCl, pH 7.4, containing 0.01% (w/v) bovine serum albumin, 200 μM chymostatin, 100 μM leupeptin, 100 μM phosphoramidon, 100 μM captopril, 250 μM bestatin, and 0.005% (v/v) 2-mercaptoethanol. For the guinea-pig lung parenchymal [^{125}I-Tyr^8]-SP binding assay, the 250 μl incubations also included 1 mM $MnCl_2$, 20–25 pM [^{125}I-Tyr^8]-SP and 75 μg of membrane protein. Non-specific binding was determined in the presence of 1 μM SP. For the guinea-pig lung parenchymal [^{125}I]-NKA binding assay, the 500 μl incubations also included 3 mM $MnCl_2$, 20–25 pM [^{125}I]-NKA and 250 μg of sucrose-purified membrane protein. For the guinea-pig tracheal [^{125}I]-NKA binding assay the 500 μl incubation also included 10 mM $MnCl_2$, 50 pM [^{125}I]-NKA and 300–500 μg of membrane protein. Non-specific binding was determined in the presence of 1 μM NKA. Aliquots of frozen membrane preparations were initially resuspended in the presence of EDTA, to give a final concentration of 1 mM in the incubation medium.

Incubations were conducted for 1 h for [^{125}I-Tyr^8]-SP binding assays and 2 h for [^{125}I]-NKA binding assays, both at room temperature. Bound and free radioligand was then separated by rapid filtration, under vacuum, through Whatman GF/B filters presoaked for 4 h, at 4°C, in 20 mM Tris/HCl, pH 7.4, containing 0.1% polyethylenimine and 0.01% (w/v) bovine serum albumin (BSA). The filters were washed with approximately 16 ml of 20 mM Tris/HCl, pH 7.4, containing 0.01% (w/v) BSA and the residual radioactivity bound to the filters determined by gamma counting, with an efficiency of approximately 80%.

In all experiments specific binding was defined as the difference between total binding and non-specific binding, determined in the presence of an excess (> 10000 fold) of the appropriate unlabelled competing ligand. Specific binding was linear with respect to both membrane protein concentration and radioligand concentration, and routinely represented 70–90% of the total radioligand bound to the membrane protein.

Data calculation

The first-order dissociation rate constant (k_{-1}) was determined as the slope obtained by linear regression analysis of plots of ln B/B_0 on the y-axis, versus incubation time in min on the x-axis, where B is the amount of radioligand bound at a given incubation time and B_0 is the amount of radioligand bound at time zero. This analysis was also employed to determine the observed association rate constant (k_{obs}) which was then expressed as k_{obs}/[radioligand].

Scatchard plot analysis was performed by linear transformation of saturation binding curves using Accufit Saturation-One Site data analysis software (Beckman Instruments Inc.) based on the linear transformation of Scatchard (1949) and Rosenthal (1967). Hill coefficients were determined as the slope obtained by linear regression analysis of plots of log $B/(B_{max}-B)$ (y-axis) versus log [radioligand] (x-axis), where B is the amount of specific binding at a given radioligand concentration and B_{max} is the maximum specific binding observed (Hill, 1910).

Sigmoidal equilibrium competition curves were analysed by custom designed software employing a non-linear least-squares curve fitting routine based on a four parameter logistic equation to determine IC_{50} values and slope factors (apparent Hill coefficients). The corresponding K_i values were calculated from the equation $K_i = IC_{50}/1 + ([\text{radioligand}]/K_D)$.

Reverse phase high performance liquid chromatography

Reverse phase high performance liquid chromatography (r.p.-h.p.l.c) was employed to assess the stability of the [^{125}I]-NKs under radioreceptor binding assay conditions. [^{125}I]-neurokinin binding assays were performed in a final incubation volume of 500 μl of 50 mM Tris/HCl, pH 7.4, essentially as described above, but omitting $MnCl_2$, which was found to interfere with r.p.-h.p.l.c. analysis. The incubation medium also contained different combinations of the following inhibitors: leupeptin (0–100 μM), phosphoramidon (0–100 μM), captopril (0–100 μM), chymostatin (0–200 μM) and bestatin (0–250 μM). Control incubations were performed both in the absence of inhibitors and the absence of membranes.

Following a 1 h or 2 h incubation at room temperature for [^{125}I-Tyr^8]-SP and [^{125}I]-NKA binding assays, respectively, the membranes were recovered by centrifugation at 150,000 *g* for 15 min, at 4°C. The supernatant containing the unbound

radioligand was removed, the resulting membrane pellet resuspended by sonication in 250 µl of 0.1% (v/v) trifluoroacetic acid (TFA) and the supernatant containing the bound radioligand recovered by a second centrifugation, conducted under the same conditions. Unbound and bound radioligand fractions were analysed by r.p.-h.p.l.c., using a Nova Pak C_{18} column (0.39 × 15 cm; Waters Associates) equilibrated in 0.1% (v/v) TFA. Following the initial 5 min wash with 0.1% (v/v) TFA, the [^{125}I]-peptide(s) was eluted with a linear gradient from 0 to 60% (v/v) acetonitrile in 0.1% TFA, developed over 60 min, at a flow rate of 1 ml min^{-1}. The radioactivity profile was monitored by on-line solid scintillation counting using a flow-through h.p.l.c. radioactivity monitor. The recovery following r.p.-h.p.l.c. was 100%.

Protein determination

Protein concentration was determined by monitoring the absorbance at 562 nm produced by the formation of protein-Cu^{1+}-bicinchoninic acid complexes in alkaline solution (Smith *et al.*, 1985), with BSA used as the standard.

Materials

Guinea-pigs were obtained from Charles River (LaSalle Québec, Canada); [^{125}I-Tyr^8]-SP (2200 Ci mmol^{-1}) and [^{125}I]-NKA (2200 Ci mmol^{-1}) were from New England Nuclear (Mississauga, Ontario, Canada); SP, NKA, neurokinin B, SP methyl ester, physalaemin, [Sar^9,Met(O$_2$)^{11}]SP, and [β-Ala^8]-NKA (4–10) were from Peninsula Laboratories, Inc. (Belmont, CA, U.S.A.); MEN 10,376 (H-Asp-Tyr-(D)Trp-Val-(D)Trp-(D)Trp-Lys-NH_2) was from IAF BioChem (Montréal, Québec, Canada); CP-99,994 ((+),(2S,3S)-3-(2-methoxybenzylamino)-2-phenylpiperidine), L-736,322 ((−),(2R,3R)-3-(2-methoxybenzylamino)-2-phenylpiperidine), RP67580 (7,7-diphenyl-2[1-imino-2(2-methoxyphenyl)-ethyl]perhydroisoindol-4-one (3aR,7aR)), RP68651 (7,7-diphenyl-2[1-imino-2(2-methoxyphenyl)-ethyl]perhydroisoindol-4-one (3aS,7aS)) and SR49868 ((S)-N-methyl-N[4-(4-acetylamino-4-phenyl piperidino)-2-(3,4-dichlorophenyl)butyl]benzamide) were synthesized by Merck Research Laboratories; E-64 and GTPγS were from Boehringer Mannheim (Laval, Québec, Canada); eledoisin, senktide, bestatin, captopril, chymostatin, leupeptin, pepstatin, phosphoramidon, phenylmethylsulphonyl fluoride, EDTA and polyethylenimine were from the Sigma Chemical Company (St. Louis, Missouri, U.S.A.); the protein determination kit was from Pierce (Rockford, U.S.A.). All other reagents were of analytical grade.

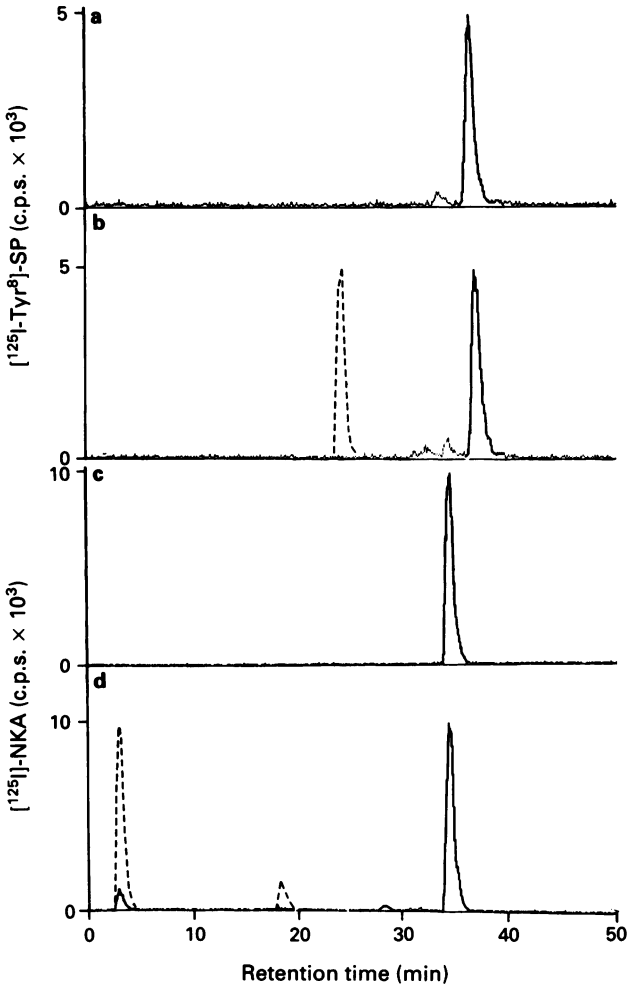

Figure 1 R.p.-h.p.l.c. analysis of [^{125}I-Tyr^8]-substance P ([^{125}I-Tyr^8]-SP) and [^{125}I]-neurokinin A ([^{125}I]-NKA) metabolism. The stability of [^{125}I-Tyr^8]-SP and [^{125}I]-NKA during radioreceptor binding assays was assessed by r.p.-h.p.l.c., as described in the Methods. (a) and (c) show the elution positions of the [^{125}I-Tyr^8]-SP and [^{125}I]-NKA standards; (b) and (d) show the profile of unbound radiolabelled material following incubation of [^{125}]-Tyr^8]-SP and [^{125}I]-NKA in the absence (- - - -) and presence (———) of an inhibitor mixture of leupeptin (100 µM), phosphoramidon (100 µM), captopril (100 µM), chymostatin (200 µM) and bestatin (250 µM). The profile obtained for bound radiolabelled material was essentially the same (data not shown).

Results

[^{125}I]-neurokinin metabolism

Both [^{125}I-Tyr^8]-SP and [^{125}I]-NKA were completely degraded when incubated with guinea-pig lung parenchymal membranes, in the absence of protease inhibitors, under the conditions used for [^{125}I]-NK binding assays (Figure 1). Both radioligands were stable when incubated in the absence of membranes. Extensive r.p.-h.p.l.c. analysis of binding assay incubations was conducted in order to establish the precise inhibitor mixture necessary to prevent [^{125}I]-NK metabolism. Protease inhibitors effective against different classes of enzymes were evaluated over a concentration range up to 500 µM, both singly and in various combinations. The following inhibitors were all found to be essential in blocking a component of [^{125}I]-NK degradation at the concentration indicated, and were routinely included in [^{125}I]-NK binding assay incubations: phosphoramidon (100 µM) for neutral endopeptidase (EC 3.4.24.11), bestatin (250 µM) for aminopeptidase, captopril (100 µM) for angiotensin coverting enzyme (EC 3.14.5.1), chymostatin (200 µM) for chymotrypsin-like serine proteases, and leupeptin (100 µM) for trypsin-like serine proteases. Thiorphan, a specific neutral endopeptidase inhibitor, was found to be equipotent with phosphoramidon in inhibiting [^{125}I]-NK breakdown, while the cysteine protease inhibitor E-64 and the aspartic protease inhibitor pepstatin had no effect. Analysis by r.p.-h.p.l.c. showed that the chosen mixture inhibited over 90% of the [^{125}I]-NK degradation previously observed, in both the unbound and bound radioligand fractions, over the binding assay incubation period (Figure 1). The inhibitor mixture also blocked over 95% of the metabolism of [^{125}I]-NKA by guinea-pig tracheal membranes.

Characterization of the NK_1 receptor in guinea-pig lung parenchymal membranes

Enhancement by divalent cations [^{125}I-Tyr^8]-SP specific binding to guinea-pig lung parenchymal membranes was enhanced in the presence of divalent cations with $Mn^{2+} > Mg^{2+} >> Ca^{2+}$ (Figure 2). The optimum divalent cation concentration was 1 mM for Mn^{2+}, which resulted in a 5–6 fold increase in [^{125}I-Tyr^8]-SP specific binding, from a baseline level of 595 ± 71 c.p.m. to 3450 ± 170 c.p.m. ($n = 3$). Divalent cation concentrations greater than 10 mM proved

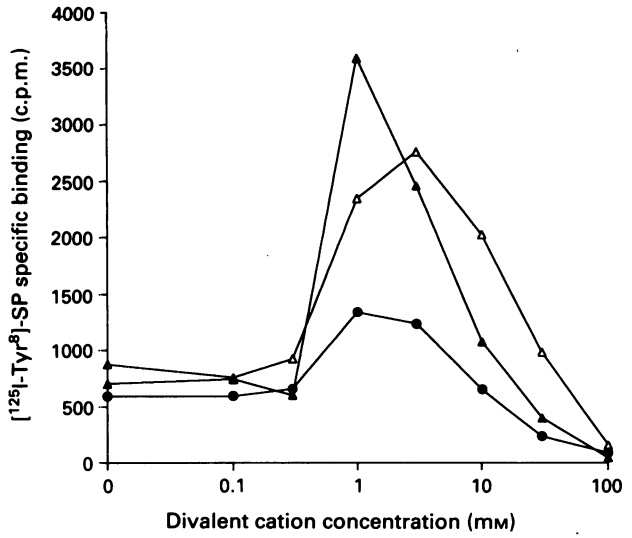

Figure 2 Effect of divalent cations on [^{125}I-Tyr^8]-substance P ([^{125}I-Tyr^8]-SP) specific binding to guinea-pig lung membranes. [^{125}I-Tyr^8]-SP binding assays were performed, as described in the Methods, in the presence of 0–100 mM $MnCl_2$ (▲), $MgCl_2$ (△) and $CaCl_2$ (●). Results are expressed as specific binding in c.p.m. as a function of divalent cation concentration. These are representative data from three experiments giving similar values.

inhibitory, with complete abolition of [^{125}I-Tyr^8]-SP specific binding observed at 100 mM, in all cases.

Rates of association and dissociation The rate of association of [^{125}I-Tyr^8]-SP to guinea-pig lung parenchymal membranes was slow, with an observed association rate constant (k_{obs}/[radioligand]) of approximately 2×10^9 min^{-1} M^{-1} ($n = 2$). Equilibrium was reached over a 1 h incubation period. Addition of a large excess of unlabelled competing SP (1 μM) resulted in a time-dependent dissociation of [^{125}I-Tyr^8]-SP, with a first-order dissociation rate constant (k_{-1}) of 0.018 min^{-1} ($n = 2$). The addition of 100 μM GTPγS with the competing SP, however, provoked complete dissociation of [^{125}I-Tyr^8]-SP binding to non-specific levels within 1 min (Figure 3).

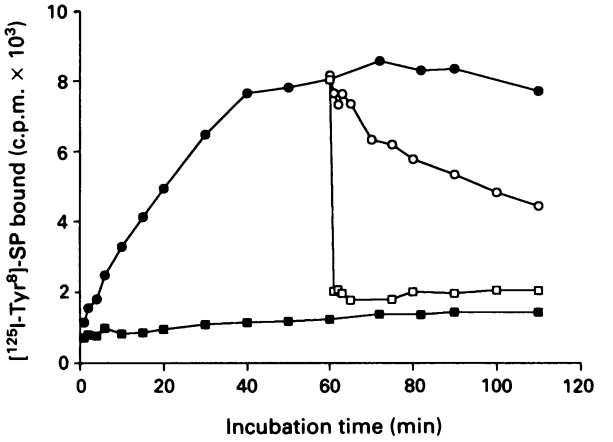

Figure 3 Association and dissociation of [^{125}I-Tyr^8]-substance P ([^{125}I-Tyr^8]-SP) binding to guinea-pig lung membranes. The rates of association of total (●) and non-specific (■) binding of [^{125}I-Tyr^8]-SP to guinea-pig lung parenchymal membranes were followed, at the required time intervals, by removing and filtering 250 μl aliquots from homogeneous binding assay mixtures incubated in the absence (●) or presence (■) of 1 μM SP. At 60 min the rate of dissociation of total binding was monitored, in the same manner, following addition of either 1 μM SP (○), or 1 μM SP with 100 μM GTPγS (□).

Analyses by Scatchard and Hill plot [^{125}I-Tyr^8]-SP specific binding to guinea-pig lung parenchymal membranes was saturable, as demonstrated by incubation with an increasing concentration (2–200 pM) of [^{125}I-Tyr^8]-SP, in the presence and absence of 1 μM unlabelled SP. Scatchard analysis of the deduced specific binding saturation curve demonstrated that [^{125}I]-Tyr^8]-SP specific binding conformed to a single binding site model, with a K_D of 46 ± 4 pM ($n = 3$) and a maximum number of binding sites (B_{max}) of 67 ± 6 fmol mg^{-1} of membrane protein ($n = 3$). Analysis of the specific binding data by Hill plot gave a straight line, with a Hill coefficient of 0.96 ± 0.01 ($n = 3$), close to unity. This confirms that [^{125}I-Tyr^8]-SP was binding in an independent manner to a site, or sites, with one apparent affinity in these preparations, with no evidence for cooperativity.

Equilibrium competition curves Tachykinin-receptor agonists and antagonists were used to compete for [^{125}I-Tyr^8]-SP specific binding to guinea-pig lung parenchymal membranes (Table 1). The most potent competing ligands were the NK_1 preferring SP and the selective NK_1 agonist [Sar^{9}Met(O$_2$)^{11}]-SP, which were equipotent, with IC_{50} values of approximately 0.1 nM. Substance P-methyl ester and the amphibian peptide physalaemin, which also interact preferentially with NK_1 receptors, were only 10–20 fold less potent than SP. In comparison, NKA, NKB and eledoisin, which bind preferentially to NK_2 and NK_3 receptors, respectively, were less effective in competing for [^{125}I-Tyr^8]-SP specific binding to guinea-pig lung parenchymal membranes, being 300–600 fold less potent than SP. Most strikingly, the highly selective NK_2 agonist [βAla^8]NKA (4–10) was considerably less potent when competing for these [^{125}I-Tyr^8]-SP specific binding sites, with an IC_{50} value of 4.4 μM, while the NK_3-selective agonist senktide (Wormser *et al.*, 1986) was inactive.

In agreement with the profile obtained for tachykinin-receptor agonists the selective NK_1-receptor antagonist CP-99,994 was a potent competing ligand for [^{125}I-Tyr^8]-SP specific binding to guinea-pig lung parenchymal membranes, with an IC_{50} value of 0.6 ± 0.1 nM (Table 1). The related **2R,3R** enantiomer L-736,322 was 1,000 fold less potent with an IC_{50} value of 633 ± 118 nM. In contrast, the NK_1 antagonist (−)-RP67580 (Garret *et al.*, 1991) was 300 fold less potent than CP-99,994 in competition assays, with an IC_{50} of 181 ± 31 nM. In this case, the related (+)-enantiomer RP68651 was totally inactive at concentrations up to 20 μM. In comparison, the NK_2-selective antagonists SR48968 and MEN 10,376 (Maggi *et al.*, 1991) were poor competitors for [^{125}I-Tyr^8]-SP specific binding to these preparations, with IC_{50} values of 1.1 μM and > 10 μM, respectively.

Characterization of [^{125}I]-neurokinin A specific binding to guinea-pig lung parenchymal membranes

Enhancement by divalent cations Initial binding assays investigating the effect of divalent cations on [^{125}I]-NKA specific binding to guinea-pig lung parenchymal membranes were conducted using the 100,000 *g* membrane fraction. Under the experimental conditions there was no detectable [^{125}I]-NKA specific binding to these preparations in the absence of divalent cations, but [^{125}I]-NKA specific binding was substantially increased in the presence of both Mg^{2+} and Ca^{2+}. Further experiments demonstrated, however, that Mg^{2+}- and Ca^{2+}-enhanced [^{125}I]-NKA specific binding was not inhibited by GTPγS, neurokinins, other than NKA, nor by receptor antagonists, and did not, therefore, represent specific binding to a tachykinin receptor (data not shown).

In contrast to the results with Mg^{2+} and Ca^{2+}, only a slight enhancement of [^{125}I]-NKA specific binding to guinea-pig lung parenchymal membranes was observed in the presence of Mn^{2+}. Further purification of the guinea-pig parenchymal lung membranes over discontinuous sucrose gradients, however, resulted in a 3–4 fold increase in the number of Mn^{2+}-dependent, [^{125}I]-NKA specific binding sites,

Table 1 Competition curves: tachykinin-receptor agonists and antagonists were used to compete for [^{125}I-Tyr^8]-substance P ([^{125}I-Tyr^8]-SP) specific binding to guinea-pig lung parenchymal membranes and [^{125}I]-neurokinin A ([^{125}I]-NKA) specific binding to sucrose-purified guinea-pig lung parenchymal membranes

	[^{125}I-Tyr^8]-SP		*[^{125}I]-NKA*	
Competing ligand	*IC_{50} (nM)*	*Slope factor*	*IC_{50} (nM)*	*Slope factor*
Agonist				
NK_1				
SP	0.083 ± 0.004 (10)	0.98 ± 0.02 (9)	0.17 ± 0.09 (3)	1.25 ± 0.05 (3)
[Sar^{9}Met(O$_2$)^{11}]SP	0.11 ± 0.01 (3)	0.92 ± 0.03 (3)	0.12 ± 0.01 (6)*	0.96 ± 0.12 (5)
SP-methyl ester	1.35 ± 0.09 (3)	0.85 ± 0.03 (3)	0.84 ± 0.42 (3)	0.58 ± 0.04 (3)
Physalaemin	1.5 ± 0.4 (3)	1.1 ± 0.2 (3)	0.3 (1)	0.73 (1)
NK_2				
NKA	31 ± 5 (3)	0.74 ± 0.04 (3)	2.3 ± 0.3 (4)	0.96 ± 0.07 (4)
[β-Ala^8]NKA (4–10)	4393 ± 472 (3)	0.73 ± 0.1 (3)	87 ± 11 (3)	0.69 ± 0.09 (3)
NK_3				
NKB	28.4 ± 0.3 (3)	0.83 ± 0.04 (3)	9 (2)	0.79 (2)
senktide	> 10000 (3)		> 10000 (3)	
Antagonist				
NK_1				
CP-99,994	0.6 ± 0.1 (3)	1.03 ± 0.02 (3)	0.47 ± 0.05 (4)*	0.64 ± 0.11 (4)
L-736,322	633 ± 118 (3)	1.05 ± 0.06 (3)	447 (2)*	0.53 (2)
RP67580	181 ± 31 (3)	0.93 ± 0.03 (3)	ND	
RP68651	> 20000 (2)		ND	
NK_2				
SR48968	1170 ± 202 (3)	0.92 ± 0.07 (3)	617 ± 71 (3)	0.66 ± 0.11 (3)
MEN 10376	> 10000 (3)		7763 ± 872 (3)	0.57 ± 0.06 (3)

IC_{50} values and slope factors (apparent Hill coefficients) were determined as described in the Methods and are shown ± s.e.mean with the number of observations given in parentheses. ND = not determined. Maximum specific binding was defined as the difference between total binding and non-specific binding determined in the presence of 1 μM of SP or NKA, respectively. The maximum inhibition of specific binding (I_{max}) was 100% in all cases except for the inhibition of [^{125}I]-NKA specific binding by [Sar^{9}Met(O$_2$)^{11}]SP, CP-99,994 and L-736,322 where I_{max} was 80% (*). The IC_{50} values and slope factors for these three competing ligands were calculated with non-specific binding determined in the presence of 1 μM of [Sar^{9}Met(O$_2$)^{11}]SP or CP-99,994 or 30 μM L-736,322, respectively.

from 0.59 ± 0.03 to 1.87 ± 0.06 fmol of [^{125}I]-NKA bound mg^{-1} membrane protein ($n = 3$). The optimal Mn^{2+} concentration for both purified and non-purified membrane preparations was 3 mM. In addition, Mn^{2+}-enhanced [^{125}I]-NKA specific binding fulfilled the criteria for binding specifically to a tachykinin receptor(s), as described in the following sections.

Rates of association and dissociation The profile obtained for the association and dissociation of [^{125}I]-NKA specific binding to sucrose-purified parenchymal membranes was directly comparable to the profile previously observed for [^{125}I-Tyr^8]-SP specific binding to guinea-pig lung parenchymal membranes. The rate of association of Mn^{2+}-dependent [^{125}I]-NKA specific binding to sucrose-purified parenchymal membranes was very slow (k_{obs}/[radioligand] = 0.8 × 10^9 min^{-1} M^{-1}, $n = 2$). In this case equilibrium was attained over a 2 h incubation at room temperature. The rate of dissociation provoked by addition of excess competing NKA (1 μM) was also slow ($k_{-1} = 0.01$ min^{-1}, $n = 2$), while the addition of GTPγS (100 μM) with the NKA, again resulted in the rapid and complete dissociation of [^{125}I]-NKA binding to non-specific levels.

Equilibrium competition curves Selected tachykinin-receptor agonists and antagonists were used to compete for [^{125}I]-NKA specific binding to sucrose-purified guinea-pig lung parenchymal membranes. The rank order of potency observed for the peptide agonists was SP = [Sar^{9}Met(O$_2$)^{11}]SP > SP-methyl ester > NKA > NKB >> [βAla^8]NKA (4–10), with senktide inactive at concentrations up to 10 μM. This was directly comparable with the rank order of potency determined for these ligands in competition for [^{125}I]-Tyr^8]-SP specific binding to guinea-pig lung parenchymal membranes (Table 1).

The results for inhibition of [^{125}I]-NKA specific binding to sucrose-purified guinea-pig lung parenchymal membranes by NK_1- and NK_2-receptor antagonists were also comparable with the data obtained for inhibition of [^{125}I-Tyr^8]-SP specific binding to guinea-pig lung parenchymal membranes by these compounds. Thus, the NK_1-selective CP-99,994 was the most potent competing ligand, displaying an IC_{50} value of 0.47 ± 0.05 nM, with the related stereoisomer L-736,322 again 1000 fold less potent. The NK_2-selective SR48968 and MEN 10,376, however, were still relatively poor in competition for [^{125}I]-NKA specific binding to these preparations, displaying IC_{50} values of 0.62 ± 0.07 μM and 7.8 ± 0.9 μM, respectively (Table 1).

The highly NK_1-selective [Sar^{9}Met(O$_2$)^{11}]SP and CP-99,994, however, only inhibited approximately 80% of the [^{125}I]-NKA specific binding to sucrose-purified guinea-pig lung parenchymal membranes (Figure 4). [^{125}I]-NKA binding assays were therefore conducted in the presence of saturating concentrations of [Sar^{9}Met(O$_2$)^{11}]SP (1 μM) or CP-99,994 (2.7 μM) to abolish the NK_1-receptor binding component. Under these conditions, the remaining [^{125}I]-NKA specific binding could be completely inhibited in a concentration-dependent manner by both [βAla^8]NKA (4–10) and SR48968, with IC_{50} values of 27 ± 7 nM ($n = 3$) and 0.23 ± 0.04 nM ($n = 4$), respectively (Figure 4), while the NK_3-selective agonist senktide was inactive at concentrations up to 10 μM.

Characterization of [^{125}I]-neurokinin A specific binding to guinea-pig tracheal membranes

[^{125}I]-NKA binding assay Due to the relatively low amount of membrane protein obtained from guinea-pig tracheal preparations, a limited series of experiments was conducted to identify the tachykinin-receptor types present in this tissue. The characteristics of [^{125}I]-NKA specific binding to guinea-pig tracheal membranes were essentially the same as those observed for sucrose-purified guinea-pig lung parenchymal membranes. [^{125}I]-NKA specific binding was enhanced by Mn^{2+} ions, although in this case the optimum cation concentration was 10 mM. In addition, the equilibrium for [^{125}I]-NKA specific binding to guinea-pig tracheal membranes was reached over a 2 h incubation period, and the dissociation of the radioligand provoked by addition of excess competing

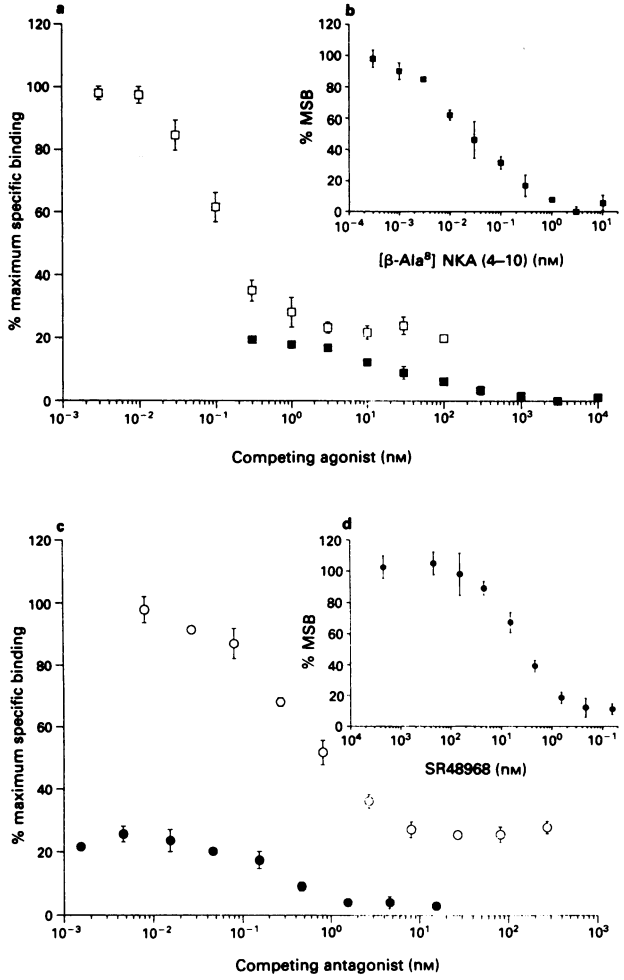

Figure 4 Competition for [^{125}I]-neurokinin A ([^{125}I]-NKA) specific binding to sucrose-purified guinea-pig lung parenchymal membranes by selective tachykinin-receptor agonists and antagonists. [^{125}I]-NKA binding assays were performed, as described in the Methods, in the presence of 3 pM–300 nM of either [Sar^9,Met(O$_2$)^{11}]SP (□), (a), or CP-99,994 (○). Residual [^{125}I]-NKA specific binding was then competed for with 0.3 nM–10 μM [β-Ala^8]NKA (4–10) (■, in the presence of 1 μM [Sar^9,Met(O$_2$)^{11}]SP, (a), or 30 pM–30 nM SR48968 (●), in the presence of 2.7 μM CP-99,994 (c). The inhibition curves for [β-Ala^8]NKA (4–10) and SR48968 expressed as a function of the percentage maximum specific binding determined in the presence of 1 μM [Sar^9,Met(O$_2$)^{11}]SP and 2.7 μM CP-99,994, respectively, are shown in (b) and (d). The IC_{50} values for competing NK_1- and NK_2-receptor agonists and antagonists were calculated on the basis of the maximum specific binding to the individual NK_1- and NK_2-receptor components.

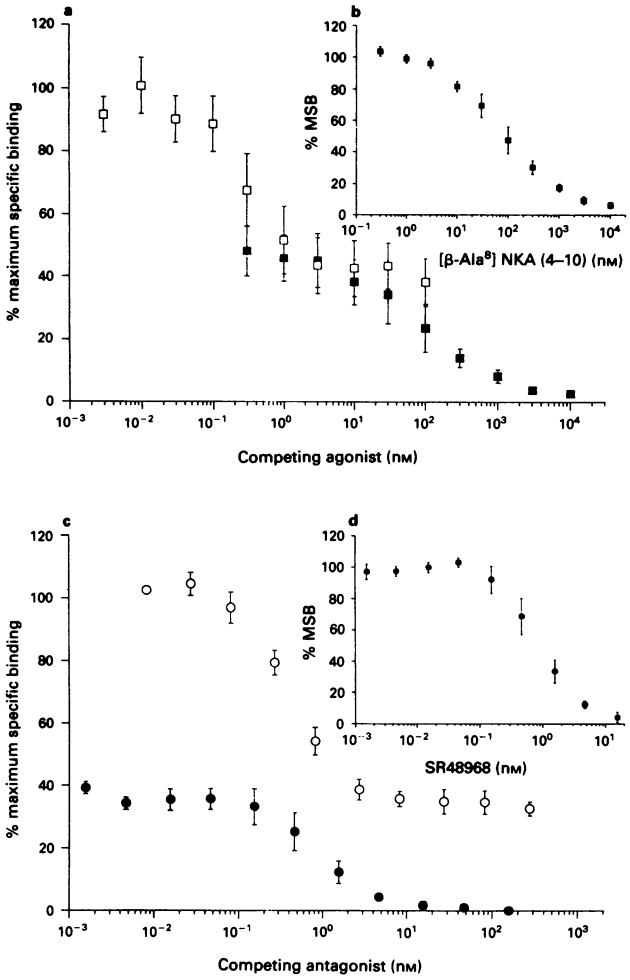

Figure 5 Competition for [^{125}I]-neurokinin A ([^{125}I]-NKA) specific binding to guinea-pig tracheal membranes by tachykinin-receptor selective agonists and antagonists. [^{125}I]-NKA binding assays were conducted as described in Figure 4. (a) Shows the concentration-dependent inhibition of [^{125}I]-NKA specific binding by [Sar^9,Met(O$_2$)^{11}]SP (□), and also [β-Ala^8]NKA (4–10) (■) when competed for in the presence of 1 μM [Sar^9,Met(O$_2$)^{11}]SP. (c) Shows the concentration-dependent inhibition of [^{125}I]-NKA specific binding by CP-99,994 (○) and also by SR48968 (●) when titrated in the presence of 2.7 μM CP-99,994. The inhibition curves for [β-Ala^8]NKA (4–10) and SR48968 expressed as a function of the percentage maximum specific binding in the presence of 1 μM [Sar^9,Met(O$_2$)^{11}]SP and 2.7 μM CP-99,994, respectively, are shown in (b) and (d). The IC_{50} values for competing NK_1- and NK_2-receptor agonists and antagonists were calculated on the basis of the maximum specific binding to the individual NK_1- and NK_2-receptor components.

NKA was significantly increased by simultaneous addition of 100 μM GTPγS.

Equilibrium competition curves Approximately 60% of [^{125}I]-NKA specific binding to guinea-pig tracheal membranes could be inhibited in a concentration-dependent manner by the NK_1-selective [Sar^{9}Met(O$_2$)^{11}]SP and CP-99,994, with IC_{50} values of 0.2 nM ($n = 2$) and 0.5 nM ($n = 2$), respectively (Figure 5). The remaining [^{125}I]-NKA specific binding component could then be completely inhibited in a concentration-dependent manner by either the NK_2-selective agonist [βAla^8]NKA (4–10) or antagonist SR-48968, with IC_{50} values of 88 nM and 0.9 nM ($n = 2$) (Figure 5).

Discussion

[^{125}I-Tyr^8]-substance P and [^{125}I]-NKA binding assays were employed to characterize NK_1 and NK_2 receptors in guinea-pig lung parenchymal and tracheal membrane preparations. Both radioligands were completely degraded by peptidases present in the tissue preparations. It is well established that neutral endopeptidase inactivates neurokinins in a variety of tissues, including guinea-pig airways (Dusser *et al.*, 1988; Devillier *et al.*, 1988; Webber, 1989). Inhibition of this enzyme alone, however, was insufficient to prevent metabolism of radiolabelled neurokinins under our experimental conditions. Thus, it was essential to include inhibitors of

angiotensin converting enzyme, aminopeptidase and several serine-protease activities. These results are in contrast to previous studies where neutral endopeptidase inhibitors either alone (Aharony *et al.*, 1991), or in combination with low concentrations of serine-protease inhibitors (Coats & Gerard, 1989; Geraghty *et al.*, 1992), appeared sufficient to maintain integrity of the radioligands used. In several studies this difference may be explained by the use of the radiolabel [^{125}I-Bolton-Hunter]SP, which is more resistant to proteolysis, particularly by aminopeptidase. In this case, however, it is highly likely that the unlabelled competing peptides will be degraded and, consequently, their potency underestimated.

The specific binding of [^{125}I-Tyr^8]-SP to guinea-pig lung parenchymal membranes was found to be of high affinity, saturable, enhanced by Mn^{2+}, dissociated by the GTP analogue GTPγS, and inhibited by tachykinin-receptor agonists with the following rank order of potency: SP = [Sar^{9}Met(O$_2$)^{11}]SP > SP-methyl ester = physalaemin > NKA >> [βAla^8]NKA (4–10) >> senktide. These results are consistent with previous studies describing the NK_1 receptor in a variety of systems, including the NK_1 monoreceptor dog carotid artery preparation (Regoli *et al.*, 1988) and in expression systems for the cloned rat and human NK_1 receptors (Hershey *et al.*, 1990; Takeda *et al.*, 1991). Our data convincingly demonstrate, therefore, that [^{125}I-Tyr^8]-SP binds to the NK_1 receptor in guinea-pig lung parenchyma.

Of particular interest is the observation that two structurally different NK_1-receptor antagonists, CP-99,994 (McLean *et al.*, 1992) and RP67580 (Garret *et al.*, 1991), display a 300 fold difference in potency at the guinea-pig lung NK_1 receptor, with K_i values of 0.4 nM and 127 nM, respectively. CP-99,994 is structurally related to CP-96345, the first selective non-peptide NK_1 antagonist to be described (Snider *et al.*, 1991). CP-96345 is approximately 100 fold more potent in competition for binding to the guinea-pig NK_1 receptor than with the rat NK_1 receptor. In contrast, RP67580 is a potent antagonist at the rat NK_1 receptor, with a K_i of 4.16 nM (Watling, 1992), but, as shown in this study, is 30 fold less active at the guinea-pig NK_1 receptor. These results, therefore, support the proposal that there is a distinct species-dependent structure-activity relationship for NK_1 antagonists (Garret *et al.*, 1991; Snider *et al.*, 1991; Appell *et al.*, 1992).

In order to characterize the NK_2 receptor in guinea-pig lung the number of detectable [^{125}I]-NKA specific binding sites in parenchymal membranes was increased 3–4 fold by purification over discontinuous sucrose gradients. The ability of tachykinin-receptor agonists and antagonists to compete for [^{125}I]-NKA specific binding was directly comparable to their potency in competition for [^{125}I-Tyr^8]-SP specific binding to parenchymal membranes. Thus, both the rank order of potency and the determined IC_{50} values were almost identical, demonstrating that the majority of [^{125}I]-NKA specific binding to sucrose-purified guinea-pig lung parenchymal membranes was also to the NK_1 receptor.

Notwithstanding this conclusion, the NK_1-receptor selective ligands [Sar^{9}Met(O$_2$)^{11}]SP and CP-99,994 were observed to inhibit only 80% of the [^{125}I]-NKA specific binding to sucrose-purified guinea-pig lung parenchymal membranes. In addition, the NK_2-preferring agonists NKA and [βAla^8]NKA (4–10) were more potent in competing for [^{125}I]-NKA specific binding than for [^{125}I-Tyr^8]-SP specific binding. Therefore, the possibility that [^{125}I]-NKA was also binding to the NK_2 receptor in sucrose-purified guinea-pig lung parenchymal membranes was investigated. When [^{125}I]-NKA binding assays were conducted in the presence of saturating concentrations of NK_1-selective ligands in order to fully occupy the NK_1 receptor, the remaining [^{125}I]-NKA specific binding sites could be inhibited in a concentration-dependent manner by the NK_2-selective ligands [βAla^8]NKA (4–10) and SR48968, but not by the NK_3-selective agonist senktide. The IC_{50} values determined for [βAla^8]NKA (4–10) and SR48968 in the presence of NK_1 ligands were in agreement with the potencies of these ligands at the NK_2 receptor (Emonds-Alt *et al.*, 1992). These data, therefore, strongly suggest the presence of a small population of NK_2 receptors in guinea-pig parenchyma and are consistent with the recent description of NK_2-mediated plasma extravasation in guinea-pig intraparenchymal airways (Tousignant *et al.*, 1993a).

Similar results were obtained investigating [^{125}I]-NKA specific binding to guinea-pig tracheal membranes. The profile of partial inhibition by selective ligands shows that this tissue contains a dual population of NK_1 and NK_2 receptors. The identification of both these tachykinin-receptor types supports similar conclusions from pharmacological studies which concluded that tachykinin-mediated contraction of guinea-pig trachea occurs as a result of activation of both NK_1 and NK_2 receptors (Ireland *et al.*, 1991).

In addition to the reports demonstrating that the NK_1 receptor type displays profound species differences, there are many studies supporting the hypothesis that species homologues of the NK_2 receptor also exist (for review see Maggi *et al.*, 1993). The rank order of potency of NK_2-receptor antagonists has led to the broad classification of the guinea-pig, rabbit and human receptors as NK_{2A} where MEN 10,207 > L-659877 > R396, and the hamster and rat receptors as NK_{2B}, where this order of potency is reversed. This classification of NK_{2A} and NK_{2B} receptors is, however, somewhat misleading as it is, as yet, essentially a reflection of species differences rather than the existence of true NK_2 receptor subtypes. Whereas there are several studies suggesting that subtypes of both NK_1 and NK_2 receptors exist within the same species, including the guinea-pig, the evidence is principally pharmacological and awaits clarification at the molecular level (Carruette *et al.*, 1992; Maggi *et al.*, 1993). In this report the small population of NK_2 receptors present in guinea-pig airways, combined with the low amounts of membrane available from tracheal tissue, has limited the study to the identification of the tachykinin receptor types present in these tissues and has precluded a detailed pharmacological characterization of these binding sites.

In summary, this paper confirms the presence of the NK_1 receptor in guinea-pig lung parenchymal membranes and, in addition, demonstrates that these tissues also express detectable levels of NK_2 receptors. We also provide evidence for the presence of both NK_1 and NK_2 receptors in guinea-pig trachea. These results are entirely consistent with functional studies describing NK_1- and NK_2-mediated events in guinea-pig airways at the level of both the parenchyma and the trachea. While the presence of mRNA coding for both NK_1 and NK_2 receptors in human lung has already been shown (Hopkins *et al.*, 1991; Graham *et al.*, 1991), this is the first unambiguous identification of NK_2 receptors in airway tissues by use of radioligand binding techniques.

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