Characterization of NK₁ and NK₂ tachykinin receptors in guinea-pig and rat bronchopulmonary and vascular systems

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1 NK₁ and NK₂ tachykinin receptors were characterized in guinea-pig and rat bronchopulmonary systems and in the vasculature of the rat by use of radioligand binding and/or functional studies.

2 The radioligands for NK₁ and NK₂ receptors (³H]-SP and [³H]-pNKA, respectively) did not label tachykinin receptors in homogenates of rat lungs or bronchi. In contrast, in the guinea-pig, [³H]-SP bound with high affinity to these tissues ($K_D = 0.23 \pm 0.08$ nM and 0.34 ± 0.05 nM, for lungs and bronchi, respectively). The total number of binding sites was 4.6 fold greater in bronchus ($B_{max} = 135 \pm 27$ fmol mg⁻¹ protein) than in lung homogenates ($B_{max} = 29.3 \pm 0.1$ fmol mg⁻¹ protein). Furthermore, this binding was markedly displaced by CP-96,345 ($pK_i = 9.5 \pm 0.1$) and RP 67580 ($pK_i = 7.6 \pm 0.1$), antagonists of NK₁ receptors, slightly displaced by SR 48968 ($pK_i = 6.6 \pm 0.1$), but not affected by actinomycin D or L-659,877, antagonists of NK₂ receptors. Specific binding of [³H]-pNKA, detected in guinea-pig bronchi ($K_D = 5.2 \pm 0.1$ nM, and $B_{max} = 203 \pm 19$ fmol mg⁻¹ protein) but not in lungs, was similarly (40 to 53%) displaced by RP 67580 (1 μ M), CP-96,345 (10 and 100 nM) or SR 48968 (10 and 100 nM). The displacement approximately doubled (87 to 91%) when SR 48968 (10 nM) was combined with either RP 67580 (1 μ M) or CP-96,345 (10 nM), but not when RP 67580 was combined with CP-96,345.

3 In urethane-anaesthetized guinea-pigs, i.v. injections of the NK₁ receptor agonists SP, [Pro⁹]-SP, [Sar⁹,Met(O₂)¹¹]-SP and septide, as well as the NK₂ receptor agonists NKA and [Lys⁵,MeLeu⁹,NLeu¹⁰]-NKA₍₄₋₁₀₎ (0.1–10 μ g kg⁻¹, i.v.), dose-dependently increased lung inflation pressure. The most potent of these peptides were septide and [Lys⁵,MeLeu⁹,NLeu¹⁰]-NKA₍₄₋₁₀₎ ($EC_{50} = 0.38 \pm 0.07$ and 0.07 ± 0.02 μ g kg⁻¹, respectively). Interestingly, septide was 130 fold less potent than SP in displacing [³H]-SP from its binding sites in the guinea-pig lung, whereas it was 14 fold more potent than SP as a bronchoconstrictor. RP 67580 (0.3–5 mg kg⁻¹, i.v.) and CP-96,345 (0.01–3 mg kg⁻¹, i.v.) dose-dependently reduced the bronchoconstriction produced by the NK₁ receptor agonists. Conversely, the NK₂ receptor antagonists actinomycin D (1–10 mg kg⁻¹, i.v.) and SR 48968 (0.03–0.3 mg kg⁻¹, i.v.) inhibited specifically the responses induced by NK₂ receptor agonists.

4 In pentobarbitone-anaesthetized rats, the NK₁ and NK₂ receptor agonists (0.01–4 μ g kg⁻¹, i.v.) produced dose-dependent hypotensive responses. The order of potency was SP = [Sar⁹,Met(O₂)¹¹]-SP = [Pro⁹]-SP > septide = NKA > [Lys⁵,MeLeu⁹,NLeu¹⁰]-NKA₍₄₋₁₀₎. RP 67580 (0.13–0.5 mg kg⁻¹, i.v.) and CP-96,345 (0.5–2 mg kg⁻¹, i.v.) antagonized in a dose-related manner (20 to 64%) the vascular effects of both NK₁ and NK₂ receptor agonists, whereas actinomycin D (3 mg kg⁻¹, i.v.) and SR 48968 (2 mg kg⁻¹, i.v.) did not. RP 67580 was approximately 4 times more potent than CP-96,345.

5 These studies indicate that NK₁ and NK₂ receptors are both present in the guinea-pig bronchopulmonary system whereas only NK₁ receptors are detectable in the rat vasculature under our experimental conditions. Furthermore, NK₁ receptors in the guinea-pig bronchopulmonary system are pharmacologically distinct from those present in the rat vascular system, since both agonist potencies and antagonist affinities differ between the two species.

Keywords: Vascular system of rat; bronchopulmonary systems of rat, guinea-pig; bronchoconstriction; hypotension; tachykinin receptor; RP 67580; CP-96,345; SR 48968; actinomycin D

Introduction

Substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) are members of a family of structurally related peptides, called tachykinins, that are involved in the regulation of various biological processes, particularly the modulation of bronchial smooth muscle contraction. These endogenous substances interact with three distinct types of tachykinin receptor, named NK₁, NK₂ and NK₃, as suggested by pharmacological and radioligand binding experiments, for which SP, NKA and NKB are the preferred endogenous agonists, respectively (Guard & Watson, 1991; Maggi *et al.*, 1993, for review). Recently, several novel, highly selective agonists of these receptors have been identified: [Pro⁹]-SP (Lavielle *et al.*, 1986), [Sar⁹,Met(O₂)¹¹]-SP (Regoli *et al.*, 1988) and septide (Wormser *et al.*, 1986) for the NK₁ receptor, [Lys⁵,MeLeu⁹,

NLeu¹⁰]-NKA₍₄₋₁₀₎ (Chassaing *et al.*, 1991) for the NK₂ receptor and senktide (Laufer *et al.*, 1986) for the NK₃ receptor. The availability of selective antagonists of these receptors, such as RP 67580 (Garret *et al.*, 1991) and CP-96,345 (Snider *et al.*, 1991) for NK₁, as well as SR 48968 (Advenier *et al.*, 1992; Emonds-Alt *et al.*, 1992) and L-659,877 (Williams *et al.*, 1988; McKnight *et al.*, 1991) for NK₂ receptors, provides an additional opportunity to characterize further the pharmacology and tissue location of these receptor sites (Watling, 1992). NK₂ receptor-mediated responses are also blocked by high doses of the antibiotic, actinomycin D (Lundberg & Lou, 1991; Delay-Goyet & Lundberg, 1991).

Tachykinins are known to contract bronchial smooth muscle (Karlsson & Persson, 1989; Frossard & Advenier, 1991, for review) and to cause hypotension (Couture *et al.*, 1989). However, the distribution of the different tachykinin receptors along the bronchopulmonary tree, as well as the partic-

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ular receptor type involved in these responses, is not well understood. Therefore, the aim of the present investigation was to determine the distribution and function of the different tachykinin receptors at the bronchopulmonary level. To this end, binding experiments on homogenates prepared from rat and guinea-pig bronchi and lungs were performed and tachykinin receptor-mediated bronchopulmonary and vascular responses were measured in guinea-pigs and rats, respectively. For these studies, we used various selective agonists and antagonists of NK₁ and NK₂ receptors.

Methods

Radioligand binding assays

Binding to NK₁ and NK₂ tachykinin receptor sites was performed using [³H]-SP and [³H]-propionyl-NKA ([³H]-pNKA), respectively, according to published procedures (Lee *et al.*, 1983; Bergström *et al.*, 1987) with slight modifications in order to increase levels of specific binding (Fardin & Garret, 1991; Garret *et al.*, 1991).

Preparation of homogenates of guinea-pig and rat bronchopulmonary tissues Male Sprague-Dawley rats (250–300 g, Iffa Credo, France) and male Dunkin-Hartley guinea-pigs (250–350 g, Charles River, France) were decapitated and their lungs and bronchi removed rapidly. The tissues were washed several times in ice-cold binding buffer which contained Tricine buffer (50 mM, pH 7.4 at 20°C), glucose (5.6 mM) and MgCl₂ (10 mM) for NK₁ or Tris buffer (50 mM, pH 7.4 at 20°C) for NK₂ receptor assays. The lungs were then freed from all non-lung tissue and weighed. Large and small bronchi were prepared by scraping off the parenchymal tissue of the lungs. Then, these tissues were cut into thin pieces and homogenized with a polytron sonicator in 15 vol (w/v) of appropriate binding buffer. Peptidase inhibitors (bacitracin 40 mg l⁻¹, leupeptin 4 mg l⁻¹ and bestatin 5 mg l⁻¹) were added to the buffer used for the homogenization and all washing steps. The homogenates were centrifuged (50,000 g for 10 min at 4°C) twice and the final pellets suspended in 5 vol (w/v) of buffer. This suspension was divided into aliquots and frozen at -80°C until use (generally 1 to 8 weeks after preparation). Protein content, measured according to the method of Smith *et al.* (1985) using bovine serum albumin (BSA) as the standard, yielded approximately 9 and 5 mg ml⁻¹ for lung and bronchus homogenates, respectively.

Binding assays On the day of experiment, the homogenate preparations were adjusted to a protein concentration of 0.3 mg ml⁻¹ by dilution with the appropriate binding buffer, containing BSA (0.02%), MnCl₂ (3 mM for NK₂ assays) and protease inhibitors [bacitracin (40 mg l⁻¹), chymostatin (4 mg l⁻¹, except for [³H]-SP assays for which bestatin 5 mg l⁻¹ was used), leupeptin (4 mg l⁻¹) and ketorphan (1 µM) or phosphoramidon (10 µM)]. The binding interaction was initiated by the addition of 1 ml of the homogenate suspension to 0.1 ml of the solution containing the ligand and 0.1 ml of binding buffer either alone (determination of total binding) or containing the competing drugs (final volume 1.2 ml). Incubations were performed at 25°C for 20 min ([³H]-SP) or 90 min ([³H]-pNKA). Non-specific binding was measured in the presence of non-radioactive SP (1 µM) and NKA (10 µM) for NK₁ and NK₂ assays, respectively.

For distribution studies, three different concentrations of [³H]-SP (0.3, 2 and 5 nM) and [³H]-pNKA (1, 3 and 6 nM) were used, whereas saturation binding assays were conducted over a ligand concentration range of 0.1–5 nM for [³H]-SP and 0.2–15 nM for [³H]-pNKA. In these studies, total and non-specific binding were measured in triplicate. Tachykinin binding was characterized by drug competition experiments. Guinea-pig lung homogenates were incubated with a single concentration of [³H]-SP (0.3 nM) and 6 to 8 concentrations

of tachykinin agonists SP, NKA, [Pro⁹]-SP, [Sar⁹, Met(O₂)¹¹]-SP, septide, [Lys⁵, MeLeu⁹, NLeu¹⁰]-NKA_(4–10) or senktide) or antagonists (CP-96,345, RP 67580, RP 68651, SR 48968, L-659,877 or actinomycin D). These experiments allowed the determination of the IC₅₀ values (concentration inducing 50% inhibition of specific binding) for each competing agent. Guinea-pig bronchi homogenates were incubated with [³H]-pNKA (1, 3 and 6 nM) in the presence of SR 48968 (100 nM) or RP 67580 (1 µM). Additional experiments were performed with [³H]-pNKA (3 nM) in the presence of SR 48968 (10 or 100 nM), RP 67580 (100 nM or 1 µM) or CP-96,345 (10 or 100 nM) or the combinations SR 48968 (10 nM) plus RP 67580 (1 µM), SR 48968 (10 nM) plus CP-96,345 (10 nM) or CP-96,345 (10 nM) plus RP 67580 (1 µM).

In all assays, incubations were terminated by rapid filtration (Skatron cell harvester), through Whatman GF/B glass fibre filters presoaked with 0.3% polyethylenimine. Filters were immediately rinsed with ice-cold buffer, and the radioactivity trapped on the filters measured by liquid scintillation counting.

Analysis of data Binding data were analysed by the curve fitting programmes LIGAND (Munson & Rodbard, 1980) and EBDA (McPherson, 1985), which allowed equilibrium dissociation constants (K_D) and total number of binding sites (B_{max}) to be determined from saturation studies, and IC₅₀ values to be determined from drug competition experiments. IC₅₀ values were then transformed into inhibition constants (K_i) according to the equation of Cheng & Prusoff (1973). These affinity values were expressed as mean pK_i values $\pm$ s.e. mean, which are the negative logarithms of the K_i values and which are reported in the result section.

Tachykinin-induced bronchoconstriction in guinea-pigs

Animals and treatments Male Dunkin-Hartley guinea-pigs (Charles River, St Aubin Lès Elbeuf, France) weighing 400–500 g were anaesthetized with an intraperitoneal injection of urethane (1.25 g kg⁻¹) and prepared according to the method of Konzett & Rössler (1940). The animals were ventilated with a respiratory pump (60 cycles min⁻¹, 10 ml air kg⁻¹). The right jugular vein was catheterized for drug injections and spontaneous breathing was suppressed by the administration of gallamine triethiodide (6 mg kg⁻¹, i.v.). The bronchial resistance to inflation was measured with a bronchospasm transducer (Ugo Basile 7020) connected to a recorder (Graptec, Mark VII). The right carotid artery was cannulated with a catheter connected to a blood pressure transducer (Gould P23XL) and the mean carotid blood pressure was recorded on the polygraph. The intratracheal cannula was clipped for 3 s before the experiment to calibrate the bronchospasm transducer. After a 15 min equilibration period, bronchial reactivity was tested with an intravenous bolus injection of acetylcholine (30 µg kg⁻¹). Only animals responding with an increase of more than 8 cmH₂O in lung inflation pressure were retained for the full experimental procedure.

In studies with agonists, animals received 10 min later three or four consecutive i.v. bolus injections of increasing doses of SP, [Pro⁹]-SP, [Sar⁹, Met(O₂)¹¹]-SP, septide, NKA or [Lys⁵, MeLeu⁹, NLeu¹⁰]-NKA_(4–10). A 10 min interval was allowed to elapse between successive doses, so that lung inflation pressure could return to pre-injection values.

In studies with antagonists, guinea-pigs received an intravenous injection of a submaximal dose of tachykinins twice. The bronchoconstrictor response to the second dose was taken as a control value. Then, animals received RP 67580, RP 68651, CP-96,345, actinomycin D, SR 48968 or their solvent, given over 1 min as 1 ml kg⁻¹, i.v. A third dose of tachykinin (the same as the previous two) was injected 5 min after treatment.

Analysis of data Experimental data are presented as means $\pm$ s.e.mean, whereas ED_{50} and ID_{50} values are reported with their s.d. Bronchoconstrictor responses are reported as the absolute increase (cmH₂O) in lung inflation pressure from baseline values. The potency of the agonists was evaluated by determining the ED_{50} , which is the dose producing 50% of the maximal response obtained with the highest dose of agonist studied ($E_{max} = 100\%$), by analysing the dose-response curve with the sigmoidal function $y = E_{max} + [(E_{min} - E_{max}) / (1 + x/ED_{50})^n]$ where y = measured bronchoconstrictor response, E_{min} = minimal effect, x = dose of agonist and n = slope coefficient (Gomeni & Gomeni, 1980). This programme allowed more than one dose-response curve to be drawn simultaneously and the $ED_{50} \pm$ s.d. to be calculated. For the antagonists, the dose inhibiting by 50% the response to a submaximal dose of agonist ($ID_{50} \pm$ s.d.) was determined by fitting simultaneously all the experimental results with the same sigmoidal function as above, where y = inhibition (%) of the bronchoconstrictor response. Statistical significance was evaluated with Student's unpaired or paired t test and the effect was assumed to be significant when $P \leq 0.05$.

Tachykinin-induced hypotension in rats

Animals and treatments Male Sprague-Dawley rats (Charles River, France) weighing 250 to 270 g were anaesthetized with sodium pentobarbitone (60 mg kg⁻¹, intraperitoneally) and artificially ventilated with room air (60 strokes min⁻¹, 10 ml kg⁻¹). The left carotid artery and the right femoral vein were cannulated for blood pressure measurement and drug administration, respectively. After a 15 min equilibration period, RP 67580, RP 68651, CP-96,345, actinomycin D, SR 48968 or vehicles were given as 10 min intravenous infusions. Ten minutes later, dose-response curves to i.v. bolus injections of SP, [Pro³]-SP, [Sar⁹, Met(O₂)¹¹]-SP, septide, NKA or [Lys⁵, MeLeu⁹, NLeu¹⁰]-NKA₍₄₋₁₀₎ were determined. Blood pressure was recorded on a polygraph (Graphtec, Mark VII) via a pressure transducer (Gould P23XL).

Analysis of data Results are reported as means $\pm$ s.e.mean of at least five rats/group. Hypotensive responses are given as maximal changes in blood pressure from baseline values. The potency of agonists was determined by calculating the dose that reduced blood pressure by 30 mmHg. This was done by computer-assisted curve fitting with the Sigma-plot programme (Jandel). The areas under the dose-(a $\rightarrow$ b)-hypotensive curves ($AUC_{(a\rightarrow b)}$), as illustrated in Figure 7, were calculated. These parameters were used to evaluate the inhibitory effects of the antagonist over the full dose-response curve (Cavero *et al.*, 1980). This inhibition was calculated as the percentage of reduction of the $AUC_{(a\rightarrow b)}$ obtained after the administration of the antagonist, taking the $AUC_{(a\rightarrow b)}$ obtained after vehicle pretreatment as 100%. The significance ($P \leq 0.05$) of the difference between responses to vehicle and to treatment was assessed by means of a Student's t test for unpaired data.

Materials

([³H]-prolyl^{2,4,3,4})-SP (specific activity 48.5–60 Ci mmol⁻¹; 1 Ci = 37 GBq) and ([³H]-propionyl-lysyl-3)-NKA (specific activity 93.8–106 Ci mmol⁻¹, synthesis on request) were obtained from Amersham (Les Ulis, France). [L-Pro³]-SP and [Lys⁵, MeLeu⁹, NLeu¹⁰]-NKA₍₄₋₁₀₎ were synthesized by Drs S. Lavielle and G. Chassaing (University Paris VI, Paris, France). RP 67580 ((3aR,7aR)-7,7-diphenyl-2-[imino-2-(2-methoxyphenyl)-ethyl]perhydroisindol-4-one), its inactive enantiomer RP 68651, and the active enantiomers CP-96,345 ((2S,3S)-cis-2-(diphenylmethyl)-N-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octan-3-amine) and SR 48968 ((S)-N-methyl-N-[4-(4-acetylaminophenyl)piperidino]-2-(3,4-dichlorophenyl)butyl]benzamide) were synthesized by Dr J.F. Peyronel and collaborators (Chemistry Department, Rhône-Poulenc Rorer, Centre de Recherche de Vitry-Alfortville,

France). SP, [Sar⁹, Met(O₂)¹¹]-SP and septide were obtained from Bachem (Bachem A.G., Bubendorf, Switzerland). NKA and L-659,877 were purchased from Novabiochem (Meudon, France) and CRB (Cambridge Research Biochemicals, Cheshire, UK), respectively. Other chemicals and reagents were obtained from Sigma (Sigma Chemical, St Louis, MO, U.S.A.) or Prolabo (Paris, France), except polyethylenimine which was obtained from Janssen Chemica (Noisy Le Grand, France). Kelatorphan was a generous gift from Prof. B. Roques (University Paris V, Paris, France).

Results

Binding studies

Effects of the metalloprotease inhibitors, phosphoramidon and kelatorphan In initial experiments, we studied the effects of either phosphoramidon (10 μ M) or kelatorphan (1 μ M) on the specific binding of [³H]-SP and [³H]-pNKA in guinea-pig tissues since it has been reported recently that phosphoramidon increases tachykinin binding in the lung (Aharony *et al.*, 1991; Geraghty *et al.*, 1991). In these assays, the peptidase inhibitors leupeptin, bacitracin, and either bestatin or chymostatin were present as described in the Methods section. In guinea-pig bronchi, kelatorphan increased the bind-

Figure 1 Distribution of tachykinin receptors labelled by [³H]-substance P ([³H]-SP) (a) and [³H]-propionyl-neurokinin A ([³H]-pNKA) (b) in homogenates of rat lung (O) and bronchus (●), and guinea-pig lung (□) and bronchus (■). The results are given as means $\pm$ s.e.mean of 3–4 independent experiments run in triplicate.

ing of [^3H]-pNKA, at concentrations of 1, 3 and 6 nM, by 270 ± 23 , 230 ± 20 and $190 \pm 10\%$ ($n = 3$), respectively. Similar increases were found with phosphoramidon. In contrast, these metalloprotease inhibitors did not significantly ($\leq 22\%$ of control values) modify [^3H]-SP (0.3, 2 and 5 nM) binding. On the basis of these results, kelatorphan (1 μM) was routinely used for all the studies described below.

Distribution of tachykinin receptors in the bronchopulmonary system In the rat, no significant specific binding of [^3H]-SP or [^3H]-pNKA to lung or bronchial tissue was detected, except when these ligands were used at high concentrations (5 or 6 nM, respectively), in which case the specific binding was slight (about 400 d.p.m. for [^3H]-SP and about 250 d.p.m. for [^3H]-pNKA).

In contrast, in the guinea-pig, [^3H]-SP displayed specific, concentration-dependent binding in both tissues. In the lungs, the specific binding of 0.3 nM [^3H]-SP was 85% of total binding, resulting in $24.4 \pm 2.4 \text{ fmol mg}^{-1}$ protein ($n = 3$, about 1000 d.p.m.); in bronchus homogenates, it resulted in $76 \pm 4 \text{ fmol mg}^{-1}$ protein ($n = 3$). Thus, the binding of [^3H]-SP was greater in the bronchi than in the lungs (Figure 1). Concentration-dependent specific binding, again predominant in the bronchi, was also obtained with [^3H]-pNKA. Only high concentrations (6 nM) of this ligand labelled lung homogenates and this occurred with some interassay variability ($9.4 \pm 2.3 \text{ fmol}$, about 700 d.p.m., $n = 3$). Moreover, within the range of concentrations used, the [^3H]-pNKA binding appeared lower (i.e. $58.5 \pm 7.0 \text{ fmol mg}^{-1}$ protein at 6 nM, $n = 4$) than that observed with [^3H]-SP in guinea-pig bronchi ($134 \pm 5 \text{ fmol mg}^{-1}$ protein at 5 nM, $n = 3$).

Figure 2 Scatchard analysis of [^3H]-substance P ([^3H]-SP) (a) and [^3H]-propionyl-neurokinin A ([^3H]-pNKA) (b) binding in guinea-pig lung (○) and bronchus (■) homogenates. Saturation studies were conducted over a concentration range of 0.1 to 5 nM for [^3H]-SP and 0.2 to 15 nM for [^3H]-pNKA. Data are taken from single representative experiments carried out in triplicate. The K_D and B_{max} values from this single experiment determined with [^3H]-SP were 0.374 nM and $38.1 \text{ fmol mg}^{-1}$ protein, respectively, for lung homogenates. The corresponding values for bronchi were 0.390 nM and 162 fmol mg^{-1} protein. For [^3H]-pNKA, the K_D and B_{max} values for bronchi were 5.8 nM and 240 fmol mg^{-1} protein, respectively.

To examine further the binding in guinea-pig tissues, saturation experiments were conducted with [^3H]-SP (0.1–5 nM) and [^3H]-pNKA (0.2–15 nM). Specific binding exhibited saturability for the two ligands and Scatchard plots obtained from typical experiments are shown in Figure 2. Assays performed with lung and bronchus homogenates showed that [^3H]-SP labelled an apparently homogeneous population of sites with high affinity. The equilibrium dissociation constants were in the same range for both tissues: $K_D = 0.23 \pm 0.08 \text{ nM}$ ($n = 3$) for lungs and $K_D = 0.34 \pm 0.05 \text{ nM}$, ($n = 2$) for bronchi. The total number of binding sites was 4.6 fold greater in bronchi ($B_{\text{max}} = 135 \pm 27 \text{ fmol mg}^{-1}$ protein) than in lung homogenates ($B_{\text{max}} = 29.3 \pm 0.1 \text{ fmol mg}^{-1}$ protein).

The transformation of the raw data obtained from experiments on [^3H]-pNKA binding to guinea-pig bronchi gave rise to linear Scatchard plots with the following equilibrium constants: $K_D = 5.2 \pm 0.1 \text{ nM}$ and $B_{\text{max}} = 203 \pm 19 \text{ fmol mg}^{-1}$ protein ($n = 3$). Thus, in guinea-pig bronchi, the total number of sites labelled by [^3H]-pNKA was 1.5 fold greater than that labelled by [^3H]-SP.

Pharmacological profile of tachykinin binding in guinea-pig lungs and bronchi The pharmacological profile of the specific binding of [^3H]-SP at a low concentration ($0.31 \pm 0.01 \text{ nM}$, $n = 8$) in guinea-pig lung homogenates was in agreement with that expected for NK_1 binding sites. SP and the two selective NK_1 receptor agonists, [Pro^9]-SP and [$\text{Sar}^9, \text{Met}(\text{O}_2)^{11}$]-SP, potently displaced specific [^3H]-SP binding, with IC_{50} values lower than 1 nM (pK_i values between 9.48 and 9.72, Table 1). Septide, another selective NK_1 receptor agonist, was found to be 130 fold less potent than SP ($\text{pK}_i = 7.60$). The selective NK_2 ([$\text{Lys}^5, \text{MeLeu}^9, \text{NLeu}^{10}$]-NKA $_{(4-10)}$) and NK_3 (senktide) receptor agonists exhibited very low affinities (at least 7000 fold lower than that of SP). However, NKA, the preferred endogenous ligand for NK_2 receptors, displaced the specific binding of [^3H]-SP with a pK_i value of 7.0; thus, it was about 500 fold less potent than SP itself.

We further characterize [^3H]-SP specific binding in the lungs by determining the affinities of several NK_1 and NK_2

Table 1 Affinities of several tachykinins and related compounds for [^3H]-substance P ([^3H]-SP, 0.3 nM) binding sites in guinea-pig lung

Competitor	pK_i
Agonists	
SP	9.72 ± 0.06
NKA	7.03 ± 0.15
[Pro^9]-SP	9.48 ± 0.09
[$\text{Sar}^9, \text{Met}(\text{O}_2)^{11}$]-SP	9.62 ± 0.07
Septide	7.60 ± 0.02
[$\text{Lys}^5, \text{MeLeu}^9, \text{NLeu}^{10}$]-NKA $_{(4-10)}$	5.86 ± 0.10
Senktide	inactive
Antagonists	
CP-96,345	9.47 ± 0.10
RP 67580	7.63 ± 0.06
RP 68651	inactive
SR 48968	6.59 ± 0.07
L-659,877	inactive
Actinomycin D	inactive

Data are expressed as pK_i values (the negative logarithm of the K_i). Results are the means $\pm$ s.e. mean of at least 3 independent determinations, each one performed in duplicate. Hill coefficients were close to 0.9 for CP-96,345 and RP 67580 and were 0.68 ± 0.04 and 0.58 ± 0.01 for septide and [$\text{Lys}^5, \text{MeLeu}^9, \text{NLeu}^{10}$]-NKA $_{(4-10)}$, respectively. Inactive indicates either no inhibition, or less than 50% inhibition, with a 10 μM concentration of the competitor ($\text{pK}_i < 5.4$).

receptor antagonists. The NK₁ antagonists used, CP-96,345 (0.01–100 nM) and RP 67580 (1 nM–3 µM), dose-dependently inhibited this binding (Figure 3). A clear enantioselectivity existed for this binding since RP 68561, the inactive enantiomer of RP 67580, exhibited no affinity for the sites labelled by [³H]-SP in the lungs. The NK₂ receptor antagonists, L-659,877 and actinomycin D, even at high concentrations (10 µM) did not displace [³H]-SP binding, whereas SR 48968, a selective NK₂ receptor antagonist, exhibited a slight affinity

for lung NK₁ receptors, but this was over 1000 fold lower than that of SP (Table 1).

The small amount of tissue obtained from guinea-pig bronchi limited our pharmacological characterization of the bronchial [³H]-pNKA binding site. The specific binding of

Figure 3 Displacement of [³H]-substance P ([³H]-SP) binding to guinea-pig lung homogenates by various tachykinin agonists: SP (Δ), septide (○), [Lys⁵, MeLeu⁹, NLeu¹⁰]-NKA₍₄₋₁₀₎ (■) and senktide (▽) (a) and antagonists: CP-96,345 (▲), RP 67580 (●), SR 48968 (□) and L-659,877 (▼) (b). Values are given as percentages of control [³H]-SP specific binding and are the means ± s.e.mean of three to four experiments performed in duplicate. Non-specific binding corresponded to 5–15% of total binding.

Figure 4 Specific binding of [³H]-propionyl-neurokinin A ([³H]-pNKA, 3 nM) to guinea-pig bronchi in the absence (controls: Con) or presence of RP 67580 (RP), CP-96,345 (CP), SR 48968 (SR) or their combination. Results are given as percentages of control [³H]-pNKA specific binding and are the means ± s.e.mean of 3 independent experiments run in duplicate. Non-specific binding corresponded to 10–20% of total binding. Statistical significance was evaluated by ANOVA followed by a Wilcoxon's test. Inhibition of binding in comparison to controls was significant ($P < 0.01$) for all groups. Significantly ($P < 0.01$) greater displacements were obtained when SR 48968 was combined with either CP-96,345 or RP 67580, than when only one antagonist was used.

Figure 5 Bronchoconstrictor effects (Δ in intratracheal pressure from baseline) produced in anaesthetized guinea-pigs by i.v. bolus injections of several NK₁ agonists: substance P (SP) (Δ), [Pro⁹]-SP (▲), [Sar⁹, Met(O₂)¹¹]-SP (●) and septide (○) (a) and NK₂ agonists: neurokinin A (NKA) (□) and [Lys⁵, MeLeu⁹, NLeu¹⁰]-NKA₍₄₋₁₀₎ (■) (b). Results are expressed as means ± s.e.mean of 4 to 6 guinea-pigs per group.

Table 2 Bronchopulmonary response to various tachykinin receptor agonists in the guinea-pig expressed as means ± s.e.mean of E_{max} (maximal increase in intratracheal pressure from baseline obtained with the highest dose of agonist studied) and means ± s.d. of ED_{50} (dose producing 50% of the E_{max}) values

Agonist	E_{max} (cmH ₂ O)	ED_{50} (μg kg ⁻¹ , i.v.)
SP	9.0 ± 0.3	5.17 ± 0.66
[Pro ⁹]-SP	9.0 ± 0.2	1.19 ± 0.26*
[Sar ⁹ , Met(O ₂) ¹¹]-SP	9.6 ± 0.2	0.72 ± 0.17*
Septide	9.4 ± 0.3	0.38 ± 0.07*
NKA	9.6 ± 0.2	0.82 ± 0.05*
[Lys ⁵ , MeLeu ⁹ , NLeu ¹⁰]-NKA ₍₄₋₁₀₎	9.5 ± 0.5	0.07 ± 0.02*

$n = 4-6$ guinea-pigs per group. *Value significantly ($P < 0.05$, Student's t test) different from that obtained in the SP group.

[³H]-pNKA was determined in the presence of 1 μ M RP 67580, a concentration that totally (over 90%) inhibited the binding of [³H]-SP to the NK₁ receptor in guinea-pig lungs. Under these conditions, the binding of [³H]-pNKA (6 nM) was decreased significantly by 43%. The presence of NK₂ binding sites was indicated by the displacement of about 50% of [³H]-pNKA binding by a relatively low concentration (100 nM) of SR 48968, which did not significantly inhibit the binding of [³H]-SP in guinea-pig lungs. Furthermore, [³H]-pNKA (3 nM) binding was similarly displaced (40 to 53%) by SR 48968 (10 and 100 nM), RP 67580 (1 μ M) and CP-96,345 (10 and 100 nM) (Figure 4). When CP-96,345 (10 nM) and RP 67580 (1 μ M) were combined, the displacement (49%) did not change. However, the combination of SR 48968 (10 nM) with either RP 67580 or CP-96,345 produced a greater displacement (91 and 87%, respectively), which was almost equal to the sum of the effects obtained with each individual antagonist (Figure 4).

Guinea-pig bronchopulmonary studies

Responses to agonists In guinea-pigs, the intravenous administration of the NK₁ receptor agonists SP, [Pro⁹]-SP, [Sar⁹, Met(O₂)¹¹]-SP and septide, and the NK₂ receptor agonists NKA and [Lys⁵, MeLeu⁹, NLeu¹⁰]-NKA₍₄₋₁₀₎ (0.1–10 μ g kg⁻¹, i.v.), dose-dependently increased lung inflation pressure (Figure 5). In this respect, [Lys⁵, MeLeu⁹, NLeu¹⁰]-NKA₍₄₋₁₀₎ and septide were the most potent compounds, being 74 and 14 fold more potent, respectively, than SP (ED₅₀ = 0.07, 0.38 and 5.17 μ g kg⁻¹, respectively). Their maximal effects did not differ significantly (Table 2). For studies with antagonists, equiactive doses of SP (5 μ g kg⁻¹, i.v.), [Pro⁹]-SP (3 μ g kg⁻¹, i.v.), [Sar⁹, Met(O₂)¹¹]-SP (2 μ g kg⁻¹, i.v.), septide (0.5 μ g kg⁻¹, i.v.), NKA (1 μ g kg⁻¹, i.v.) and [Lys⁵, MeLeu⁹, NLeu¹⁰]-NKA₍₄₋₁₀₎ (0.15 μ g kg⁻¹, i.v.) were chosen (Δ inflation pressure = 7.5 $\pm$ 0.3, 8.6 $\pm$ 0.3, 8.6 $\pm$ 0.9, 8.6 $\pm$ 0.3, 7.2 $\pm$ 0.4 and 8.5 $\pm$ 0.4 cmH₂O, respectively, n = 8–11 guinea-pigs/group).

Effects of antagonists None of the antagonists studied modified the baseline bronchomotor tone. CP-96,345 (0.01–3 mg kg⁻¹, i.v.) dose-dependently reduced the bronchoconstriction produced by SP, [Pro⁹]-SP, [Sar⁹, Met(O₂)¹¹]-SP and septide (ID₅₀ = 0.86 $\pm$ 0.25, 0.11 $\pm$ 0.02, 0.07 $\pm$ 0.02 and 0.024 $\pm$ 0.004 mg kg⁻¹, i.v., respectively, n = 4 to 5 guinea-pigs per dose). In contrast, RP 67580 (0.3–5 mg kg⁻¹, i.v.) did not affect SP-evoked bronchoconstriction but inhibited the pulmonary responses to [Pro⁹]-SP, [Sar⁹, Met(O₂)¹¹]-SP and septide (ID₅₀ = 3.30 $\pm$ 0.69, 3.52 $\pm$ 0.36 and 0.75 $\pm$ 0.11 mg kg⁻¹, i.v., respectively), whereas RP 68651 (5 mg kg⁻¹, i.v.), the inactive enantiomer of RP 67580, failed to antagonize septide-induced bronchoconstriction. Neither actinomycin D

(10 mg kg⁻¹, i.v.) nor SR 48968 (1 mg kg⁻¹, i.v.) modified the response induced by SP or septide (n = 4 to 6/group).

Responses to NKA and to [Lys⁵, MeLeu⁹, NLeu¹⁰]-NKA₍₄₋₁₀₎ were not affected by either RP 67580 (5 mg kg⁻¹, i.v.) or CP-96,345 (3 mg kg⁻¹, i.v.). Conversely, actinomycin D (1–10 mg kg⁻¹) and SR 48968 (0.03–0.3 mg kg⁻¹) dose-dependently inhibited the effects induced by NKA (ID₅₀ = 1.94 $\pm$ 0.46 and 0.07 $\pm$ 0.01 mg kg⁻¹, i.v., respectively) and by [Lys⁵, MeLeu⁹, NLeu¹⁰]-NKA₍₄₋₁₀₎ (ID₅₀ = 5.5 $\pm$ 1.1 and 0.08 $\pm$ 0.02 mg kg⁻¹, i.v., respectively, n = 4 to 6/group).

Rat vascular studies

Responses to agonists In rats, the NK₁ (SP, [Pro⁹]-SP, [Sar⁹, Met(O₂)¹¹]-SP and septide) and NK₂ (NKA and [Lys⁵, MeLeu⁹, NLeu¹⁰]-NKA₍₄₋₁₀₎) receptor agonists induced dose-dependent hypotension (Figure 6). The rank order of potency, established for doses decreasing blood pressure by 30 mmHg (Table 3), was SP = [Sar⁹, Met(O₂)¹¹]-SP = [Pro⁹]-SP > septide = NKA > [Lys⁵, MeLeu⁹, NLeu¹⁰]-NKA₍₄₋₁₀₎. SP was 2.7, 7.3 and 70 fold more potent than septide, NKA and [Lys⁵, MeLeu⁹, NLeu¹⁰]-NKA₍₄₋₁₀₎, respectively.

Effects of antagonists The intravenous infusion of RP 67580 (2 mg kg⁻¹), RP 68651 (10 mg kg⁻¹), actinomycin D (3 mg kg⁻¹) or SR 48968 (2 mg kg⁻¹) did not modify blood pres-

Table 3 Vascular response to various tachykinin receptor agonists in rats

Agonist	ED ₃₀ mmHg (μ g kg ⁻¹ , i.v.)
SP	0.189 $\pm$ 0.054
[Pro ⁹]-SP	0.148 $\pm$ 0.029
[Sar ⁹ , Met(O ₂) ¹¹]-SP	0.165 $\pm$ 0.025
Septide	0.628 $\pm$ 0.164*
NKA	1.054 $\pm$ 0.379*
[Lys ⁵ , MeLeu ⁹ , NLeu ¹⁰]-NKA ₍₄₋₁₀₎	20.67 $\pm$ 3.88*

Results are expressed in terms of ED₃₀ mmHg $\pm$ s.e.mean, which is the dose producing a blood pressure decrease of 30 mmHg. Initial MAP values were not significantly different from one group to another (from 124 $\pm$ 3 to 131 $\pm$ 2 mmHg, n = 5–6 rats per group).

*Value significantly (P < 0.05, Student's t test) different from that obtained in the SP group.

Figure 6 Hypotensive effects (Δ MAP) produced in anaesthetized rats by i.v. bolus injections of several NK₁ agonists: substance P (SP) (Δ), [Pro⁹]-SP ($\blacktriangle$), [Sar⁹, Met(O₂)¹¹]-SP ($\bullet$) and septide ($\circ$) (a) and NK₂ agonists: neurokinin A (NKA) ($\square$) and [Lys⁵, MeLeu⁹, NLeu¹⁰]-NKA₍₄₋₁₀₎ ($\blacksquare$) (b). Results are expressed as means $\pm$ s.e.mean of 4 to 6 rats per group.

Figure 7 Effects of RP 67580 (A) and CP-96,345 (B) on [Sar⁹, Met(O₂)¹¹]-substance P-induced hypotension in anaesthetized rats (n = 4–6 rats/group). The different treatments [(RP 67580, 0.13 (○) or 0.5 (●) mg kg⁻¹, CP 96,345, 0.5 (Δ) or 2 (▲) mg kg⁻¹ and vehicle (▼)] were infused over a 10 min period, 10 min before the agonist. Similar displacements of the dose-response curves to substance P (SP), [Pro⁹]-SP and septide were obtained with RP 67580 (0.13–0.5 mg kg⁻¹) and CP-96,345 (0.5–2 mg kg⁻¹, i.v.). AUC_{a→b} represents the area under the dose-response curve for doses ranging from the lowest (a) to the highest (b) dose studied.

Table 4 Hypotensive responses of several agonists expressed as the area under the dose-response curve ($AUC_{a \rightarrow b} \pm$ s.e.mean) in rats pretreated with antagonists (AUC_{ant}) or their vehicle (AUC_{veh}), as graphically represented in Figure 7 ($n = 5-6$ rats/group)

Agonist	Dose $a \rightarrow b$ ($\mu\text{g kg}^{-1}$, i.v.)	Vehicle — —	RP 67580 (mg kg^{-1})		$AUC_{a \rightarrow b}$ (% of inhibition) CP-96,345 (mg kg^{-1})		Actinomycin D (mg kg^{-1}) 3	SR 48968 (mg kg^{-1}) 2
			0.13	0.5	0.5	2		
SP	0.016→1	35 ± 1	28 ± 2* (20)	16 ± 2* (54)	25 ± 1* (29)	19 ± 1* (46)	36 ± 2 (0)	35 ± 2 (0)
[Pro ⁹]-SP	0.016→1	34 ± 2	26 ± 2* (24)	22 ± 2* (35)	26 ± 2* (24)	19 ± 1* (44)	ND	ND
[Sar ⁹ , Met(O ₂) ¹¹]-SP	0.063→1	34 ± 2	25 ± 1* (26)	15 ± 1* (56)	28 ± 3 (18)	14 ± 1* (59)	ND	ND
Septide	0.063→4	151 ± 10	115 ± 11* (24)	64 ± 8* (58)	104 ± 7* (31)	60 ± 5* (60)	ND	ND
NKA	0.016→1	25 ± 1	16 ± 2* (36)	12 ± 1* (52)	17 ± 1* (32)	9 ± 1* (64)	24 ± 2 (4)	24 ± 2 (4)
[Lys ⁵ , MeLeu ⁹ , NLeu ¹⁰]-NKA ₍₄₋₁₀₎	0.25→16	334 ± 23	ND	191 ± 19* (43)	ND	203 ± 26* (39)	ND	371 ± 34 (0)

Antagonist effects are given, in parentheses, according to $(AUC_{veh} - AUC_{ant}) \times 100$. The $AUC_{a \rightarrow b}$ are reported in arbitrary units.

*Value significantly ($P < 0.05$, Student's t test) different from that obtained in the vehicle-treated group.

ND = not determined.

sure, whereas CP-96,345 (2 mg kg^{-1}) caused significant hypotension (from 126 ± 2 to 85 ± 3 mmHg at the end of the 10 min infusion), which faded 10 min after ending the administration. This transitory effect may be attributed to the ability of CP-96,345 to interact with Ca^{2+} channels (Schmidt *et al.*, 1992). RP 67580 ($0.13-0.5 \text{ mg kg}^{-1}$) and CP-96,345 ($0.5-2 \text{ mg kg}^{-1}$) each antagonized to a similar degree the hypotensive effects of SP, [Pro⁹]-SP, [Sar⁹, Met(O₂)¹¹]-SP or septide (Table 4, Figure 7). In this respect, RP 67580 was approximately four times more potent than CP-96,345. However, SP-induced hypotension was not modified by RP 68651, the inactive enantiomer of RP 67580, at doses up to 10 mg kg^{-1} , i.v., by actinomycin D (3 mg kg^{-1} , i.v.) or by SR 48968 (2 mg kg^{-1} , i.v.). Finally, RP 67580 (0.5 mg kg^{-1}) and CP-96,345 (2 mg kg^{-1}) inhibited the vascular effects of NKA and [Lys⁵, MeLeu⁹, NLeu¹⁰]-NKA₍₄₋₁₀₎, whereas actinomycin D (3 mg kg^{-1} , i.v.) and SR 48968 (2 mg kg^{-1} , i.v.) did not (Table 4).

Discussion

The existence of NK₁ receptors in the guinea-pig lung is strongly supported by the results of previous (Castairs & Barnes, 1986; Aharony *et al.*, 1991; Geraghty *et al.*, 1991; Morimoto *et al.*, 1992) and present radioligand binding studies with [³H]-SP. Since most of the available data on tachykinin receptors in the bronchopulmonary system were obtained by studying the entire lung, one goal of the present work was to assess whether tachykinin binding sites are present in the bronchi. A higher density of NK₁ binding sites (4.6 fold) was found in the guinea-pig bronchus, as compared with the entire lung. The apparent affinity of [³H]-SP for guinea-pig lungs ($K_D = 0.23 \text{ nM}$) is close to that found for bronchi ($K_D = 0.34 \text{ nM}$) and is virtually the same as that reported by Morimoto *et al.* (1992) for the lung ($K_D = 0.27 \text{ nM}$).

Furthermore, we were able to obtain evidence for the first time, to our knowledge, that NK₂ binding sites exist in the bronchi. These results are in agreement with available functional studies showing that both NK₁ and NK₂ receptors mediate bronchoconstriction in the guinea-pig (Maggi *et al.*, 1991; see also Maggi *et al.*, 1993). A major problem for identifying NK₂ sites is the lack of sufficiently selective ligands for this receptor. This problem was brought to light by Geraghty *et al.* (1991), who reported that a low concen-

tration (50 pM) of the NK₂ ligand, [¹²⁵I]-iodohistidyl-NKA, labelled an NK₁-type receptor in guinea-pig lungs. However, the binding of [³H]-pNKA that we observed in guinea-pig bronchi was compatible with a simultaneous labelling of NK₁ and NK₂ sites since it was displaced to a similar degree (approximately 50%) by low concentrations of the selective antagonists of both NK₁ receptors (RP 67580 and CP-96,345) and NK₂ receptors (SR 48968). Although we have no direct evidence that would exclude the possibility that the inhibition of [³H]-pNKA binding by the NK₁ antagonists is due to their affinity for NK₂ receptors in the tissues studied, we consider this very unlikely because RP 67580, at a concentration of $10 \text{ }\mu\text{M}$, does not modify the binding of [³H]-pNKA to the rat duodenum (Garret *et al.*, 1991), nor does CP-96,345 display significant affinity for NK₂ binding sites (Snider *et al.*, 1991). Furthermore, the combination of SR 48968 with either CP-96,345 or RP 67580 resulted in a greater inhibition of this binding, which was almost equal to the sum of the inhibitory effects produced by each individual compound. That [³H]-pNKA labelled both NK₁ and NK₂ receptors is further supported by the absence of additional effects when RP 67580 and CP-96,345, each at a concentration inhibiting significantly [³H]-pNKA binding, were combined.

The present results, which extend those of Hua *et al.* (1984), also clearly indicate that stimulation of NK₁ or NK₂ receptors with appropriate agonists produces bronchoconstriction in guinea-pigs. We found that the NK₁ receptor agonists, SP, [Pro⁹]-SP, and [Sar⁹, Met(O₂)¹¹]-SP all displaced [³H]-SP from its binding sites in guinea-pig lungs with a similar degree of potency, and all produced bronchoconstriction. Furthermore, we antagonized the effects of these agonists with novel, selective antagonists of these receptors. RP 67580, which exhibited a 50 fold lower affinity for [³H]-SP binding sites than CP-96,345, was also approximately 30 times less potent than CP-96,345 in inhibiting NK₁ receptor agonist-induced bronchoconstriction in guinea-pigs.

Interestingly, septide, another selective NK₁ receptor agonist, was 130 fold less potent than SP in displacing [³H]-SP from its binding sites in the guinea-pig lung, whereas it was 14 fold more potent than SP as a bronchoconstrictor. We have already observed that septide was 350 to 7000 fold less potent than SP in inhibiting binding to NK₁ sites in various animal species and tissues (Fardin *et al.*, 1993). The poor correlation between affinity and functional activity has already been reported for the guinea-pig ileum, where septide is as potent as SP in producing contraction, whereas its affinity

for NK₁ receptors (labelled by either [³H]-SP or [³H]-Pro⁹-SP) is at least 200 fold lower than that of SP (Wormser *et al.*, 1986; Fardin & Garret, 1991; Petit *et al.*, 1992). A possible explanation for these findings is that septide may stimulate a subtype of the NK₁ receptor for which [³H]-SP is not the best ligand (Fardin & Garret, 1991; Petit *et al.*, 1992). This suggestion is supported by the finding of Petit *et al.* (1992) showing that GR 71251, a selective NK₁ peptide antagonist (Ward *et al.*, 1990), is 25 fold more potent in antagonizing the contractile effects of septide ($pK_B = 7.9$) than those of Pro⁹-SP ($pK_B = 6.5$) in the guinea-pig ileum. However, if a specific 'septide receptor site' exists, the present as well as previous results (Garret *et al.*, 1991; Carruette *et al.*, 1992) indicate that RP 67580 and CP-96,345 can block the activation of both the classical NK₁ and also the putative septide receptor sites. Moreover, recent studies involving the use of a recombinant rat NK₁ receptor, have yielded evidence that septide interacts with the NK₁ receptor at a site distinct from that of SP (Pradier *et al.*, 1993).

In the rat, SP and NKA, even at very high i.v. doses, produced only weak and not easily reproducible bronchoconstrictor responses (Joos *et al.*, 1988). These results suggest that NK₁ and NK₂ receptors are either absent or functionally silent (e.g. absence of coupling) in this species. We favour the former suggestion since, in contrast to what was observed with guinea-pigs, [³H]-SP and [³H]-pNKA specific binding was not detectable in either rat lungs or bronchi under experimental conditions identical to those used for guinea-pigs. However, it has been reported that some NK₁ agonists increase plasma protein extravasation in the rat airways (Saria *et al.*, 1983; Abelli *et al.*, 1991). Therefore, we cannot exclude the possibility that tachykinin receptors are present in the bronchopulmonary region of this species, but only in some discrete areas, particularly at the vascular level. Such species variations have also been found by experiments on isolated tracheal preparations, which failed to demonstrate tachykinin receptor-mediated contractile responses in the rat but did reveal them in the guinea-pig, rabbit and pig (Sheldrick *et al.*, 1990). However, this conclusion is perhaps not entirely valid for the cardiovascular system, since both the rat and guinea-pig respond to NK₁ receptor stimulation with a decrease in blood pressure (Goel & Biggs, 1987). That SP, [Pro⁹]-SP, [Sar⁹, Met(O₂)¹¹]-SP and septide interact with the NK₁ receptor is supported by the ability of RP 67580 and CP-96,345 to antagonize the hypotensive effects of these peptides.

Even though it is known that endopeptidases are present in the lung, the potency of SP as a bronchoconstrictor was only slightly (2.2 fold) enhanced in animals pretreated with phosphoramidon, an inhibitor of the enzyme that inactivates SP (data not shown). Thus, that septide was more potent than SP cannot be totally accounted for by the endogenous degradation of SP. Furthermore, although septide was significantly less potent than SP as a hypotensive agent, similar doses of NK₁ receptor antagonists (RP 67580 or CP-96,345) were needed to antagonize the effect of an equiactive dose of SP or septide. In contrast, septide was not only more potent than SP in inducing bronchospasm in guinea-pigs, but the dose of CP-96,345 inhibiting its effects was 30 fold smaller than that inhibiting the effects of an equiactive dose of SP.

Furthermore, in the rat cardiovascular system, CP-96,345 was found to be less potent than RP 67580 as an antagonist of NK₁ agonist-induced responses. Thus, RP 67580 appears to block NK₁ receptors better in the rat vasculature than in the guinea-pig bronchopulmonary system. The reverse holds true for CP-96,345. Therefore, although NK₁ receptors are present in the rat vascular system, they are not the identical counterpart of those in guinea-pig bronchi. Our *in vivo* results are in agreement with *in vitro* functional and binding studies which have demonstrated that CP-96,345 is a weaker NK₁ antagonist in the rat and mouse than in guinea-pig and human preparations and that the reverse holds true for RP 67580 (Beresford *et al.*, 1991; Fardin *et al.*, 1992).

These findings could be explained by the subtle difference in the amino-acid sequence of the rat and guinea-pig NK₁ receptors, which nevertheless have a high degree of homology (94.6%) (Gorbulev *et al.*, 1992). Indeed, for human and rat NK₁ receptors, the differences in affinities between RP 67580 and CP-96,345 are due to amino-acid differences at position 116 and 290 of the NK₁ receptor (Fong *et al.*, 1992; Pradier *et al.*, 1992). Interestingly, at those two positions, the amino-acids in the human and guinea-pig receptors are identical. An analogous case is the recent demonstration that the human 5-HT_{1B} receptor can be transformed into the rodent 5-HT_{1B} receptor by replacing threonine with asparagine at residue 355 (Oksenberg *et al.*, 1992). This single change renders the pharmacology of those two receptors essentially identical.

As already mentioned, we have also demonstrated the specific binding of [³H]-pNKA to NK₂ receptor sites in guinea-pig bronchi. This biochemical observation is confirmed by functional studies which demonstrate that NKA and [Lys⁵, MeLeu⁹, NLeu¹⁰]-NKA₍₄₋₁₀₎ produce bronchoconstriction in the guinea-pig, which is blocked specifically by the NK₂ receptor antagonists, actinomycin D and SR 48968. In the rat, the NK₂ receptor agonists, NKA and [Lys⁵, MeLeu⁹, NLeu¹⁰]-NKA₍₄₋₁₀₎ decreased blood pressure. This observation may indicate the presence of NK₂ receptors in the vascular system of the rat; moreover, NKA and a more selective NK₂ receptor agonist, NKA₍₄₋₁₀₎, have been reported to be poorly hypotensive peptides (Couture *et al.*, 1989). However, in the present study, the hypotensive effects evoked by NKA and [Lys⁵, MeLeu⁹, NLeu¹⁰]-NKA₍₄₋₁₀₎ were not modified by the selective NK₂ receptor antagonists, actinomycin D and SR 48968, but were antagonized by the NK₁ receptor antagonists, RP 67580 and CP-96,345. Thus, the rat NK₁ vascular receptor can also be stimulated by NK₂ receptor agonists, suggesting that the active site (or sites) of the NK₁ receptor on the rat vascular system discriminates poorly NK₁ from NK₂ agonists.

In conclusion, radioligand binding and functional studies indicate that NK₁ and NK₂ receptors are present in the bronchopulmonary system of the guinea-pig. A functional NK₁ receptor is present in the rat vasculature, but it differs from that of the guinea-pig bronchopulmonary system, since NK₁ receptor agonists exhibit differential potencies in inducing bronchoconstriction in guinea-pigs and hypotension in rats. The possible activation of NK₂ receptors in guinea-pigs does not appear to explain the differences in potency, since NK₂ antagonists did not modify the responses to NK₁ agonists at the bronchopulmonary level under our experimental conditions. Furthermore, RP 67580 is a more potent antagonist of the rat than of the guinea-pig NK₁ receptor, whereas the reverse relationship exists for CP-96,345, another non-peptide antagonist of NK₁ receptors. Finally, the NK₁ vascular receptor in the rat does not discriminate between NK₁ and NK₂ receptor agonists, since the effects of these substances can be inhibited by NK₁ antagonists but not by NK₂ antagonists. However, this is not the case for the bronchoconstrictor responses to NK₁ and NK₂ receptor agonists which are discriminated only by their respective antagonists. Biochemical (Beresford *et al.*, 1991; Fardin *et al.*, 1992; 1993) and molecular (Gorbulev *et al.*, 1992) studies have indicated that the human and guinea-pig NK₁ receptors resemble each other. Therefore, the optimization of chemical series of NK₁ receptor antagonists for human application should employ experimental animal preparations, such as the bronchopulmonary system of the guinea-pig, in which the NK₁ receptors have the same pharmacological characteristics as the human NK₁ receptor.

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References

- ABELLI, L., MAGGI, C.A., ROVERO, P., DEL BIANCO, E., REGOLI, D., DRAPEAU, G. & GIACHETTI, A. (1991). Effect of synthetic tachykinin analogues on airway microvascular leakage in rats and guinea-pigs: evidence for the involvement of NK-1 receptors. *J. Auton. Pharmacol.*, **11**, 267–275.
- ADVENIER, C., EMONDS-ALT, X., VILAIN, P., GOULAOUIC, P., PROIETTO, V., VAN BROECK, D., NALINE, E., NELIAT, G. & LE FUR, G. (1992). SR 48968, a potent and selective non-peptide antagonist of the neurokinin A (NK₂) receptor. *Br. J. Pharmacol.*, **105**, 77P.
- AHARONY, D., CATANESE, C.A. & WOODHOUSE, D.P. (1991). Binding of the novel ligand [4,5-³H-Leu¹⁰] Substance P to high-affinity NK₁ receptors on guinea-pig lung membranes: Modulation by GTP analogue and sulphydryl modifying agents. *J. Pharmacol. Exp. Ther.*, **259**, 146–155.
- BERESFORD, I.J.M., BIRCH, P.J., HAGAN, R.M. & IRELAND, S.J. (1991). Investigation into species variants in tachykinin NK₁ receptors by use of the non-peptide antagonist, CP-96,345. *Br. J. Pharmacol.*, **104**, 292–293.
- BERGSTROM, L., BEAUJOUAN, J.C., TORRENS, Y., SAFFROY, M., GLOWINSKI, J., LAVIELLE, S., CHASSAING, G., MARQUET, A., D'ORLEANS-JUSTE, P., DION, S. & REGOLI, D. (1987). [³H]-neurokinin A labels a specific tachykinin-binding site in the rat duodenal smooth muscle. *Mol. Pharmacol.*, **32**, 764–771.
- CARRUETTE, A., MOUSSAOUI, S.M., CHAMPION, A., COTTEZ, D., GONJOT, P. & GARRET, C. (1992). Comparison in different tissue preparations of the in vitro pharmacological profile of RP 67580, a new non-peptide substance P antagonist. *Neuropeptides*, **23**, 245–250.
- CASTAIRS, J.R. & BARNES, P.J. (1986). Autoradiographic mapping of substance P-receptors in lung. *Eur. J. Pharmacol.*, **127**, 295–296.
- CAVERO, I., GOMENI, R., LEFEVRE-BORG, F. & ROACH, A.G. (1980). Comparison of mianserin with desipramine, maprotiline and phentolamine on cardiac presynaptic and vascular postsynaptic α -adrenoceptors and noradrenaline reuptake in pithed normotensive rats. *Br. J. Pharmacol.*, **68**, 321–332.
- CHASSAING, G., LAVIELLE, S., LOEUILLET, D., ROBILLIARD, P., CARRUETTE, A., GARRET, C., BEAUJOUAN, J.C., SAFFROY, M., PETITET, F., TORRENS, Y. & GLOWINSKI, J. (1991). Selective agonists of NK₂ binding sites, highly active on rat portal vein (NK₂ bioassay). *Neuropeptides*, **1**, 91–95.
- CHENG, Y.C. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (I₅₀) of an enzymatic reaction. *Biochem. Pharmacol.*, **22**, 3099–3108.
- COUTURE, R., LANEUVILLE, O., GUIMOND, C., DRAPEAU, G. & REGOLI, D. (1989). Characterization of the peripheral action of neurokinins and neurokinin receptor selective agonists on the rat cardiovascular system. *Naunyn Schmied. Arch. Pharmacol.*, **340**, 547–557.
- DELAY-GOYET, P. & LUNDBERG, J.M. (1991). Dactinomycin is a competitive neurokinin-2 receptor antagonist. *Biochem. Biophys. Res. Commun.*, **180**, 1342–1349.
- EMONDS-ALT, X., VILAIN, P., GOULAOUIC, P., PROIETTO, V., VAN-BROECK, D., ADVENIER, C., NALINE, E., NELIAT, G., LE FUR, G. & BRELIERE, J.C. (1992). A potent and selective non-peptide antagonist of the neurokinin A (NK₂) receptor. *Life Sci.*, **50**, 101–106.
- FARDIN, V., FOUCAULT, F., BOCK, M.D., FLAMAND, O., JOLLY, A., CLERC, F. & GARRET, C. (1993). Variations in affinities for the NK₁ receptor: differences between the non-peptide substance P antagonists RP 67580 and CP-96,345 and the agonist septide. *Regul. Pept.*, **46**, 300–303.
- FARDIN, V., FOUCAULT, F., BOCK, M.D. & GARRET, C. (1992). Binding profile of RP 67580, a new non-peptide substance P antagonist: Comparison with CP-96,345. *Br. J. Pharmacol.*, **105**, 80P.
- FARDIN, V. & GARRET, C. (1991). Species differences between (³H)-Substance P binding in rat and guinea-pig shown by the use of peptide agonists and antagonists. *Eur. J. Pharmacol.*, **201**, 231–234.
- FONG, T.M., YU, H. & STRADER, C.D. (1992). Molecular basis for the species selectivity of the neurokinin-1 receptor antagonists CP-96,345 and RP 67580. *J. Biol. Chem.*, **267**, 25668–25671.
- FROSSARD, N. & ADVENIER, C. (1991). Tachykinin receptors and the airways. *Life Sci.*, **49**, 1941–1953.
- GARRET, C., CARRUETTE, A., FARDIN, V., MOUSSAOUI, S., PEYRONEL, J.F., BLANCHARD, J.C. & LADURON, P.M. (1991). Pharmacological properties of a potent and selective non-peptide substance P antagonist. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 10208–10212.
- GERAGHTY, D.P., MUSSAP, C.J. & BURCHER, E. (1991). Radioiodinated substance P, neurokinin A and eledoisin bind predominantly to NK₁ receptors in guinea pig lung. *Mol. Pharmacol.*, **41**, 147–153.
- GOEL, V. & BIGGS, D.F. (1987). Comparison of the bronchoconstrictor and cardiovascular effects of some tachykinins in guinea-pigs. *Life Sci.*, **40**, 1007–1015.
- GOMENI, R. & GOMENI, C. (1980). A conversational graphic program for the analysis of the sigmoid curve. *Comput. Biomed. Res.*, **13**, 489–499.
- GORBULEV, V., AKHUNDOVA, A., LUZIUS, H. & FAHRENHOLZ, F. (1992). Molecular cloning of substance P receptor cDNA from guinea-pig uterus. *Biochim. Biophys. Acta*, **1131**, 99–102.
- GUARD, S. & WATSON, S.P. (1991). Tachykinin receptor types: classification and membrane signaling mechanisms. *Neurochem. Int.*, **18**, 149–165.
- HUA, X., LUNDBERG, J.M., THEODORSSON-NORHEIM, E. & BRODING, E. (1984). Comparison of cardiovascular and bronchoconstrictor effects of substance P, substance K and other tachykinins. *Naunyn Schmied. Arch. Pharmacol.*, **328**, 196–201 and 361 (erratum).
- JOOS, G.F., PAUWELS, R.A. & VAN DER STRAETEN, M.E. (1988). The mechanism of tachykinin-induced bronchoconstriction in the rat. *Am. Rev. Respir. Dis.*, **137**, 1038–1044.
- KARLSSON, J.A. & PERSSON, C.G.A. (1989). Novel peripheral neurotransmitters and control of the airways. *Pharmacol. Ther.*, **43**, 397–423.
- KONZETT, H. & RÖSSLER, R. (1940). Versuchsanordnung zu Untersuchungen an der Bronchialmuskulatur. *Arch. Exp. Path. Pharmacol.*, **195**, 71–74.
- LAUFER, R., GILON, C., CHOREV, M. & SELINGER, Z. (1986). Characterization of a neurokinin B receptor site in rat brain using a highly selective radioligand. *J. Biol. Chem.*, **261**, 10257–10263.
- LAVIELLE, S., CHASSAING, G., JULIEN, S., MARQUET, A., BERGSTROM, L., BEAUJOUAN, J.C., TORRENS, Y. & GLOWINSKI, J. (1986). Specific recognition of SP and NKB receptors by analogues of SP substituted at positions 8 and 9. *Eur. J. Pharmacol.*, **125**, 461–462.
- LEE, C.M., JAVITCH, J.A. & SNYDER, S.H. (1983). [³H]-Substance P binding to salivary gland membranes: regulation by guanyl nucleotides and divalent cations. *Mol. Pharmacol.*, **23**, 563–569.
- LUNDBERG, J.M. & LOU, Y.P. (1991). Dactinomycin inhibits non-cholinergic bronchoconstriction by capsaicin-sensitive sensory nerves in the guinea-pig by antagonizing neurokinin 2 receptor activation. *Acta Physiol. Scand.*, **141**, 141–142.
- MAGGI, C.A., PATACCHINI, R., ROVERO, P. & GIACHETTI, A. (1993). Tachykinin receptors and tachykinin receptor antagonists. *J. Auton. Pharmacol.*, **13**, 23–93.
- MAGGI, C.A., PATACCHINI, R., ROVERO, P. & SANTICIOLI, P. (1991). Tachykinin receptors and non cholinergic bronchoconstriction in the guinea-pig isolated bronchi. *Am. Rev. Resp. Dis.*, **144**, 363–367.
- MCKNIGHT, A.T., MAGUIRE, J.J., ELLIOTT, N.J., FLETCHER, A.C., FOSTER, A.C., TRIDGETT, R., WILLIAMS, B.J., LONGMORE, J. & IVERSEN, L.L. (1991). Pharmacological specificity of novel, synthetic, cyclic peptides as antagonists at tachykinin receptors. *Br. J. Pharmacol.*, **104**, 355–360.
- MCPHERSON, G.A. (1985). Analysis of radioligand binding experiments. A collection of computer programs for the IBM PC. *J. Pharmacol. Methods*, **14**, 213–228.
- MORIMOTO, H., MURAI, M., MAEDA, Y., HAGIWARA, D., MIYAKE, H., MATSUO, M. & FUJII, T. (1992). FR 113680: a novel tripeptide substance P antagonist with NK₁ receptor selectivity. *Br. J. Pharmacol.*, **106**, 123–126.
- MUNSON, P.J. & RODBARD, D. (1980). A versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.*, **107**, 220–239.
- OKSENBERG, D., MARSTERS, S.A., O'DOWD, B.F., JIN, S., PEROUTKA, S.J. & ASHKENAZI, A. (1992). A single amino-acid difference confers major pharmacological variation between human rodent 5-HT_{1B} receptors. *Nature*, **360**, 161–163.

- PETITET, F., SAFFROY, M., TORRENS, Y., LAVIELLE, S., CHASSAING, G., LOEUILLET, D., GLOWINSKI, J. & BEAUJOUAN, J.C. (1992). Possible existence of a new tachykinin receptor subtype in the guinea-pig ileum. *Peptides*, **13**, 383–388.
- PRADIER, L., EMILE, L., HABERT, E., MERCKEN, L., LE GUERN, S., MORAS, V., LOQUET, I., CLOT, J., FARDIN, V., DOBLE, A. & MAYAUX, J.F. (1992). Molecular characterization of the NK₁ antagonist species selectivity on rat and human recombinant receptors. *Soc. Neurosci. Abst.*, **18**, 400.
- PRADIER, L., MENAGER, J., LE GUERN, J., BOCK, M.D., HEUILLET, E., FARDIN, V., DOBLE, A. & MAYAUX, J.F. (1993). Septide: an agonist of the NK₁ receptor acting at a site distinct from substance P. *Br. J. Pharmacol.*, **110**, 57P.
- REGOLI, D., DRAPEAU, G., DION, S. & COUTURE, R. (1988). New selective agonists for neurokinin receptors: pharmacological tools for receptor characterization. *Trends Pharmacol. Sci.*, **9**, 290–295.
- SARIA, A., LUNDBERG, J.M., SKOFITSCH, G. & LEMBECK, F. (1983). Vascular protein leakage in various tissues induced by substance P, capsaicin, bradykinin, serotonin, histamine and by antigen challenge. *Naunyn Schmied. Arch. Pharmacol.*, **324**, 212–218.
- SHELDRIK, R.L.G., BALL, D.I. & COLEMAN, R.A. (1990). Characterization of the neurokinin receptors mediating contraction of isolated tracheal preparations from a variety of species. *Agents Actions* (Suppl.), **31**, 205–209.
- SCHMIDT, A.W., MCLEAN, S. & HEYM, J. (1992). The substance P receptor antagonist CP-96,345 interacts with Ca²⁺ channels. *Eur. J. Pharmacol.*, **219**, 491–492.
- SMITH, P.K., KROHN, R.I., HERMANSON, G.T., MALLIA, A.K., GARTNER, F.H., PROVENZANO, M.D., FUJIMOTO, E.K., GOEKE, N.M., OLSON, B.J. & KLENK, D.C. (1985). Measurement of protein with bicinchoninic acid. *Anal. Biochem.*, **150**, 76–85.
- SNIDER, R.M., CONSTANTINE, J.W., LOWE III, J.A., LONGO, K.P., LEBEL, W.S., WOODY, H.A., DROZDA, S.E., DESAI, M.C., VINICK, F.J., SPENCER, R.W. & HESS, H.J. (1991). A potent nonpeptide antagonist of the substance P (NK1) receptor. *Science*, **251**, 435–437.
- WARD, P., EWAN, G.B., JORDAN, C.C., IRELAND, S.J., HAGAN, R.M. & BROWN, J.R. (1990). Potent and highly selective neurokinin antagonists. *J. Med. Chem.*, **33**, 1848–1851.
- WATLING, K.J. (1992). Nonpeptide antagonists herald new era in tachykinin research. *Trends Pharmacol. Sci.*, **13**, 266–269.
- WILLIAMS, B.J., CURTIS, N.R., MCKNIGHT, A.T., MAGUIRE, J., FOSTER, A. & TRIDGETT, R. (1988). Development of NK-2 selective antagonists. *Regul. Pept.*, **22**, 189–194.
- WORMSER, U., LAUFER, R., HART, Y., CHOREV, M., GILON, C. & SELINGER, Z. (1986). Highly selective agonists for substance P receptor subtypes. *EMBO J.*, **5**, 2805–2808.

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