Differences in the distribution and characteristics of tachykinin NK₁ binding sites between human and guinea pig lung

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- 1 The distribution and characteristics of tachykinin NK₁ binding sites have been compared in human and guinea pig lung using quantitative *in vitro* receptor autoradiography with [¹²⁵I]-Bolton Hunter-labelled substance P ([¹²⁵I]-BH-SP). In addition, the effects on these sites of ovalbumin sensitization and challenge have been determined in guinea pig lung.
- 2 [¹²⁵I]-BH-SP bound specifically and with high affinity to microvascular endothelium in both human and guinea pig lung, but to bronchial smooth muscle and pulmonary artery media in only guinea pig lung.
- 3 Specific binding of [¹²⁵I]-BH-SP to guinea pig bronchial smooth muscle was positively correlated with airway diameter in the range 150–800 µm and was less dense in trachea than in main bronchi.
- 4 [¹²⁵I]-BH-SP binding was inhibited by tachykinins with rank orders of affinity of SP > NKA > NKB (human microvessels) and SP > NKA = NKB (guinea pig bronchi and pulmonary arteries). NKA displayed a higher affinity for [¹²⁵I]-BH-SP binding sites in human microvessels than in guinea pig tissues ($P < 0.0001$), indicating differences in selectivity for tachykinins between human and guinea pig NK₁ receptors.
- 5 In both human and guinea pig lung, [¹²⁵I]-BH-SP binding was inhibited by the specific tachykinin receptor antagonists FK888 (NK₁ selective antagonist) and FK224 (mixed NK₁/NK₂ antagonist), with FK888 displaying equal affinity to SP and > 500 times higher affinity than FK224. SP, NKA, NKB and FK888 exhibited similar affinities for [¹²⁵I]-BH-SP binding sites in both guinea pig arteries and bronchi.
- 6 Similar distributions, densities and characteristics of [¹²⁵I]-BH-SP binding sites were demonstrated in ovalbumin-sensitized and -challenged guinea-pig lung and in naive animals.
- 7 Differences in the distribution and characteristics of NK₁ binding sites labelled with [¹²⁵I]-BH-SP between guinea pig and human lung suggest limitations in the use of guinea pig models for studying roles of tachykinins in pulmonary disease. However, the similar microvascular distributions of NK₁ binding sites in human and guinea pig lung suggest that the selective tachykinin receptor antagonists FK888 and FK224 may be useful in the management of airway inflammation in man.

Keywords: Substance P; receptor; autoradiography; asthma; bronchoconstriction; endothelium; tachykinin antagonists.

Introduction

Tachykinins, including substance P (SP) and neurokinin A (NKA), have been implicated in the regulation of airway tone and in the induction of oedema and mucous secretion, as well as in the control of inflammatory cell function and pulmonary vascular tone (Frossard & Advenier, 1991). Tachykinin receptors have been classified into three main types, referred to as NK₁, NK₂ and NK₃, for which SP, NKA and neurokinin B (NKB) are the preferred endogenous agonists respectively, although considerable cross-reactivity occurs (Frossard & Advenier, 1991).

Both NK₁ and NK₂ receptors can mediate bronchoconstriction in the guinea pig (Maggi *et al.*, 1991), although endogenous tachykinins, released by agents such as capsaicin or by nerve stimulation, cause bronchoconstriction predominantly via NK₂ receptors (Maggi *et al.*, 1991; Ballati *et al.*, 1992; Renzetti *et al.*, 1992). In human airways, tachykinin-induced bronchoconstriction is predominantly NK₂ receptor mediated (Advenier *et al.*, 1987; Joos *et al.*, 1987). By contrast, in animals, airway oedema induced by exogenous or endogenously released tachykinins is mediated almost entirely by NK₁ receptors (Lembeck *et al.*, 1992;

Murai *et al.*, 1993), and NK₁ receptors have been implicated in airway oedema induced by environmental agents such as cigarette smoke and allergen challenge (Delay-Goyet *et al.*, 1992; Bertrand *et al.*, 1993). NK₁ receptor activation also increases mucous secretion in human and guinea pig airways (Rogers *et al.*, 1989; Kuo *et al.*, 1990).

Because of these widespread effects on pulmonary function, it has been suggested that tachykinins play a role in human airway diseases such as asthma (Barnes, 1991). Recently, metabolically stable, selective tachykinin receptor antagonists have been developed as potential therapeutic agents for the treatment of human airway disease. These include the macrocyclic peptide, mixed NK₁/NK₂ receptor antagonist FK224 (*N*-[*N*²-[*N*-[2,3-didehydro-*N*-methyl-*N*-[3-(2-pentylphenyl)-propionyl]-L-threonyl]tyrosyl-L-leucynyl]-D-phenylalanyl]-L-allo-threonyl]-L-asparaginyl]-L-serine-*-v*-lactone) (Morimoto *et al.*, 1992) and the selective dipeptide NK₁ receptor antagonist, FK888 (*N*²-[(4*R*)-4-hydroxy-1-(1-methyl-1*H*-indol-3-yl)carbonyl-L-prolyl]-*N*-methyl-*N*-phenylmethyl-3-(2-naphthyl)-L-alaninamide) (Fujii *et al.*, 1992). However, differences between experimental models and man have made interpretation of data derived from animals difficult. SP, for example, is a more potent mediator of bronchoconstriction in the commonly used models of airway

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hyperreactivity in guinea pigs than in man (Advenier *et al.*, 1987).

Such species differences may be attributable to different distributions or pharmacological specificities of tachykinin receptor subtypes between species. Initial reports suggested similar distributions of [125 I]-BH-SP binding sites in human and guinea pig lung (Carstairs & Barnes, 1986), although a subsequent study found abundant binding to guinea pig airway smooth muscle but little or none to that in human lung (Goldie, 1990). [125 I]-BH-SP binding to guinea pig lung membranes has characteristics of NK₁ receptors (Geraghty *et al.*, 1992), and interspecies differences have recently been observed in the pharmacological profile of NK₁ receptors (Beresford *et al.*, 1991; Fujii *et al.*, 1992; Walsh *et al.*, 1993). The pharmacological characteristics of [125 I]-BH-SP binding sites in human lung have not, however, previously been reported or compared with those in the guinea-pig.

In addition to interspecies differences, responsiveness to SP may change during pulmonary disease. Following allergen challenge, guinea pigs become more sensitive to the bronchoconstrictor actions of SP with a leftward shift of the dose-response curve (Nieri *et al.*, 1992). It is at present unknown, however, whether antigen challenge modulates tachykinin receptor expression or if this increased sensitivity is due to other factors such as changes in peptide degradation or concomitant changes in prostaglandin release.

Since guinea pigs are commonly used as models of airway hyperreactivity in the development of pharmacological agents, we have compared the distribution and characteristics of [125 I]-BH-SP binding sites between guinea pig and human lung and have compared naive with ovalbumin-sensitized and -challenged guinea pigs. We have, furthermore, investigated the interaction with these sites of two tachykinin receptor antagonists, FK224 and FK888, recently developed as potential therapeutic agents for the treatment of human airway disease.

Methods

Tissues

Normal human lung was obtained from regions which were both macroscopically and microscopically normal from pneumonectomy samples removed for bronchial carcinoma ($n = 11$) and at autopsy ($n = 2$). The autoradiographic localization of [125 I]-BH-SP binding sites was investigated in samples of trachea, main/hilar bronchus and peripheral lung from two, ten and five cases respectively, and quantitative analysis performed on main and hilar bronchi.

Male Dunkin-Hartley guinea pigs were killed by overdose with sodium pentobarbitone (60 mg kg⁻¹), their tracheae and both lungs rapidly removed, and the lungs inflated by intra-tracheal insufflation with Tissue-Tek (Miles, Elkhart, IN, U.S.A.) diluted 1:1 with phosphate-buffered saline. Unfixed tissues were mounted on cork blocks with Tissue-Tek, snap frozen in melting isopentane and stored at -20°C. Sections (10 µm) were thaw mounted onto Vectabond-pretreated slides (Vector laboratories, Peterborough, U.K.) and used immediately or stored at -20°C with silica gel for up to 4 days before use.

Hypersensitive guinea pig model

Male Dunkin-Hartley strain guinea pigs ($n = 9$) were sensitized and challenged to ovalbumin by a modification of the method of Hutson *et al.* (1988). Animals were sensitized on two occasions (day 0 and 7) by 3 min exposure to an aerosol of 1% ovalbumin solution (w/v) in 0.9% saline, generated by a Wright nebulizer. Exposure was performed via an endotracheal tube inserted under transient anaesthesia induced by an injection of hypnorm (fentanyl citrate 0.315, fluanisone 10 mg ml⁻¹, 0.1 ml kg⁻¹ s.c.) followed by exposure to

methoxyflurane vapour. The use of an endotracheal tube avoids nasal deposition of the allergen and so restricts responses to the lower airways (Varley *et al.*, 1991a). Challenge of sensitized animals was performed on day 14, when they were exposed to an aerosol of 2% ovalbumin solution for 5 min, also administered via endotracheal tube while under anaesthesia. Animals were pretreated with mepyramine (15 mg kg⁻¹ i.p.) 30 min prior to challenge to prevent death due to anaphylactic shock. Control animals included guinea pigs which were not sensitized and were challenged with normal saline rather than ovalbumin (naive group, $n = 6$), sensitized but not challenged (sensitized group, $n = 6$), or were not sensitized but challenged with ovalbumin (challenged group, $n = 6$).

Following challenge, lower airways responses were measured in sensitized and challenged animals and in naive animals using an automated, computer-controlled whole-body plethysmograph (Varley *et al.*, 1991b). Measurements of lung function were taken at 15, 30, 45, 60 and 90 min after challenge, at which point the animals were killed by overdose of sodium pentobarbitone (60 mg kg⁻¹). The mean weight of animals at death was 621 g (95% CI 596-646).

[125 I]-BH-SP in vitro autoradiography

Quantitative *in vitro* autoradiography, using [125 I]-BH-SP, was performed as previously described (Wharton *et al.*, 1993). Sections of guinea pig lung (10 µm thick) were preincubated for 20 min in 10 mM HEPES buffer, pH 7.4, containing 130 mM NaCl, 4.7 mM KCl, 5 mM MgCl₂, 1 mM ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and 0.005% (v/v) polyethylenimine (buffer A). Excess buffer was removed and sections were incubated for 45 min with [125 I]-BH-SP in buffer B (buffer A plus 1% bovine serum albumin, 0.1% bacitracin, 100 µM chymostatin, 100 µM leupeptin and 10 µM phosphoramidon). Radioligand comprised 0.25 nM [125 I]-BH-SP alone (total binding) or together with an excess (1 µM) of unlabelled SP (non-specific binding). Sections were subsequently washed twice for 5 min at 4°C in buffer A and rinsed in distilled water before being rapidly dried under a stream of cold air. Binding to 10-µm-thick sections of human lung was assessed using a modification of the method of Carstairs & Barnes (1986). Incubation times and ligand concentrations were as for guinea pig lung, but, for human lung, buffers comprised 50 mM Tris-HCl, pH 7.4, with 3 mM MnCl₂ and 0.0025% polyethylenimine (buffer A) with buffer B containing, in addition, 1% bovine serum albumin, 0.1% bacitracin, 100 µM chymostatin, 100 µM leupeptin and 10 µM phosphoramidon. Except where stated, incubations were performed at 22°C.

Quantification of autoradiographic images

Radiolabelled tissue sections were apposed to radiosensitive film (Hyperfilm- 3 H, Amersham International, U.K.) and exposed for 3 days (guinea pig lung) or 5 days (human lung) at 4°C. Films were developed in Kodak D19 for 3 min at 15°C. Autoradiographic images were analysed using an IBAS 386 image processing system (Kontron, Watford, U.K.). Bronchi and pulmonary arteries were distinguished by comparison of films with corresponding tissue sections stained with haematoxylin and eosin. Binding density was derived from grey values by comparison with 10-µm-thick 125 I-labelled standards (Amersham International, U.K.) co-exposed with each film and corrected for the activity date of the radioligand.

Bronchus and pulmonary artery diameters were measured interactively on autoradiograms using the IBAS 386 image processing system. Diameters were taken as the maximum width perpendicular to the long axis of the structure, measured between the mid-points of the muscle layers, as indicated by localization of specific binding.

Microautoradiography

Tissue sections labelled with [¹²⁵I]-BH-SP were fixed in modified Bouin solution for 1 h at room temperature, washed in phosphate-buffered saline, rinsed in distilled water and dried in a flow of cold air. Sections were dipped in radiosensitive emulsion (Ilford K5) at 42°C and dried at room temperature. Following exposure for 3–5 weeks at 4°C, dipped slides were developed as for film autoradiograms, then counterstained with haematoxylin and eosin, dehydrated and mounted in dibutylphthalate polystyrene xylene (DPX, Raymond Lamb, London, U.K.).

Pharmacological characterization of [¹²⁵I]-BH-SP binding sites

Specific binding of [¹²⁵I]-BH-SP was defined as total binding minus non-specific binding (in the presence of 1 μM SP). Saturability of binding was measured by 'cold' saturation using total SP concentrations over the range 0.25–10.25 nM. Sections were incubated with 0.25 nM [¹²⁵I]-BH-SP together with increasing concentrations of unlabelled SP and total bound ligand (radiolabelled plus unlabelled) calculated as $([*L] + [L]) \times *B/[*L]$, where $[*L]$ is the concentration of free [¹²⁵I]-BH-SP, $[L]$ is the concentration of free unlabelled SP, and $*B$ is the measured bound [¹²⁵I]-BH-SP.

Specificity of [¹²⁵I]-BH-SP binding was measured by inhibition studies using unlabelled ligands, including SP and the related tachykinins NKA and NKB. In addition, inhibition studies were performed using the tachykinin receptor antagonists FK224 and FK888 (Fujisawa Pharmaceuticals, Osaka, Japan). NKB, FK224 and FK888 were each initially dissolved in dimethylsulphoxide (DMSO) and subsequently diluted in incubation buffer to give a maximum concentration of DMSO of 0.001%. In separate experiments, DMSO was found not to affect [¹²⁵I]-BH-SP binding at concentrations up to 0.005%.

Data analysis

Equilibrium constants were derived from specific binding data by iterative non-linear regression, assuming single-site models, using GraphPAD Inplot 4 (GraphPAD Software, San Diego, CA, U.S.A.). For guinea-pigs, equilibrium constants were derived for each of 2–6 bronchi and 1–4 pulmonary arteries per animal. Equilibrium binding densities (B_{eq}) were obtained with 0.25 nM [¹²⁵I]-BH-SP, and equilibrium dissociation constants (K_d) and maximal binding densities (B_{max}) were derived from binding inhibition studies using 0.25 nM [¹²⁵I]-BH-SP and 0–1000 nM unlabelled SP (DeBlasi *et al.*, 1989). Values for binding inhibition constants (K_i) and Hill coefficients were derived by fitting to sigmoid curves. K_i values were derived from IC_{50} values for each structure using the K_d value calculated for that structure in a consecutive series of sections (Cheng & Prusoff, 1973). Descriptive data are expressed as geometric means (95% confidence interval) of six guinea pigs per group. For human lung, the 12 microvessels per section with the most dense specific binding were quantified, and data are expressed as geometric means (95% confidence intervals) of 5–6 cases. The relationships between equilibrium binding density and structure diameter in peripheral lung tissues were determined for each case by linear regression. B_{eq} and B_{max} values were compared between naive and ovalbumin-challenged animals by stepwise multiple regression incorporating structure diameter and using the Statistical Package for Social Sciences (SPSS) software version 4.0 (Chicago, IL, U.S.A.). Comparisons of K_i values (which were not significantly correlated with structure diameter) and between different structures were made using analysis of variance (ANOVA) and Student's *t*-test with Bonferroni corrections on geometric data. Comparisons between bronchi and pulmonary arteries were made on data from separate experiments to those for comparisons

between naive and ovalbumin-sensitized and -challenged animals.

Immunohistochemistry

To confirm the binding of [¹²⁵I]-BH-SP to bronchial microvessels, consecutive sections to microautoradiograms were immunostained for von Willebrand factor (vWF) or platelet-endothelial cell adhesion molecule (PECAM, CD31) using previously characterized rabbit polyclonal antibodies (A082; Dako, High Wycombe, U.K.) or mouse monoclonal antibodies (JC170; Dako, High Wycombe, U.K.) respectively, and the avidin-biotin complex (ABC) method of Hsu *et al.* (1981). Cryostat sections (10 μm) were fixed in acetone for 5 min at 4°C, incubated with primary antibody for 2 h at room temperature, then with biotinylated goat anti-rabbit IgG (for vWF) or biotinylated horse antimouse IgG (for PECAM) for 30 min, avidin-biotin complex for 30 min and finally developed in diaminobenzidine, dehydrated and mounted in DPX.

Materials

[¹²⁵I]-BH-SP (Specific activity 2000 Ci mmol⁻¹), [¹²⁵I]-labelled standards and Hyperfilm-[³H] were obtained from Amersham International, U.K. FK224 (*N*-[*N*'-*N*-[*N*-[2,3-didehydro-*N*-methyl-*N*-[*N*-[3-(2-pentylphenyl)-propionyl]-L-threonyl]tyrosyl-L-leucynyl]-D-phenylalanyl]-L-*allo*-threonyl]-L-asparaginyl]-L-serine-*v*-lactone) and FK888 (*N*'-[(4*R*)-4-hydroxy-1-(1-methyl-1*H*-indol-3-yl)carbonyl-L-prolyl]-*N*-methyl-*N*-phenylmethyl-3-(2-naphthyl)-L-alaninamide) were kindly provided by Fujisawa Pharmaceuticals, Osaka, Japan. Other unlabelled peptides, enzyme inhibitors and enzyme-free bovine serum albumin were obtained from Sigma Chemical Co., Poole, U.K. Antibodies to vWF and PECAM were purchased from Dako, High Wycombe, U.K., and biotinylated goat anti-rabbit affinity-purified IgG biotinylated horse anti-mouse affinity-purified IgG and avidin-biotin complex were from Vector laboratories, Peterborough, U.K.

Results

Localisation of [¹²⁵I]-BH-SP binding sites

Human lung Specific binding of [¹²⁵I]-BH-SP was demonstrated to the endothelium of bronchial microvessels between the airway epithelium and smooth muscle, and in the connective tissue around cartilage (Figures 1a and 2a–d). Specific binding of [¹²⁵I]-BH-SP to microvessels was also observed in sections of human trachea and in main and hilar bronchus, but not in peripheral lung. No specific binding was observed to the smooth muscle of bronchus, trachea or pulmonary artery at any level. Specific binding of [¹²⁵I]-BH-SP in the region of bronchial and tracheal glands appeared to be localized to microvessels, as demonstrated by the distribution of vWF- and PECAM-like immunoreactivity, rather than to glandular elements themselves (Figure 2c and d). Binding of [¹²⁵I]-BH-SP to human lung under identical conditions to those used for guinea pig lung also revealed specific binding only to bronchial microvessels. Non-specific binding was, however, higher under these conditions.

Guinea pig lung Specific binding of [¹²⁵I]-BH-SP was localized in both naive and ovalbumin-challenged guinea pig lung to bronchial and tracheal smooth muscle (Figures 1c and e and 2e). On emulsion-dipped preparations, specific equilibrium binding of [¹²⁵I]-BH-SP to larger peripheral airways (diameter > 500 μm) appeared denser than that to smaller peripheral airways (diameter < 250 μm). Specific binding was observed to the endothelium of bronchial microvessels, both immediately beneath the epithelium and in connective tissue outside the cartilage of trachea and main bron-

chi. The distribution of [125 I]-BH-SP binding to bronchial microvessels corresponded to that of vWF-immunoreactive endothelia. Specific binding in the region of tracheal mucous glands was localized to microvessels rather than to glandular elements. Specific binding was also observed to the media of pulmonary arteries in peripheral lung (Figure 1e), but not in central pulmonary arteries. Specific binding was observed to ganglia in the oesophagus and intense non-specific binding was observed to airway epithelium and to eosinophils in some specimens. Polyethylenimine at 0.005% did not significantly inhibit specific [125 I]-BH-SP binding, but considerably reduced non-specific binding to eosinophils. Specific binding was not observed to airway epithelium.

Characterisation of [125 I]-BH-SP binding sites

Human lung Specific binding of [125 I]-BH-SP to human bronchial microvessels was saturable and of high affinity (Table 1 and Figure 3). Specific binding was inhibited by tachykinins with a rank order of affinities SP > NKA > NKB (Table 1 and Figure 4a). The selective tachykinin receptor antagonists inhibited specific binding, FK888 having > 500 times higher affinity than FK224 and equal affinity to SP (Table 1 and Figure 4b).

Guinea pig lung Specific binding of [125 I]-BH-SP to guinea pig bronchi and pulmonary arteries was also saturable and of

Figure 1 Binding of 0.25 nM [125 I]-Bolton Hunter-labelled substance P to human (a and b) and guinea-pig (c-f) lung. (a and b) Human bronchus showing specific punctate binding to microvessels in the submucosa and peribronchial regions. No specific binding is observed to bronchial smooth muscle. C, non-specific binding to bronchial cartilage; L, bronchial lumen. (c and d) Guinea pig trachea and hilar region showing specific binding to tracheal and hilar airway smooth muscle (m), to subepithelial microvessels (v) and to oesophagus (o). (e and f) Intrapulmonary structures in guinea pig lung with specific binding to bronchial smooth muscle (large arrows) and pulmonary artery media (small arrows). (a, c and e) Total binding and (b, d and f) non-specific binding in the presence of 1 μ M unlabelled substance P. Reversal prints of film autoradiograms. Bars = 2 mm.

high affinity (Table 1 and Figure 3). Specific binding to each structure was inhibited by tachykinins with the rank order of affinities SP > NKA = NKB (Table 1 and Figures 4c and 5). Binding was also inhibited by the tachykinin receptor antagonists FK224 and FK888, with FK888 having > 500 times higher affinity than FK224 (Table 1 and Figure 4d). No significant differences were found between bronchi and pulmonary arteries for affinities of SP, NKA, NKB or FK888. Bronchial microvessels demonstrated a similar order of affinities for these compounds, as assessed subjectively on emulsion-dipped preparations, to that found by quantification for bronchi and pulmonary arteries.

No significant differences in K_i values were found between human bronchial microvessels and guinea pig bronchi for the antagonists FK224 and FK888, but the agonist NKA had higher affinity in human bronchial microvessels than in guinea pig bronchi (Table 1).

Equilibrium binding of [¹²⁵I]-BH-SP to airway smooth muscle varied through the bronchial tree, with most dense binding to large, central bronchi and less dense binding to trachea ($t = 3.19$, $P = 0.004$). Increasing bronchial diameter in peripheral lung sections was associated with increasing B_{eq} (Table 2) and with increasing maximal binding density [slope of $\log B_{max}$ vs. mean bronchial diameter = 0.7 (95% CI 0.02–1.4) ($\log \text{amol mm}^{-2}$) mm^{-1} , $R^2 = 0.14$, $P = 0.04$]. No significant associations were observed between K_i values and bronchial diameter. Equilibrium binding of [¹²⁵I]-BH-SP to pulmonary arteries in peripheral lung was positively correlated with pulmonary artery diameter (Table 2).

Hypersensitive guinea pig model Ovalbumin challenge of sensitized guinea pigs induced significant and transient increases in airway resistance from 15 to 30 min after challenge, as compared with sensitized animals challenged

Figure 2 Microscopic localization of [¹²⁵I]-Bolton Hunter-labelled substance P binding in human (a–d) and guinea pig (e and f) lung. (a) Specific binding to submucosal microvessels (arrows) in human bronchus. No specific binding is observed to epithelium (open arrows) or bronchial glands (g). (b) Bronchial artery showing endothelial localization of binding. (c) Section of human lung showing bronchial microvessels (arrows), gland (g) and muscle (m). (d) Film autoradiogram corresponding to (c). Specific binding is restricted to microvessels, rather than to glandular elements or smooth muscle. (e) Specific binding to guinea pig bronchial smooth muscle (m), but not to bronchial glands or epithelium. Non-specific binding is observed over eosinophils (small arrows). (f) Specific binding to guinea pig bronchial microvessels (arrows). (a, b, e, and f) are emulsion-dipped preparations with haematoxylin counterstain. (c) shows immunostaining for PECAM with haematoxylin counterstain. Bars = 100 µm.

Table 1 Characteristics of [¹²⁵I]-Bolton Hunter-labelled substance P binding to human and naive guinea pig lung

	Human lung Microvessels	Bronchus	Guinea pig lung Pulmonary artery
$B_{\max}$ (amol mm ⁻²)	96 (37 to 251)	916 ^d (608 to 1377)	206 ^d (131 to 324)
K_i values (nM)			
Substance P	0.8 ^{ab} (0.5 to 1.1)	1.0 ^{ab} (0.7 to 1.3)	0.5 ^{ab} (0.3 to 0.7)
FK888	0.8 ^b (0.3 to 1.7)	0.9 ^b (0.7 to 1.3)	0.7 ^b (0.3 to 1.7)
Neurokinin A	13.3 ^{ac} (5.6 to 31.9)	336 ^{ac} (231 to 488)	215 ^{ac} (150 to 307)
Neurokinin B	439 ^a (149 to 1294)	179 ^a (114 to 284)	126 ^a (74 to 213)
FK224	434 ^b (263 to 714)	619 ^b (408 to 938)	NA
Hill coefficients			
Substance P	-1.3 (-1.2 to -1.5)	-1.4 (-1.2 to -1.6)	-1.2 (-0.9 to -1.6)
FK888	-1.0 (-0.7 to -1.4)	-0.8 (-0.7 to -1.0)	-0.8 (-0.5 to -1.2)
Neurokinin A	-1.0 (-0.7 to -1.4)	-0.9 (-0.8 to -1.0)	-1.0 (-0.8 to -1.2)
Neurokinin B	-1.2 (-0.9 to -1.7)	-1.2 (-1.0 to -1.5)	-1.1 (-0.9 to -1.4)
FK224	-1.3 (-1.0 to -1.5)	-1.3 (-1.1 to -1.5)	NA

Values expressed as geometric means (95% CI) of six cases per group. In both human and guinea pig lung ^aneurokinin A and neurokinin B had lower affinities than substance P (SP) ($P < 0.001$) and ^bSP and FK888 had equal affinities, and each had higher affinity than FK224 ($P < 0.001$). ^cNeurokinin A had higher affinity for specific [¹²⁵I]-BH-SP binding sites on human bronchial microvessels than on guinea pig bronchus or pulmonary artery ($P < 0.001$). No significant differences in K_i values were found between bronchi and pulmonary arteries. ^dMaximal binding to guinea pig bronchi was significantly greater than to pulmonary arteries ($P < 0.0001$). NA, not assessed.

with saline (Figure 6). Airway resistance had returned to baseline and control values by the time of sacrifice at 90 min.

The localization of [¹²⁵I]-BH-SP binding sites in ovalbumin-sensitized and-challenged guinea pig lung was similar to that in naive animals, in sensitized and in challenged controls. Comparisons of equilibrium binding of [¹²⁵I]-BH-SP to airway smooth muscle at the carina revealed no significant differences between the four groups [B_{eq} values for sensitized and challenged group, 29 (95% CI 22–38) amol mm⁻²; naive group, 28 (95% CI 15–53) amol mm⁻²; sensitized group, 31 (95% CI 13–74) amol mm⁻²; challenged group, 21 (95% CI 22–38) amol mm⁻²; $P = 0.59$, one-way ANOVA]. Subsequent comparisons were made between naive and ovalbumin-sensitized and -challenged groups. The relationships between equilibrium binding density of [¹²⁵I]-BH-SP and bronchial or pulmonary artery diameter were similar for naive and sensitized/challenged animals (Table 2) and no significant differences in $B_{\max}$ or K_i values were observed between disease groups (Table 3).

Discussion

We have found major differences in the distribution of [¹²⁵I]-BH-SP binding sites between guinea pig and human lung. Bronchial microvessels bound [¹²⁵I]-BH-SP specifically in both species, but, whereas binding was dense to bronchial smooth muscle and pulmonary arteries in guinea pig lung, these structures did not show specific binding in human lung. These results are similar to those described by Goldie (1990), but contrast with the apparent similarities between species implied by Carstairs & Barnes (1986). We have used both fresh surgical and post-mortem tissues, and sampled both peripheral and central airways. It seems unlikely, therefore, that the differences observed resulted from variations in the methods of tissue collection or from regional heterogeneity in human lung. Furthermore, since binding sites with characteristics of NK₁ receptors were identified on bronchial microvessels in human lung, we conclude that our tissue samples were adequately preserved for receptor autoradiography. It remains possible that human bronchi and pulmonary arteries possess NK₁ receptors, but at levels below the sensitivity of our autoradiographic method. However, our data are consistent with findings that human bronchi are less sensitive to NK₁ agonists than are guinea pig bronchi, and we suggest that such differences are due to lower levels of NK₁ receptors in human bronchial smooth muscle.

Figure 3 Saturation curves for [¹²⁵I]-Bolton Hunter-labelled substance P binding to human bronchial microvessels (▲), guinea pig bronchi (●) and guinea pig pulmonary arteries (○). Each point represents the mean ($\pm$ s.e. mean) of six cases.

The localization of [¹²⁵I]-BH-SP binding sites to bronchial microvessels in both guinea-pig and human trachea and central bronchi confirms previous findings (Hoover & Hancock, 1987; Goldie, 1990) and is consistent with a role for SP in control of vascular tone and permeability. We have demonstrated that these sites have features of NK₁ receptors with similar specificities to microvascular endothelial [¹²⁵I]-BH-SP binding sites characterized in other human tissues such as synovium (Walsh *et al.*, 1992; 1993). A similar localization of [¹²⁵I]-BH-SP binding to bronchial microvessels has been reported in rats, in which binding site distribution correlates with SP-induced plasma extravasation, suggesting a role in oedema formation (Sertl *et al.*, 1988). Airway oedema is an important component of airway obstruction, both in asthma and in chronic airflow limitation, but is not susceptible to acute reversal by conventional bronchodilator agents. Inhibition of airway oedema by NK₁ receptor antagonists, including FK888, has been demonstrated in animal models (Eglezos *et al.*, 1991; Lei *et al.*, 1992; Delay-Goyet *et al.*, 1992; Hirayama *et al.*, 1993; Murai *et al.*, 1993). Our demonstration that human bronchial microvessels also possess NK₁ binding sites and that [¹²⁵I]-BH-SP binding is inhibited by the tachykinin receptor antagonists FK888 and FK224 suggests that NK₁ antagonists may be of value in the control of airway oedema in humans.

Figure 4 Inhibition of specific [¹²⁵I]-Bolton Hunter-labelled substance P binding to human bronchial microvessels (a and b) and to guinea-pig bronchi (c and d) by unlabelled agonists and antagonists. (a and c) the rank orders of affinities for substance P (SP, ●), neurokinin A (NKA, ▲) and neurokinin B (NKB, ■) are SP > NKA > NKB in human lung, and SP > NKB = NKA in guinea pig bronchi, NKA having significantly higher affinity in human microvessels than in the guinea pig ($P < 0.0001$). (b and d) FK888 (■) and FK224 (▲) inhibit binding with similar affinities in both human microvessels and guinea pig bronchi, with FK888 having approximately equal affinity to SP (●) and higher affinity than FK224. Values represent means ($\pm$ s.e. mean) of six cases, specific binding expressed as per cent of that in absence of unlabelled ligand.

Figure 5 Inhibition of specific [¹²⁵I]-Bolton Hunter-labelled substance P binding to guinea pig pulmonary arteries by unlabelled substance P (●), neurokinin A (▲), neurokinin B (■) and FK888 (□). No significant differences were found between affinities between guinea pig pulmonary arteries and bronchi. Values represent means ($\pm$ s.e. mean) of six cases, specific binding expressed as per cent of that in absence of unlabelled ligand.

Guinea pig bronchi, in contrast to human airways, demonstrate clear bronchoconstrictor responses to NK₁ receptor agonists. In some species, bronchial responsiveness to NK₁ receptor agonists is greater in peripheral rather than central segments of main bronchi, and greater in central than peripheral lung strips (Black *et al.*, 1990; Wong *et al.*, 1992). The regional distribution of [¹²⁵I]-BH-SP binding sites noted in the current study in guinea pig central airways is similar to that previously reported in the rabbit (Black *et al.*, 1990). Furthermore, the decrease in the density of [¹²⁵I]-BH-SP bind-

ing sites with decreasing intrapulmonary airway diameter indicates that regional heterogeneity in small- and medium-sized airway responses to SP may result from variations in NK₁ receptor densities. The biological importance of differential regulation of bronchial tone at different levels of the bronchial tree remains to be determined.

Guinea pig pulmonary vasculature responds to tachykinins *in vivo* through actions on multiple receptors (Maggi *et al.*, 1990). Endothelium-dependent vasodilation, as in the systemic circulation, is NK₁ receptor mediated, while endothelium-independent vasoconstriction is predominantly NK₂ receptor mediated. Specific binding of [¹²⁵I]-BH-SP to the media of guinea pig pulmonary arteries has previously been noted, but not characterized (Carstairs & Barnes, 1986; Burcher *et al.*, 1989). Our findings suggest that this binding is to NK₁ rather than to NK₂ receptors, and to sites very similar to NK₁ receptors present in bronchial smooth muscle. A precise functional role for NK₁ receptors in the media of pulmonary arteries remains to be determined, but the available data suggest differences in peptide regulation between systemic and pulmonary vascular beds.

NKA and NKB displayed similar affinities for [¹²⁵I]-BH-SP binding sites in guinea pig lung, as previously found in membrane preparations (Geraghty *et al.*, 1992). In human lung, however, NKA displayed higher affinity than NKB, suggesting that guinea pig and human NK₁ receptors differ in their sensitivity to agonists. The rank order of affinities of unlabelled tachykinins and antagonists for specific microvascular [¹²⁵I]-BH-SP binding sites in human lung was identical to that previously demonstrated for microvessels in human synovium (Walsh *et al.*, 1992; 1993). These latter studies used HEPES buffer containing the same concentrations of cations as those used for guinea pig lung in this study, suggesting that differences in selectivity between human and guinea pig [¹²⁵I]-BH-SP binding sites were not due to the different buffers used.

Table 2 Relationship between specific equilibrium binding density of [¹²⁵I]-Bolton Hunter-labelled substance P and bronchial or pulmonary artery diameter in naive and ovalbumin-sensitized and -challenged guinea pigs

	Bronchus		Pulmonary artery	
	Naive	Ovalbumin-sensitized/ challenged	Naive	Ovalbumin-sensitized/ challenged
R^2	0.48 (0.30 to 0.67)	0.41 (0.26 to 0.56)	0.29 (0.05 to 0.53)	0.26 (0.18 to 0.34)
Gradient [(log amol mm ⁻¹) mm ⁻¹]	2.1 (1.7 to 2.6)	2.3 (1.4 to 3.1)	2.1 (1.1 to 3.1)	1.8 (0.7 to 2.9)
y intercept (log amol mm ⁻²)	0.8 (0.4 to 1.1)	0.7 (0.2 to 1.1)	0.1 (-0.2 to 0.4)	-0.2 (-0.5 to 0.1)

Values represent means (95% CI) of six cases per group. Values of R^2 , gradient and y intercept were derived for each case in each group by linear regression analysis of log₁₀ equilibrium binding density (abscissa) against structure diameter (in mm). All bronchial or pulmonary artery profiles with diameters between 0.2 and 0.8 mm were quantified ($n = 8-20$ per case). Significant positive relationships were observed between equilibrium binding density and bronchial and pulmonary artery diameters in both naive and experimental lung, with no overall differences between disease groups.

Figure 6 Changes in airway resistance (R_{aw}) in ovalbumin-sensitized guinea pigs following challenge via an intratracheal tube with aerosolized ovalbumin (●) or saline (▲). (a and b) The increase in R_{aw} was significantly greater 15 and 30 min following challenge with ovalbumin than following saline ($^aP < 0.01$ and $^bP < 0.05$).

The tachykinin receptor antagonists FK888 and FK224 displaced [¹²⁵I]-BH-SP binding from both human and guinea pig pulmonary tissues. Recently, Miyayasu *et al.* (1993) demonstrated that [³H]-FK888 binds to guinea pig bronchial smooth muscle and to sites with characteristics of NK₁ receptors in guinea pig lung. In our studies, human and guinea pig [¹²⁵I]-BH-SP binding sites had similar affinities for the antagonists FK224 and FK888. By contrast, FK888 displays much lower affinity for [¹²⁵I]-BH-SP binding sites in rat tissues than in guinea pig lung and human synovial microvessels (Fujii *et al.*, 1992; Walsh *et al.*, 1993). Similar orders of affinities between species have been observed for the non-peptide NK₁ receptor antagonist CP-96,345 (Beresford *et al.*, 1991). It seems likely that further interspecies differences in NK₁ receptors may be uncovered by the development of novel ligands in the future, and testing of potential therapeutic compounds on human NK₁ receptors will remain an important aspect of drug development.

Intraspecies NK₁ receptor subtypes have been suggested by *in vivo* work performed in guinea pig ileum (Petitet *et al.*, 1992), but not, so far, confirmed in binding studies. Quantitative *in vitro* receptor autoradiography permits the independent characterization of multiple structures in a single tissue simultaneously incubated under identical conditions. We found no evidence, however, that [¹²⁵I]-BH-SP binds to different receptor subtypes in different structures in guinea pig lung.

The presence of [¹²⁵I]-BH-SP binding sites in hypersensitive and challenged guinea-pig airways suggests that these animals are, like naive animals, susceptible to the actions of NK₁ receptor agonists and antagonists on airway function.

Table 3 Characteristics of [¹²⁵I]-Bolton Hunter-labelled substance P binding to bronchi from naive and ovalbumin-sensitized and -challenged guinea pigs

	Naive	Ovalbumin-sensitized/ challenged
B_{max} (amol mm ⁻²)	427 (275 to 676)	724 (386 to 1380)
K_i values (nM)		
Substance P	1.5 (0.9 to 2.3)	1.8 (1.2 to 2.7)
FK888	1.1 (0.6 to 1.8)	1.2 (0.7 to 2.0)
FK224	619 (408 to 938)	607 (472 to 847)
Hill coefficients		
Substance P	-1.0 (-0.9 to -1.2)	-1.2 (-1.0 to -1.5)
FK888	-1.1 (-1.0 to -1.2)	-1.3 (-1.0 to -1.6)
FK224	-1.3 (-1.1 to -1.5)	-1.4 (-1.2 to -1.7)

Values expressed as geometric means (95% CI) of six animals from each group. No significant differences in B_{max} or K_i values, or in Hill coefficients, were observed between naive and ovalbumin-sensitized and challenged animals.

However, after taking account of regional variations in NK₁ receptor distribution in guinea pig lung, we found no significant differences in [¹²⁵I]-BH-SP binding to bronchial smooth muscle between naive and ovalbumin-challenged hypersensitive guinea pigs. In particular, we found no evidence for changes in equilibrium or maximal binding densities for [¹²⁵I]-BH-SP, or in affinity for SP, FK224 or FK888 following ovalbumin sensitization and challenge. The increased sensitivity to SP observed following ovalbumin challenge in hypersensitive guinea pigs (Neiri *et al.*, 1992) may not be caused by receptor up-regulation or changes in receptor affinity, but rather by changes in peptide-degrading enzymes, or by potentiation by other mediators released during the challenge.

In conclusion, [¹²⁵I]-BH-SP binds with high affinity to NK₁ binding sites present on human bronchial microvessels and, in the guinea pig, present on bronchi, pulmonary arteries and microvessels. Interspecies differences are apparent between the guinea pig and human in terms both of the distribution of binding sites and the affinities of tachykinin agonists for these sites, particularly NKA, but not the tachykinin antagonists tested here. Our data support a potential role for NK₁ receptor antagonists such as FK224 and FK888 in the therapeutic control of airway function, but suggest that NK₁ receptor antagonists are more likely in man to be of benefit in controlling airway microvascular function and inflammation rather than to have direct effects on airway tone.

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