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Autoradiographic localization of tachykinin NK_2 and NK_1 receptors in the guinea-pig lung, using selective radioligands

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Abstract

The distribution of tachykinin receptors in guinea-pig airways was studied using newly developed selective radioligands, [^{125}I][Lys^5,Tyr(I_2)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10) for NK_2 receptors and [^{125}I]Bolton-Hunter-[Sar^9,Met(O_2)^{11}]substance P for NK_1 receptors. Optimum incubation times were 60 and 45 min for tachykinin NK_2 and NK_1 sites, respectively, in slide-mounted sections of guinea-pig lung. Bacitracin (40 μg/ml) greatly reduced specific binding of [^{125}I][Lys^5,Tyr(I_2)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10), whereas phosphoramidon (1 and 10 μM) and bacitracin (40 μg/ml) significantly increased the specific binding of [^{125}I]Bolton-Hunter-[Sar^9,Met(O_2)^{11}]substance P. Dense specific binding of [^{125}I]Bolton-Hunter-[Sar^9,Met(O_2)^{11}]substance P occurred over bronchial smooth muscle of large and small airways, with moderate binding on bronchial epithelium and over pulmonary arterial smooth muscle. Moderate specific binding of [^{125}I][Lys^5,Tyr(I_2)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10) was associated with bronchial smooth muscle of mainly large airways but not with other histological regions. This is the first autoradiographic report of (a low density of) tachykinin NK_2 binding sites on airway smooth muscle and supports the potent actions of NK_2 receptor ligands as contractile agents in guinea-pig isolated bronchi.

Keywords: Autoradiography; NK_2 receptor; [^{125}I][Lys^5,Tyr(I_2)^7,MeLeu^9,Nle^{10}]Neurokinin A-(4–10); Airway; Substance P

1. Introduction

Tachykinins are a widely distributed family of neuropeptides, found in both the central nervous system and peripheral tissues, including the respiratory tract (Otsuka and Yoshioka, 1993). In the airways, nerve fibres containing tachykinins are found close to blood vessels, within the bronchial smooth muscle layer and around local tracheobronchial ganglion cells (Lundberg et al., 1984; Martling, 1987). Tachykinins cause effects, such as bronchoconstriction, increased vascular permeability and stimulation of mucus secretion, in several species, including man (Solway and Leff, 1991; Frossard and Advenier, 1991). Neurokinin A is more potent than substance P in contracting airway smooth muscle in vivo and in vitro (Hua et al., 1985; Zeng et al., 1994) and neurokinin A also causes bronchoconstriction in asthmatics, whereas substance P is without effect (Joos et al., 1987). Tachykinins released during inflammation modulate cholinergic transmission by accelerating the pre-junctional release of acetylcholine (Black et al., 1990b). Thus, the symptoms described in asthma, i.e. bronchial inflammation, bronchoconstriction, vasodilatation, bronchial oedema, mucosecretion, chemotaxis and activation of inflammatory cells, can be mimicked by administration of tachykinins (Barnes, 1989; De Jongste et al., 1991; Frossard and Advenier, 1991).

Three main types of tachykinin receptors have been described. These are classified as NK_1 for substance P preferring receptors, NK_2 for neurokinin A preferring receptors and NK_3 for neurokinin B preferring receptors (Mussap et al., 1993; Maggi et al., 1993). Tachykinins interact with the receptors via the conserved amidated C-terminus (Mussap et al., 1993). These receptors have been cloned, in several species (Ohkubo and Nakanishi, 1991), with cDNA encoding the tachykinin NK_1 and NK_2 receptors isolated from the guinea-pig (Gorbulev et al., 1992; Aharony et al., 1994). The NK_1 receptor from guinea-pig shows 95–97% homology with the rat and human NK_1 receptors (Gorbulev et al., 1992). Species differences are more pronounced in the NK_2 receptor, where variations in pharmacology (Maggi et al., 1993), length and amino-acid sequence (Gerard et al., 1990; Aharony et al., 1994) have been described.

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In this study, tachykinin binding sites in guinea-pig airways were examined autoradiographically using highly selective NK_1 and NK_2 receptor radioligands. The tachykinin NK_1-selective [^{125}I]Bolton-Hunter-[Sar^9, Met(O_2)^{11}]substance P has been characterized in rat brain, rat submandibular gland (Lew et al., 1990) and guinea-pig lung (Burcher et al., 1995). [^{125}I][Lys^5,Tyr(I_2)^7,MeLeu^9, Nle^{10}]neurokinin A-(4–10) is a more recently developed radioligand which recognises tachykinin NK_2 receptors in guinea-pig lung (Zeng et al., 1994). Its unlabelled chemical equivalent is potent in causing contraction of isolated guinea-pig hilus bronchi (Zeng et al., 1994), rat fundus (Burcher et al., 1993) and human detrusor smooth muscle (Zeng et al., 1995). In addition to being highly selective, this radioligand has the advantage of being resistant to degradation by peptidases. The lung, particularly the bronchial epithelium and submucosa, contains peptidases which are partly responsible for degradation of tachykinins (Solway and Leff, 1991). These include neutral endopeptidase, which inactivates substance P and neurokinin A, and angiotensin converting enzyme, which cleaves substance P but not neurokinin A (Skidgel et al., 1984; Hooper et al., 1985; Wang et al., 1991).

2. Materials and methods

2.1. Radioligands

[^{125}I]Bolton-Hunter-[Sar^9,Met(O_2)^{11}]substance P (specific activity 2000 Ci/mmol), was made using [^{125}I]Bolton-Hunter reagent and purified by high-performance liquid chromatography (HPLC) as previously described (Lew et al., 1990). [^{125}I]Bolton-Hunter-[Sar^9,Met(O_2)^{11}]substance P eluted at 28 ± 0.2% acetonitrile and showed very high specific binding (96 ± 0.2%) when tested with rat submandibular gland membranes (Lew et al., 1990).

[^{125}I][Lys^5,Tyr(I_2)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10) was prepared by the chloramine T method, using 1 μCi of [^{125}I]sodium iodide and the reaction mixture was purified by reverse-phase HPLC (Burcher et al., 1993). [^{125}I][Lys^5, Tyr(I_2)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10) eluted in 1 peak of radioactivity, corresponding to 30 ± 0.7% acetonitrile. In rat fundus membranes (Burcher et al., 1993), this fraction showed high specific binding (85 ± 7%).

2.2. Autoradiographic studies – preparation of sections

English short-haired guinea-pigs (weight 350–450 g) were killed by a blow to the back of the head and exsanguinated. The lungs were removed and quickly frozen in liquid nitrogen, wrapped in foil and stored at −80°C until required. Serial sections (20 μm) of guinea-pig lung were then cut and thaw-mounted onto gelatin-coated slides, packed into boxes with dessicant and dried overnight at −20°C.

2.3. Autoradiographic studies – radiolabelling of sections

Sections of guinea-pig lung were pre-incubated in 3 × 5-min changes of buffer (50 mM Tris-HCl, 0.02% bovine serum albumin, pH 7.4, at 25°C) to remove endogenous ligand from tissue. Slides to demonstrate total binding were then incubated at 25°C for the appropriate amount of time (as determined by time-course studies) in 50 mM Tris-HCl buffer containing 0.02% bovine serum albumin, 3 mM $MnCl_2$, at pH 7.4, with appropriate peptidase inhibitors, and 60 pM [^{125}I]Bolton-Hunter-[Sar^9, Met(O_2)^{11}]substance P or 100 pM [^{125}I][Lys^5,Tyr(I_2)^7, MeLeu^9,Nle^{10}]neurokinin A-(4–10). Slides to demonstrate non-specific binding were co-incubated with 1 μM unlabelled [Sar^9,Met(O_2)^{11}]substance P or [Lys^5,Tyr(I_2)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10).

After incubation, all slides were washed for a pre-determined length of time in 50 mM Tris-HCl buffer, containing 0.02% bovine serum albumin and 3 mM $MnCl_2$, pH 7.4, at 4°C and subsequently rinsed twice in ice-cold distilled water, dried rapidly in cold air and fixed in paraformaldehyde vapour at 70°C for 30 min.

2.4. Autoradiographic studies – optimization of conditions

Using an initial time of incubation of 60 min for each radioligand, lung sections to demonstrate total and non-specific binding were incubated at 25°C in the absence and presence of a number of peptidase inhibitors at various concentrations, before being washed, dried, scraped off and amount of radioactivity bound determined using a Wallac gamma spectrometer. Data (specific dpm and percentage of control specific dpm) were analysed by analysis of variance followed by Bonferroni's test. The level of significance was set at $P < 0.05$.

In order to determine the optimum incubation times, lung sections to demonstrate total and non-specific binding were then incubated with radioligand, in incubation buffer with appropriate peptidase inhibitors for varying periods of time. Slides were then processed as above to determine the amount of radioactivity bound.

Finally, test sections were incubated in incubation buffer of appropriate peptidase inhibitor content for the appropriate time as determined above and the optimum time of post-incubation washing in cold buffer determined for each radioligand.

2.5. Autoradiographic studies – visualization of binding sites

For visualization at the macroscopic level, radiolabelled slide-mounted sections were apposed to X-omat K ultro-

film for 5–14 days in light-tight cassettes (Lew et al., 1990). Labelled sections were also fixed in paraformaldehyde vapour at 70°C for 30 min, then dipped in LM-1 Amersham light microscopy emulsion at 42°C and allowed to expose in the dark at 4°C for 5–14 days. The labelled slides were then developed and stained with pyronin Y (pH 7.4) and observed under light- and dark-field microscopy (Burcher et al., 1986). Adjacent unlabelled sections were fixed, stained with haematoxylin and eosin, or other specific stain, and examined histologically.

2.6. Materials

[Sar^9,Met(O$_2$)^{11}]substance P was purchased from Auspep (Melbourne, Australia). Concentrated solutions (500 μM) were stored with 0.01 M acetic acid (containing 1% β-mercaptoethanol) at −20°C until use. [Lys^5,Tyr(I$_2$)^7, MeLeu^9,Nle^{10}]neurokinin A-(4–10) was donated by Dr. S. Lavielle (Université Pierre et Marie Curie, Paris, France). [^{125}I]Bolton-Hunter reagent was purchased from NEN (Dupont, Australia) and [^{125}I]sodium iodide and LM-1 photographic emulsion purchased from Amersham (UK).

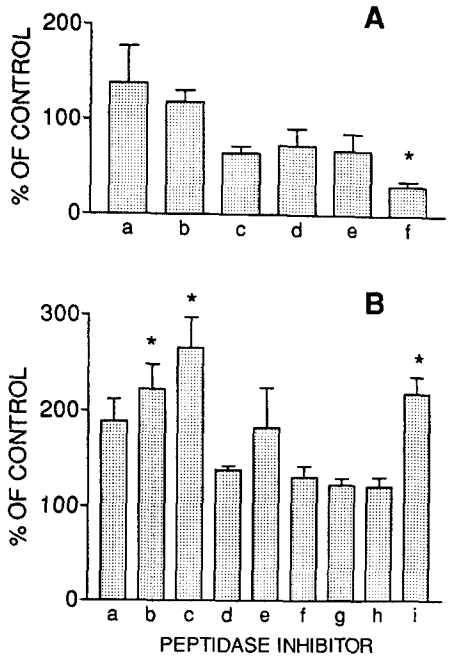

Fig. 1. Effect of peptidase inhibitors on specific binding to sections of guinea-pig lung labelled with (A) [^{125}I][Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10) and (B) [^{125}I]Bolton-Hunter-[Sar^9,Met(O$_2$)^{11}]substance P. Data represent specific dpm bound, expressed as a percentage of that determined in replicate sections incubated without peptidase inhibitors, and are mean ± S.E.M. obtained in sections from 3–6 animals. * $P < 0.05$ for significance of difference compared with control (analysis of variance followed by Bonferroni's test). A: phosphoramidon 1 μM (a); phosphoramidon 10 μM (b); chymostatin 4 μg/ml (c); captopril 1 μM (d); bacitracin 4 μg/ml (e); bacitracin 40 μg/ml (f). B: phosphoramidon at 0.1 μM (a), 1 μM (b) and 10 μM (c); captopril at 1 μM (d) and 10 μM (e); leupeptin 4 μg/ml (f); chymostatin at 4 μg/ml (g) and 40 μg/ml (h); bacitracin 40 μg/ml (i).

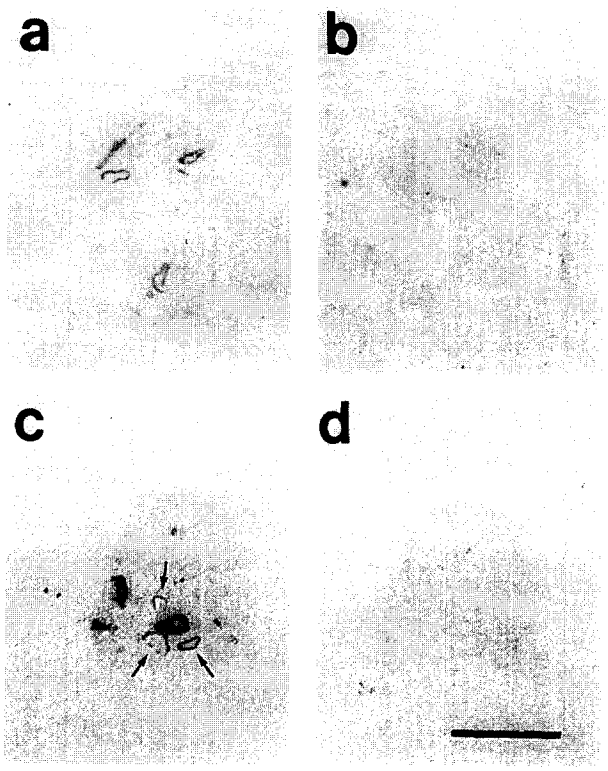

Fig. 2. X-omat K ultrofilm images of sections of guinea-pig lung labelled with [^{125}I][Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10) (a,b) or [^{125}I]Bolton-Hunter-[Sar^9,Met(O$_2$)^{11}]substance P (c,d). Moderate binding of [^{125}I][Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10) is associated with bronchi (a) but not with other histological features, as verified by subsequent staining of sections with pyronin Y. Very dense binding of [^{125}I]Bolton-Hunter-[Sar^9,Met(O$_2$)^{11}]substance P was associated with small bronchi (dark rings in c), with moderate binding seen over adjacent branches of the pulmonary artery (indicated by small arrows in c). Non-specific binding of both radioligands was very low, and was defined in adjacent sections co-incubated with 1 μM of [Lys^5,Tyr(I$_2$)^7, MeLeu^9,Nle^{10}]neurokinin A-(4–10) (b) or [Sar^9,Met(O$_2$)^{11}]substance P (d). Exposure time 12 days (a,b) and 14 days (c,d). Bar 5 mm.

Phosphoramidon, leupeptin, chymostatin, captopril, bacitracin and all histochemical stains were purchased from Sigma (USA), paraformaldehyde powder from ICN Biomedical (USA) and X-omat K ultrofilm and D19 developer from Kodak (Australasia). All other reagents and chemicals were of analytical grade.

3. Results

3.1. Optimization of incubation conditions for autoradiographic studies

3.1.1. Time-course studies

[^{125}I][Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10) was incubated with sections of guinea-pig lung ($n = 3$) at 30, 45, 60 and 90 min. There was a rise in total binding with time, with a corresponding increase in non-specific

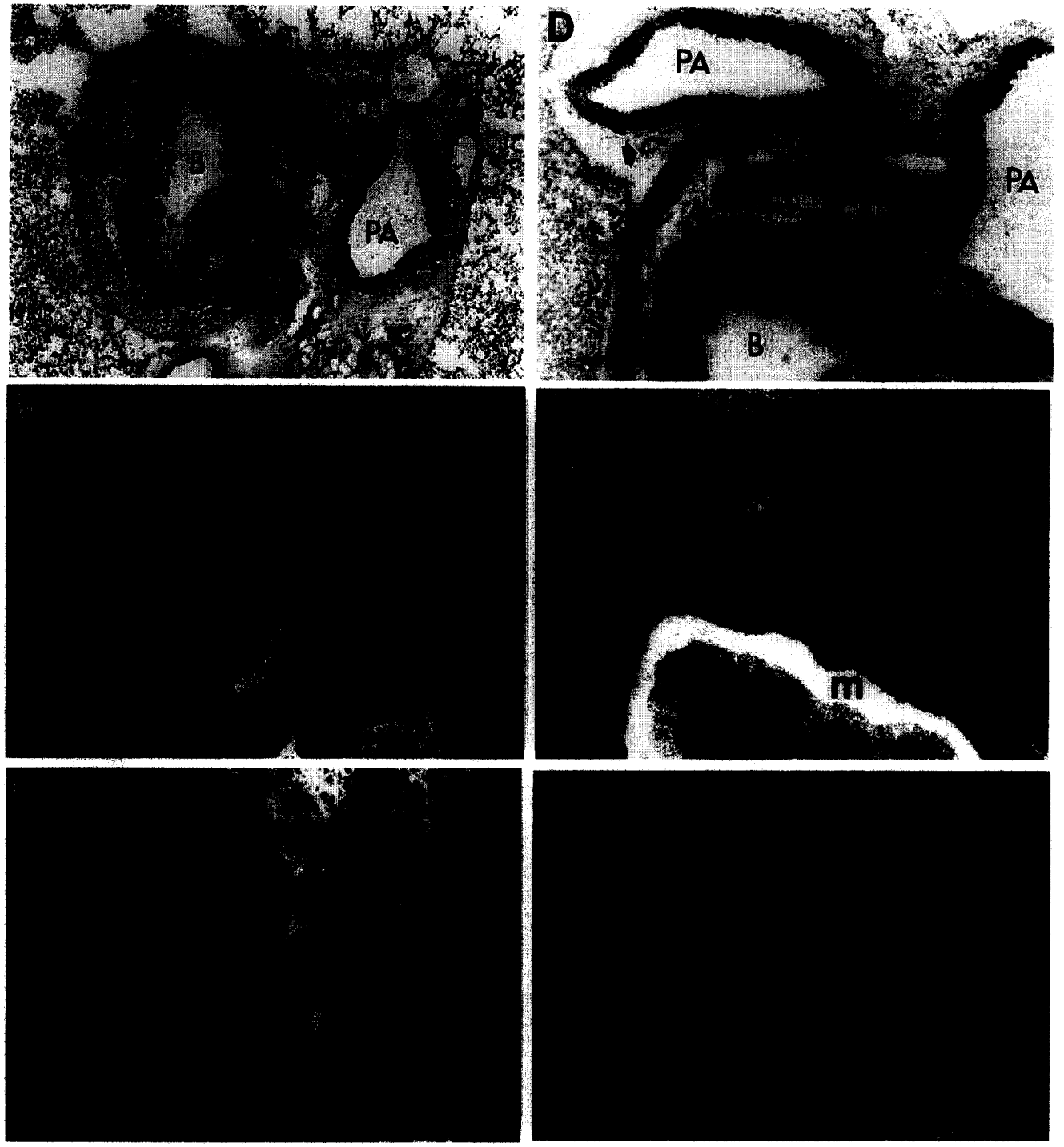

Fig. 3. Low-power photomicrographs of sections of guinea-pig lungs, showing a major bronchus and branches of the pulmonary artery. Sections were labelled with [^{125}I][Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10) (B,C) or [^{125}I]Bolton-Hunter-[Sar^9,Met(O$_2$)^{11}]substance P (D,E,F), dipped in emulsion and stained with pyronin Y. Dark-field photomicrographs (B,C,E,F) demonstrate total (B,E) and non-specific (C,F) binding, with silver grains appearing as small white dots in dark-field. A and D are light-field photomicrographs, with A representing an adjacent histological section and D being a light-field view of the section depicted in E. They show a major bronchus (labelled with B in lumen) with folds of epithelium (e) and associated plates of cartilage (c) and branches of the pulmonary artery (PA). B shows moderately dense binding over the smooth muscle layer (m) of the bronchus. E shows very dense binding over the smooth muscle layer of the bronchus, with moderate binding associated with the epithelium (e) of the bronchus and the vascular smooth muscle (v) of the artery. Small blood vessels (wide arrows in A and D) are seen near cartilage plates and bronchial smooth muscle, but no binding is associated with these (B,E). In A–C, adipose tissue (AT) and the pulmonary artery endothelium (arrowed) show reflection of light in dark-field panels, but show no silver grains. Non-specific binding is shown in adjacent sections co-incubated with 1 μM of [Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10) (C) or [Sar^9,Met(O$_2$)^{11}]substance P (F). Exposure time 10 days. Bar 200 μm.

binding. Specific binding reached a maximum at 60 min (74 ± 37 dpm; 46 ± 16%).

Sections of guinea-pig lung ($n = 3$) were incubated with [^{125}I]Bolton-Hunter-[Sar^9,Met(O$_2$)^{11}]substance P for 15, 30, 45, 60 and 90 min. Specific binding reached a maximum at 45 min (635 ± 300 dpm; 66 ± 13%).

3.1.2. Effect of peptidase inhibitors

Sections of guinea-pig lung ($n = 3$–6) were incubated with [^{125}I][Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10) in the presence and absence of various concentrations of peptidase inhibitors. Fig. 1A indicates that phosphoramidon, captopril and chymostatin had no effect on bind-

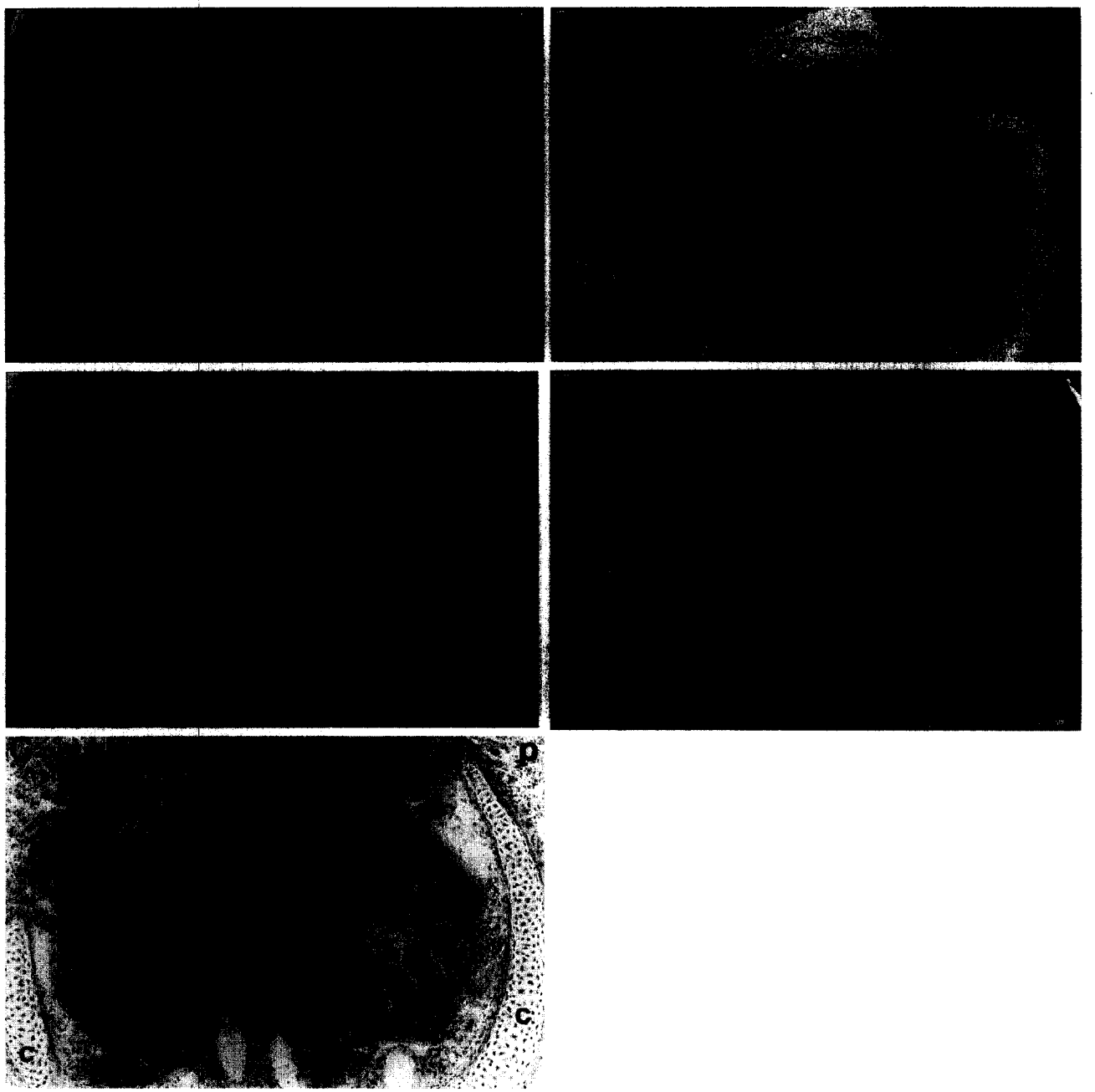

Fig. 4. A, B, D and E are dark-field photomicrographs of adjacent sections of one guinea-pig lung, labelled with [^{125}I][Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10) (A,B) or [^{125}I]Bolton-Hunter-[Sar^9,Met(O$_2$)^{11}]substance P (D,E), and stained with pyronin Y. A demonstrates binding of [^{125}I][Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10) to bronchial smooth muscle only. D demonstrates dense binding of [^{125}I]Bolton-Hunter-[Sar^9,Met(O$_2$)^{11}]substance P to bronchial smooth muscle with weaker binding to respiratory epithelium. C is a near-adjacent section stained with haematoxylin and eosin. Neither radioligand labelled cartilage or small blood vessels in the bronchial wall. Non-specific binding is shown in adjacent sections co-incubated with 1 μM of [Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10) (B) or [Sar^9,Met(O$_2$)^{11}]substance P (E). Non-specific binding occurs to eosinophils (arrowed) in B. sm, bronchial smooth muscle; e, epithelium, c, cartilage, p, parenchymal tissue. Exposure time 14 days. Bar 100 μm.

ing, whilst a high (40 μg/ml) but not a low (4 μg/ml) concentration of bacitracin significantly decreased specific binding, to 31% of control. Subsequent studies using this radioligand took place without any peptidase inhibitors.

Sections of guinea-pig lung were similarly incubated with [^{125}I]Bolton-Hunter-[Sar^9,Met(O$_2$)^{11}]substance P in the presence of a number of peptidase inhibitors (Fig. 1B). Leupeptin and chymostatin were without effect. Phosphoramidon (1 and 10 μM) and bacitracin (40 μg/ml) caused a significant increase in specific binding, compared with control. Captopril (10 μM) also caused a significant increase in specific dpm (data not shown) although this was not significant when expressed as percentage of control (Fig. 1B). These 3 inhibitors were used in subsequent autoradiographic experiments.

3.1.3. Effect of post-incubation wash time

After incubation with [^{125}I][Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10), sections of guinea-pig lung ($n = 3$) were washed in ice-cold Tris-HCl buffer for different durations to determine the effect on total and non-specific binding. The optimum wash time was 4 × 4 min, with specific binding reaching a maximum of 66 ± 25 dpm (67 ± 6%).

Sections of guinea-pig lung ($n = 3$) were incubated with [^{125}I]Bolton-Hunter-[Sar^9,Met(O$_2$)^{11}]substance P under standard conditions and washed at 4 different intervals in cold buffer. Prolonged wash reduces the total binding in sections as well as substantially decreasing the amount of non-specific binding. The optimum wash time was 4 × 6 min, corresponding to specific binding of 690 ± 190 dpm (67 ± 12%).

3.2. Autoradiographic studies

3.2.1. Visualization of tachykinin NK$_2$ sites

Autoradiographic localization of NK$_2$ binding sites with [^{125}I][Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10) on film shows a distinct band around major bronchi (Fig. 2a) but no other features. Non-specific binding was negligible (Fig. 2b).

A more detailed examination was possible in labelled sections, emulsion-dipped and stained with pyronin Y. Present in all sections were bronchi with associated plates of cartilage, bronchioles and branches of pulmonary artery and vein (Fig. 3). Moderately dense specific binding of [^{125}I][Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10) was seen over the smooth muscle of the bronchi (Figs. 3–5), with weaker binding on smooth muscle of bronchioles (Fig. 6). No binding was seen on the bronchial or bronchiolar epithelium, pulmonary vein, pulmonary endothelium, bronchial blood vessels or parenchymal tissue. Adjacent sections co-incubated with 1 μM [Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10), representing non-specific binding, showed no localization of silver grains apart from non-specific binding to eosinophils (Fig. 4B).

3.2.2. Visualization of tachykinin NK$_1$ sites

Fig. 2c,d depicts film images of [^{125}I]Bolton-Hunter-[Sar^9,Met(O$_2$)^{11}]substance P binding to sections of guinea-

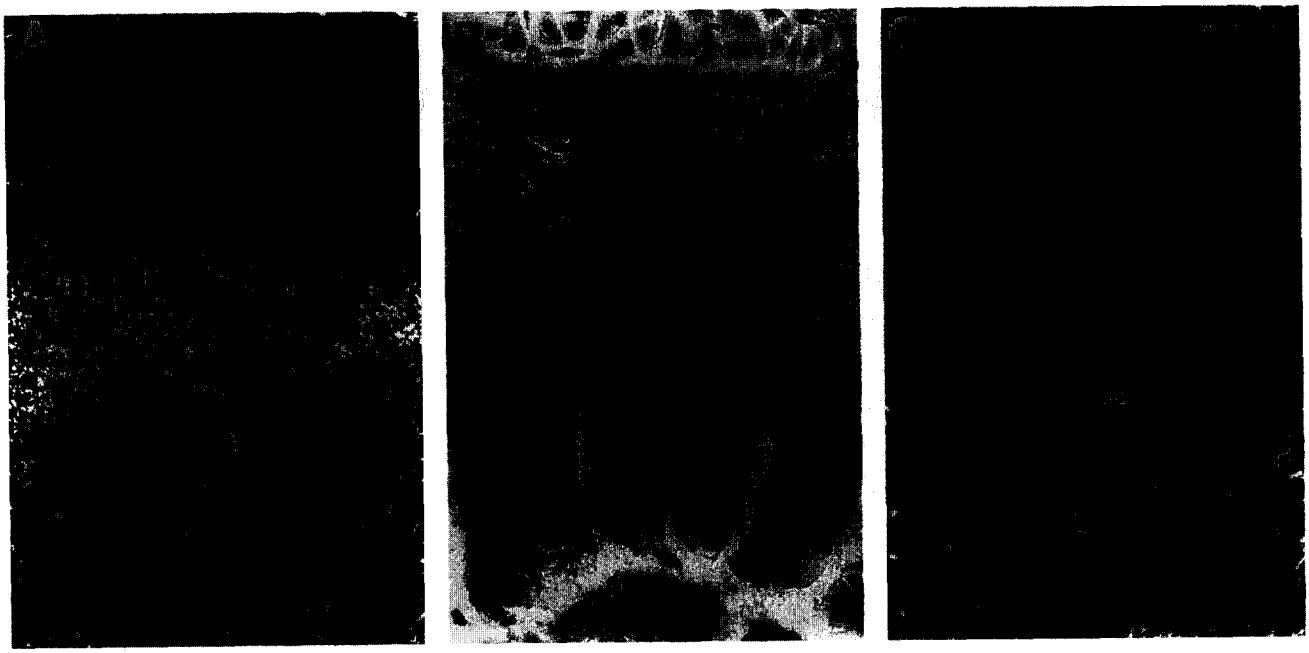

Fig. 5. A and B are dark-field and corresponding light-field high-power photomicrographs of lung section labelled with [^{125}I][Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10), showing part of the bronchial wall. Specific binding is seen over bronchial smooth muscle (sm) but not over epithelium (e) or cartilage (c). C is a dark-field photomicrograph of an adjacent section co-incubated with 1 μM of [Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10) to demonstrate non-specific binding. Exposure time 14 days. Bar 100 μm. In A and C, the bronchial epithelium shows reflection.

Fig. 6. A represents a light-field photomicrograph of a histological section, and B is a dark-field photomicrograph of an adjacent section of guinea-pig peripheral lung, labelled with [^{125}I][Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10). B shows binding (arrowed) to a large bronchiole (left) but not to a pulmonary artery (partially shown on right). Specific binding occurs only to the bronchiolar smooth muscle (B). C is a dark-field photomicrograph of an adjacent section co-incubated with 1 μM [Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10) to demonstrate non-specific binding. m, bronchial smooth muscle; e, epithelium; lu, lumen of pulmonary artery; p, parenchymal tissue; v, vascular smooth muscle. The bronchial epithelium and parenchymal tissue shows reflection (B,C). Exposure time 14 days. Bar 100 μm.

pig lung. Dense binding was associated with bronchi and moderately dense binding with branches of the pulmonary artery (Fig. 2c). Non-specific binding was very low (Fig. 2d).

Photomicrographs of emulsion-coated sections of lung show dense binding over the smooth muscle layer of the bronchus (Figs. 3 and 4). Moderate binding was associated with the epithelium of the bronchus (Fig. 4) and over the muscularis layer (tunica media) but not the intima of the accompanying branches of the pulmonary artery (Fig. 3). Moderate binding was seen over the smooth muscle of bronchioles but not over the accompanying small arteries. No specific binding was seen over small vessels of the bronchial circulation, cartilage or branches of the pulmonary vein. Non-specific binding was low.

4. Discussion

4.1. Peptidases

The aim of optimizing conditions for each radioligand in guinea-pig lung was to achieve maximum specific binding as well as minimum non-specific binding. A number of enzymes, including aminopeptidases and endopeptidases, degrade both substance P (Palmieri et al., 1985) and neurokinin A (Nau et al., 1986) with site-specific mode of action (Lazarus et al., 1987; Kim et al., 1992). The use of radioligands based on peptidase-resistant analogues should result in greater accuracy in autoradiographic studies. With [^{125}I][Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10), none of the peptidase inhibitors used enhanced specific binding, confirming our earlier observations that [Lys^5,MeLeu^9,Nle^{10}]neurokinin A-(4–10) is resistant to neutral endopeptidase in guinea-pig airways (Zeng and Burcher, 1994). However, our finding that phosphoramidon showed significant enhancement of specific binding of [^{125}I]Bolton-Hunter-[Sar^9,Met(O$_2$)^{11}]substance P suggests that cleavage by neutral endopeptidase still occurs with this analogue of substance P. Captopril also showed a minor enhancement of specific binding, suggesting that angiotensin converting enzyme, present in membranes from lung and pulmonary endothelial cells (Johnson et al., 1982), can also degrade [^{125}I]Bolton-Hunter-[Sar^9,Met(O$_2$)^{11}]substance P. Some enhancement of [^{125}I]Bolton-Hunter-[Sar^9,Met(O$_2$)^{11}]substance P binding was also seen with bacitracin, which inhibits an endopeptidase from rat brain (Bergmann and Bauer, 1986) and enhances responses of isolated lung strips to substance P (Webber and Foreman, 1984). This effect at NK$_1$ receptors is in contrast to its effect in significantly reducing specific binding with [^{125}I][Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10). This adverse effect of bacitracin, in reducing specific binding of tachykinin NK$_2$ receptor radioligands, has previously been reported by our laboratory (Mussap and Burcher, 1993). Bacitracin has similar effects elsewhere, in reducing [^{125}I]VIP binding in a concentration-dependent manner (Izzo et al., 1991).

4.2. Localization of tachykinin NK_2 receptors

Although only limited autoradiographic studies have been performed to date using [^{125}I][Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10), homogenate binding studies indicate that this is a ligand with high affinity, efficacy and specificity for tachykinin NK_2 receptors in guinea-pig airways (Zeng et al., 1994) and human detrusor muscle (Zeng et al., 1995). The chemical equivalent of the radioligand [Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10) had a similar potency in contracting guinea-pig isolated bronchi to that shown in binding assays (Zeng et al., 1994). In the present study, [^{125}I][Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10) bound mainly to the bronchial smooth muscle of guinea-pig airways. This confirms functional data showing that neurokinin A and analogues act on airway smooth muscle by interactions with tachykinin NK_2 receptors (cf. Frossard and Advenier, 1991). No binding to bronchial epithelium was seen, in accordance with previous functional studies from this laboratory showing that the contractile effect of the related selective tachykinin NK_2 receptor agonist [Lys^5,MeLeu^9, Nle^{10}]neurokinin A-(4–10) was epithelium-independent (Zeng and Burcher, 1994).

This is the first report of the localization of tachykinin NK_2 binding sites on bronchial smooth muscle in the guinea-pig. Earlier studies have used [^{125}I]Bolton-Hunter neurokinin A (Burcher et al., 1986) and [^{125}I]neurokinin A (Mussap and Burcher, 1993) as tools for autoradiographic visualization of tachykinin NK_2 receptors. These radioligands have been very suitable in demonstrating localization of NK_2 receptors in species, such as rat and human (Burcher et al., 1986; Mussap and Burcher, 1993; Mantyh, 1991). However, when applied to guinea-pig airways there has been much less success, in spite of the extensive literature demonstrating NK_2 receptor-mediated contraction of airways (Frossard and Advenier, 1991). In autoradiographic studies, weak specific binding of [^{125}I]neurokinin A on guinea-pig bronchial muscle was characterized as binding to tachykinin NK_1 receptors (Burcher et al., 1989) and this was confirmed by homogenate binding studies (Geraghty et al., 1992). Our hypothesis that addition of bulky iodinated groups may impair biological activity of neurokinin A was disproved by our recent finding that [^{127}I]Bolton-Hunter-neurokinin A was a full agonist in isolated guinea-pig bronchi with potency 10-fold higher than substance P (Burcher et al., 1995).

The differences in ability of tachykinin NK_2 receptor radioligands to bind to NK_2 receptors in different species may well be due to species differences between NK_2 receptors. Profound differences in pharmacology exist, revealed by use of some NK_2 receptor antagonists in isolated preparations (cf. Maggi et al., 1993). The NK_2 receptor has been cloned in 6 species to date and shows variation in size and amino-acid sequence (Gerard et al., 1990; Aharony et al., 1994). The difference in length occurs at the cytoplasmic C-terminal region and species differences in amino-acid sequence are found here as well as at the N terminus (Aharony et al., 1994). Studies utilising site-directed mutagenesis at the tachykinin NK_2 receptor are now beginning, with 4 residues found critical for agonist binding at the human receptor (Bhogal et al., 1994).

Another factor which has hampered detection of tachykinin NK_2 receptors in guinea-pig airways is likely to be the low numbers of receptors. The low NK_2 receptor density seen in this study and in our previous binding study (Zeng et al., 1994) supports observations by Aharony et al. (1994) using molecular biological techniques. Although these workers were successful in isolating tachykinin NK_2 receptor cDNA from guinea-pig lung, they reported difficulties in detecting NK_2 receptor mRNA using polymerase chain reaction, due to low receptor expression.

4.3. Localization of tachykinin NK_1 receptors

The present study demonstrates the suitability of the radioligand [^{125}I]Bolton-Hunter-[Sar^9,Met(O$_2$)^{11}]substance P for localization of NK_1 receptors in guinea-pig airways. The advantage of using the selective analogue of substance P, is its ability to interact only with tachykinin NK_1 receptors. Specific binding of [^{125}I]Bolton-Hunter-[Sar^9,Met(O$_2$)^{11}]substance P was very dense over the bronchial smooth muscle and respiratory epithelium of all airways. Moderately dense binding was observed over the muscularis layer of pulmonary arteries. No specific binding occurred over bronchial arteries or small vessels of the bronchial wall. These studies confirm data from earlier experiments using [^{125}I]Bolton-Hunter-substance P, the original iodinated radioligand which labels bronchial smooth muscle in guinea-pig lung (Carstairs and Barnes, 1986; Watkins and Burcher, 1987; Hoover and Hancock, 1987) as well as smooth muscle of rabbit distal airways (Black et al., 1990a). Similar results were seen in peripheral lung with [^{3}H]CP96,345, the NK_1 receptor antagonist, although this also labelled alveolar walls (Zhang et al., 1995).

4.4. Correlation with functional studies

The finding of both tachykinin NK_2 and NK_1 binding sites on guinea-pig airway smooth muscle is excellent agreement with a number of functional studies showing that neurokinin A, substance P and their analogues contract airway smooth muscle (Ireland et al., 1991; Maggi et al., 1991; Zeng and Burcher, 1994). The presence of NK_1 binding sites on bronchial epithelium, shown here and in previous studies, is in accordance with reports that substance P has actions on the airway epithelium (Tschirhart and Landry, 1986).

In the present study, the density of silver grains over

both small and larger airways, as well as the specific dpm bound, was clearly greater for [^{125}I][Sar^9,Met(O$_2$)^{11}]substance P compared with [^{125}I][Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10). This probably reflects the higher affinity of the former, compared with the latter, radioligand for their respective binding sites in guinea-pig airways. Our earlier data using these same radioligands in guinea-pig lung membranes showed that NK_1 binding sites were of substantially higher affinity (K_D 0.26 nM, Burcher et al., 1995) than NK_2 sites (K_D 1.3 nM, Zeng et al., 1994), with both sites having a low B_{max}.

However, there are discrepancies in apparent affinity and potency between functional studies in isolated airways and data from membrane binding and autoradiographic studies. The higher affinity of tachykinin NK_1, compared with NK_2, receptors seen in binding studies is in marked contrast to the potency of the corresponding agonists as smooth muscle spasmogens. In guinea-pig isolated bronchi, neurokinin A and NK_2 analogues, including [Lys^5,MeLeu^9,Nle^{10}]neurokinin A-(4–10), are 1–2 orders of magnitude more potent as contractile agonists than substance P and NK_1 receptor agonists, including [Sar^9,Met(O$_2$)^{11}]substance P (Frossard and Advenier, 1991; Zeng et al., 1994).

Several factors could explain this lack of correlation between functional and radioligand binding studies. One possibility could be the opposing actions of substance P and analogues, in causing bronchial smooth muscle contraction but also causing NK_1 receptor-mediated release of bronchorelaxant substances from the airway epithelium (Tschirhart and Landry, 1986; Frossard and Advenier, 1991). Our data from guinea-pig isolated bronchi only partly support this hypothesis. We found that epithelial denudation had no effect on responses to [Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10), but did enhance the contractile potency of [Sar^9,Met(O$_2$)^{11}]substance P 2.4-fold, with pD_2 values in denuded bronchi 9.15 and 8.38, respectively (Zeng and Burcher, 1994). Other relevant factors include difference in airway size and cell populations in the 2 types of study (cf. Burcher et al., 1995) as well as possibly weaker mechanisms of post-receptor coupling for airway NK_1 receptors.

Substance P is a vasodilator and potent mediator of plasma extravasation in a number of vascular beds (Otsuka and Yoshioka, 1993). The absence of NK_1 sites on small blood vessels of the bronchial wall does not suggest a role for substance P in the lung bronchial microcirculation. However, appreciable expression of tachykinin NK_1 rather than NK_2 binding sites was seen on the smooth muscle but not endothelium of branches of the pulmonary artery. The few studies using the guinea-pig isolated pulmonary artery have found that both substance P and neurokinin A cause relaxation of the intact vessel (Saria et al., 1987), via NK_1 receptors (Maggi et al., 1990). Relaxation to substance P was transient and endothelium-dependent; in endothelium-denuded preparations a small transient contraction was seen (Maggi et al., 1990). The contractile response could be mediated by the smooth muscle NK_1 sites observed here.

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References

Aharony, D., J. Little, C. Thomas, S. Powell, M. Downey-Jones and A. Graham, 1994, Isolation and characterization of neurokinin A receptor cDNAs from guinea-pig lung and rabbit pulmonary artery, J. Recept. Res. 14, 399.

Barnes, P.J., 1989, Airway neuropeptides: role in fine tuning and in disease?, Trends Pharmacol. Sci. 4, 116.

Bergmann, A. and K. Bauer, 1986, A membrane bound substance P degrading enzyme from rat brain, NIDA Res. Monogr. 75, 283.

Bhogal, N., D. Donnelly and J.B. Findlay, 1994, The ligand binding site of the neurokinin 2 receptor. Site-directed mutagenesis and identification of neurokinin A binding residues in the human neurokinin 2 receptor, J. Biol. Chem. 269, 27269.

Black, J.L., L.M. Diment, C.L. Armour, L.A. Alouan and P. Johnson, 1990a, Distribution of substance P receptors in rabbit airways, functional and autoradiographic studies, J. Pharmacol. Exp. Ther. 253, 381.

Black, J.L., P.R.A. Johnson, L.A. Alouan and C.L. Armour, 1990b, Neurokinin A with K^+ channel blockade potentiates contraction to electrical stimulation in human bronchus, Eur. J. Pharmacol. 180, 311.

Burcher, E., T. Badgery-Parker, X.-P. Zeng and S. Lavielle, 1993, Characterisation of a novel, selective radioligand, [^{125}I][Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10), for the tachykinin NK_2 receptor in rat fundus, Eur. J. Pharmacol. 233, 201.

Burcher, E., S.H. Buck, W. Lovenberg and T.L. O'Donohue, 1986, Characterization and autoradiographic localization of multiple tachykinin binding sites in gastrointestinal tract and bladder, J. Pharmacol. Exp. Ther. 236, 819.

Burcher, E., D.J. Watkins and N.M. O'Flynn, 1989. Both neurokinin A and substance P bind to NK_1 receptors in guinea pig lung, Pulm. Pharmacol. 1, 201.

Burcher, E., X.-P. Zeng, J. Strigas, D.P. Geraghty and S. Lavielle, 1995, Tachykinin receptors in guinea-pig airways: characterization using selective ligands, Can. J. Physiol. Pharmacol. 73, 915.

Carstairs, J.R. and P.J. Barnes, 1986, Autoradiographic mapping of substance P receptors in lung, Eur. J. Pharmacol. 127, 295.

De Jongste, J.C., R.C. Jongejan and K.F. Kerrebijn, 1991, Control of airway caliber by autonomic nerves in asthma and in chronic obstructive pulmonary disease, Am. Rev. Respir. Dis. 143, 1421.

Frossard, N. and C. Advenier, 1991, Tachykinin receptors and the airways, Life Sci. 49, 1941.

Geraghty, D.P., C.J. Mussap and E. Burcher, 1992, Radioiodinated substance P, neurokinin A, and eledoisin bind predominantly to NK_1 receptors in guinea pig lung, Mol. Pharmacol. 41, 147.

Gerard, N.P., R.L. Eddy, T.B. Shows and C. Gerard, 1990, The human neurokinin A (substance K) receptor, J. Biol. Chem. 265, 20455.

Gorbulev, V., A. Akhundova, H. Luzius and F. Fahrenholz, 1992, Molecular cloning of substance P receptor cDNA from guinea-pig uterus, Biochim. Biophys. Acta 1131, 99.

Hooper N.M., A.J. Kenny and A.J. Turner, 1985, The metabolism of neuropeptides. Neurokinin A (substance K) is a substrate for endopeptidase-24.11 but not for peptidyl dipeptidase A (angiotensin-converting enzyme), Biochem. J. 231, 357.

Hoover, D.B. and J.C. Hancock, 1987, Autoradiographic localization of substance P binding sites in guinea-pig airways, J. Auton. Nerv. Syst. 19, 171.

Hua, X.-Y., E. Theodorsson-Norheim, E. Brodin, J.M. Lundberg and T. Hökfelt, 1985, Multiple tachykinins (neurokinin A, neuropeptide K and substance P) in capsaicin-sensitive sensory neurons in the guinea-pig, Regul. Pept. 13, 1.

Ireland, S.J., F. Bailey, A. Cook, R.M. Hagan, C.C. Jordan and M.L. Stephens-Smith, 1991, Receptor mediated tachykinin-induced contractile responses in guinea-pig trachea, Br. J. Pharmacol. 103, 1463.

Izzo, R.S., R.A. Scioione, C. Pellecchia and R.S. Lokchander, 1991, Binding and internalization of VIP in rat intestinal epithelial cells, Regul. Pept. 33, 21.

Johnson, A.R., M. John and E.G. Erdos, 1982, Metabolism of vasoactive peptides by membrane-enriched fractions from human lung tissue, pulmonary arteries and endothelial cells, Ann. N.Y. Acad. Sci. 384, 72.

Joos, G.F., R. Pauwels and M. Van der Straeten, 1987, Effect of inhaled substance P and neurokinin A on the airways of normal and asthmatic subjects, Thorax 42, 779.

Kim, Y.-A., B. Shriver, T. Quay and L.B. Hersh, 1992, Analysis of the importance of arginine 102 in neutral endopeptidase (enkephalinase) catalysis, J. Biol. Chem. 267, 12330.

Lazarus, S.C., D.B. Borson, W.M. Gold and J.A. Nadel, 1987, Inflammatory mediators, tachykinins and enkephalinase in airways, Int. Arch. Allergy Appl. Immunol. 82, 372.

Lew, R., D.P. Geraghty, G. Drapeau, D. Regoli and E. Burcher, 1990, Binding characteristics of [^{125}I]Bolton-Hunter [Sar^{9},Met(O_2)^{11}]substance P, a new selective radioligand for the NK_1 receptor, Eur. J. Pharmacol. 184, 97.

Lundberg, J.M., T. Hökfelt, C.-R. Martling, A. Saria and C. Cuello, 1984, Substance P-immunoreactive sensory nerves in the lower respiratory tract of various mammals including man, Cell Tissue Res. 235, 251.

Maggi, C.A., R. Patacchini, L. Quartara, P. Rovero and P. Santicioli, 1991, Tachykinin receptors in the guinea-pig isolated bronchi, Eur. J. Pharmacol. 197, 167.

Maggi, C.A., R. Patacchini, F. Perretti, M. Tramontana, S. Manzini, P. Geppetti and P. Santicioli, 1990, Sensory nerves, vascular endothelium and neurogenic relaxation of the guinea-pig isolated pulmonary artery, Naunyn-Schmiedeberg's Arch. Pharmacol. 342, 78.

Maggi, C.A., R. Patacchini, P. Rovero and A. Giachetti, 1993, Tachykinin receptors and tachykinin antagonists, J. Auton. Pharmacol. 13, 23.

Mantyh, P.W., 1991, Substance P and the inflammatory and immune response, Ann. N.Y. Acad. Sci. 632, 263-271.

Martling, C.-R., 1987, Sensory nerves containing tachykinins and CGRP in the lower airways, Acta Physiol. Scand. Suppl. 563, 1.

Mussap, C.J. and E. Burcher, 1993, Characterization and autoradiographic localization of tachykinin receptors in rat fundus, J. Pharmacol. Exp. Ther. 266, 1043.

Mussap, C.J., D.P. Geraghty and E. Burcher, 1993, Tachykinin receptors: a radioligand binding perspective, J. Neurochem. 60, 1987.

Nau, R., G. Schäfer, C.F. Deacon, T. Cole, D.V. Agoston and J.M. Conlon, 1986, Proteolytic inactivation of substance P and neurokinin A in the longitudinal muscle layer of guinea pig small intestine, J. Neurochem. 47, 856.

Ohkubo, H. and S. Nakanishi, 1991, Molecular characterization of the three tachykinin receptors, Ann. N.Y. Acad. Sci. 632, 53.

Otsuka, M. and K. Yoshioka, 1993, Neurotransmitter functions of mammalian tachykinins, Physiol. Rev. 73, 229.

Palmieri, F.E., J.J. Petrelli and P.E. Ward, 1985, Vascular plasma membrane aminopeptidase M, Biochem. Pharmacol. 34, 2309.

Saria, A, C.R. Martling, E. Theodorsson-Norheim, R. Gamse, X.Y. Hua and J.M. Lundberg, 1987, Coexisting peptides in capsaicin-sensitive sensory neurons: release and actions in the respiratory tract of the guinea-pig, Acta Physiol. Hung. 69, 421.

Skidgel R.A., S. Engelbrecht, A.R. Johnson and E.G. Erdos, 1984, Hydrolysis of substance P and neurotensin by converting enzyme and neutral endopeptidase, Peptides 5, 769.

Solway, J. and A.R. Leff, 1991, Sensory neuropeptides and airway function, J. Appl. Physiol. 71, 2077.

Tschirhart, E. and Y. Landry, 1986, Airway epithelium releases a relaxant factor: demonstration with substance P, Eur. J. Pharmacol. 132, 103.

Wang, L., S. Ahmad, I.F. Benter, A. Chow, S. Mizutani and P.E. Ward, 1991, Differential processing of substance P and neurokinin A by plasma dipeptidyl(amino)peptidase IV, aminopeptidase M and angiotensin converting enzyme, Peptides 12, 1357.

Watkins, D.J. and E. Burcher, 1987, Autoradiographic localization of substance (SP) binding sites in guinea-pig respiratory tract, in: Substance P and Neurokinins, eds. J.L. Henry et al. (Springer, New York) p. 87.

Webber, S.E. and J.C. Foreman, 1984, The effect of substance P and related peptides on the guinea-pig lung strip, Agents Actions 14, 425.

Zeng, X.-P. and E. Burcher, 1994, Use of selective antagonists for further characterization of tachykinin NK-2, NK-1 and possible 'septide-selective' receptors in guinea-pig bronchus, J. Pharmacol. Exp. Ther. 290, 1295.

Zeng, X.-P., S. Lavielle and E. Burcher, 1994, Evidence for tachykinin NK-2 receptors in guinea-pig airways from binding and functional studies, using a new selective radioligand, [^{125}I][Lys^{5},Tyr(I_2)^{7},Me-Leu^{9},Nle^{10}]-NKA(4–10), Neuropeptides 26, 1.

Zeng, X.-P., K.H. Moore and E. Burcher, 1995, Characterization of tachykinin NK_2 receptors in human urinary bladder, J. Urol. 153, 1688.

Zhang, X.-L., J.C.W. Mak and P.J. Barnes, 1995, Characterization and autoradiographic mapping of [^{3}H]CP96,345, a nonpeptide selective NK_1 receptor antagonist in guinea pig lung, Peptides 5, 867.