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Quantitative autoradiography of peripheral opioid binding sites in rat lung

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Abstract

Previous studies in our laboratory have characterized non-conventional opioid binding sites in membrane preparations from both rat and human lung. The studies described in this paper utilized autoradiography to investigate the regional distribution of these [^{3}H]morphine binding sites within rat lungs. Specific binding of [^{3}H]morphine was saturable and Rosenthal analysis of tissue section wipes revealed the presence of both high-affinity and low-affinity opioid binding sites. The mean ± S.E.M. binding affinity and the mean ± S.E.M. density values for the low-affinity binding site ($K_d = 217 \pm 160$ nM, $B_{max} = 12 \pm 8$ pmol/mg protein) were similar to the values obtained in our previous whole-rat lung membrane binding assays ($K_d = 187 \pm 36$ nM, $B_{max} = 13.5 \pm 2$ pmol/mg protein) (Cabot, P.J., P.R. Dodd, T. Cramond and M.T. Smith, 1994, Eur. J. Pharmacol. 268, 247). Quantitative autoradiography showed that the highest density of opioid binding sites appeared to be present within the alveolar wall (13.2 ± 0.8 pmol/mg protein). A significantly lower ($P < 0.05$) density of binding was also observed in the smooth muscle of the trachea and main bronchi (5.5 ± 2.1 pmol/mg protein). However, no morphine binding sites were evident in the smooth muscle surrounding the smaller airways and pulmonary vasculature within the lobes of the rat lung. It remains to be investigated whether the opioid binding sites located within the trachea and main bronchi of the rat airways are the prejunctional opioid receptors on C-afferent nerve fibres which modulate the release of potent inflammatory neuropeptides.

Keywords: Morphine; Dyspnea; Quenching; Autoradiography; Opioid

1. Introduction

Our previous studies have demonstrated the presence of opioid binding sites in homogenate preparations of both rat and human lung (Cabot et al., 1994). These morphine binding sites were shown to have a binding affinity approximately 100-fold lower and a binding density approximately 100-fold higher than the respective values obtained in homogenate preparations of rat brain. The presence of opioid binding sites in the lung may explain why low doses of nebulized opioids are so effective in the treatment of dyspnoea and chronic inflammatory bronchoconstriction. In preliminary studies in humans, nebulized opioids have been shown to relieve dyspnoea effectively (Farncombe and Charter, 1994). Similarly, studies in animals have shown that nebulized morphine appears to relieve inflammatory bronchoconstriction via lung opioid receptors rather than by a central opioid mechanism (Karlsson et al., 1990; Belvisi et al., 1990).

Opioids have been shown to act upon capsaicin-sensitive nerve fibres to inhibit the release of inflammatory neuropeptides, such as substance-P, neurokinin A and calcitonin gene-related peptide (Ray et al., 1991). These neuropeptide-containing nerve fibres have been shown to innervate even the most peripheral areas within the lung, consistent with the view that chronic bronchoconstriction is caused by neurogenic inflammation in the lower airways (Barnes et al., 1990; Laduron, 1984). Therefore, the distribution of opioid binding sites which have been proposed to modulate the release of the inflammatory neuropeptides, substance P, calcitonin gene-related peptide, and neurokinin A from peripheral nerve fibres in the lung, should have a distribution similar to that of the C-afferent nerve fibres containing these inflammatory neuropeptides. Our preliminary distribution studies performed on rat lung ho-

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mogenates prepared separately from the upper and lower airways showed that the lower airways have a much greater density of opioid binding sites than do the upper airways (Cabot et al., 1994). However, more detailed analysis of opioid binding site distribution within the lung, requires autoradiography.

Therefore, the purpose of the present study was to investigate the possible variable distribution of morphine binding sites within rat lung using autoradiography and to verify the values for morphine binding affinity and density obtained from our previous homogenate binding studies (Cabot et al., 1994).

2. Materials and methods

2.1. Materials

[^{3}H]Morphine (75.5 μCi/mmol) was purchased from New England Nuclear (Sydney, Australia). Hexamethonium · HBr, Naloxone · HCl, [D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]enkephalin (DAMGO), Hepes and Tris buffer were purchased from Sigma (Sydney, Australia). Morphine · HCl was purchased from the Pharmacy Department (Royal Brisbane Hospital). Tissue Tek was purchased from Miles (Indiana, USA). Ethical clearance for this study was obtained from The University of Queensland Animal Experimentation Ethics Committee.

2.2. Tissue preparation

Adult male Wistar rats (250 g) were decapitated and their lungs removed and inflated with Tissue Tek: 2.5 mM Hepes/Tris buffer (1 : 4). Whole inflated lungs were frozen rapidly in the inflation medium and stored at −70°C until further use. Lung sections (8–14 μm) were cut on a cryostat (−20°C) (Microtome, FSE, Australia), thaw-mounted onto gelatin-coated slides (−20°C) and stored in a moist ice-cold (4°C) environment for immediate use.

2.3. Binding studies

Binding studies were initiated by the addition of 200 μl of the incubation buffer to each section for 45 min at 4°C, as determined by preliminary equilibrium binding studies. The incubation buffer comprised 2.5 mM Hepes/Tris buffer (pH 7.5), 3 nM [^{3}H]morphine and a range of concentrations (0.1 nM–100 μM) of unlabelled ligands. Non-specific binding was determined in the presence of 100 μM unlabelled ligand. The binding experiments were performed at 4°C in a moist environment. The binding reaction was terminated by washing the slides 6 times (1-s dips) in ice-cold buffer with one final wash in ice-cold water to prevent buffer crystal formation which may cause image fogging. Sections were either wiped and placed in 4 ml of scintillant (Optiphase-Hisafe 3, Wallac, Australia) and counted on a scintillation counter (Packard 2700TR, Australia), or allowed to dry (4°C) and exposed to Hyperfilm-^{3}H (Amersham, Australia) for 4–5 weeks at −70°C in x-ray cassettes. Commercially prepared [^{3}H] microscales (Amersham, Australia) were exposed to film at the same time as that of radiolabelled lung tissue sections to permit quantitative analysis (see Section 2.6 for further discussion). Autoradiographic film was developed under dark room conditions using D-19 developer (Kodak) for 5 min followed by X-ray fixer (Kodak) for 5 min, then rinsed thoroughly with water. Rosenthal analysis was performed using the non-linear least squares regression programs EBDA (McPherson, 1983) and LIGAND (Munson and Rodbard, 1980).

2.4. Determination of binding site instability

Storage of sections at either −20 or −70°C resulted in total loss of specific opioid binding. Attempts to maintain binding site integrity by replacing oxygen with nitrogen also failed to maintain specific binding. Therefore, all binding studies were carried out on fresh lung sections thawed to 4°C after sectioning and maintained at that temperature throughout the binding assay. Furthermore, it was necessary to keep the sections moist throughout most procedures in order to maintain binding. When drying was required, it was performed using a small fan in a dry ice bath.

2.5. Establishment of equilibrium binding and washing time

Determination of equilibrium binding of [^{3}H]morphine to sections of rat lung was performed by incubating lung tissue sections with 3 nM [^{3}H]morphine and corresponding sections with both labelled and unlabelled morphine (100 μM). Binding was assessed at a range of times over a 1.5-h period (Fig. 1). Similarly, the optimization of washing time was determined in a manner analogous to binding equilibrium time. As expected, the low-affinity nature of the binding site meant that it was necessary for section washes to be multiple rapid (1 s) single dips into buffer baths (4°C) to optimize specific binding. The lowest levels of non-specific binding without sacrificing specific binding were found with 6 buffer rinses followed by a single water rinse.

2.6. Preparation of tritium calibration standards for rat lung tissue sections and assessment of differential quenching

Tissue equivalent scales for the estimation of tritium quenching were prepared in sections (14 μm) of rat lung containing both lobe and tracheal tissue. Sections were thawed to room temperature to denature opioid binding sites. Known quantities of [^{3}H]morphine were added to

serial sections in the concentration range of 300–8500 dpm per section. Following a 15-min incubation period in a dry environment, the sections were dipped (1 s) in water to remove the excess radioligand that had not been absorbed by the tissue according to the method described by Kuhar and Unnerstall (1990). Labelled sections were then exposed to film as described in section 2.2. After exposure to film (4 weeks), the sections were wiped to determine the amount of radiolabel absorbed by the tissue. Optical densities and area of image were measured using the ImagePro v 1.1 image analysis system (Sci-Tech, Melbourne, Australia).

2.7. Image analysis

Autoradiographic images were quantified by densitometric analysis using the image-analysis software which employs a 256-level grey scale. Levels of tritium bound were determined by calibration with co-exposed commercial scales of brain homogenates. Measurements were adjusted for the differential quenching by lung tissue with prepared standards of [^{3}H]morphine incorporated into lung sections co-exposed with the commercial standards. After image densities and areas were quantified, individual disintegrations per min (dpm) were determined by interpolation from calibration scales adjusted for quenching and changes in specific activity due to radiolabel decay, as described by Kuhar and Unnerstall (1990). Protein estimation was performed on lung homogenates and expressed as mg protein/g of wet weight of tissue. Calibration of sections for protein concentration was performed as described by Kuhar and Unnerstall (1990). Specifically, the area and wet weight of tissue sections were measured for a set of representative sections and calibrated with the protein estimates obtained from protein assays of separate homogenates of rat lung lobes and rat trachea, according to the method described by Lowry et al. (1951).

3. Results

3.1. Assessment of equilibrium binding

Equilibrium binding was assessed over 90 min and optimal binding was found to be achieved by 45 min (Fig. 1). Loss of equilibrium binding was observed after 1 h of incubation, presumably as a result of the apparent instability of the lung opioid binding site.

3.2. Specific and saturable binding

Section wipes of morphine binding to rat lung tissue sections showed saturable specific binding (Fig. 2). Rosenthal analysis produced binding parameter estimates (mean ± S.E.M.) for a two-site fit. The relatively low density of the high-affinity binding site ($K_d = 18 \pm 7$ nM, $B_{max} =$ 440 ± 113 fmol/mg protein) is consistent with our previous findings in homogenates prepared from the upper airways of the rat lung ($K_d = 39 \pm 16$ nM, $B_{max} = 310 \pm 150$ fmol/mg protein, Cabot et al., 1994). Similarly, the estimates (mean ± S.E.M.) of binding affinity and density for the low-affinity binding site were 217 ± 160 nM and

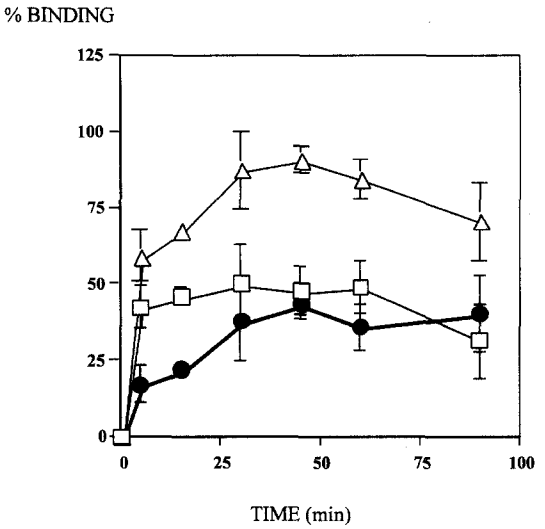

Fig. 1. Equilibrium binding was assessed by incubating sections (14 μm) with 3 nM [^{3}H]morphine and 100 μM unlabelled morphine for a range of time periods ($n = 4$). The sections were washed to terminate the binding reaction and wiped and counted to assess total (△) and non-specific binding (□) at each time point. Subtraction of non-specific binding from total binding revealed that specific binding (●) had reached equilibrium by 45 min.

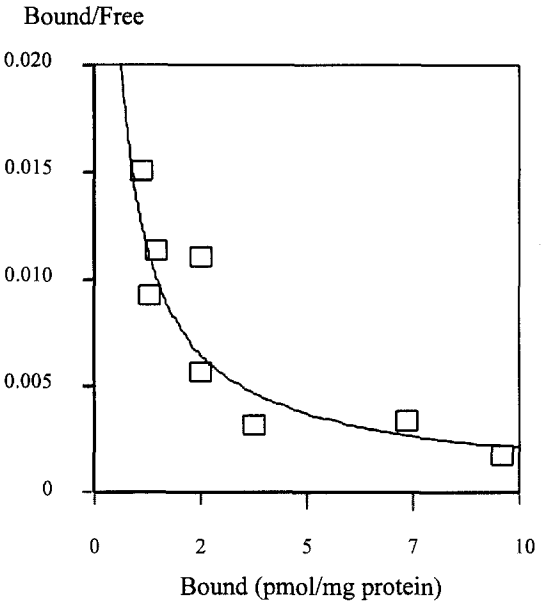

Fig. 2. Rosenthal analysis of lung sections incubated with 3 nM [^{3}H]morphine and a range of concentrations of unlabelled morphine ($n = 3$). Sections were rinsed (1-s dips), wiped and then counted. Binding site affinity and density were estimated using least squares regression using the curve-fitting analysis software, LIGAND, which revealed a more significant two-site fit to the data than a single-site fit ($P < 0.05$).

12 ± 8 pmol/mg protein, respectively. These values were similar to those obtained for morphine binding to homogenates prepared from the lower airways of rats in our previous studies (Cabot et al., 1994). Levels of non-specific binding were relatively high (47%) but efforts to reduce levels of non-specific binding without sacrificing specific binding were unsuccessful. However, differences in optical densities between total and non-specific binding were sufficient for unambiguous interpretation of the data (Fig. 3).

3.3. Assessment of differential quenching effects in the lung

Regional differences in tissue quenching were assessed by measuring the optical densities of film exposed to

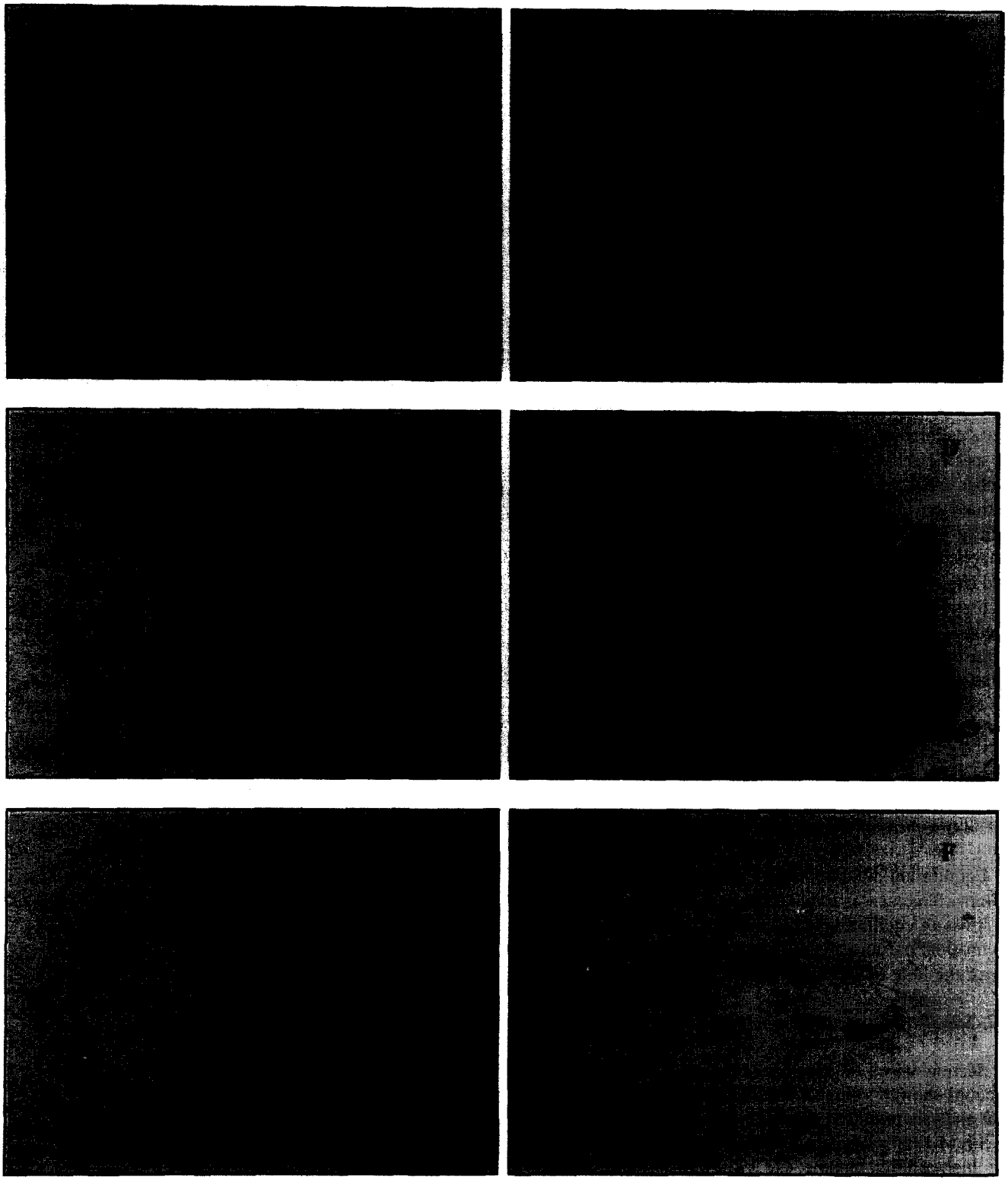

Table 1
Quantitative analysis of autoradiographical images of [^{3}H]morphine binding to sections of rat lung

Ligand	Specific binding		Density
	dpm/mm^2	fmol/mm^2	pmol/mg protein
Rat lower airways			
[^{3}H]Morphine-morphine	62.3 ± 9.00	0.334 ± 0.05	12.6 ± 1.6
[^{3}H]Morphine-naloxone	48.0 ± 5.54	0.280 ± 0.03	11.4 ± 1.3
[^{3}H]Morphine-hexamethonium	68.7 ± 7.5	0.40 ±0.04	15.7 ± 1.4
Rat trachea			
[^{3}H]Morphine-morphine	18 ± 7.5	0.11 ±0.04	5.5 ± 2.1

Using calibrated tritium scales, specific binding was determined from the optical densities of autoradiographical images for at least four images obtained from three individual animals. Density values were calculated by conversion to fmol of [^{3}H]morphine and then adjusted for protein concentration, estimated at 36 and 40 μg protein/mg of lung tissue for rat trachea and lobe, respectively. The mean area of section (58 and 174 mm^2 for trachea and lobe, respectively) and depth of tritium penetration (5 μm) were incorporated into the final calculation of binding site density.

sections of rat lung which contained both trachea and lobe. It is apparent from the prepared scales that there is less quenching in sections from the trachea than for sections from the lobes (90.3 ± 4.4% of tracheal value normalized to 100%). However, there was no observable differential quenching across the lung lobes. The 9.7% difference in quenching observed between trachea and lobe may be a significant factor when comparing binding site densities between these two regions of the rat airways. Therefore, estimates of binding densities were corrected for this effect, as described by Kuhar and Unnerstall (1990).

3.4. Quantitative and qualitative autoradiography

Image analysis of autoradiograms permitted the estimation of binding site densities for the displacement of [^{3}H]morphine by naloxone, morphine and hexamethonium (Table 1). Quantitation of autoradiograms revealed a similar binding site density to that obtained from Rosenthal analysis of tissue sections wiped and counted (Table 1). Furthermore, these values were consistent with the values of binding site density that we had determined previously in homogenate binding studies (Cabot et al., 1994). Calculation of densities of binding sites as a function of protein concentration required adjustment for a depth of 5 μm, this being the depth of penetration of β-radiation in tissue sections that does not undergo significant quenching (Hudson, 1993). Evidence confirming this effect was obtained by comparison of images of sections cut both at thicknesses of 8 μm and 14 μm. Although the levels of total and non-specific binding were reduced for section wipes in the thinner sections, the optical density values from either sets of sections were not significantly different ($P > 0.05$).

Hexamethonium, a ganglionic blocker, completely displaced [^{3}H]morphine binding on lung sections (Table 1) in an analogous manner to that observed in our previous lung homogenate binding studies (Cabot et al., 1994). In addition, our previous homogenate binding studies demonstrated that the μ-opioid peptide, DAMGO, did not displace [^{3}H]morphine from the rat lung binding sites. Similarly, our autoradiographical studies have also found that [^{3}H]morphine was not displaced by DAMGO ($n = 3$) from specific binding sites in sections of rat lung, as there was no difference between the distribution of optical densities for the autoradiographs of either [^{3}H]morphine or [^{3}H]morphine plus DAMGO.

The possible variability in the characteristics of opioid binding sites within the rat lung was investigated using image analysis. Non-specific levels of opioid binding were observed in pulmonary vasculature and around bronchioles within the lungs (Fig. 3, cf. panels D and F). Specific binding sites appeared to be predominantly located in the alveolar walls (Fig. 3, panel D), with significant levels of opioid binding also located in the smooth muscle of the trachea and main bronchi (Fig. 3, panel C). It is apparent that there are two distinct populations of morphine binding sites within the rat lung. The first and most abundant group of binding sites appear to be present within the alveolar walls (Fig. 3, panel D). The second group of binding sites appears to line the smooth muscle within the trachea and main bronchi close to the lumen (Fig. 3, panel C). The lack of morphine binding sites within the smooth muscle sur-

Fig. 3. Panel A shows the Cresyl violet-stained histological section depicting a vertical cross-section of the upper and lower trachea. The cartilage and smooth muscle (SM) within the trachea (T) are clearly visible as well as a portion of the upper lobe. Similarly, panel B shows the Cresyl violet-stained histological section obtained from the right lobe of a Wistar rat. This section clearly shows pulmonary vasculature (V) and a secondary or tertiary bronchiole (B), scale bar represents 1 mm for both panels A and B. Panel C shows the corresponding autoradiograph of total [^{3}H]morphine binding to the section depicted in panel A. There is a high density of binding sites in the rat lobe as well as a lower density of binding sites distributed throughout the smooth muscle of the trachea. Panel D shows the Hyperfilm-^{3}H image of total binding to panel B, and demonstrates the high distribution of morphine binding sites within the alveolar tissue. In addition, very low levels of morphine binding sites are observed around the pulmonary vasculature and bronchioles (V, B). Panels E and F demonstrate non-specific binding determined in adjacent sections to the sections depicted in panels C and D, respectively, by incubation with 100 μM naloxone for panel E and 100 μM unlabelled morphine in panel F.

rounding the vasculature within the lobes (Fig. 3, panel D) is in contrast to the abundance of these sites in the smooth muscle of the trachea and main bronchi (Fig. 3, panel C) and clearly differentiates the two populations of morphine binding sites.

4. Discussion

The binding of [^{3}H]morphine to sections of rat lung showed similar characteristics to that obtained in our previously reported lung homogenate binding studies (Cabot et al., 1994). Specific binding was saturable and Rosenthal analysis of lung section wipes revealed the presence of both high-affinity (mean ± S.E.M. $K_d = 18 \pm 7$ nM) and low-affinity (mean ± S.E.M. $K_d = 217 \pm 160$ nM) binding sites. The binding affinity and density of morphine for the low-affinity site ($K_d = 217 \pm 160$ nM, $B_{max} = 12 \pm 8$ pmol/mg protein) were similar to the respective values obtained in our previous studies in membrane preparations of rat ($K_d = 270 \pm 43$ nM, $B_{max} = 38 \pm 6$ pmol/mg protein) and human peripheral airways ($K_d = 230 \pm 90$ nM, $B_{max} = 3.4 \pm 1.3$ pmol/mg protein) (Cabot et al., 1994). In contrast, the high-affinity binding site had a closer affinity (mean ± S.E.M. $K_d = 18 \pm 7$ nM) to that of the morphine binding sites reported for the rat brain (mean ± S.E.M. $K_d = 1.3 \pm 0.1$ nM) (Tejwani et al., 1991).

Using quantitative autoradiography, the densities of opioid binding sites on sections of rat lung were found to be similar to the density values obtained from Rosenthal analysis of both tissue section wipes and our previously published values obtained from lung homogenate binding studies (Cabot et al., 1994). The uncharacteristic specific displacement of [^{3}H]morphine by hexamethonium previously observed in our lung homogenate binding studies was also confirmed by autoradiography in these studies. In fact, hexamethonium (100 μM) showed complete displacement of [^{3}H]morphine in an identical manner to that of naloxone on lung tissue sections. Consistent with our previous homogenate binding studies (Cabot et al., 1994) the μ-opioid receptor agonist DAMGO did not displace [^{3}H]morphine from sections of the lobe of rat lung, further indicating the non-conventional nature of these binding sites. However, the possibility that the binding sites in the trachea and main bronchi are shared by both morphine and DAMGO in a manner analogous to the opioid binding sites within the brain, remains to be investigated.

Morphine binding sites in sections obtained from rat lung appeared to be concentrated in the alveolar wall. In addition, a much lower density of binding was also evident within the smooth muscle of the trachea and main bronchi. However, the smooth muscle around the pulmonary arteries and bronchioles showed little specific binding within the lobes of the rat lung. The high density of opioid binding sites within the alveolar wall is consistent with the results of early electron micrograph studies which showed that axons of sensory nerve fibres innervated the alveolar wall of both rat and human lung (Merick and Reid, 1971; Fox et al., 1980). These studies also identified possible receptor sites on what appeared to be presynaptic nerve terminals. The high distribution of opioid binding sites throughout the alveolar tissue is also consistent with the evidence supporting the presence of J-receptors in the peripheral regions of the lung, close to small arteries and veins and eliciting potent effects on airway reflexes (Paintal, 1973).

More recently, studies using electrical field stimulation of excised guinea-pig lungs have shown that morphine inhibits non-adrenergic-non-cholinergic bronchoconstriction by the apparent activation of prejunctional μ-opioid receptors on C-afferent nerve fibres within the trachea (Frossard and Barnes, 1987). These sensory nerve fibres have been shown to contain neuropeptides, such as substance P, neurokinin A and calcitonin gene-related peptide, which when released evoke plasma extravasation, increase mucus secretion and have potent effects upon mast cells (Barnes, 1992). Substance P-containing nerve fibres have been shown to be in a range of locations within the airways of humans and animals, such as the epithelial lining, close to pulmonary vasculature and within bronchiolar smooth muscle (Lundberg et al., 1984). However, the distribution of the lower-affinity opioid binding sites within the lobes of the rat lung were not close to pulmonary vasculature or within smooth muscle, which indicates that the peripheral opioid binding sites within the alveolar walls are unlikely to be primarily located on substance P-containing nerve fibres. However, the opposite is likely to be true for the opioid binding sites found within the smooth muscle of the trachea and main bronchi. The location of these sites is consistent with the location of substance P-containing sensory nerve fibres (Lundberg et al., 1984) and may represent a different population of opioid binding sites (possibly, the high-affinity morphine binding sites found in this study) that play an active role in the non-adrenergic-non-cholinergic control of the trachea and main bronchi. Production of specific antibodies to the low-affinity opioid binding site in the peripheral airways would permit further definition of the exact cellular location of this binding site within the alveolar walls.

Tritium quenching is a common problem often overlooked in most quantitative autoradiographic studies. A number of theories have been proposed regarding the causes of quenching in autoradiography with β-radiating compounds, such as differential quenching occurring in sections of brain tissue as a consequence of varying levels of myelin, chromatin and lipid density (Zilles et al., 1990). The other major theory relates to differential tissue shrinkage across a section resulting in different apparent levels of tritium because of the low penetration of β-radiation (Nissanov and McEachron, 1991). In our studies, partial quenching was observed in the lobes of the rat lung. There was no apparent difference within the lobes themselves but

there was a significant difference between the densities in the lobes and the density in the trachea on the same section. The quenching had its greatest effect on low and very high values of optical density of the autoradiograms. Left uncorrected, this would have resulted in significant errors in the apparent binding densities calculated from non-calibrated scales.

In conclusion, we have further characterized the distribution of opioid binding sites within the rat lung. These binding sites were found predominantly in the alveolar wall, with possibly a second population within the trachea and main bronchi localized in smooth muscle. The alveolar population of opioid binding sites may exist as a receptor component of a cellular mechanism within the alveolar cell wall, and are possibly associated with J-receptors, close to very small vasculature within the alveolar walls of rat and human lungs. The opioid binding sites within the trachea and main bronchi are possibly prejunctional opioid receptors on C-afferent nerve fibres modulating the release of potent inflammatory neuropeptides, such as substance P, as proposed by Barnes et al. (1990). Whether the two apparent populations of opioid binding sites within rat airways act similarly or cause different effects by activation of distinct nerve fibres or pathways is unknown and requires further investigation. Given the high density of the low-affinity rat lung opioid binding sites and the relatively low resolution of autoradiographical analysis, the exact cellular localization of opioid binding sites is not possible using this technique. Further studies with antibodies raised against the low-affinity lung opioid binding sites may provide further insight into their exact cellular location.

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