Autoradiographic localization of β-adrenoceptors in pig lung using [^{125}I]-iodocyanopindolol

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1   The binding of the β-adrenoceptor radioligand [^{125}I]-iodocyanopindolol (I-CYP) has been studied in pig lung parenchyma and the distribution of binding sites visualised by light microscopic autoradiography.

2   I-CYP binding was saturable (maximum binding capacity $B_{max} = 51 \pm 3$ fmol mg^{-1} protein), involving sites with high affinity (dissociation constant $K_D = 73 \pm 10$ pM).

3   Specific I-CYP binding was displaceable both by β-adrenoceptor agonists (($-$)-isoprenaline $>$ ($-$)-adrenaline $>$ ($\pm$)-fenoterol $>$ ($-$)-noradrenaline $>$ ($+$)-isoprenaline $>$ ($\pm$)-RO363) and antagonists (($\pm$)-propranolol $>$ ICI-118551 $>$ atenolol), indicating a predominance of β_2-adrenoceptors. Further analysis showed that displacement data for the β_1-selective antagonist atenolol and the β_2-selective antagonist ICI-118551 were fitted best to a 2 binding site model and that both β_1- and β_2-adrenoceptors were present in pig lung in the ratio 28:72 respectively.

4   Autoradiographic grains were localized over tissue and were most dense over alveolar walls $>$ vascular endothelium $>$ vascular smooth muscle $>$ bronchial smooth muscle = bronchial epithelium. Atenolol (10^{-5}M) caused a 31% reduction in specific grain density over alveolar wall tissue, while a 10 fold lower concentration of ICI-118551 (10^{-6}M) caused a 50% decrease. These results are consistent with binding data in pig lung parenchyma demonstrating a mixed population of β-adrenoceptors with a predominance of the β_2 subtype.

5   Approximately 95% of the parenchymal β-adrenoceptors were associated with the alveolar wall as a mixed population of the β_1 and β_2 subtypes in the ratio 30:70 respectively. A greater proportion of the β-adrenoceptors associated with bronchial and vascular smooth muscle seemed to be of the β_2 subtype.

6   It is possible that the previously described relaxant responses of the pig lung parenchyma strip to β-agonists, mediated via β_2-adrenoceptors, resulted from the sum of reactivities in airway and vascular smooth muscle together with relaxation of alveolar interstitial cells.

Introduction

The lung parenchyma strip is a heterogeneous preparation containing bronchiolar, alveolar and vascular tissue, all of which may contribute to its contractility and relaxant responsiveness to drugs (Evans & Adler, 1981; Bertram *et al.*, 1983a). We have previously shown that β_2-adrenoceptors mediate relaxation of the pig lung parenchyma strip (Goldie *et al.*, 1982). However, both β_1- and β_2-adrenoceptors have been revealed in both membrane binding studies and in autoradiographic studies in lung parenchyma from the rat (Xue *et al.*, 1983), rabbit (Rugg *et al.*, 1978) and guinea-pig (Carswell & Nahorski, 1983). It was of interest to determine the distribution of β-adrenoceptors in pig lung parenchyma to identify those tissue structures upon which β_2-adrenoceptors

reside. It is these structures which must constitute that component of the pig lung strip which respond functionally to relaxant β-agonists. The possible existence of parenchymal β_1-adrenoceptors was also investigated.

Methods

Tissue preparation

Central lung lobes were obtained from pigs freshly slaughtered at a local abattoir. Lobes were inflated by tracheal instillation of OCT embedding medium diluted 1:4 with 0.9% w/v NaCl solution (saline).

Inflated lobes were snap frozen in isopentane, quenched with liquid nitrogen and tissue blocks (approximately $10 \times 10 \times 10$ mm) cut and stored at $-75°C$ until required. Serial frozen tissue sections ($10 \mu m$, autoradiographs; $16 \mu m$, kinetic experiments) were cut at $-30°C$ from a total of 20 blocks from 7 separate lungs and mounted and thawed onto acid washed, gelatinised glass slides. Sections were stored at $-75°C$ for up to 2 weeks before use without loss of radioligand binding capacity.

Radioligand binding studies

Slides with tissue sections were incubated at $22°C$ for 5–180 min in Tris-HCl buffer (170 mM; pH 7.6) containing [^{125}I]-iodocyanopindolol (I-CYP, $\sim$ 2000 Ci mmol^{-1}, 10–320 pM) and the protease inhibitor phenylmethylsulphonylfluoride (PMSF, $10 \mu M$). Specific binding of I-CYP to β-adrenoceptors was defined as I-CYP binding which was displaceable by 200 μM ($-$)-isoprenaline.

Kinetic experiments Lung sections ($16 \mu m$) were incubated with I-CYP (50 pM) for 150 min in the presence or absence of the β-adrenoceptor antagonists ($\pm$)-propranolol (10^{-9}–10^{-5}M; non-selective), atenolol (6×10^{-6}–10^{-2}M; β_1-selective) or ICI-118551 (6×10^{-8}–10^{-4}M; β_2-selective) or the β-adrenoceptor agonists, ($-$)-isoprenaline (10^{-8}–10^{-4}M), ($+$)-isoprenaline (10^{-6}–10^{-2}M), ($-$)-noradrenaline (10^{-7}–10^{-3}M), ($-$)-adrenaline (10^{-7}–10^{-4}M), ($\pm$)-fenoterol (3×10^{-8}–10^{-4}M; β_2-selective) and RO363 (3×10^{-5}–10^{-4}M; β_1-selective). After incubation with ligands, sections were washed once in buffer at $22°C$ for 1 min and again for 2×15 min periods and then quickly rinsed in distilled water. Sections were then wiped from slides with glass fibre filter paper (Whatman, GF/A) and the radioactivity counted in a Packard gamma counter (Model 5650). Twelve tissue sections from blocks from 3 different lungs were used for the estimation of each data point. Protein content in adjacent sections in each tissue block was estimated by the method of Lowry *et al.*, (1951). Preliminary binding constants were obtained in the saturation experiments using Scatchard and Hill analyses. Final binding parameter estimates and their errors were estimated by non-linear least squares regression analysis using the computer programme NONLIN (Metzler, 1969). In competition experiments, parameter estimates were obtained for 1 and 2 site binding models using the programme MLAB (N.I.H., U.S.A.).

Autoradiography Tissue sections ($10 \mu m$) were incubated with I-CYP (50 pM) for 150 min in the absence or presence of 200 μM ($-$)-isoprenaline or the β-adrenoceptor antagonists atenolol (10^{-7}–10^{-4}M) or

ICI-118551 (10^{-8}–10^{-5}M) and washed as described for kinetic experiments. Sections were then rapidly dried under a stream of cold dry air.

Coverslips (type 0) were coated at $46°C$ with NTB-3 nuclear track emulsion (Eastman Kodak Co., Rochester, N.Y.) diluted 1:1 with distilled water, air dried for 3 h and stored at $4°C$. Emulsion coated coverslips were attached to one end of tissue slides with cyanoacrylate adhesive and stored in dessicated light-tight X-ray cassettes at $4°C$ for 3 days. The emulsion was developed in Dektol (Kodak) diluted 1:1 with distilled water for 3 min and fixed for 3 min with Rapidfix (Kodak) diluted 1:4 with distilled water. The tissue was stained with Gill's haematoxylin for 30 s. After air drying, the coverslips were re-apposed to the slides and the tissue mounted in DePeX medium (BDH). Sections were viewed with a Zeiss photomicroscope under light and dark-field illumination using a $\times$ 10 objective.

Quantitation of I-CYP binding sites in pig lung parenchyma was achieved by manual counting of autoradiographic grains visualised on dark-field photomicrographs of fields described over tissue areas of alveolar wall, airway and vascular smooth muscle, airway epithelium and vascular endothelium. Three hundred and forty-two fields, each with an area of approximately 1000 μm^2 were chosen within 28 tissue sections from a pig lung. Sections were labelled with I-CYP in the absence or presence of 200 μM ($-$)-
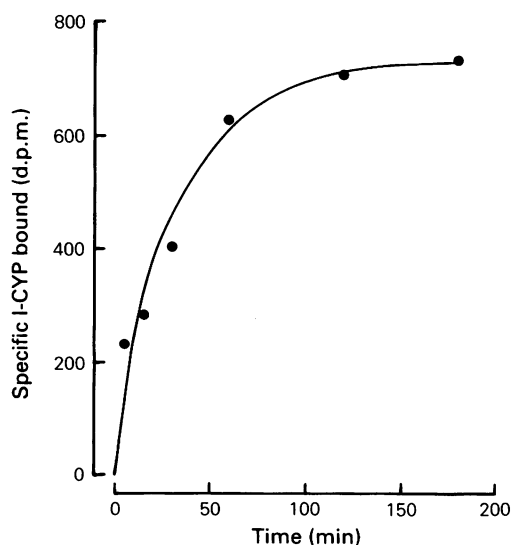
Figure 1 Time-dependent increase in specific [^{125}I]-iodocyanopindolol (I-CYP; 50 pM) binding in $16 \mu m$ sections of pig lung parenchyma. Each point represents the mean of data from 2 separate lung samples (4 tissue sections per lung sample).

isoprenaline, or the β-adrenoceptor antagonists ICI-118551 and atenolol.

The probability (P) of differences between mean values for autoradiographic grain densities was determined using Student's two-tailed, non-paired t test and was considered significant if $P < 0.05$.

Drugs

Drugs used were $(-)$-3-[^{125}I]-iodocyanopindolol (Amersham); $(-)$-isoprenaline hydrochloride, $(+)$-isoprenaline bitartrate, $(-)$-noradrenaline bitartrate, $(-)$-adrenaline bitartrate, $(\pm)$-propranolol hydrochloride (Sigma); $(\pm)$-fenoterol hydrobromide (Boehringer Ingelheim); RO363 ($(\pm)$-1-(3,4-dimethoxyphenyethylamino)-3-(3,4-dihydroxyphenoxy)-2-propanol)oxalate; synthesized at the Victorian College of Pharmacy, Melbourne, Australia); atenolol, ICI-118551 (erythro-DL-1-(7-methylindan-4-yloxy)-3-(isopropylaminbutan-2-ol) hydrochloride; ICI). Stock solutions of the catecholamines were freshly prepared in saline containing ascorbic acid 20 µg ml^{-1}.

Results

Characteristics of I-CYP binding

The specific binding of I-CYP in pig lung parenchyma

reached equilibrium at between 120 and 180 min (Figure 1). Subsequent binding experiments were performed using an equilibration time of 150 min. Binding of I-CYP was saturable, involving a single population of non-interacting sites (mean ± s.e.mean Hill coefficient, $n_H = 0.933 \pm 0.052$). Specific binding sites had a high affinity for I-CYP (dissociation constant, $K_D = 73.4 \pm 10.0$ pM) and the maximum binding capacity (B_{max}) was 51 ± 3 fmol mg^{-1} protein (Figure 2). Each data point on the saturation curve is the mean of 12 observations derived from sections from 3 separate lung samples. Specific binding of I-CYP in pig lung parenchyma was displaceable by both β-agonists and antagonists (Figure 3). The potency order for displacement as determined by concentrations of competing β-agonists producing 50% reduction in specific binding (IC$_{50}$) was $(-)$-isoprenaline $>$ $(-)$-adrenaline $>$ $(\pm)$-fenoterol $>$ $(-)$-noradrenaline $>$ $(+)$-isoprenaline $>$ $(\pm)$-RO363. Data for $(+)$-fenoterol is not shown in Figure 3. The potency order for β-antagonists was $(\pm)$-propranolol $>$ ICI-118551 $>$ atenolol. Competition for specific I-CYP binding sites by isoprenaline was stereo-selective with the laevo isomer approximately 270 times more potent than the dextro isomer. $(-)$-Adrenaline was 4 times more potent than $(-)$-noradrenaline indicating the predominance of β$_2$-adrenoceptors. Displacement data for the β$_1$-selective antagonist atenolol and for the β$_2$-selective antagonist ICI-118551, were fitted best to
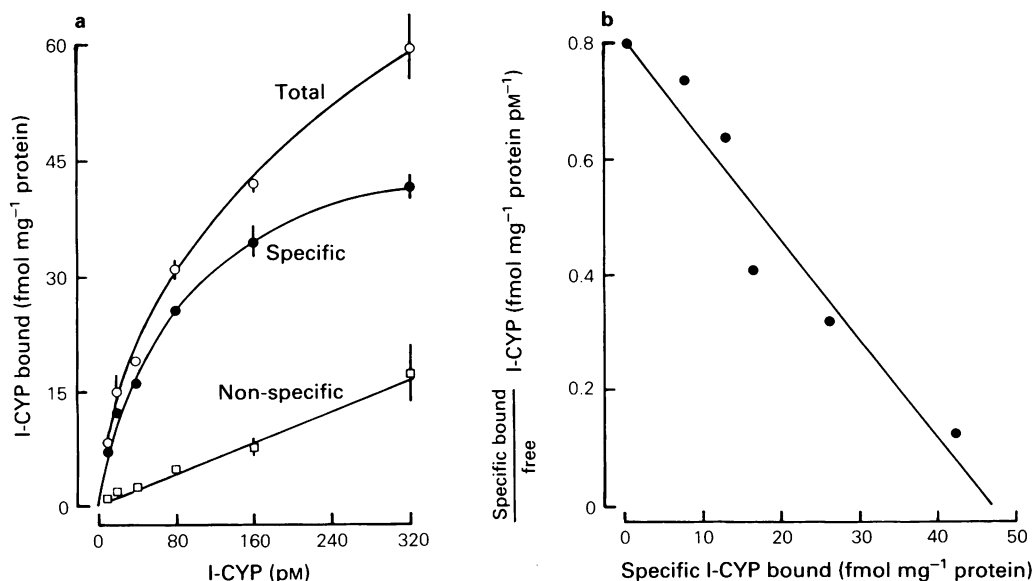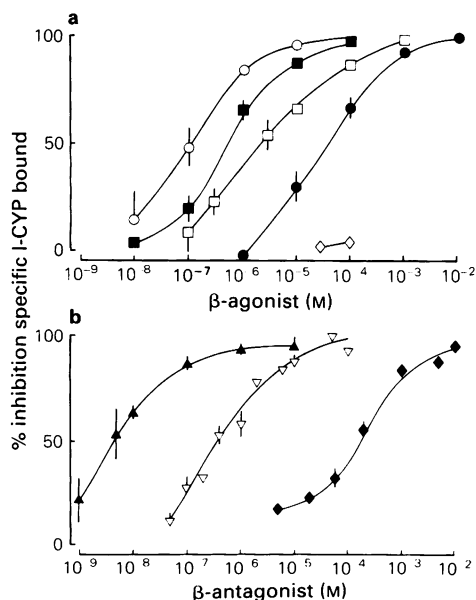

Figure 2 (a) Saturation of [^{125}I]-iodocyanopindolol (I-CYP) binding in 16 µm sections of pig lung parenchyma. Each point represents the mean of data from 3 separate lung samples (4 tissue sections per lung sample) and vertical lines indicate s.e.mean. Non-specific I-CYP binding was taken as that occurring in the presence of 200 µM $(-)$-isoprenaline. Specific binding = total minus non-specific binding. (b) Scatchard analysis of specific I-CYP binding.

a 2 binding site model indicating that both β_1- and β_2-adrenoceptors were present in pig lung. Hill coefficients significantly less than unity were obtained for atenolol ($n_H = 0.426 \pm 0.053$) and for ICI-118551 ($n_H = 0.721 \pm 0.100$). Computer analysis (MLAB) of displacement data for atenolol showed that β_1- and β_2-adrenoceptors were present in the proportions $31.4 \pm 8.1\%$ and $68.6 \pm 8.2\%$ respectively. ICI-118551 displacement data determined the proportions as $25.3 \pm 12.4\%$ β_1 and $74.7 \pm 12.3\%$ β_2. Thus on average, 28% of the β-adrenoceptors were of the β_1 subtype, while 72% were β_2-adrenoceptors.
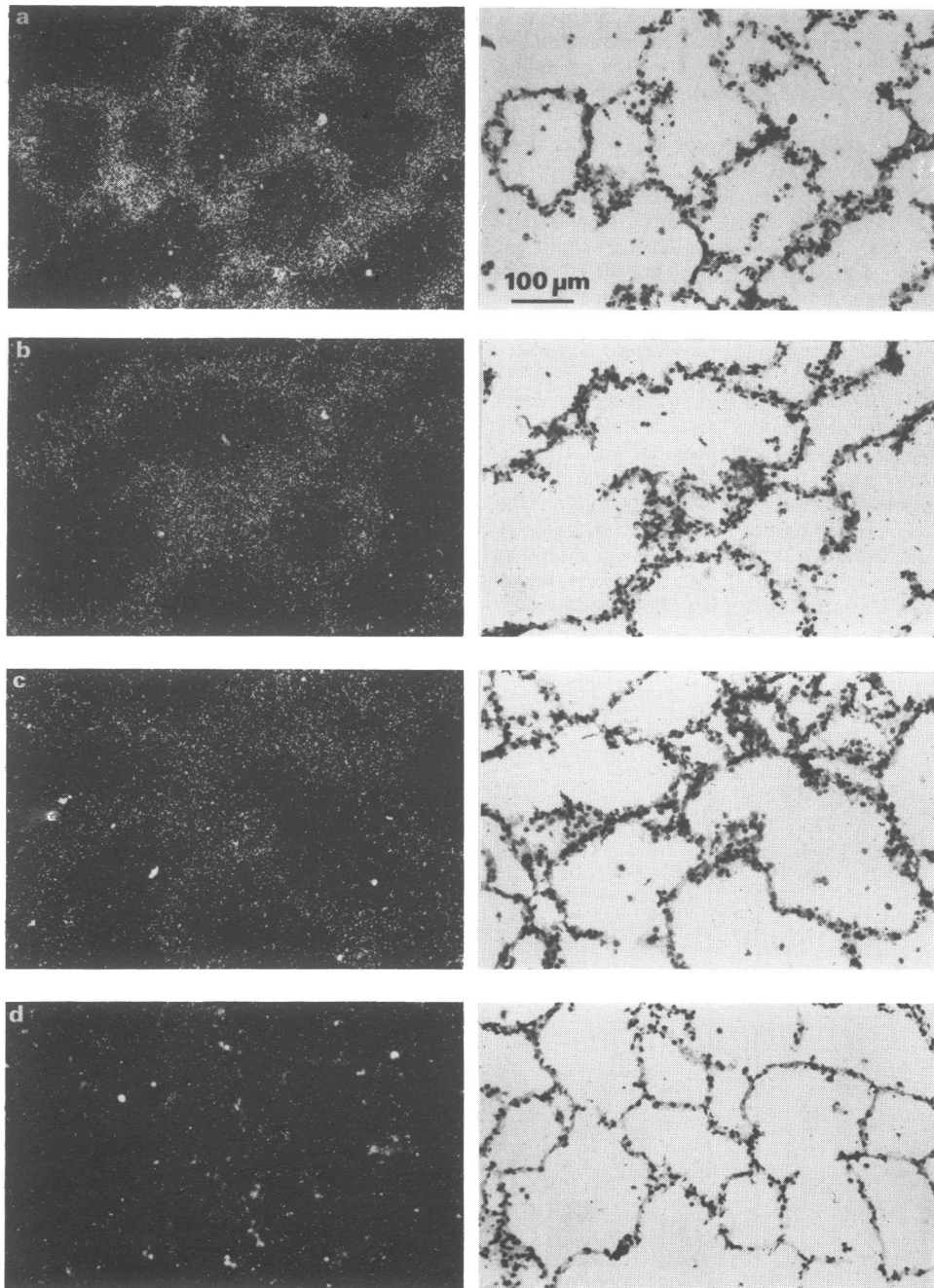
Autoradiography

Autoradiographic grains representing I-CYP binding sites, were localized with different densities over several tissue types within the lung parenchyma including alveolar wall, vascular and airway smooth muscle, vascular endothelium and airway epithelium (Figure 4). Labelling over alveolar walls was dense and

Figure 3 Concentration-effect relationships for the displacement of specific [^{125}I]-iodocyanopindolol (I-CYP; 50 pM) binding in 16 μm sections of pig lung parenchyma by: (a) the β-adrenoceptor agonists (+)-isoprenaline (●), (−)-isoprenaline (○), (−)-noradrenaline (□), (−)-adrenaline (■) and (±)-RO363 (◇); (b) the β-adrenoceptor antagonists (±)-propranolol (▲), ICI-118551 (▽) and atenolol (◆). Each point represents the mean of data from at least 3 separate lung samples (4 tissue sections per lung sample).
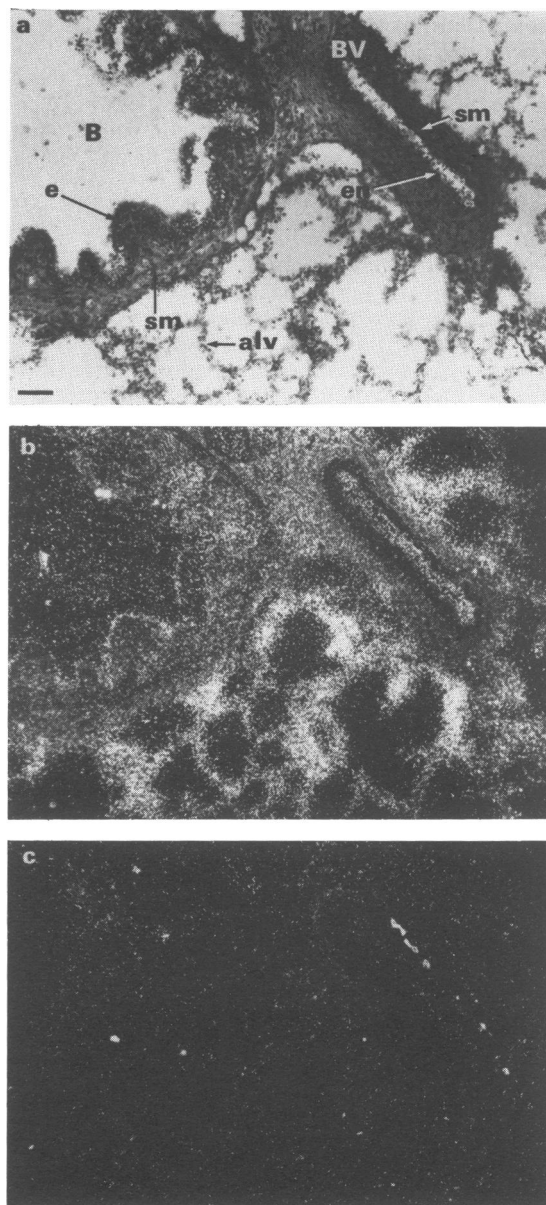
Figure 4 (a) Bright-field photomicrograph of a 10 μm frozen section of pig lung parenchyma. B = bronchiole, BV = blood vessel, e = epithelium, en = endothelium, sm = smooth muscle, alv = alveolar wall. Bar = 100 μm. (b) Dark-field photomicrograph of the above section showing the distribution and localization of autoradiographic grains derived from [^{125}I]-iodocyanopindolol (I-CYP, 50 pM) binding. (c) Dark-field photomicrograph showing the distribution of non-specific autoradiographic grains in the next serial section incubated with I-CYP (50 pM) and (−)-isoprenaline (200 μM).

Figure 5 Dark-field (left-hand panels) and bright-field (right-hand panels) photomicrographs of 10 μm frozen sections of pig lung parenchyma showing the localization of autoradiographic grains derived from [^{125}I]-iodocyanopindolol (I-CYP, 50 pM) binding in alveolar wall in the absence (a), or the presence of (b) atenolol, 10^{-5}M; (c) ICI-118551, 10^{-6}M or (d) (−)-isoprenaline, 200 μM. Bar = 100 μm.

uniform, giving no indication that I-CYP binding was selective for particular cell types. Sections incubated with I-CYP in the presence of 200 μM (−)-isoprenaline demonstrated low levels of uniform non-specific binding.

The effects of the β-adrenoceptor antagonists atenolol (β_1-selective) and ICI-118551 (β_2-selective) on total I-CYP binding in alveolar wall tissue are shown in Figure 5. Atenolol (10^{-5}M, Figure 5b) and ICI-118551 (10^{-6}M, Figure 5c) caused reductions in specific grain density of 30.8 ± 6.7% and 49.9 ± 4.3% respectively. Atenolol and ICI-118551 reduced grain counts over tissue to background levels at 10^{-3}M and 10^{-5}M respectively, concentrations where non-selective displacement was expected.

The relative densities of β-adrenoceptors (as determined by autoradiographic grain counting) were estimated over alveolar wall, vascular endothelium and smooth muscle, and airway epithelium and smooth muscle (Figure 6). Alveolar wall tissue was the most densely labelled tissue type followed by vascular endothelium, although this was 32% lower than that over alveolar wall. The grain density over vascular smooth muscle was 61% lower than that over alveolar wall tissue, but was twice as high as that over airway
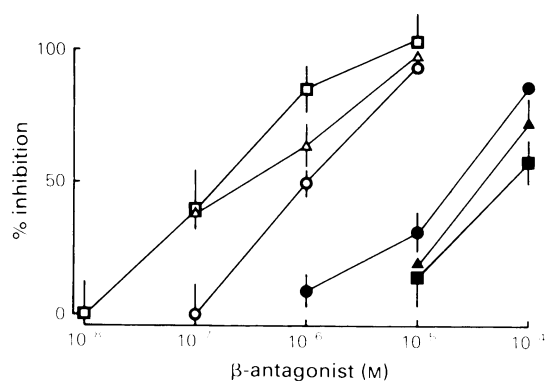
Figure 7 Concentration-effect relationships between the β_1-selective antagonist atenolol (solid symbols), the β_2-selective antagonist ICI-118551 (open symbols) and reduction in the densities of autoradiographic grains (% inhibition) derived from [^{125}I]-iodocyanopindolol (I-CYP, 50 pM) binding in alveolar wall tissue (●,○), airway smooth muscle (■,□) and vascular smooth muscle (▲,△) in a sample of pig lung. Vertical lines represent s.e.mean of 5–14 field measurements.
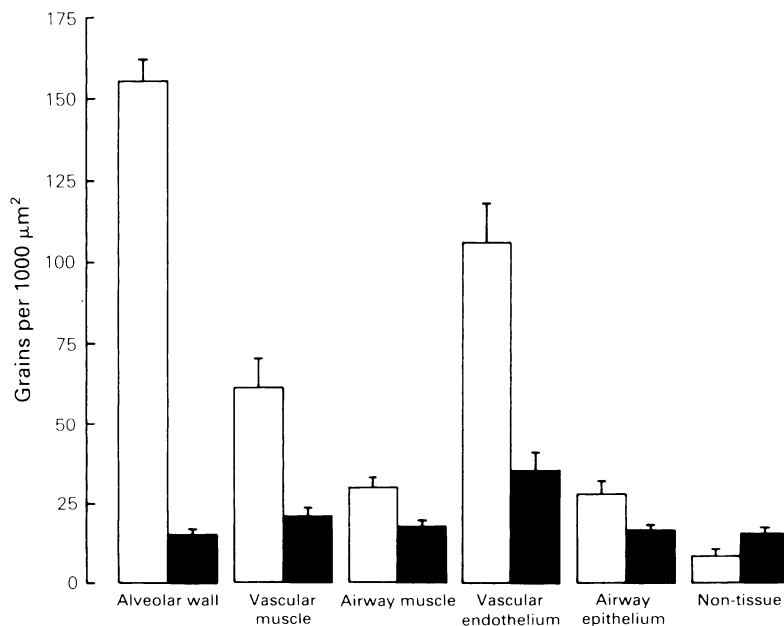
Figure 6 Mean densities of specific (open columns) and non-specific (filled columns) autoradiographic grains (grains per 1000 μm^2) derived from [^{125}I]-iodocyanopindolol (I-CYP, 50 pM) binding in alveolar wall tissue (specific, $n = 44$ fields; non-specific, $n = 28$ fields), vascular ($n = 14;9$) and airway smooth muscle ($n = 24;18$), vascular endothelium ($n = 15;9$), airway epithelium ($n = 24;16$) and over non-tissue (airway lumen; $n = 24;37$) in a sample of pig lung. Vertical bars on columns represent s.e.mean.

smooth muscle and epithelium. Grain densities over connective tissue elements, including bronchial cartilage, were very low and approximately equivalent to those over non-tissue areas.

The effects of atenolol (10^{-7}–10^{-4}M) and of ICI-118551 (10^{-8}–10^{-5}M) on I-CYP binding were assessed in terms of reductions in autoradiographic grain densities over alveolar wall tissue and airway and vascular smooth muscle (Figure 7). ICI-118551 was 10 times and 5 times more potent in airway and vascular smooth muscle respectively, than in alveolar wall tissue. Conversely, atenolol was 2–3 times less potent in these tissues than in alveolar wall. These data are consistent with the existence of both β_1- and β_2-adrenoceptors in alveolar wall tissue, while more homogeneous populations of β_2-adrenoceptors exist in airway and vascular smooth muscle.

Discussion

The present study documents the tissue localization of β-adrenoceptors in pig lung parenchyma using the potent, highly selective radioligand I-CYP (Engel et al., 1981; Munzel et al., 1983). Kinetic experiments showed that I-CYP was bound to a saturable population of sites with high affinity. Both β-adrenoceptor-selective agonists and antagonists displaced specific I-CYP binding. Furthermore, competition for I-CYP binding sites by isoprenaline was stereoselective, in that the laevo isomer was 270 times more potent than the dextro isomer, a finding similar to that of Carstairs et al., (1984) in human lung. Taken together, these results indicate that specific I-CYP binding in pig lung was selective for β-adrenoceptors.

The heterogeneous nature of lung tissue was reflected in the different autoradiographic grain densities observed over different tissue types within the lung. The highest grain density was associated with alveolar wall tissue, as previously observed in both human (Carstairs et al., 1984) and rat (Conner & Reid, 1984) lung. In contrast, in the ferret (Barnes et al., 1982) and rabbit (Barnes et al., 1984), bronchial smooth muscle contained a significantly greater density of β-adrenoceptors than did alveolar wall tissue. The density of β-adrenoceptors over vascular smooth muscle was twice that observed over airway smooth muscle and epithelium. While epithelial β-adrenoceptors do not appear to be directly relevant to the expression of mechanical responses of airway smooth muscle they are known to mediate increases in the active transport of chloride ions (Davis et al., 1979), increases in the velocity of airway mucous transport (Mossberg et al., 1976a,b) and to influence epithelial cell differentiation (Jones & Reid, 1979). Very low levels of specific binding were detected over connective tissue elements and cartilage. With the exception of the low grain density measured over airway epithelium, the relative grain densities over particular tissue types in pig lung were similar to those determined in human lung (Carstairs et al., 1984).

Competition binding experiments demonstrated that β_2-adrenoceptors predominate in pig lung parenchyma. However, further analysis of data from the present study, describing the displacement of specific I-CYP binding by atenolol and ICI-118551 revealed that β_1-adrenoceptors constituted 28% of the total population. Manual counting of autoradiographic grains indicated that the density of β-adrenoceptors was approximately 2.5 times and 5 times greater over alveolar wall tissue than over vascular and airway smooth muscle respectively. Volume density analysis has previously shown that human lung parenchyma is 78% alveolar wall, 8% vascular smooth muscle and only 3% airway smooth muscle, with the remaining 11% consisting mainly of cartilage and connective tissue elements (Bertram et al., 1983a,b).

Assuming that the composition of pig lung parenchyma is similar to human lung parenchyma, approximately 95% of the total population of β-adrenoceptors must have been associated with the alveolar wall. This is in close agreement with the value of 97% established in rat lung alveolar wall (Conner & Reid, 1984). Given that approximately 28% of the total population of parenchymal β-adrenoceptors were of the β_1 subtype, it is clear that the great majority of these were associated with the alveolar wall. Thus alveolar tissue contained both β_1- and β_2-adrenoceptors approximately in the proportion 30:70, respectively. Autoradiographic grain counting demonstrated a smaller potency difference between ICI-118551 and atenolol in alveolar tissue than in bronchial and vascular smooth muscle (Figure 6). This indicates a higher percentage of β_2-adrenoceptors in smooth muscle than in alveolar tissue.

We have previously shown that β-agonist-induced relaxation of the pig lung parenchyma strip was mediated via β_2-adrenoceptors (Goldie et al., 1982). It is often assumed that relaxation of lung strips reflects the reactivity of airway smooth muscle rather than of vascular or alveolar components. However, in view of the very low volume density of airway smooth muscle in lung parenchyma as well as the relatively sparse population of β_2-adrenoceptors in the peripheral airways, it seems likely that relaxant response of peripheral blood vessels may also contribute to β-agonist-induced relaxation of the lung parenchyma strip.

Our data suggest that the majority of alveolar β-adrenoceptors are located on cells which do not subserve mechanical responses. This is consistent with data showing that β-agonists stimulate the release of surfactant from alveolar type II cells (Dobbs & Mason, 1979; Giannopoulos, 1980) and may regulate fluid absorption from the alveolar spaces in the new-

born (Walters & Olver, 1978). However, a small population of potentially contractile alveolar interstitial cells has been described in several species of lung (Kapanci *et al.*, 1974) and these may contain β_2-adrenoceptors. Since the majority of the lung parenchyma tissue consists of alveolar wall, such interstitial cells may also contribute to the relaxant responsiveness of the lung strip to β-agonists.

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