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High affinity [^{3}H]formoterol binding sites in lung: characterization and autoradiographic mapping

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Abstract

Agonist binding to the β_2-adrenoceptors and its mapping were studied using the newly developed radioligand [^{3}H]formoterol. The results of [^{3}H]formoterol saturation binding and formoterol inhibition of [^{3}H]formoterol binding were consistent with binding to a single class of receptors ($K_d = 1.34 \pm 0.15$ nM, $B_{max} = 154.9 \pm 8.0$ fmol/mg protein in guinea pig lung membranes, $n = 8$; $K_d = 1.05 \pm 0.17$ nM, $B_{max} = 67.8 \pm 8.1$ fmol/mg protein in human lung membranes, $n = 5$) and competition assays with other agonists and antagonists disclosed only a single class of site. The nonhydrolyzable GTP analogue GTPγS caused a reduction in both K_d and B_{max}, indicating that the receptors labelled by [^{3}H]formoterol are coupled to a guanine nucleotide binding regulatory protein. Receptor mapping of [^{3}H]formoterol binding sites shows that β_2-adrenoceptors were widely distributed in both guinea pig and human lung, with dense labelling over airway epithelium and uniformly over alveolar walls, and sparse labelling of airway and vascular smooth muscle. In addition, submucosal glands were also sparsely labelled in human bronchus. The distribution of β_2-adrenoceptors was similar to the pattern previously described with non-selective radiolabelled antagonists in the presence of selective β_1-adrenoceptor antagonists.

Keywords: Formoterol; β_2-Adrenoceptor; Autoradiography; Lung

1. Introduction

There is a high density of β-adrenoceptors in lungs and autoradiographic studies have shown that these receptors are localized to many different cell types (Barnes et al., 1982; Carstairs et al., 1984; 1985). Receptor binding studies have demonstrated that β_2-adrenoceptors predominate over β_1-adrenoceptors in homogenates in guinea pig and human lung (Engel, 1981; Carstairs et al., 1985), and autoradiographic studies have localized β_2-adrenoceptors to epithelium, airway smooth muscle, submucosal glands and pulmonary vascular smooth muscle and endothelium (Carstairs et al., 1985). β_2-adrenoceptors mediate many aspects of lung function including relaxation of airway and pulmonary vascular smooth muscle (Goldie et al., 1982; Zaagsma et al., 1983), regulation of microvascular permeability (Erjefalt and Persson, 1986), stimulation of mucus gland secretion (Phipps et al., 1982), epithelial ion transport (Knowles et al., 1984), surfactant release from type II pneumocytes (Dobbs and Mason, 1979), inhibition of mediator release from mast cells (Church and Hiroi, 1987) and modulation of cholinergic neurotransmission (Rhoden et al., 1988).

β_2-Adrenoceptor agonists such as salbutamol and terbutaline are widely used as bronchodilators in the treatment of asthma (Barnes, 1989; Burrows and Lebowitz, 1992). These selective drugs have the advantage over nonspecific β-adrenoceptor agonists such as isoprenaline, with fewer cardiac side-effects (Svedmyr and Löofdahl, 1987). Recently, inhaled β_2-adrenoceptor agonist with a prolonged bronchodilator effect, such as formoterol and salmeterol, has been developed (Löofdahl and Chung, 1991). In clinical studies, formoterol was found to have a long duration of bronchodilation (over 12 hours) when given by inhalation but when given by the oral route its duration of action was similar to that of salbutamol (Löofdahl and Sved-

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myr, 1989). The aim of the present study was to evaluate the binding of [^{3}H]formoterol to guinea pig and human lung using a conventional membrane binding assay as well as receptor autoradiography.

2. Materials and methods

2.1. Materials

[^{3}H]Formoterol (1.52 TBq/mmol) was synthesized by CIBA-GEIGY (Basel, Switzerland). Salmeterol, formoterol and CGP-20712A, (±)-(2-hydroxy-5-[2-((2-hydroxy-3-(4-((1-methyl-4-trifluoromethyl)1*H*-imidazole-2-yl-)phenoxy)propyl)amino)ethoxy]benzamidemonomethane sulfonate, were kindly supplied by CIBA-GEIGY. ICI-118,551, erythro-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol, was a generous gift from Imperical Chemical Industries (Macclesfield, UK). Salbutamol, propranolol, pindolol, atenolol, (−)-isoprenaline and GTPγS were obtained from Sigma (Poole, UK). Filtron-X was from National Diagnostics (Hull, UK). Other substances were from standard commercial sources. Salmeterol and formoterol were dissolved in 1 N HCl at 1 mM and 50% absolute alcohol respectively. Further dilutions and solutions of all other compounds were made in buffer.

Human lung tissue was obtained at the time of resection for lung carcinoma or from heart-lung transplantation donors. Tissue distant from any pathological process was dissected. Lungs were also obtained from male Dunkin-Hartley guinea pigs and were dissected immediately after death.

2.2. Methods

2.2.1. Tissue preparation

For membrane binding assays, lung tissue was disrupted with a Polytron homogenizer in 10 volumes of ice-cold 25 mM Tris-HCl buffer (pH 7.4) containing 0.32 M sucrose for 30 s at setting 6. Debris was sedimented by 10 min of centrifugation at $1000 \times g$ at 4°C, after which membranes were sedimented at 40000 $\times g$ for 20 min at 4°C. Membranes were resuspended and washed twice. The final pellets were resuspended in 25 mM Tris-HCl buffer (pH 7.4) to give a protein concentration of 1–3 mg/ml. Aliquots were snap-frozen in liquid nitrogen and stored at −80°C until use. Protein determination was carried out according to the method of Lowry et al. (1951) with bovine serum albumin as a standard.

For receptor autoradiography, small pieces of human and guinea pig peripheral lung were inflated with tissue-embedding medium (Tissue-Tek OCT compound) diluted 1:4 with phosphate-buffered saline (PBS) before freezing. Segmental human bronchi and guinea pig trachea were also dissected out and frozen. Serial sections (8–16 μm thick) were cut on a Bright cryostat at −30°C, thaw-mounted onto gelatin-coated glass slides and stored at −80°C until use.

2.2.2. Radioligand binding assay

[^{3}H]Formoterol binding assays were performed in a final incubation volume of 0.25 ml of 25 mM Tris-HCl (pH 7.4) at 25°C for 45 min, which was shown to reach equilibrium. The reaction was initiated by addition of lung membranes (approximately 200–300 μg of protein). For saturation experiments, membrane suspensions were incubated with increasing concentrations (0.0625–8 nM) of [^{3}H]formoterol in the absence (total binding) or in the presence of 10 μM ICI-118,551 (a selective β_2-adrenoceptor antagonist; nonspecific binding)(Dickinson et al., 1981). For competition experiments, the displacement of binding of 1.0 nM [^{3}H]formoterol was performed by adding increasing concentrations of agonists and antagonists to the membrane suspension. Incubation was terminated by rapid filtration under vacuum through pre-soaked Whatman GF/B glass fibre filters. The filters were washed three times with 5 ml ice-cold buffer and residual radioactivity bound to the filters was measured by liquid scintillation counting in 4 ml Filtron-X at 48% counter efficiency. All incubations were carried out in triplicate.

Specific binding was defined as the difference between total and nonspecific binding determined in the presence of 10 μM ICI-118,551. Under these experimental conditions the membrane preparations bound approximately 5–10% of the [^{3}H]formoterol added to the incubation. At 1.0 nM [^{3}H]formoterol, specific binding was linear with respect to membrane protein and routinely represented 75–90% and 50–65% of the [^{3}H]formoterol binding to guinea pig and human lung membranes respectively. Guinea pig lung membranes were used in all experiments, since they have high and consistent specific binding.

2.2.3. Autoradiography

This was performed using the method as previously described (Carstairs et al., 1985). Sections (8–16 μm) were warmed to room temperature, washed for 5 min in incubation buffer (25 mM Tris-HCl and 0.25% polypep; pH 7.4), and incubated with 1.0 nM [^{3}H]formoterol at 25°C for 45 min. Nonspecific binding was determined by incubating adjacent sections with the same concentration of [^{3}H]formoterol and 10 μM ICI-118,551. After incubation, slides were washed twice for 5 min in ice-cold buffer, rinsed in cold distilled water to remove any buffer salts, rapidly dried in a stream of cold air, and stored with desiccant under vacuum overnight. Glass coverslips that had been coated with stripping film (AR10, Kodak) were fixed to one end of the slides with cyanoacrylate adhesive and held in

contact with the sections by butterfly clips. Slides were exposed to the emulsion for 3–4 months in desiccated light-tight boxes at 4°C. The emulsion was developed in Kodak D-19 developer and fixed. Sections were stained with 1% cresyl fast violet. The slides were viewed under a Zeiss microscope equipped with light- and dark-field illumination.

2.2.4. Data analysis

Raw dpm were processed by the computer program EBDA (McPherson, 1983), in conjunction with the nonlinear iterative curve-fitting program LIGAND (Munson and Rodbard, 1980). The F test was used to determine whether the data fitted a multiple-site model rather than a single-site model. $P < 0.05$ was considered to be statistically significant. Data are expressed as the mean ± standard error of the mean.

Differences between treated groups were compared to controls and changes were considered significant when $P < 0.05$, using Student's paired t-test.

3. Results

3.1. Binding studies

[^{3}H]formoterol specific binding to guinea pig and human lung membranes was saturable, as determined by incubating membrane preparations with increasing concentrations of [^{3}H]formoterol, in the absence and presence of 10 μM ICI-118,551. Scatchard analysis revealed a single class of sites with dissociation constants (K_d) of 1.34 ± 0.15 nM and 1.05 ± 0.17 nM, maximum number of binding sites (B_{max}) of 154.9 ± 8.0 fmol/mg protein and 67.8 ± 8.1 fmol/mg protein in guinea pig lung ($n = 8$) and human lung ($n = 5$) respectively. Although several saturation experiments showed evidence of two sites, LIGAND analysis failed to give statistically significant two-site fits. These results suggest that [^{3}H]formoterol binds specifically, and with high affinity in guinea pig and human lung membranes.

The binding characteristics of [^{3}H]formoterol to guinea pig lung membranes were further examined in competition studies using several β-adrenoceptor agonists and antagonists, in order to assess whether these binding sites displayed the selectivity profile expected for β_2-receptors. Table 1 shows the slope factors (pseudo-Hill coefficients) and LIGAND values for equilibrium dissociation constants (K_d values) of competitors for binding of [^{3}H]formoterol, using a one-site binding model. The order of potency of agonists for [^{3}H]formoterol binding was formoterol > salmeterol > (−)-isoprenaline > salbutamol (Fig. 1A). A single-site model was the most suitable to describe competition data for non-selective β-adrenoceptor antagonists, pindolol and propranolol, and selective β_2-adrenoceptor antagonist, ICI-118,551 against [^{3}H]formoterol binding sites (Fig. 1B). In some experiments, computer analysis of the competition data for agonists and antagonists gives a better two-site model but not statistically signif-

Table 1
Competition by β-adrenoceptor agonists and antagonists against [^{3}H]formoterol binding in guinea pig lung membranes

Competitor	K_d (nM)	Slope factor
Agonist		
Formoterol	0.86 ± 0.03	0.92 ± 0.04
Salmeterol	7.79 ± 1.85	0.99 ± 0.06
(−)-Isoprenaline	12.7 ± 3.2	0.89 ± 0.08
Salbutamol	101.3 ± 7.0	0.84 ± 0.02
Antagonist		
Pindolol	1.07 ± 0.27	0.92 ± 0.03
Propranolol	3.56 ± 0.47	0.94 ± 0.08
ICI-118, 551	4.11 ± 1.66	0.90 ± 0.02
Atenolol	ND [a]	
CGP-20712A	ND [a]	

Values for equilibrium dissociation constant (K_d) were generated by LIGAND and are the mean ± S.E.M. of three to four curves analyzed simultaneously. Slope factors were generated by EBDA and are the mean ± S.E.M..
[a] ND, negligible inhibition of binding at 10 μM.

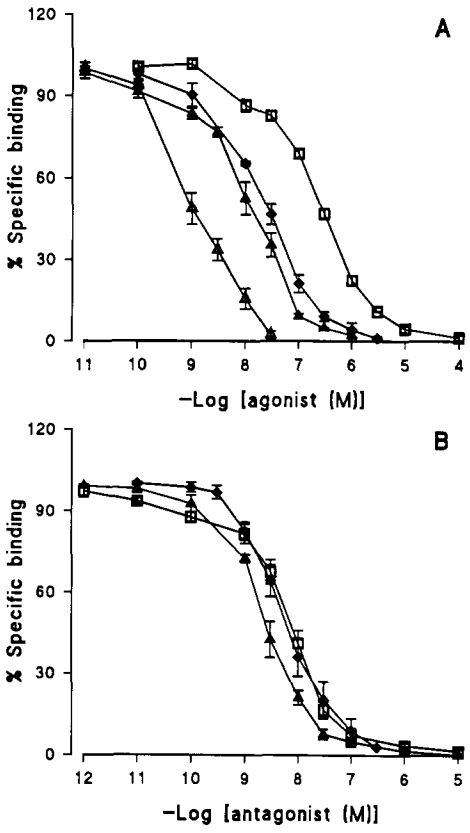

Fig. 1. Competition curves for β-agonists (A), and β-antagonists (B) against [^{3}H]formoterol binding in guinea pig lung membranes. Each point represents the mean ± S.E.M. of three to four determinations. Δ, formoterol; Δ, salmeterol and pindolol respectively; ♦, (−)-isoprenaline and ICI-118,551 respectively; □, salbutamol and propranolol respectively.

icant improvement of the one-site fit by the LIGAND program ($P > 0.05$, F-test). The selective β_1-adrenoceptor antagonists, atenolol and CGP-20712A produced negligible inhibition of [^{3}H]formoterol specific binding at 10 μM (Table 1). These results indicate that the [^{3}H]fomoterol binding is specific for β_2-adrenoceptors.

It has been well established that guanine nucleotides reduce high affinity agonist binding to G-protein-coupled β-adrenoceptors (De Lean et al., 1980). GTPγS, a nonhydrolyzable GTP analogue, inhibited approximately 85% specific binding at 1.0 nM [^{3}H]formoterol (Fig. 2). The IC_{50} (half-maximal inhibition) for GTPγS was 3 μM.

The mechanism of the inhibition by GTPgS was further investigated in equilibrium-saturation experiments conducted in the absence or presence of 100 μM GTPγS. The saturation curves and Scatchard plots were shown in Fig. 3 (A and B). Scatchard analysis of equilibrium binding of [^{3}H]formoterol to guinea pig lung membranes in the absence or presence of GTPγS resulted in linear plots. In three paired experiments, we observed a 2.8-fold change in affinity (K_d values were 1.51 ± 0.18 nM and 4.28 ± 1.11 nM, $P < 0.05$ for control and GTPγS, respectively), with a significant change in B_{max} (150.2 ± 13.2 fmol/mg protein and 110.7 ± 8.8 fmol/mg protein, respectively)($n = 3$). Interestingly, in one out of three batches, a two-site model could be fitted to the data for control. However, the fit was not statistically better ($P > 0.05$, F test) than a single-site model, thus accepting the latter model for all three experiments.

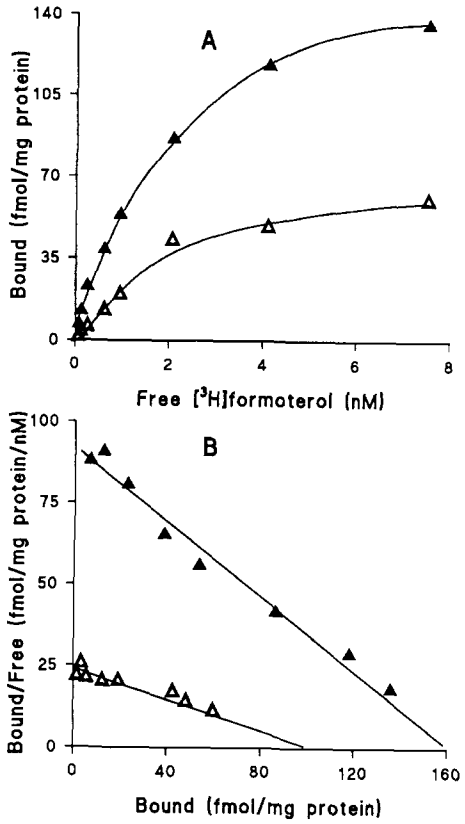

Fig. 3. Saturation curves (A) and Scatchard plots (B) of [^{3}H]formoterol binding in the absence (Δ) and presence (Δ) of 100 mM GTPγS to guinea pig lung membranes. Data are from a representative experiment that was repeated twice with similar results.

3.2. Receptor autoradiography

In agreement with previous findings, autoradiographic analysis showed that in both guinea pig and human lung, specific [^{3}H]formoterol binding sites were most dense in airway epithelium and alveolar walls. Moderate labelling was seen in airway and vascular smooth muscle in both species. Low levels of labelling were also seen over submucosal glands in human bronchi. There was no specific labelling pattern with [^{3}H]formoterol in the presence of excess of ICI-118,551 (Figs. 4 and 5).

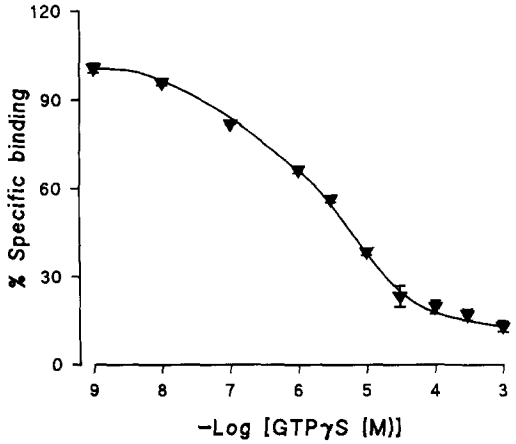

Fig. 2. Competition curve of [^{3}H]formoterol binding to guinea pig lung membranes by GTPγS. Each point represents the mean $\pm$ S.E.M. of three experiments.

4. Discussion

The present study demonstrates the existence of high affinity [^{3}H]formoterol binding sites in guinea pig and human lung membranes. The maximal number of [^{3}H]formoterol binding sites (B_{max}) is in agreement with 70% of total β-adrenoceptors being β_2-adrenoceptors in lung, as shown by previous studies using labelled non-selective antagonist such as [^{125}I]iodocyanopindolol in competition with selective β_1- and β_2-adrenoceptor antagonists (Engel, 1981; Carstairs et al., 1985). In addition, these binding sites were only displaced by non-selective β-adrenoceptor antagonists and selective β_2-adrenoceptor antagonists but not selective β_1-adrenoceptor antagonists, giving further support for β_2-adrenoceptors.

The inhibitory effect of GTPγS (up to 1 mM) on [^{3}H]formoterol binding observed in this study, can be interpreted as fractional occupancy of a low-affinity form of the receptors by [^{3}H]formoterol. This phenomenon of guanine nucleotide has been described with other receptors coupled to G-proteins, such as substance P receptors (Luber-Narod et al., 1990). In the presence of GTPγS, Scatchard analysis showed decreases in both K_d and B_{max}, in agreement with previous reports (Buck and Shatzer, 1988; Aharony et al., 1991; Johansson et al., 1992). These results suggest the presence of either two GTPγS-sensitive receptor/G-protein complexes with differential stability or differential accessibility of GTPγS to its binding sites on the G-protein. We cannot distinguish between these two possibilities but it is likely that this phenomenon underlies the dissociation kinetics in the presence of GTPγS. It is known that guanine nucleotides increase the rate of dissociation, resulting the formation of a low-affinity state. In this study, the loss of [^{3}H]formoterol binding sites could also be due to our filtration procedure which is too slow to detect all of the low-affinity, rapidly dissociable binding sites. The β-receptor-G_s complex apparently dissociates in the presence of a GTP analogue, which is associated with a loss of receptors in the agonist-specific high affinity state (Tolkovsky and Levitzki, 1978).

The results from autoradiography of the human and guinea-pig lung, agree with the previous findings (Carstairs et al, 1985; Gatto et al., 1987; Engels et al., 1989), demonstrating the greatest density of β_2-adrenoceptors in the airway epithelium and over the alveolar walls. The labelling of airway and vascular smooth muscle was less dense, confirming the differential distribution of β_2-adrenoceptors in different lung cells. Labelling over submucosal glands was reluctantly sparse, confirming our previous observations that these structures express predominantly β_1-adrenoceptors.

In conclusion, the results of this study demonstrate that [^{3}H]fomoterol binds specifically and has high affinity to β_2-adrenoceptors. The data are also consistent with the hypothesis that β_2-adrenoceptors are coupled to guanine nucleotide-binding protein and can exist in at least two interconvertible and guanine nu-

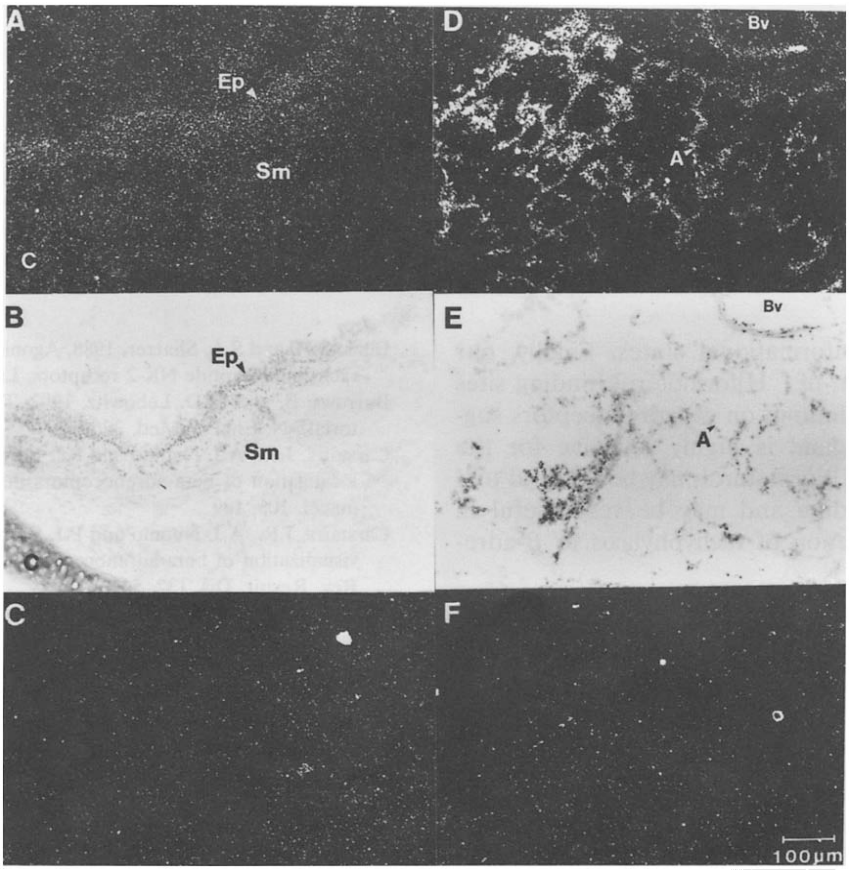

Fig. 4. Distribution of [^{3}H]formoterol binding sites in guinea pig trachea and peripheral lung. (B and E) Brightfield photomicrographs of trachea (B) and peripheral lung (E) stained with 1% cresyl fast violet. (A, C, D and F) Darkfield photomicrographs of adjacent sections showing the distribution of autoradiographic grains after incubation with [^{3}H]formoterol alone (A and D) or with [^{3}H]formoterol in the presence of 10 μM ICI-118,551 (C and F). Labelling was dense over airway epithelium (Ep) and alveolar walls (A), moderate over the wall of pulmonary blood vessel (Bv) and sparse over airway smooth muscle (Sm).

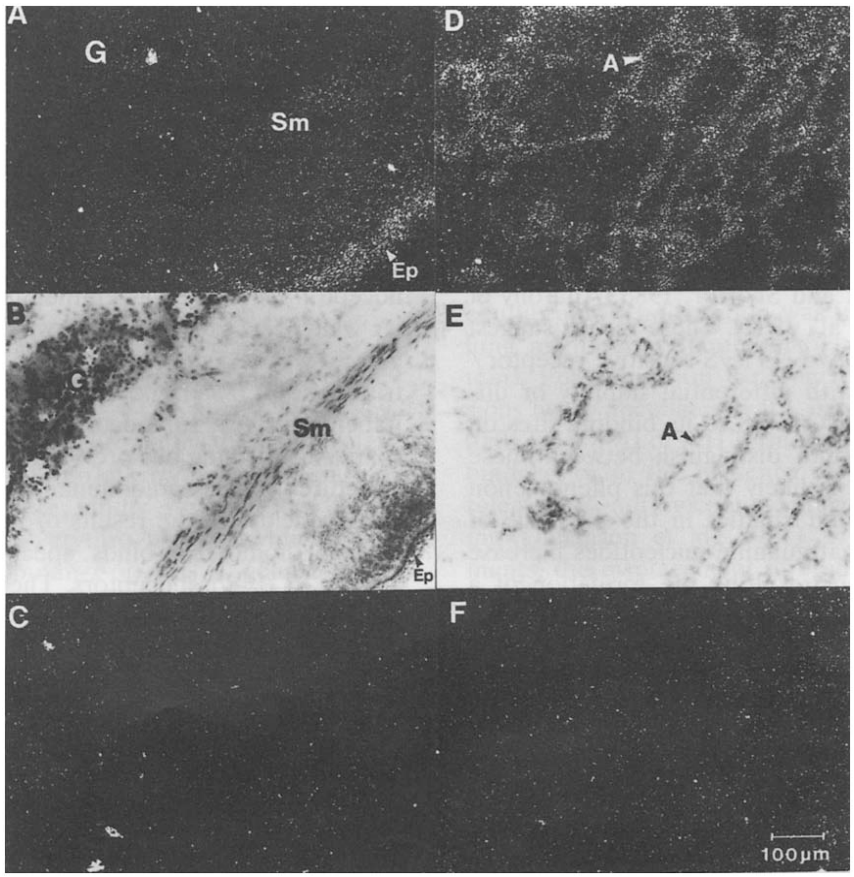

Fig. 5. Distribution of [^{3}H]formoterol binding sites in human bronchus and peripheral lung. (B and E) Brightfield photomicrographs of bronchus (B) and peripheral lung (E) stained with 1% cresyl fast violet. (A, C, D and F) Darkfield photomicrographs of adjacent sections showing the distribution of autoradiographic grains after incubation with [^{3}H]formoterol alone (A and D) or with [^{3}H]formoterol in the presence of 10 μM ICI-118,551 (C and F). Labelling was dense over airway epithelium (Ep) and alveolar walls (A) and sparse over airway smooth muscle (Sm) and submucosal glands (G).

cleotide-regulated conformational states. Finally, our findings of localization of [^{3}H]formoterol binding sites similar to previously findings on β_2-adrenoceptors suggest that this radioligand is highly selective for the β_2-adrenoceptors. [^{3}H]Formoterol may be a useful tool to assess agonist binding and may be very useful in studying the phenomenon of tachyphylaxis to β-adrenoceptor agonists.

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