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Suc-[Glu^9, Ala^{11,15}]-endothelin-1 (8–21), IRL 1620, identifies two populations of ET_B receptors in guinea-pig bronchus

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1 The pharmacological properties of endothelin receptors (ETR) were investigated in guinea-pig bronchus by comparing binding and functional results.

2 In binding assays, both the ET_B agonists, endothelin-3 (ET-3) and N-suc-[Glu^9,Ala^{11,15}]ET-1(8–21) (IRL 1620), and the antagonist, N-cis-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine (BQ 788), showed biphasic inhibition curves of [^{125}I]-endothelin-1 (ET-1) binding to bronchus membranes prepared from intact or epithelium-deprived tissue. IRL 1620 did not completely displace specifically [^{125}I]-ET-1 bound to these tissue preparations. In the presence of the ET_A-selective antagonist, cyclo(-D-Trp-D-Asp-L-Pro-D-Val-L-Leu) (BQ 123, 1 μM), IRL 1620 displacement curves were shallow but a complete inhibition was reached at a concentration of 1 μM. Both curves were better represented by two-site models. In addition, BQ 788 competition curves became monophasic when binding experiments were performed in the presence of 1 μM BQ 123. The non-selective agonist, ET-1, and BQ 123 inhibited [^{125}I]-ET binding to bronchus membranes in dose-dependent fashions with monophasic curves.

3 The contracting activity of IRL 1620 (0.55 nM–1.6 μM) was tested on multiple-ring bronchial preparations pretreated with peptidase and cyclo-oxygenase inhibitors. BQ 788 shifted IRL1620 concentration-response curves to the right while BQ 123 did not influence bronchial responsiveness. In addition, a potentiation of the maximal response to the agonist was observed in BQ 788 treated bronchial rings. This effect was abolished by tissue pretreatment with N$^{\omega}$-nitro-L-argininemethylester (L-NAME) or epithelium removal but not by pretreatment with atropine or iberiotoxin.

4 Our results demonstrate that guinea-pig bronchus contains two populations of ET_B receptors with different affinities for the ET_B-selective agonist, IRL 1620. One ET_B receptor population appears to activate bronchial muscle contraction while another on epithelial cells causes muscle relaxation through the release of nitric oxide (NO).

Keywords: Endothelin receptors; ET_B receptors; endothelins; guinea-pig bronchus

Abbreviations: ET-1, endothelin-1; ET-2, endothelin-2; ET-3, endothelin-3; ETR, endothelin receptor; ETs, endothelins; IRL 1620, N-Suc-[Glu^9,Ala^{11,15}]-endothelin-1 (8–21); BQ 788, N-cis-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine; BQ 123, cyclo(-D-Trp-D-Asp-L-Pro-D-Val-L-Leu); L-NAME, N$^{\omega}$-nitro-L-argininemethylester; Ach, acetylcholine; HEPES, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]; EDTA, ethylenediaminetetracetic acid; PMSF, phenylmethanesulphonylfluoride; BSA, bovine serum albumin; DMSO, dimethyl sulphoxide

Introduction

Endothelins (ETs)[1], a family of three closely related peptide hormones, endothelin-1, -2, and -3 (ET-1, ET-2, and ET-3), were originally recognized as potent modulators of vascular functions participating in both vasoconstriction and vasodilatation. Thereafter, the presence of ETs was demonstrated in several other tissues of different species including humans, where they exert various physiological effects (for a review see: Rubanyi & Polokoff, 1994; Webb *et al*., 1998). All activities of ETs are mediated through the interaction with specific cell surface receptors (for a review see: Bax & Saxena, 1994; Ohlstein *et al*., 1996; Pollock *et al*., 1995). Two ETR subtypes, termed ET_A and ET_B, have been identified, cloned and expressed from rat (Lin *et al*., 1991; Sakurai *et al*., 1990; Sugiura *et al*., 1989), bovine (Arai *et al*., 1990; Saito *et al*., 1991), and human tissues (Adachi *et al*., 1991; Cyr *et al*., 1991; Ogawa *et al*., 1991; Sakamoto *et al*., 1991). Both receptor subtypes belong to the large receptor family characterized by seven transmembrane-spanning regions which regulate distinct intracellular signalling pathways through the activation of G proteins. The two subtypes can be distinguished by different rank orders of affinity toward the three ET isopeptides. The ET_A receptor binds ET-1, ET-2 and ET-3 with decreasing affinities whereas the ET_B receptor shows a similar affinity for all three isopeptides. An additional classification of both receptor subtypes as ET_{A1}, ET_{A2}, ET_{B1}, and ET_{B2} has been proposed on the basis of their affinities for agonists or antagonists (Karaki *et al*., 1994; Sudjarwo *et al*., 1994).

The ET_A receptor mediates contraction of arterial smooth muscles, while the ET_B receptor exhibits either contractile or relaxing effects depending on the vascular bed examined (Pollock *et al*., 1995). Selective activation of ET_B receptors on endothelial cells causes relaxation of rat aorta through the release of nitric oxide (Karaki *et al*., 1993). However,

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activation of ET_B receptors on venous smooth muscles gives rise to vasoconstriction (Moreland *et al*., 1994).

ETs modulate airway activities of different species including humans (Hay *et al*., 1993; Knott *et al*., 1995). These peptides are potent constrictors of airways both *in vivo* and *in vitro* (Hay *et al*., 1993; Touvay *et al*., 1990). In guinea-pig trachea and bronchus, the contraction induced by ET-1 appears to involve a mixed population of ETRs (Hay *et al*., 1993). In addition, regional differences of ETR subtype distribution seem to exist in guinea-pig airways, with bronchus expressing mainly ET_B receptor subtypes (Hay *et al*., 1993). In guinea-pig trachea, functional (Battistini *et al*., 1993) and autoradiographic studies (Tschirhart *et al*., 1991) have shown the presence of ETRs on the epithelium. Functional data reported by Battistini *et al*. (1993) have also indicated that in guinea-pig trachea, ET-1 activates ET_A receptors on epithelial cells, inducing the release of prostanoids, while airway contraction is mediated *via* ET_B receptors probably present on smooth muscles. Recently, it has been suggested (Yoneyama *et al*., 1995b) that ETs modulate the airway tone by acting not only directly on airway smooth muscle, but also on the cholinergic nerve, to modulate acetylcholine (ACh) release.

The pathophysiological role of ETs in asthma and other respiratory disorders has received some attention (Hay *et al*., 1993). The presence of ET_B receptors in guinea-pig and human bronchus mediating smooth muscle contraction is well established (Battistini *et al*., 1994; Hay *et al*., 1993; 1998; Hay & Luttman, 1997). However, in guinea-pig this receptor population has not been characterized for its biochemical and pharmacological features. Two subtypes of ET_A and ET_B receptors seem to mediate smooth muscle contraction of rabbit trachea (Yoneyama *et al*., 1995a). Similarly, the ET_B receptor population in guinea-pig bronchus cannot be homogeneous. As guinea-pig bronchus is frequently used as an experimental model to test the activity of new anti-asthmatic drugs, characterization of its ET_B receptor population is pivotal for elucidating the pathophysiological significance of ETs in asthma. Thus, we investigated the biochemical and pharmacological properties of ETRs in guinea-pig bronchus using selective agonist and antagonist ligands.

Methods

Male Dunkin-Hartley guinea-pigs weighting 300–400 g were killed by cervical dislocation and rapidly bled. The trachea and bronchus were dissected out, placed in Krebs buffer (mM) (NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.18, KH_2PO_4 1.18, $NaHCO_3$ 25, glucose 11.5), and trimmed of connective tissue and fat. Then bronchi were excised from the trachea. Some bronchus preparations were denuded of their epithelium by gentle rubbing of the internal surface with a wet cotton swab.

Tissue preparation for binding assays

Tissue was homogenized in 20 vol of ice-cold 20 mM N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid] (HEPES)-Tris buffer, pH 7.4, containing 5 mM ethylenediaminetetracetic acid (EDTA), 0.1 mM benzamidine, 0.1 mM bacitracin, 0.1 mM phenylmethanesulphonylfluoride (PMSF), and 1 $\mu g\ ml^{-1}$ leupeptin (buffer A) using a Polytron homogenizer. The homogenate was centrifuged at $48{,}000 \times g$ for 15 min at 4°C. The resulting pellet was resuspended in 20 vol of ice-cold buffer A and homogenized. The homogenate was filtered through four layers of cheesecloth and centrifuged at $48{,}000 \times g$ for 15 min at 4°C. The membrane pellet was stored in aliquots at −80°C until the time of assay. Protein content was determined by the method of Lowry *et al*. (1951), using bovine serum albumin (BSA) as standard.

Binding assay

[^{125}I]-ET-1 binding assays were performed as described by Cody *et al*. (1995), with some modifications. Briefly, bronchus membranes (~20 μg protein) were incubated with [^{125}I]-ET-1 (20–30 pM) in 0.25 ml of 20 mM Tris-HCl buffer, pH 7.4 at 37°C, containing 2 mM EDTA, 0.1 mM bacitracin, 0.1 mM PMSF, 1 $\mu g\ ml^{-1}$ leupeptin, 5 $\mu g\ ml^{-1}$ aprotinin (buffer B) and 0.08 $mg\ ml^{-1}$ BSA for 2 h at 37°C. After incubation, reactions were stopped with 3 ml of ice-cold 50 mM Tris-HCl, pH 7.3 at 4°C, containing 0.1 mM bacitracin (buffer C). Membrane-bound radioactivity was separated from the free

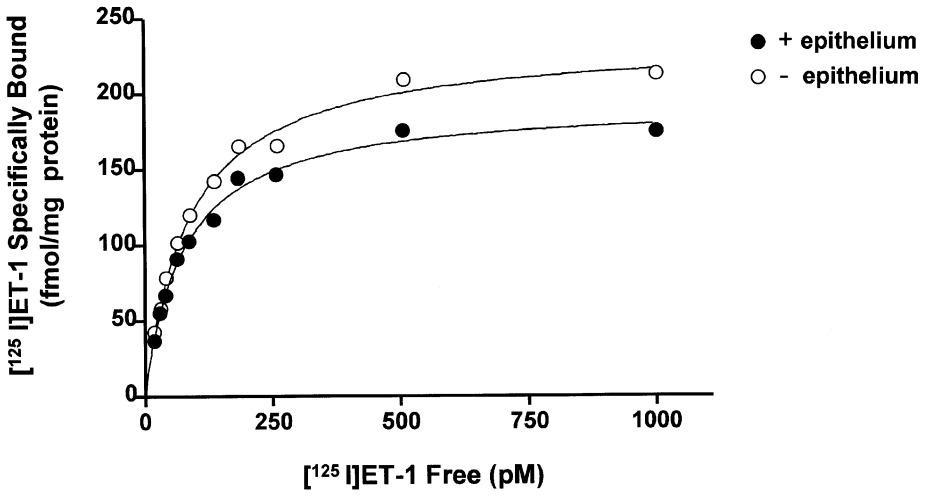

Figure 1 Equilibrium binding of [^{125}I]-ET-1 to guinea-pig bronchus membranes with and without epithelium. Saturation isotherms were obtained by transformation of dilution binding data. Membranes (20 μg of protein) were incubated with [^{125}I]-ET-1 (20 pM) in the presence or absence of increasing concentrations of unlabeled ET-1 ranging from 0.005–500 nM as described in 'Methods'. Non-specific binding was measured in the presence of 100 μM ET-1. Values shown are means from a representative experiment performed in duplicate and repeated three to four additional times with similar results. For this experiment, the K_D and B_{max} values in membranes with epithelium were 76.1 pM and 194 fmol mg^{-1} protein whereas the K_D and B_{max} values in membranes without epithelium were 87.7 pM and 236 fmol mg^{-1} protein. Curves are based on results of computer analysis with GraphPad program.

ligand by filtration through Whatman GF/C filters that had been presoaked in buffer C containing 2 mg ml^{-1} BSA. The filters were washed three times with 3 ml of buffer C and then counted in a γ-counter. Non-specific binding was defined in the presence of 100 nM ET-1.

Dilution and competition binding experiments were performed incubating membranes with [^{125}I]-ET-1 (as above) in the presence and absence of various concentrations of the indicated compounds: ET-1 (0.013–3 nM), ET-3 (0.0006–500 nM), BQ 788 (0.1–1000 nM), BQ 123 (0.25–1000 nM), and IRL 1620 (0.005–1000 nM). Stock solutions of ET-1 and ET-3 were prepared in buffer B while the other compounds were dissolved in dimethyl sulphoxide (DMSO) and then diluted in buffer B to the proper concentration. In the assay, the final concentration of DMSO never exceeded 0.2%. In some competition experiments, membranes (~25 μg protein) and [^{125}I]-ET-1 (30 pM) were incubated with 1 μM BQ 123 or IRL 1620 in 0.25 ml of buffer B containing 0.08 mg ml^{-1} BSA in the presence and absence of increasing concentrations of IRL 1620 (0.005–1000 nM), BQ 788 (0.1–1000 nM) or BQ 123 (0.25–1000 nM). All compounds were in 1% DMSO while in the assay DMSO concentration was 0.4%.

Functional assays

Bronchial specimens were cut to obtain multiple rings preparations, in accordance with the method described for aortic tissues (Calderone *et al*., 1996). At the end of the experiments, the presence or the absence of the epithelium was assessed by histological examination of the tissue.

All the bronchial preparations were placed in a 10 ml organ bath containing Krebs solution at 37°C, oxygenated (95% O_2/ 5% CO_2) and loaded with 1.5 g. The equilibration period was 1 h and washings were made every 15 min. Then the contractile responses to stimulants were recorded by an isometric transducer (7003, Basile, Varese, Italy) connected to a microdynamometer (7050, Basile, Varese, Italy). Two cumulative dose-response curves were obtained in the same preparation. The second curve performed in the presence or absence (control) of an antagonist (BQ 788 or BQ 123) was expressed in terms of percentage maximal response obtained in the first curve of the same preparation which was always carried out in the absence of any antagonist. The use of an internal control curve to IRL 1620 in the same preparation allowed us to obtain more reproducible and less dispersed experimental data than expressing them as per cent of an external standard (40 mM KCl) response (data not shown). A period of 1 h elapsed between the two curves. Both IRL1620 dose-response curves were preceded by tissue pretreatment with peptidase inhibitors (1 μM thiorfan, 1 μM bestatin and 1 μM captopril) and a cyclo-oxygenase inhibitor (10 μM indomethacin) 20 and 30 min before starting each experiment, respectively.

Antagonist activity was evaluated by adding single concentrations of each drug to the preparation 30 min before the second curve. The curves obtained in the presence of antagonists were compared with those achieved in the absence of the antagonist in different preparations from the same animal. The efficacy of BQ 788 (a selective ET_B antagonist) and BQ 123 (a selective ET_A antagonist) were expressed as pK_B values.

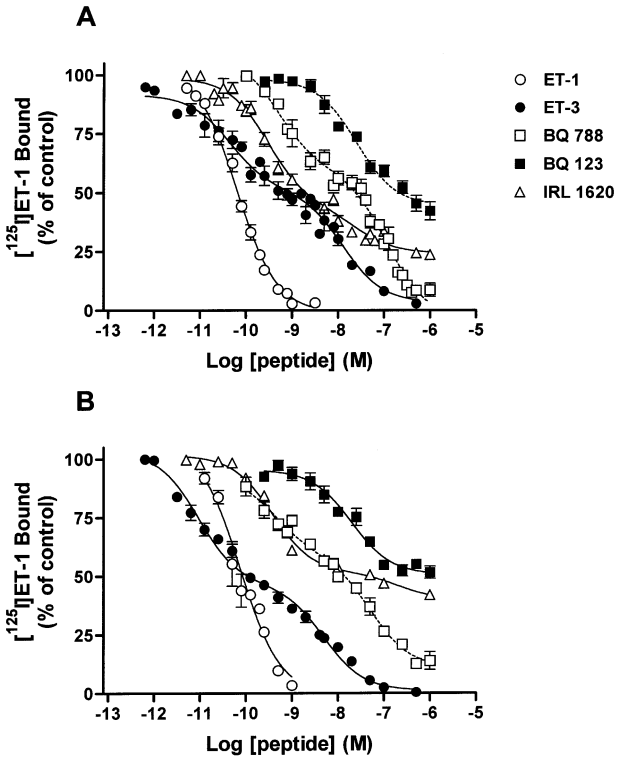

Figure 2 Competition of [^{125}I]-ET-1 binding to guinea-pig bronchus membranes with and without epithelium by unlabelled ET-1, ET-3, BQ 788, BQ 123 or IRL 1620. Intact (A) or epithelium-deprived (B) bronchus membranes (20 μg of protein) were incubated in duplicate with [^{125}I]-ET-1 (20–30 pM) in the presence and absence of increasing concentrations of ligands, as described in 'Methods'. Non-specific binding was measured in the presence of 100 nM ET-1. A non-linear regression analysis of the GraphPad computer program was used to fit the dose-response curves and derive IC_{50} values. The data points represent the means±s.e.mean of at least three independent experiments.

Table 1 Inhibition of [^{125}I]-ET-1 binding to guinea-pig bronchus membranes with and without epithelium by agonist and antagonist ligands

Ligand	K_H (nM)	R_H (%)	K_L (nM)	R_L (%)	R_N (%)
+epithelium					
ET-1	0.04±0.006	100	–	–	–
ET-3	0.02±0.004	45	8.0±0.5	55	–
IRL 1620	0.2±0.06	56	17.0±0.5	21	23
BQ 788	0.3±0.04	44	80.4±0.5	56	–
BQ 123	18.1±0.6	57	–	–	43
– epithelium					
ET-1	0.06±0.005	100	–	–	–
ET-3	0.008±0.001	55	3.8±0.6	45	–
IRL 1620	0.3±0.06	47	146.4±0.1	11	42
BQ 788	0.3±0.04	41	39.2±0.5	59	–
BQ 123	14.9±0.1	48	–	–	52

Membranes (20 μg of protein) were incubated with [^{125}I]-ET-1 (20–30 pM) in the presence and absence of increasing concentrations of ligands, as described in 'Methods'. A non-linear regression analysis of the GraphPad computer program was used to fit the dose-response curves and derive the IC_{50} values. The IC_{50} values were converted to K_i values by the Cheng & Prusoff equation (1973). K_H and K_L are the K_i values for the high- and low-affinity sites while R_H and R_L indicate their respective percentage of distribution. R_N represents the estimated percentage of binding sites which are not blocked by the competing ligand. All K_i values are expressed as means±s.e.mean of at least three experiments, each performed in duplicate.

Analysis of data

Biochemical and functional data were analysed by non-linear least squares fitting, using the GraphPad Prism Version 2.0 computer program (GraphPad Software, San Diego, CA, U.S.A.). A non-linear multipurpose curve-fitting computer program (EBDA/LIGAND, Elsevier-Biosoft) (McPherson, 1985) was used to analyse and transform dilution experiments of [^{125}I]-ET-1 with unlabelled ET-1. Displacement and dilution saturation curves were also analysed using the GraphPad Prism Version 2.0 computer program. Single- and multiple-site models were statistically compared to determine the best fit, and differences between models were tested by comparing the residual variance, using a partial F test and a significance level of $P<0.05$. The IC_{50} values obtained from the displacement curves were converted to K_i values by the Cheng & Prusoff equation (1973). Values represent the means ± s.e.mean of at least three experiments each performed in duplicate.

Functional data are expressed as mean values ± s.e.mean of 4–6 experiments. The pK_B values were calculated as −log [antagonist]/X-1, where X is the ratio of the agonist concentration required to elicit 50% of the maximal contraction in the presence of the antagonist, compared with that induced in its absence. Statistical analysis was performed by means of the Bonferroni test.

Materials

[^{125}I]-ET-1 (2000 Ci mmol^{-1}) was purchased from Amersham Corp. (Buckinghamshire, U.K.). ET-1 and ET-3 were obtained from Alexis Corp. (Läufelfingen, Switzerland) while BQ 788, BQ 123 and IRL 1620 were from Research Biochemicals Inc. (Natick, MA, U.S.A.). Bacitracin, benzamidine and PMSF were products of Fluka Chemie AG (Buchs, Switzerland). Aprotinin and leupeptin were purchased from Boehringer-Mannheim (Mannheim, Germany). Thiorfan, captopril, bestatin, indomethacin, atropine and L-NAME were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Iberiotoxin was obtained from Bachem Biochimie SARL (Voisins-Le-Bretonnex C., France). Other agents and reagents were from standard commercial sources.

Results

Binding assays

[^{125}I]-ET-1 bound to guinea-pig bronchus membranes with or without epithelium in a specific manner. Membrane protein concentrations (20–30 μg) were chosen in the linear range of the protein concentration curves. Time course experiments suggested that [^{125}I]-ET-1 binding reached equilibrium by 2 h at 37°C at the ligand and protein concentrations used (data not shown).

Dilution experiments of [^{125}I]-ET-1 with unlabelled ET-1 and transformation of data demonstrated that specific binding was saturable (Figure 1). Analysis of these

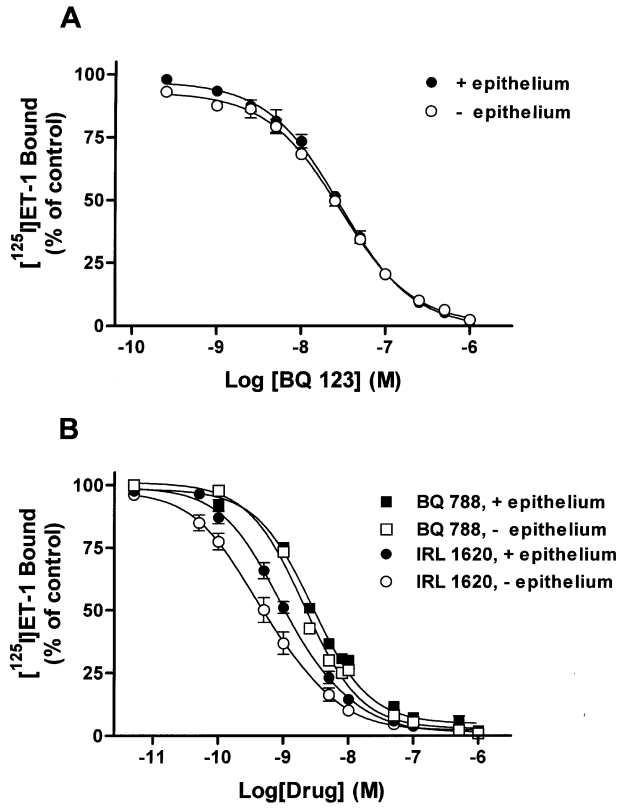

Figure 3 Competition of [^{125}I]-ET-1 binding to guinea-pig bronchus membranes with and without epithelium by BQ 123 (A), IRL 1620 (B) or BQ 788 (B) in the presence of 1 μM IRL 1620 or BQ 123, respectively. Membranes (~25 μg of protein) were incubated with 30 pM [^{125}I]-ET-1 and 1 μM IRL 1620 or BQ 123 in the presence or absence of increasing concentrations of BQ 123 or IRL 1620, as described in 'Methods'. Non-specific binding was measured in the presence of 100 nM ET-1. In the presence of 1 μM IRL 1620 specific binding in membranes with and without epithelium was 16±1± and 12±4 fmol mg^{-1} protein while in the presence of 1 μM BQ 123 specific binding was 28±2 and 27±6 fmol mg^{-1} protein, respectively. A non-linear regression analysis of the GraphPad computer program was used to fit the dose-response curves and derive IC_{50} values. The data points represent the means ± s.e.mean of at least three independent experiments.

Table 2 Effects of 1 μM BQ 123 or IRL 1620 on inhibition of [^{125}I]-ET-1 binding to guinea-pig bronchus membranes with and without epithelium by ET$_B$- or ET$_A$-selective ligands

Ligand	K_H (nM)	R_H (%)	K_L (nM)	R_L (%)
+epithelium				
IRL 1620	0.5±0.05	82	6.3±0.2	18
BQ 788	1.9±0.5	100	–	–
BQ 123	19.8±0.7	100	–	–
−epithelium				
IRL 1620	0.2±0.06	55	1.7±0.6	45
BQ 788	1.5±0.6	100	–	–
BQ 123	20.4±0.7	100	–	–

Membranes (~25 μg of protein) were incubated with [^{125}I]-ET-1 (30 pM) in the presence of 1 μM BQ 123 or IRL 1620 with and without increasing concentrations of ET$_B$- or ET$_A$-selective ligands (IRL 1620, BQ 788 or BQ 123), as described in 'Methods'. In the presence of 1 μM BQ 123, specific binding was 28±2 and 27±6 fmol mg^{-1} protein in membrane with and without epithelium while in the presence of 1 μM IRL 1620 specific binding was 16±1 and 12±4 fmol mg^{-1} protein, respectively. In the corresponding control membranes, specific binding was 43±2 and 43±7 fmol mg^{-1} protein. A non-linear regression analysis of the GraphPad computer program was used to fit the dose-response curves and derive the IC_{50} values. The IC_{50} values were converted to K_i values by the Cheng & Prusoff equation (1973). K_H and K_L are the K_i values for the high- and low-affinity sites while R_H and R_L indicate their respective percentage of distribution. All K_i values are expressed as means ± s.e.mean of at least three experiments, each performed in duplicate.

saturation data using the non-linear curve fitting techniques of EBDA/LIGAND and GraphPad Prism computer programs revealed that the best fits observed were for one-site models. In bronchus membranes with epithelium, the derived K_D and B_{max} values were 67.2 ± 8.9 pM and 195 ± 1 fmol mg^{-1} protein ($n=3$) while in membranes without epithelium, K_D and B_{max} values were 71.6 ± 6.5 pM and 124 ± 37 fmol mg^{-1} protein ($n=4$), respectively.

As expected, the displacement curves of [^{125}I]-ET-1 binding by unlabelled ET-1 in membranes with or without epithelium were monophasic (Figure 2A and B) and the best fits were for one-site models. The IC_{50} value in membranes with epithelium was 63.0 ± 9.0 pM, while in those without epithelium the IC_{50} value was 79.0 ± 7.0 pM. On the contrary, ET-3 showed biphasic inhibition of [^{125}I]-ET-1 binding to both types of membranes. Analysis of displacement curves indicated a significantly better fit for two-site than one-site models (Figure 2A and B) in both membrane preparations. In membranes with epithelium, ET-3 displaced 45% of [^{125}I]-ET-1 binding sites with high affinity and 55% with low affinity while in epithelium-deprived membranes, the high- and low-affinity sites were 55 and 45% of total binding sites, respectively (Table 1).

To further characterize ETRs in guinea-pig bronchus, inhibition of [^{125}I]-ET-1 binding by an ET_A-selective antagonist (BQ 123) and an ET_B-selective antagonist (BQ 788) or agonist (IRL 1620) were investigated. BQ 788, IRL 1620 and BQ 123 displaced [^{125}I]-ET-1 binding to membranes with and without epithelium in a concentration-dependent manner (Figure 2A and B). BQ 788 showed biphasic inhibition curves in both membrane preparations. Non-linear regression analysis of these data revealed significantly better fits for two-site than one-site models. The relative distribution between high-and low-affinity sites was similar in both membrane preparations (Table 1). The ET_B-selective agonist, IRL 1620, displaced [^{125}I]-ET-1 binding in both types of membranes with biphasic patterns but approximately 20–40% of specific binding was not inhibited even at concentrations as high as 1 μM. These competition curves were better represented by two-site than one-site models (Figure 2A and B). In membranes prepared from intact tissue, IRL 1620 displaced 56% of [^{125}I]-ET-1 binding sites with high affinity and 21% with low affinity while 23% of sites were not blocked (Table 1). In epithelium-deprived membranes, the high-and low-affinity sites were 47 and 11%, respectively while 42% of [^{125}I]-ET-1 binding sites were not inhibited (Table 1). In membranes with and without epithelium, the ET_A-selective antagonist, BQ 123, displayed monophasic competition curves (Figure 2A and B) with IC_{50} values of 25.1 ± 0.8 nM and 19.9 ± 0.8 nM, respectively. However, BQ 123 did not block 40–50% of [^{125}I]-ET-1 binding sites in both membrane preparations, even at concentrations as high as 1 μM. Table 1 summarizes the percentage distribution of binding sites and inhibition constants (K_i) for all compounds tested as competitors of [^{125}I]-ET-1 binding to bronchus membranes with and without epithelium.

Next, we attempted to identify the presence of ET_A and ET_B receptor subtypes by performing [^{125}I]-ET-1 competition binding experiments in the presence of the ET_B-selective ligand, IRL 1620 (to block all ET_B receptors) (Figure 3A), or the ET_A-selective antagonist, BQ 123 (to block all ET_A receptors) (Figure 3B). In the presence of 1 μM IRL 1620, [^{125}I]-ET-1 binding decreased by 63 and 72% compared with controls in intact and epithelium-deprived membranes, respectively. Competition curves of BQ 123, which reached complete inhibition at concentrations of 1 μM, were monophasic and better represented by one-site models (Figure 3A). The derived IC_{50} values in membranes with and without epithelium were almost identical, 28.6 ± 0.9 nM and 28.9 ± 0.9 nM, respectively. Similar displacement experiments were performed with BQ 788 or IRL 1620 in the presence of 1 μM BQ 123. Specific binding in the presence of the ET_A-selective antagonist decreased by 35 and 37% compared with controls in intact and epithelium-deprived membranes, respectively. BQ 788 inhibited [^{125}I]-ET-1 binding with monophasic patterns represented by one-site models in both tissue preparations (Figure 3B). The derived IC_{50} values in membranes with and without epithelium were very similar, 2.8 ± 0.8 nM and 2.1 ± 0.8 nM, respectively. Competition curves of IRL 1620 were monophasic but slightly shallow (Figure 3B). Specific [^{125}I]-ET-1 binding in both membrane preparations was completely inhibited by 1 μM IRL 1620. Non-linear regression analysis of competition data revealed that two-site models produced significantly better fits than did one-site models. In membrane with epithelium, IRL 1620 displaced 82% of [^{125}I]-ET-1 binding sites with high affinity and 18% with low affinity while in epithelium denuded membranes, the high- and low-affinity sites were 55 and 45%, respectively (Table 2). Table 2 summarizes the percentage distribution of binding sites and K_i values for IRL 1620, BQ 788 and BQ 123 obtained after blocking either ET_A (1 μM BQ 123) or ET_B (1 μM IRL 1620) receptors in both types of membrane preparations.

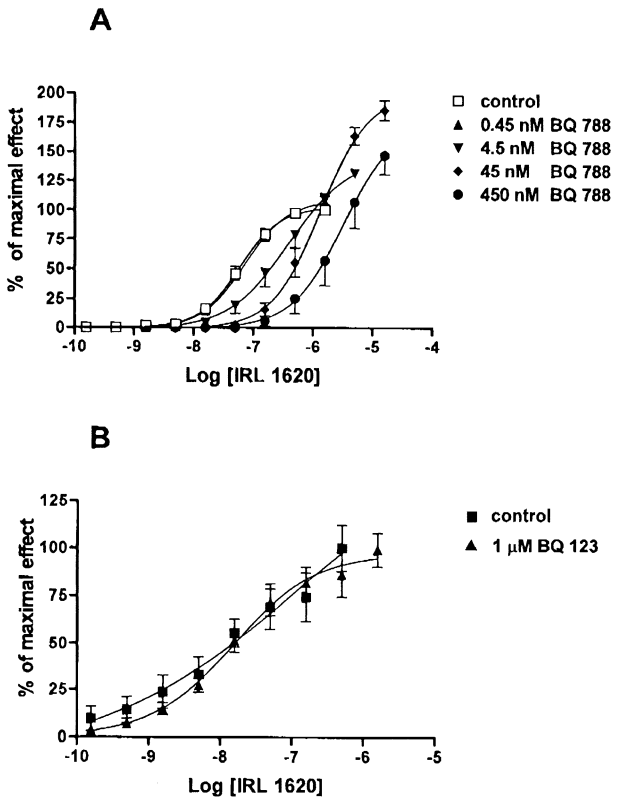

Figure 4 Contractions of guinea-pig isolated bronchus to IRL 1620 in the presence and absence of increasing concentrations of the ET_B-selective antagonist, BQ 788 (A), or the ET_A-selective antagonist, BQ 123 (B). Each point represents the mean ± s.e.mean of 4–6 experiments. Where no error bar is visible, error falls within the limits of the symbol.

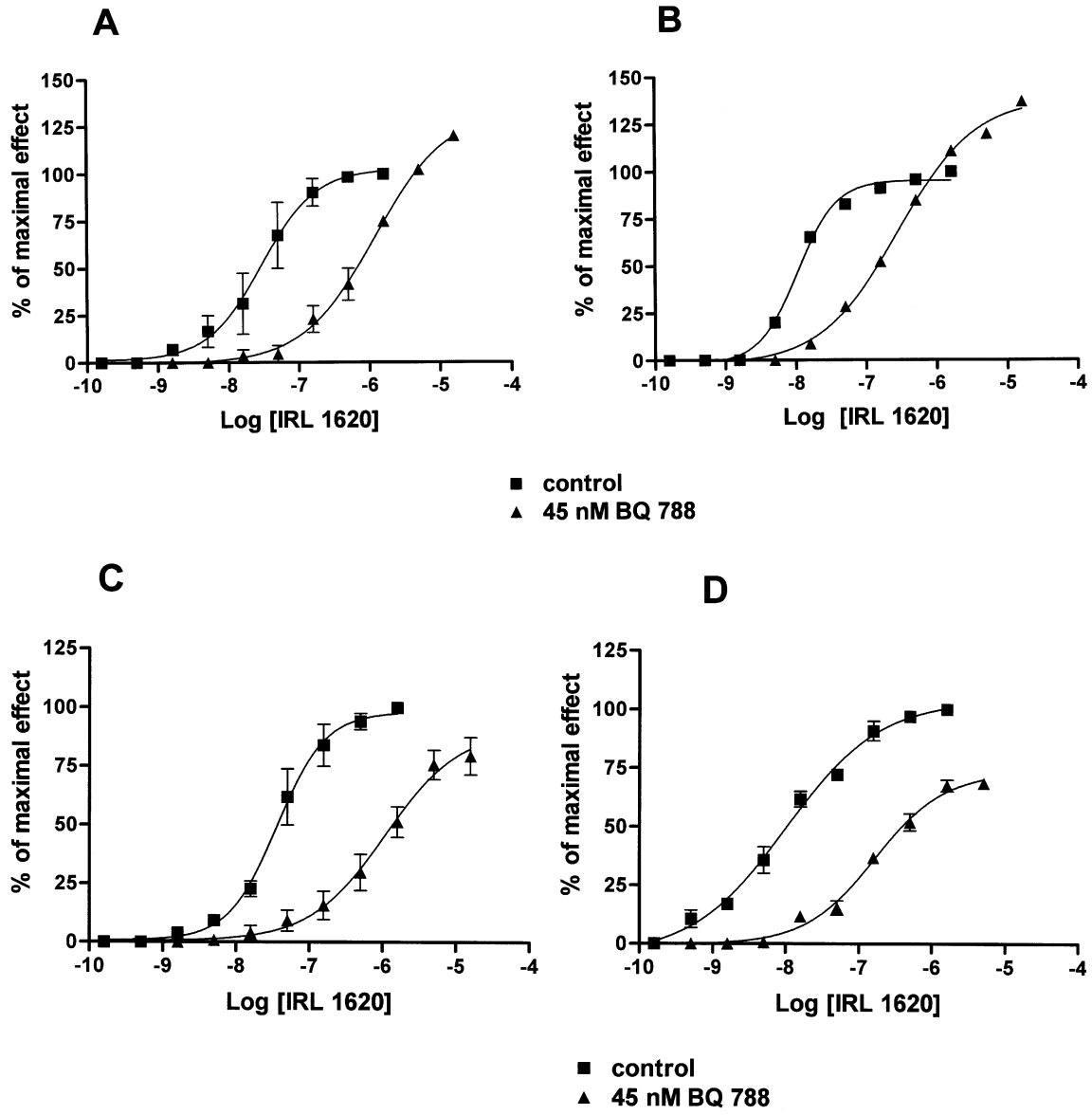

Figure 5 Contraction curves of guinea-pig isolated bronchus to IRL 1620 in the presence and absence (control) of 45 nM BQ 788. (A) Tissue was treated with various doses of the agonist in the presence and absence (control) of BQ 788 (45 nM) plus atropine (1 μM). (B and C) All dose-response curves were carried out in the presence of iberotoxin (200 nM) (B) or L-NAME (100 μM) (C). (D) Both curves were obtained using epithelium-deprived preparations. Each point represent the mean $\pm$ s.e.mean of six experiments. Where no error bars are shown, error falls within the limits of the symbol.

Functional tests

IRL 1620 developed a contracting activity in a dose-range from 0.55 nM – 1.6 μM. The concentration-response curve was sigmoidal and reached its maximum at 1.6 μM. The second curve of the agonist carried out in the same preparation was found to be slightly shifted to the right and a decrease in the maximum response was observed with respect to the first one (data not shown).

The antagonistic action of BQ 788 was assayed on the concentration-response curve to IRL 1620, since this contractile effect is generally attributed to the activation of the ET_B receptor (Battistini *et al.*, 1994). In a dose-range between 0.45 nM and 0.45 μM, BQ 788 shifted the concentration-response curve of IRL 1620 to the right in a dose-dependent fashion (Figure 4A). The pK_B values were calculated for each concentration of the antagonist and the mean value was 8.2 ± 0.4. The action of BQ 788 not only consisted in a progressive shift to the right of the IRL 1620 concentration-response curve, but a significant potentiation of the maximal response to the agonist was also observed (Figure 4A) at three different concentrations of the antagonist.

The effects of BQ 123 on the IRL 1620 concentration-response curve were tested to assess the role of ET_A receptors in the agonist stimulant action. The ET_A antagonist did not modify the IRL 1620 contracting activity, since it failed to shift the agonist curve up to 0.1 μM, or to affect the maximal response (Figure 4B).

In an attempt to clarify the mechanism of the BQ 788 potentiating effect on IRL 1620-induced contraction, we tested the effect of the ET_B antagonist in the presence of different blocking agents such as the muscarinic antagonist atropine, the NO-synthase inhibitor, L-NAME, and the K^+-channel antagonist, iberiotoxin. As shown in Figure 5, atropine and iberiotoxin failed to inhibit the potentiation of IRL 1620 activity due to 45 nM BQ 788. On the contrary, in the presence of L-NAME, no potentiation of the maximal response to IRL 1620 was observed, thus showing that the agonist activates

ET_B receptor subtypes coupled to the production and release of NO. Further experiments were carried out on bronchial specimens denuded of the epithelium, to evaluate the role of the epithelial layer as a source of NO. In epithelium-deprived preparations, BQ 788 shifted the concentration-response curve to IRL 1620 as observed in intact preparations, but no increase in the intrinsic activity was observed (Figure 5). The relative mean pK_B value was 8.4 ± 0.3, not significantly different from that obtained in intact tissues. In agreement with this evidence L-NAME pretreatment induced a potentiation of IRL 1620 mediated contraction similar to that obtained in the presence of 45 nM BQ 788. The maximal effect in the presence of L-NAME or BQ 788 (45 nM) was 138 ± 9.3 and 140.0 ± 6.3% of their respective control.

Discussion

This study demonstrates the presence of ET_A and ET_B binding sites in membranes prepared from guinea-pig bronchus. ET_B receptors are involved in modulating bronchial muscle contraction. Two functional populations of ET_B receptors with different affinities for IRL 1620 have been identified.

To investigate the presence of ETR subtypes in bronchus membranes we analysed [^{125}I]-ET-1 competition binding with unlabelled ET-1, ET-3, IRL 1620, BQ 788, and BQ 123. Competition studies with ET-1 indicate the presence of a single class of binding sites in membranes prepared from intact and epithelium-deprived bronchi. However, it is well known that ET-1 shows a similar binding affinity for both ET_A and ET_B receptors (Elshourbagy *et al*., 1993; Hori *et al*., 1992). By contrast, ET-3 displays an approximately 100 fold lower affinity for ET_A than ET_B receptors (Sakurai *et al*., 1992). Indeed, competition curves for ET-3 are clearly biphasic, indicating the presence of both receptor subtypes in guinea-pig bronchus. From these competition curves, similar amounts of these two subtypes appear to be represented. Competition experiments using ET_A- or ET_B-selective ligands confirm the presence of at least two ETR subtypes. However, the displacement curves for both an ET_B-selective antagonist, BQ 788, and an agonist, IRL 1620, are biphasic, suggesting the existence of two ET_B receptor populations with a different affinity for these ligands. Whereas BQ 788 shows a complete inhibition, IRL 1620 does not completely inhibit specific [^{125}I]-ET-1 binding, indicating a greater selectivity of this agonist for the ET_B receptor in binding assays. Removal of the airway epithelium does not modify the biphasic pattern of these competition curves. However, some variations of the inhibition constant values and percentage distributions between high- and low-affinity sites are detectable comparing the results obtained with intact and epithelium-deprived bronchus membranes. We can speculate that the presence of epithelial cells releasing ET-1 and/or a phosphoramidon-sensitive peptidase (Rubanyi & Polokoff, 1994) has some effects on [^{125}]-ET-1 binding to membranes, and thus on the displacement potency of agonist and antagonist ligands. On the other hand, various functional studies have shown that removal of the epithelium increases the responsiveness of isolated guinea-pig trachea (Hay *et al*., 1993; Tschirhart *et al*., 1991) and bronchus (Maggi *et al*., 1989) to ETs.

A further characterization of ET_B receptor populations has been obtained by performing competition experiments with either IRL 1620 or BQ 788 in the presence of a fixed concentration of BQ 123 (1 μM) to saturate ET_A receptors. In intact and epithelium-deprived bronchus membranes, BQ 788 competition curves are represented by one-site models. The derived K_i values are similar to IC_{50} values reported for BQ 788 inhibition of [^{125}I]-ET-1 binding to human Girardi heart cells and porcine cerebellar membranes (Ishikawa *et al*., 1994). Thus, BQ 788 recognizes a single population of ET_B receptors in bronchus membranes. IRL 1620 displacement curves in both types of membranes are best fitted by two-site models, supporting the presence of two populations of ET_B binding sites. Removal of the airway epithelium determines a shift to the left of the competition curve and a more interesting modification of the high- and low-affinity site distribution. The high-affinity ET_B binding sites for IRL 1620 show a relative decrease after epithelium removal. However, the K_i values for these sites are 10 – 30 fold higher than that reported for IRL 1620 inhibition of [^{125}I]-ET-1 binding to porcine lung membranes (Takai *et al*., 1992).

The component of ET_A receptors has been further characterized evaluating the effect of the ET_A-selective antagonist, BQ123, on [^{125}I]-ET-1 binding in the presence of a fixed concentration of IRL 1620 (1 μM) to saturate ET_B receptors. In all conditions, this antagonist binds to a single class of binding sites, which do not seem to be localized on the epithelial layer.

Our functional studies using IRL1620 have not shown concentration-response curves with biphasic patterns, thus suggesting that only one ET_B receptor subtype is involved in guinea-pig bronchial smooth muscle contraction. However BQ 788 determined a concentration-related shift to the right of the agonist concentration-response curve, together with a potentiating effect of the maximal response. This potentiation suggests that IRL 1620 has also a bronchial relaxing activity which is blocked by BQ 788 in a dose-dependent fashion. The results obtained with denuded preparations or L-NAME-pretreated tissues indicate that IRL 1620 activates a population of ET_B receptors localized in the epithelial layer, which acts by releasing NO. The existence of ET_B receptors which modulate synthesis and release of NO from endothelial cells has been suggested (Karaki *et al*., 1993). Moreover the relaxant activity of ET-1 on guinea-pig isolated trachea seems to be mediated by NO release from epithelial cells (Filep *et al*., 1993; Hadj-Kaddour *et al*., 1996). Thus, our data support the presence of functionally different ET_B receptors which regulate guinea-pig bronchus smooth muscle contraction. Two ET_B receptor populations with different affinities for IRL 1620 are also evident in binding assays. However, we cannot exclude the presence of ET_B receptors on other bronchus cell types.

Functional studies in guinea-pig trachea (Battistini *et al*., 1994) have suggested the presence of ET_A receptors sensitive to high doses of BQ 123 in the epithelium which are responsible for transient relaxation through the release of prostanoids. Our binding studies do not support the presence of ET_A receptors in bronchus epithelium but we cannot exclude the existence of low-affinity binding sites for this ET_A-selective antagonist. The presence of a BQ 123-insensitive ET_A receptor involved in venous and tracheal smooth muscle contraction (Sudjarwo *et al*., 1994) has been reported. In guinea-pig trachea and lung parenchyma, contraction to ET-1 has been shown to be in part mediated by ET_A receptors (Battistini *et al*., 1994), through the release of cyclo-oxygenase metabolites in the latter tissue. In our preparation, BQ 123 ineffectiveness on IRL 1620 induced contraction may be the consequence of tissue pretreatment with a cyclo-oxygenase inhibitor as indomethacin. However, pharmacological studies have also shown that BQ 123 is unable to affect the ET-1-induced contraction of guinea-pig bronchus (Battistini *et al*., 1994; Hay *et al*., 1993) but shifts the concentration-response curve of ET-1 in porcine isolated bronchus (Goldie *et al*., 1996) and human bronchi (Fukuroda

et al., 1996), suggesting that ETR subtype distribution differs among different species and portions of the respiratory system.

Yoneyama *et al*. (1995b) have proposed that in rabbit trachea, ETs modulate airway tone by acting not only directly on smooth muscles but also on the cholinergic nerve to modulate ACh release. In our functional tests, the ineffectiveness of atropine in modulating IRL 1620-mediated bronchial contraction does not support a role of ET_B receptors in ACh release from nerve terminals. However, we should consider that the lack of a basal cholinergic tone may hide an ET_B-mediated effect.

Numerous pharmacological investigations (Battistini *et al*., 1994; Hay *et al*., 1993; Hay & Luttman, 1997; Maggi *et al*., 1989) have indicated that ET_B receptors mediate contraction of ET-1 in guinea-pig bronchus. Recently, Hay *et al*. (1998) have shown that in human bronchus, ET_B receptors are mainly involved in mediating smooth muscle contraction by endothelins. Our binding and functional data confirm the presence of ET_B receptors and their major role in bronchus contraction. In addition, two ET_B receptor populations functionally involved in smooth muscle activity are detectable. The two populations differ for their affinities to an ET_B-selective agonist, have opposite functional effects and appear to be located on smooth muscle and epithelial cells.

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