Functional and Binding Characterization of Endothelin Receptors in Human Bronchus: Evidence for a Novel Endothelin B Receptor Subtype?

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ABSTRACT

Binding and functional studies were conducted to elucidate the receptor subtypes mediating contractions of human bronchus induced by endothelin (ET) receptor ligands. Binding experiments in human bronchial smooth muscle membrane preparations revealed the presence of ETA and ETB receptors in the ratio of approximately 40:60. In the presence of the combination of 1 μM BQ-123 (ETA receptor antagonist) and 1 μM S6c (ETB receptor agonist) or BQ-788 (ETB receptor antagonist) about 10 to 20% of [125I]-ET-1 binding remained. ET-1 (nonselective agonist), ET-3 (ETB receptor-prefering agonist), S6c, IRL 1620 or BQ-3020 (ETB receptor-selective agonists) potently contracted human bronchus. SB 209670 (10 μM) (ETA/ETB receptor antagonist) antagonized ET-1-induced contractions (pKb = 6.1), whereas, BQ-788 (3 μM), RES-701 (10 μM) or BQ-123 (3 μM) were without effect. The combination of BQ-788 (3 μM) and BQ-123 (3 μM) did not influence ET-1 concentration-response curves. Contractions elicited by IRL 1620 or BQ-3020, but not S6c or ET-3, were sensitive to inhibition by BQ-788 (0.03-3 μM). Based on the potent contractile effects of ETA receptor-selective agonists, and the lack of inhibitory effect of BQ-123, ET ligand-induced contractions in human bronchus appear to be mediated via ETA receptor subtype(s). However, contractions induced by ET-1, ET-3 or S6c are not sensitive to classical ETB receptor antagonists such as BQ-788. Furthermore, a residual component (about 10-20%) of the binding of radiolabeled ET agonists is resistant to various ET ligands. Collectively, these data suggest the presence of a novel ETB receptor subtype which may mediate contraction induced by some ET ligands in human bronchus.

ET-1 is a 21-amino acid peptide which was isolated and purified from porcine cultured aortic endothelial cells in 1988 by Yanagisawa et al. (1988). This novel paracrine hormone was demonstrated to be a member of a mammalian family of peptides, whose other members are ET-2 (two amino acid substitution from ET-1) and ET-3 (six amino acid substitution from ET-1) (Inoue et al., 1989; Masaki et al., 1992). Although recognized initially for its effects in the cardiovascular system, subsequent extensive research demonstrated an array of activities of ET-1. For example, a growing body of literature from in vitro and in vivo studies indicates that ET-1 produces diverse effects in the lung, many of which may be relevant to a pathophysiological role. ET-1 is released from various cells in the lung, including epithelium, endothelium and macrophages, and enhanced expression and/or levels of ET-1 has been detected in various pulmonary diseases. Accordingly, it has been speculated that ET-1 contributes significantly to the pathogenesis of several pulmonary disorders, most notably asthma and pulmonary hypertension (Hay et al., 1993a; Hay and Goldie, 1995; Michael and Markowitz, 1996).

The biological actions of the ETs are mediated via an interaction with membrane-associated receptors that belong to the superfamily of seven transmembrane spanning, G protein-coupled receptors (Masaki et al., 1992; Sakurai et al., 1992). To date, two mammalian receptors, designated ETA and ETB, have been cloned and characterized: the ETA receptor has a higher affinity for ET-1 or ET-2 compared to ET-3, and the ETB receptor has equivalent affinity for the three ET ligands (Masaki et al., 1992; Arai et al., 1990; Warner et al, 1993). The present ET receptor subtype classification may be incomplete, with functional evidence for the existence of additional ET receptors, including subtypes of ETA and ETB receptors (Sokolovsky et al., 1992; Warner et al., 1993; Bax and Saxena, 1994; Douglas et al., 1995). However, significant controversy exists in the area of ET receptor classification, and, in view of the limitations in classifying receptor families based only on functional data, expansion of

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ABBREVIATIONS: ET-1, endothelin-1; ET-3, endothelin-3; S6c, sarafotoxin S6c; ETA, endothelin A receptor subtype; ETB, endothelin B receptor subtype; pKb, -log dissociation equilibrium constant for antagonist; Kd, apparent dissociation constant for radiolabeled drug.
the present characterization of ET receptors will require additional molecular biological, operational and structural information (Kenakin et al., 1992).

Both ETA and ETB receptors are widely distributed in the mammalian lung (Hay et al., 1993a; Hay and Goldie, 1995; Michael and Markewitz, 1996). Activation of ETA receptors in human airways is responsible for ET-1-induced prostanooid release (Hay et al., 1993b) and smooth muscle proliferation (Panettieri et al., 1996), whereas stimulation of ETB receptors potentiates cholinergic nerve-induced contraction (Fernandes et al., 1996). The most recognized activity of ET-1 in human lung is potent bronchoconstrictor, which is a hallmark of asthma (Hay et al., 1993a, b, c; Hay and Goldie, 1995; Goldie et al., 1995; Michael and Markewitz, 1996). This response appears to be predominantly mediated via ETA receptor activation (Goldie et al., 1995; Hay et al., 1993c), although there may be a contribution from non-ETB receptors, including ETA receptors (Goldie et al., 1995; Fukuroda et al., 1996).

The primary aim of our study was to elucidate—from binding and functional experiments utilizing ligands selective for ETA and ETB receptors—the ET receptor subtypes responsible for contractions of human bronchus elicited by various ET agonists, including ET-1 and ET-3. The ligands used were the ETB receptor-selective agonists, S6c (Williams et al., 1991), BQ-3020 (Ihara et al., 1992), the ETB receptor-selective antagonists, BQ-788 (Ishikawa et al., 1994) and RES-701 (Tanaka et al., 1994), the combined, nonpeptide ETA and ETB receptor antagonist, SB 209670—which has high affinity for the ETA receptor and lower but significant affinity for the ETB receptor (Ohlstein et al., 1994a, b), and the ETA receptor-selective antagonist BQ-123 (Ihara et al., 1992).

**Methods**

**Tissue Preparation**

All studies were conducted using human lung tissue which was provided by the International Institute for the Advancement of Medicine (IIAM, Exton, PA) and the National Disease Research Interchange (NDRI, Philadelphia, PA). Lungs, which were obtained from organ donors who had no known history of respiratory disorders, were received within 24 hr of their removal. Bronchial tissue was removed from the lung by placing a glass probe within individual segments and dissecting away lung parenchymal, fat, connective and vascular tissues. For binding and contraction studies first and second generation and first to fifth generation bronchial tissues (approximately 4-15 mm diameter), respectively, were used; using this classification the main bronchus is regarded as the first generation airway.

**Binding Studies**

[125I]-ET-1 (specific activity of 2200 Ci/mmol), [125I]-ET-3 (specific activity of 2200 Ci/mmol), [125I]-IRL 1620 (specific activity of 2200 Ci/mmol), were obtained from New England Nuclear (Boston, MA).

**Preparation of membranes.** Human lung tissues from nine individuals were used in these studies. Bronchial strips, isolated from lung as described above, were stored at -70°C before use. Membranes were prepared by homogenizing the tissues (1 g/10 ml buffer) in buffer A [20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 0.25 M sucrose, 100 µg/ml phenylmethylsulfonylfluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin] for 5 x 15 sec, with 10-sec intervals, at a setting of 80 using a Tekmar Tissumizer (model TR-10 Polytron; Cincinnati, OH). The homogenates were centrifuged for 15 min at 4°C at 1,000 x g. The supernatants were decanted and filtered through cheesecloth and then centrifuged for 30 min at 4°C at 40,000 x g. The resulting pellets were resuspended in buffer B [50 mM Tris-HCl (pH 7.5) and 20 mM MgCl2], frozen in liquid N2 in small aliquots, and stored at -70°C until use. Protein was determined by Bradford method using Biorad reagents (Biorad Laboratories, Hercules, CA).

**Radioligand binding.** Binding of [125I]-ET-1, [125I]-ET-3 or [125I]-IRL 1620 to membranes prepared from human lung was measured in duplicate after 60-min incubation at 30°C in buffer B containing 0.05% bovine serum albumin. Membrane protein (2-6 µg/tube) was added to tubes containing either buffer (total binding), buffer plus unlabeled ET ligand (1 µM; nonspecific binding) or buffer plus competitor compound. The reactions were started by the addition of 0.05 or 0.3 nM of radioligand. After the incubation, the reactions were stopped with 3.0 ml cold buffer containing 50 mM Tris-HCl (pH 7.5) and 10 mM MgCl2. Membrane-bound radioactivity was separated from free ligand by filtering through Whatman GF/C filter paper (Clifton, NJ) presoaked in 0.1% bovine serum albumin. The filters were washed four times with 3-m1 buffer, using a Brandel cell harvester (Brandel Research and Development Laboratories, Gaithersburg, MD). Filter papers were counted in a gamma-counter (Apx 10/600 series; ICN Biomedicals, Costa Mesa, CA); with an efficiency of 75%. Saturation binding experiments were performed using increasing concentrations of the radioligand (0.03-6.0 nM) in the absence (total binding) or presence (nonspecific binding) of an appropriate unlabeled ligand (1 µM) and processed as described above. Competition binding experiments were performed using 0.05 or 0.3 nM of radioligand in the absence and presence of various concentrations of the compound under study. In a typical experiment, nonspecific binding was between 5 to 30% of total binding, depending on the radioligand used and its concentration. Time-course experiments demonstrated that, at the ligand and protein concentrations used, [125I]-ET-1, [125I]-ET-3 and [125I]-IRL 1620 binding reached steady state by 60 min at 30°C.

**Contraction Studies**

The bronchial tissues were dissected into strip preparations and then centrifuged in 10-m1 water-jacketed organ baths containing Krebs-Henseleit solution and connected via silk suture to Grass FT03C force displacement transducers (Grass Instruments Co., Quincy, MA). Mechanical responses were recorded isometrically by MP100WSAcknowledge data acquisition system (BIOPAC Systems, Goleta, CA) run on Macintosh computers. Tissues were equilibrated under approximately 2 g resting load for at least 1 hr, and washed every 15 min with fresh Krebs-Henseleit solution, before the start of each experiment. The composition of the Krebs-Henseleit solution, which was gassed with 95% O2:5% CO2 and maintained at 37°C, was (mM): NaCl 113.0, KCl 4.8, CaCl2 2.5, KH2PO4 1.2, MgSO4 1.2, NaHCO3 25.0 and glucose 5.5.

After the equilibration period, and before construction of agonist concentration-response curves, tissues were exposed to 10 µM carbachol to test for tissue viability. After plateau of this contraction, tissues were washed several times over 30 to 60 min until the tension returned to baseline levels. The preparations were then left for at least 30 min before the start of the experiment.

**Concentration-response curves.** ET-1, S6c, ET-3, BQ-3020 or IRL 1620 concentration-response curves were obtained by their cumulative addition to the organ bath in 3-fold increments. Each drug concentration was left in contact with the preparation until the response reached a plateau before addition of the subsequent agonist concentration. At the end of the experiment, tissues were exposed again to 10 µM carbachol, and agonist-induced responses in each tissue were expressed as a percentage of this reference contraction ("% postcarbachol maximal"). In experiments examining the effects of antagonists, tissues were exposed to the appropriate compound for 30 min before addition of contractile agonists. Only one agonist concentration-response curve was generated per tissue. In each tissue the response to 10 µM carbachol at the end of the experiment was larger than that obtained at the start of the study ("precarbachol
maximum). However, none of the compounds examined produced an effect on the ratio of postcarbachol maximum: pre-carbachol maximum, indicating that they do not exert a nonselective effect on smooth muscle tone (data not shown).

**Analysis of data: Binding studies.** Saturation binding experiments were analyzed by nonlinear regression analysis of untransformed data using the Lundon 1 program (Lundon Software, Inc., Cleveland, OH). Competition curves were analyzed using the Graph pad or Lundon 2 program. Single and multiple site models were statistically compared to determine the best fit, and differences among models were tested by comparing the residual variance using an F test and a significance level of P < .05. All binding experiments were conducted using membrane preparations from three individuals; the variation between different experiments was 10 to 15%.

**Analysis of data: Contraction studies.** Agonist-induced responses for each tissue were expressed as a percentage of the reference contraction (10 μM carbachol) obtained at the end of the experiment. Concentration-response curves were analyzed by nonlinear least squares regression (Ohlstein et al., 1994a) and geometric mean EC50 values (pD2 values) for agonists were calculated from linear regression analyses of data. Evidence suggests that some ET ligands do not interact with their receptors in a classical manner that will result in a reversible competitive interaction between agonist, antagonist and receptor (Marsault et al., 1991; Waggoner et al., 1992; Ohlstein et al., 1995). However, to compare the activity of compounds in this study and also with literature reports, antagonist potencies were calculated assuming a classical competitive interaction, and expressed as pK B; pKB = - log (agonist)/X-1, where X is the ratio of agonist concentration required to elicit 50% of the maximal contraction in the presence of the antagonist compared with that in its absence. Results for control- and treated-tissues were analyzed for differences in both the pD2s (-log EC50 s) and the maximal contractile responses. All data are given as the mean or mean ± S.E. mean and n represents the number of tissues studied in a particular group. Statistical analysis was conducted using analysis of variance (Fisher’s protected least square difference) or Student’s two-tailed t test for paired samples, where appropriate, with a P < .05 regarded as significant.

**Drugs.** The following drugs were used: Endothelin-1, endothelin-3, sarafotoxin S6c, IRL 1620 (Suc-[Glu9, Ala11,15]-ET-1(8-21)) and BQ-3020 (N-acetyl-[Ala11,15]-ET-1(6-21)) were purchased from Peninsula Laboratories (Belmont, CA) or American Peptide Co. (Sunnyvale, CA). Carbachol and dimethylsulfoxide were purchased from Sigma Chemical Co. (St. Louis, MO) and BQ-123 (cyclo(-b-Asp-L-Pro-D-Val-L-Leu-D-Trp-)) from American Peptide Co. SB 209670 (+)-18,2R,3S)-3-(2-carboxyethoxy-4-methoxyphenyl)-1,3,4-methylene dihydroxyphenyl)-5-(prop-1-ol)oxindane-2-carboxylic acid), and BQ-788 (N-cis,2,6-dimethylpipеридиноБэфен-1,7-метиленилактам-1-метилкарбонылтрифенил-1-норлеуцина) were synthesized by the Department of Medicinal Chemistry, SmithKline Beecham Pharmaceuticals. RES-701 (cyclo(Gly4-Asp9)Gly-Asn-Trp-His-Gly-Thr-Ala-Pro-Asp-Trp-Phe-Asn-Tyr-Tyr-Trp) was a generous gift from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). All other chemicals were of the highest grade available.

**Results**

**Binding Studies.**

The following agonists and antagonists, which are outlined below.

- **[125I]-ET-1, [125I]-ET-3 and [125I]-IRL 1620, respectively.** Subsequent studies were conducted at 30°C with an incubation time of 60 min and a membrane protein concentration of 2 to 6 μg/assay tube. GTP·S (1-100 μM) was without effect on the binding of radiolabeled ET-1, ET-3 and IRL-1620 (data not shown).

Scatchard transformations of the specific binding data from saturation binding experiments yielded a single class of high affinity binding site for all three ligands (fig. 1). The apparent dissociation constants (Kd) were 48.7 ± 9.0 pM (n = 3), 40.0 ± 3.6 pM (n = 3) and 91.0 ± 13.0 pM (n = 3), and the maximum binding sites were 1080 ± 406 fmol/mg protein, 519 ± 207 fmol/mg protein, 436 ± 191 fmol/mg protein, for [125I]-ET-1, [125I]-ET-3 and [125I]-IRL-1620, respectively (fig. 1). These data suggest the presence of both ETA and ETB receptors, as [125I]-ET-3 and [125I]-IRL-1620, which are ETB receptor-selective ligands, labeled approximately 50 and 40% of the number of binding sites for radiolabeled ET-1, which binds with equivalent affinity to both ETA and ETB receptors. This proposal was supported by the results of competition binding experiments using receptor subtype-selective agonists and antagonists, which are outlined below.

The results of experiments investigating the ability of various concentrations (1 pM-0.1 μM) of unlabeled ET-1, S6c, BQ-3020 and IRL 1620 to inhibit [125I]-ET-1 binding to human bronchial smooth muscle membranes are presented in figure 2A. Although unlabeled ET-1 displayed a monophasic competition curve with close to 100% inhibition of [125I]-ET-1 binding, the maximum inhibition observed with the ETB receptor-selective agonists, IRL 1620, BQ-3020 and S6c (all 1 μM), was 50%. These data suggest the existence of ETB and also non-ETB (probably ETA) receptors. This postulate is supported by the data from studies examining the competition curve generated using BQ-123, an ETA receptor-selective antagonist, which indicated a maximum inhibition, with a concentration of 1 μM, of approximately 40% (n = 3) (fig. 2B). Furthermore, in the same series of experiments, SB 209670, a nonpeptide, combined ETA/ETB receptor antagonist, produced a concentration-dependent competition binding of [125I]-ET-1 with a maximum inhibition of about 75%, at a concentration of 1 μM (n = 3; fig. 2B). These binding results provide evidence that human bronchus has both ETA and ETB receptors in the ratio of about 40:60.

Similar competition binding experiments were performed...
Fig. 2. Inhibition of [\(^{125}\text{I}\)]-ET-1 binding to human bronchial membranes by unlabeled (A) ET-1 ( ), S6c ( ), BQ-3020 ( ), IRL 1620 ( ) and (B) BQ-788 ( ) and BQ-123 ( ). Membranes were incubated with 0.3 nM [\(^{125}\text{I}\)]-ET-1 and increasing concentrations of unlabeled ligands for 60 min at 30°C. Bound and free ligands were separated as explained in “Methods.” [\(^{125}\text{I}\)]-ET-1 binding observed in the absence of cold competitor was normalized as 100%, and that in the presence of 1 \(\mu\)M unlabeled ET-1 was regarded as 0%, and the binding in the presence of the various concentrations of compounds studied was expressed as a percentage of these reference binding values. The data are presented as the mean ± S.E.M. of three experiments done in duplicate.

using [\(^{125}\text{I}\)]-ET-3, which preferentially binds to ET\(_B\) receptors (Huggins et al., 1993), and [\(^{125}\text{I}\)]-IRL 1620, which selectively binds to ET\(_B\) receptors (Nambi et al., 1994). Although BQ-123, in concentrations up to 1 \(\mu\)M, had no effect on [\(^{125}\text{I}\)]-ET-3 binding, BQ-3020, RES-701 and BQ-788 and SB 209670 (10 \(\mu\)M-1 \(\mu\)M) displayed monophasic competition curves (fig. 3A), indicating that [\(^{125}\text{I}\)]-ET-3 was binding to ET\(_B\) receptors in these preparations.

The competition binding data obtained from studies exploring the effects of these ligands against binding of [\(^{125}\text{I}\)]-IRL 1620 are presented in figure 3B. As expected, BQ-123 (1 \(\mu\)M-1 \(\mu\)M) did not inhibit [\(^{125}\text{I}\)]-IRL 1620 binding (fig. 3B). Although BQ-3020, RES-701, BQ-788 or SB 209670 (1 \(\mu\)M-1 \(\mu\)M) displayed comparable affinities when tested against [\(^{125}\text{I}\)]-ET-3 binding (fig. 3A), there was a significant difference in the affinities of these ligands for competition of [\(^{125}\text{I}\)]-IRL 1620 binding. Thus, the rank order potency was BQ-3020 > SB 209670 > RES-701 > BQ-788, with respective IC\(_{50}\) of 0.27, 3.3, 92 and 530 nM, respectively (fig. 3B). In addition, the competition curve for SB 209670 plateaued at 60% inhibition. Furthermore, not only SB 209670, but all the ET\(_B\) receptor-selective ligands studied, at concentrations up to 1 \(\mu\)M, also failed to inhibit completely [\(^{125}\text{I}\)]-ET-3 or [\(^{125}\text{I}\)]-IRL-1620 binding, with about 15 to 20% residual binding. These data suggest that there is a proportion of ET\(_B\) receptor binding that is insensitive to BQ-788, IRL 1620, RES-701 and BQ-3020. This latter proposal was supported by the results of studies examining the effects of combinations of ligands selective for ET\(_A\) and ET\(_B\) receptors against binding of [\(^{125}\text{I}\)]-ET-1 (fig. 4). Although BQ-123 (1 \(\mu\)M) alone blocked about 40% of [\(^{125}\text{I}\)]-ET-1 binding, and S6c or BQ-788 alone inhibited about 60 and 80%, respectively, of [\(^{125}\text{I}\)]-ET-1 binding, it was notable that the combination of BQ-123 and S6c or BQ-788 did not abolish [\(^{125}\text{I}\)]-ET-1 binding, with about 10 or 20% of [\(^{125}\text{I}\)]-ET-1 binding remaining, respectively (fig. 4A). The data obtained with [\(^{125}\text{I}\)]-ET-3 confirm that this binding site is an ET\(_B\) receptor subtype because addition of any of the ET\(_B\) receptor-selective ligands (S6c, BQ-788, IRL 1620 or BQ-3020), at a concentration of 1 \(\mu\)M, inhibited about 80-90% of the binding, with approximately 10 to 20% unaffected (fig. 4B), although BQ-123 (1 \(\mu\)M) was without significant effect on [\(^{125}\text{I}\)]-ET-3 binding (10 ± 10% inhibition; \(P > .05\)) (fig. 4B).

Contraction Studies

Contractile effects of ET-1, ET-3, S6c, IRL 1620 and BQ-3020. ET-1, ET-3, S6c, IRL 1620 or BQ-3020 potently contracted human isolated bronchus (table 1). S6c was more potent (20- to 160-fold) than ET-1, ET-3, IRL 1620 or BQ-3020 (\(P < .0001\)), and ET-1 was more potent than IRL 1620 or BQ-3020 (\(P < .05\)). Thus, ET-3 was more potent than BQ-3020 (\(P < .05\)). However, all the agonists possessed equivalent efficacies (table 1).

Effects of antagonists against ET-1-, ET-3-, S6c-, BQ-3020- or IRL 1620-induced contractions. ET-1. SB 209670 (3 \(\mu\)M) antagonized ET-1-induced contractions with a \(pK_A\) of 6.1 (\(n = 11\)) (table 2; fig. 5A). In contrast, BQ-788 (3 \(\mu\)M), RES-701 (10 \(\mu\)M) or BQ-123 (3 \(\mu\)M) did not inhibit responses induced by ET-1 (table 2; figs. 5B-D). In fact, RES-701 potentiated ET-1-induced contractions, producing a shift to the left (4.3-fold) in the agonist concentration-response curve (fig. 5C).

ET-3. The effects of the antagonists against responses elicited by ET-3 were similar to those obtained against ET-1-induced contractions. Thus, SB 209670 (3 \(\mu\)M) inhibited responses induced by lower concentrations of ET-3, although there was no significant effect on the agonist \(pD_2\) (\(P = .1\)) (fig. 6A). BQ-788 (3 \(\mu\)M), RES-701 (10 \(\mu\)M) or BQ-123 (3 \(\mu\)M) were without effect on ET-3-induced responses (table 2; figs. 6B D).

S6c, BQ-3020 or IRL 1620. The effects of the antagonists were examined against contractions produced by the ET\(_B\) receptor-selective ligands, S6c, BQ-3020 and IRL 1620. SB
ET Receptor Subtypes in Human Airways

Comparison of the potencies and efficacies of ET-1, ET-3, S6c, BQ-3020 or IRL 1620 in human bronchus

<table>
<thead>
<tr>
<th>Agonist</th>
<th>n</th>
<th>( pD_2 ) (–log M)</th>
<th>Maximum Contraction (% 10 ( \mu \text{M} ) carbachol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET-1</td>
<td>11</td>
<td>7.4 ± 0.2*</td>
<td>56.5 ± 5.5</td>
</tr>
<tr>
<td>ET-3</td>
<td>9</td>
<td>7.4 ± 0.2*</td>
<td>55.4 ± 7.1</td>
</tr>
<tr>
<td>S6c</td>
<td>13</td>
<td>8.7 ± 0.2*</td>
<td>68.2 ± 16.8</td>
</tr>
<tr>
<td>BQ-3020</td>
<td>7</td>
<td>6.5 ± 0.4</td>
<td>55.3 ± 6.7</td>
</tr>
<tr>
<td>IRL 1620</td>
<td>7</td>
<td>6.8 ± 0.1</td>
<td>64.2 ± 6.5</td>
</tr>
</tbody>
</table>

Results are presented as \( pD_2 \) (–log M) and maximum contraction (% reference contraction, 10 \( \mu \text{M} \) carbachol) and are given as mean ± S.E.M.

* Significant versus BQ-3020 or IRL 1620, \( P < .05 \).

** Significant versus BQ-3020, \( P < .05 \).

*** Significant vs. ET-1, ET-3, BQ-3020 and IRL 1620, \( P < .001 \).

209670 (3 \( \mu \text{M} \)) inhibited S6c-induced contractions with a potency \( pD_{50} = 6.4 \) similar to that for inhibition of responses elicited by ET-1 (table 2; fig. 7A). BQ-123 (3 \( \mu \text{M} \)), BQ-788 (3 \( \mu \text{M} \)) or RES-701 (10 \( \mu \text{M} \)) were without effect on S6c concentration-response curves (figs. 7B-D).

The most striking observation from this series of experiments was the sensitivity of contractions induced by IRL 1620, and to a lesser extent BQ-3020, to inhibition by BQ-788, and also SB 209670 (table 2; fig. 8). Thus, even at a low concentration of 0.03 \( \mu \text{M} \), BQ-788 or SB 209670 antagonized IRL 1620-induced contractions, producing a shift to the right in the concentration-response; there was no effect on the maximum response. Higher concentrations (0.3 or 3 \( \mu \text{M} \)) of BQ-788 or SB 209670 produced further antagonism of responses produced IRL 1620, which was reflected by a reduction in the maximum response; 3 \( \mu \text{M} \) BQ-788 or SB 209670 essentially abolished the contractions (figs. 8A, C). Similarly, BQ-788 or SB 209670, at concentrations of 0.3 or 3 \( \mu \text{M} \), produced significant antagonism of BQ-3020-induced contractions (figs. 8B, D).

RES-701 (10 \( \mu \text{M} \)) was without effect on BQ-3020- or IRL 1620-induced contractions (table 2; figs. 9A and B). BQ-123 (3 \( \mu \text{M} \)) did not influence IRL 1620 concentration-response curves (table 2; fig. 9C), but significantly inhibited BQ-3020-induced contractions (table 2; fig. 9D).

**Effects of combinations of antagonists against ET ligand-induced contractions.** As with either compound alone, the combination of BQ-788 (3 \( \mu \text{M} \)) and BQ-123 (3 \( \mu \text{M} \)) was without effect on ET-1 concentration-response curves (fig. 10A). Similarly, the combination of BQ-788 and BQ-123 did not influence contractions produced by S6c (fig. 10B).

**Discussion.**

ET-1 has been proposed to play a prominent pathophysiological role in asthma, in part because of its ability to mimic features of the disease including bronchoconstriction (Hay et al., 1993a; Hay and Goldie, 1995; Michael and Markowitz, 1996). It remains to be determined unequivocally which ET receptors mediate the activities of this mediator in human lung. In this study binding and functional experiments were performed to characterize the receptors mediating contractions of human bronchus produced by ET ligands, including ET-1. The major findings are 1) binding experiments in human bronchial smooth muscle membrane preparations revealed the presence of both ETA and ETB receptors in the ratio of about 40:60; 2) in the presence of ET ligands, selective for ETA and ETB receptors, about 10 to 20% of ETA/ETB receptor antagonist) antagonized ET-1-induced contractions, whereas, BQ-788 or RES-701 (ETB receptor antagonists) or BQ-123 (ETA receptor antagonist) were without effect. The combination of BQ-788 and BQ-123 did not influence ET-1 concentration-response curves; 4) contractions elicited by the ETB receptor-selective agonists BQ-3020 or IRL 1620, but not S6c, were sensitive to inhibition by BQ-788 but not RES-701. Collectively, these data suggest the presence of a novel ET receptor subtype which may mediate contraction induced by some ET ligands in human bronchus and also indicate that the potencies of some antagonists depend on the specific ET ligand.

The effects of the ETs are mediated via seven transmembrane spanning, G protein-coupled receptors of which two designated ETA and ETB have been cloned (Masaki et al., 1992; Sakurai et al., 1992). Both ETA and ETB receptors are found in human lung (Hay et al., 1993a; Hay and Goldie, 1995; Michael and Markowitz, 1996), where their activation produces ET-1-induced bronchoconstriction (Hay et al., 1993b) and smooth muscle proliferation (Panettieri et al., 1996), and potentiation of cholinergic nerve-induced contraction (Fernandes et al., 1996), respectively. Furthermore, ET-1 potently produces bronchoconstriction in human isolated airways (Hay et al., 1993a, b, c; Hay and Goldie, 1995; Goldie et al., 1995; Michael and Markowitz, 1996). In the initial report exploring the effects of antagonists on responses produced by ET-1 in human bronchus, based on the lack of activity of BQ-123, an ETA receptor antagonist (Ihara et al., 1992), and the potent contractile effects of S6c, an ETB receptor-selective agonist (Williams et al., 1991), it was proposed that ET-1-induced contractions were produced by stimulation of ETB receptors (Hay et al., 1993c). The results of our study provide additional support for this postulate, in that, unlike BQ-123, SB 209670, a nonpeptide antagonist for ETA and ETB receptors (Ohlstein et al., 1994a, b), inhibited ET-1-
induced contractions. However, the potency of SB 209670 (pKB = 6.1, Kₐ = 760 nM) was less than that anticipated from the results of previous binding (Kᵢ = 18 nM for inhibition of human ET₂ receptors) and functional studies (Kᵢ = 199 nM for inhibition of ET₁-induced contractions in rabbit pulmonary artery) (Ohlstein et al., 1994b). In addition, responses produced by ET-1, and also ET-3 and S6c, were not inhibited by the ETB receptor antagonists, BQ-788 (Ishikawa et al., 1994) and RES-701 (Tanaka et al., 1994). This would suggest that ET-1-induced contractions in human bronchus are mediated by stimulation of an ET₂ receptor that, unlike those in some other tissues, is insensitive to BQ-788 (Ishikawa et al., 1994) and RES-701 (Tanaka et al., 1994; Gellai et al., 1996).

Some of the functional results are at odds with those published previously by Fukuroda et al. (1996). Thus, it was reported that in human bronchus BQ-788 potently antagonized S6c-induced contractions (pKB of 7.5 with 1 μM BQ-788) whereas no effect was observed in the present study, even at a concentration of 10 μM. Furthermore, in the former study it was demonstrated that the combination of BQ-23 and BQ-788 antagonized contractions produced by ET-1; however, only minor effects (11-fold shift in the ET-1 curve) were observed with a high concentration (10 μM) of both compounds (Fukuroda et al., 1996). In both studies BQ-123 was without effect on ET-1- or S6c-induced responses and BQ-788 alone was without effect on ET-1 concentration-response curves. The reason(s) for the differences in some of the results between the two studies is unknown, but may be, at least in part, due to differences in the airway size used: 4 to 15 mm diameter in our study vs. 2 to 3 mm diameter in the investigation by Fukuroda et al. (1996). Functional and binding studies have provided evidence for regional differences in the relative distribution of ET receptor subtypes in mammalian lung (Hay and Goldie, 1995) including humans (Goldie et al., 1995; Knott et al., 1995) and guinea pigs (Hay et al., 1993c).

### TABLE 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>ET-1</th>
<th>ET-3</th>
<th>S6c</th>
<th>BQ-3020</th>
<th>IRL 1620</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB 209670</td>
<td>6.1</td>
<td>N.E.</td>
<td>6.4</td>
<td>8.0</td>
<td>8.4</td>
</tr>
<tr>
<td>(3 μM)</td>
<td>(3 μM)</td>
<td>(3 μM)</td>
<td>(3 μM)</td>
<td>(0.3 μM)</td>
<td>(0.3 μM)</td>
</tr>
<tr>
<td>BQ-788</td>
<td>N.E.</td>
<td>N.E.</td>
<td>N.E.</td>
<td>7.7</td>
<td>7.8</td>
</tr>
<tr>
<td>(3 μM)</td>
<td>(3 μM)</td>
<td>(3 μM)</td>
<td>(0.3 μM)</td>
<td>(0.3 μM)</td>
<td></td>
</tr>
<tr>
<td>RES 701 (all 10 μM)</td>
<td>L.S.</td>
<td>N.E.</td>
<td>N.E.</td>
<td>6.2</td>
<td>N.E.</td>
</tr>
</tbody>
</table>

Results are expressed as pKB; the n values and antagonist concentrations are given in parentheses. N.E., No effect. L.S., Potentiation, reflected by leftward shift in the concentration-response curve.

a Estimated pKB, as there was a marked reduction in the agonist-induced maximum response.
in tissues from asthmatic and nonasthmatic subjects are mediated by ETB receptors (insensitive to BQ-123). In addition, it was also demonstrated, from cross-desensitization studies with S6c, that a component of the ET-1-induced contraction appeared to be due to activation of a non-ETB receptor, that was also resistant to BQ-123 (Goldie et al., 1995).

Collectively, the functional data from this study as well as previous reports would suggest that responses induced by ET-1 in human airways are mediated predominantly via activation of an ETB receptor population that is not sensitive to classical ETB receptor antagonists (Goldie et al., 1995; Hay et al., 1993c), with a contribution from non-ETB receptors, the extent of which depends on airway size (Goldie et al., 1995; Fukuroda et al., 1996).

In this study although ETB receptor-selective ligands, such as BQ-3020, RES-701 and BQ-788, displayed similar affinities against [125I]-ET-3 binding, they had different affinities against [125I]-IRL 1620 binding. The reason for this difference is not known, although one postulate is that human bronchus may have two subtypes of ETB receptors, and that [125I]-ET-3 and [125I]-IRL 1620 display the same affinity for both subtypes (based on single site Scatchard plots for both ligands). The ability of BQ-788 to inhibit contractions induced by IRL 1620, but not ET-3, supports this hypothesis.

Differences were noted in the results of some binding and functional studies. For example, BQ-788 was a more potent inhibitor of the binding of [125I]-ET-3 than [125I]-IRL 1620, whereas in functional studies BQ-788 was without effect on responses produced by ET-3, but potently inhibited IRL 1620-induced contractions. Such discrepancies between the radioligand binding and functional potencies may be related to the pseudoirreversible binding nature of the binding of...
some ET ligands (Marsault et al., 1991; Waggoner et al., 1992; Ohlsten et al., 1995; Nambi and Pullen, 1995), e.g., creating nonequilibrium conditions which may affect the estimation of receptor dissociation constants. Although [125I]-ET-3 and [125I]-IRL-1620 bind to ETB receptors with high affinity, the binding characteristics of these two agonists are very different (Nambi and Pullen, 1995). Thus, although [125I]-ET-3 binding is essentially irreversible, [125I]-IRL-1620 binding is reversible. It is of note that there is a fraction of [125I]-ET-3 binding that is not sensitive to ETA receptor-selective or ETB receptor-selective ligands. Although this is a small proportion of binding sites (10-20% of total specific binding), it was a consistent finding, and was demonstrated under circumstances where all ETA and ETB receptors would have been expected to have been blocked by the combination of receptor subtype-selective ligands. An intriguing question is whether this fraction of receptors may mediate, at least in part, contraction induced by ET-1, ET-3 and S6c?

In our study it was noteworthy that the potencies of SB 209670 and BQ-788 were markedly dependent on the ET ligand under study. For example, 0.3 μM BQ-788 potently inhibited contractions elicited by IRL 1620 or BQ-3020 (pKb = 7.8 and 7.7, respectively), whereas a 10-fold higher concentration of antagonist was without effect on responses elicited by S6c and ET-3, which also produce contraction via stimulation of ETB receptors. The mechanism(s) underlying this phenomenon, which has been shown previously (Warner et al., 1993; Hay et al., 1996; Kizawa et al., 1994), is unknown but postulates include: 1) there may be two populations of ETB receptors for which different ET receptor antagonists have different affinities: one is sensitive to ET-1, ET-3 and S6c but insensitive to BQ-788, and the other is sensitive to IRL 1620, BQ-3020 and BQ-788; 2) ET-1, S6c, ET-3, IRL 1620 and BQ-3020 may interact with different binding domains within a single population of ETB receptors, and receptor antagonists, such as SB 209670 or BQ-788, may have differential affinities for these domains (Hiley et al., 1992); 3) a single population of ETB receptor may exist in different conformation states, as has been proposed previously for the angiotensin II receptor (Robertson et al., 1994): an active state coupled to contraction, and an inactive state that is not coupled to contraction. Although, based on the marked differential potencies of agonists against contractions produced by various ET ligands in human bronchus, it is tempting to postulate the existence of ETB receptor subtypes in this tissue, caution should be exerted in proposing to expand receptor classification based only on binding and functional data (Kenakin et al., 1992).

Despite inhibiting binding of [125I]-ET-1, [125I]-ET-3 and [125I]-IRL 1620, RES-701 did not antagonize responses produced by any of the ET ligands in human bronchus, and in fact potentiated contractions induced by ET-1. These data would support the proposal that RES-701 does not antagonize the contractile ETB receptor (ETB-like?) but selectively inhibits ETB-mediated vasodilation (Nambi and Pullen, 1995), which has been proposed to be mediated via activation of ETB-like receptors (Masaki et al., 1992; Sokolovsky et al., 1992; Huggins et al., 1993). The potentiating influence of RES-701 may be due to inhibition of an ET receptor that normally inhibits contraction. BQ-123 was without effect on responses produced by ET-1, ET-3, S6c or IRL 1620, but, rather surprisingly, antagonized BQ-3020-induced contractions. The reason for this is not known, but BQ-123 has been reported to inhibit some ET ligand-induced contractions that are not thought to be due to activation of classical ET receptors (Bax and Saxena, 1994).

In summary, consideration of the extensive binding and functional data from our study suggests that there are two subtypes of ETB receptors that mediate ET ligand-induced contraction in human bronchus: one that is sensitive to ET-1, ET-3 and S6c but insensitive to BQ-788, and another that is insensitive to IRL 1620, BQ-3020 and BQ-788. Of particular interest was the observation that a component (about 20%) of the binding of radiolabeled ET-1, ET-3 and IRL 1620 was resistant to displacement by various ET ligands, including various ETB receptor-selective agonists and antagonists. However, extension of the present ET receptor classification in human lung will require confirmation from additional studies, including molecular biological, structural and operational experiments.

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References