Nonpeptide Endothelin Receptor Antagonists. IX. Characterization of Endothelin Receptors in Guinea Pig Bronchus With SB 209670 and Other Endothelin Receptor Antagonists

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ABSTRACT

In this study the endothelin (ET) receptors mediating contractions produced by ET-1, ET-3 and the selective ETB ligands sarafotoxin 6c (S6c) and BQ-3020 in guinea pig bronchus were investigated using SB 209670, a nonpeptide, mixed ETA/ETB receptor antagonist, and the peptide ET receptor antagonists BQ-123 (ETA receptor-selective), BQ-788 (ETB receptor-selective) and RES-701 (ETB receptor-selective). SB 209670 (10 μM) antagonized concentration-response curves induced by ET-1 (pK_B = 6.1). In contrast, BQ-788 (10 μM) and BQ-123 (10 μM), either alone or in combination, were without significant effect on ET-1 concentration-response curves. SB 209670 (10 μM) and BQ-788 (10 μM) antagonized S6c concentration-response curves with pK_B values of 6.6 and 5.5, respectively, whereas RES-701 (10 μM) and BQ-123 (10 μM) were without effect. SB 209670 (10 μM) was about a 10-fold less potent antagonist of contractions produced by ET-3 (pK_B = 5.4) than of those elicited by S6c. BQ-788 (10 μM), RES-701 (10 μM) and BQ-123 (10 μM) were without effect on ET-3 concentration-response curves. BQ-788 (10 μM) had similar potencies for inhibition of contractions induced by S6c (pK_B = 5.8) and BQ-3020 (pK_B = 6.25). These data indicate that contractions induced by ET-1, ET-3, S6c and BQ-3020 in guinea pig bronchus appear to be mediated predominantly via stimulation of ETB receptors. However, these receptors are not very sensitive to the standard ETB receptor antagonists BQ-788 and RES-701, which suggests that responses produced by these ligands in this tissue involve activation not of the classical ETB receptor, but rather of an atypical ET receptor population. The results also provide additional evidence that the potencies of ET receptor antagonists depend upon the specific ET agonist.

ET-1, a member of a family of 21-amino acid peptides (Yanagisawa et al., 1988; Inoue et al., 1989; Masaki et al., 1992), exerts several effects in the lung, including contraction of airway smooth muscle and pulmonary vascular smooth muscle (Hay et al., 1993a; Hay and Goldie, 1995). Furthermore, increased expression and levels of ET-1 are detected in various lung diseases. On the basis of these observations, it has been proposed that ET-1 plays a significant role in the pathophysiology of pulmonary disorders, asthma in particular (Springall et al., 1991; Hay et al., 1993a; Hay and Goldie, 1995).

In the search for therapeutics for pulmonary disease that are based on antagonizing the effects of ET-1, it will be important to determine what ET receptor subtypes are responsible for the diverse effects of this mediator in the lung. Pharmacological, biochemical and molecular biological studies indicate the presence of distinct receptor subtypes (Yanagisawa and Masaki, 1989; Arai et al., 1990; Masaki et al., 1992; Sakurai et al., 1992; Hay et al., 1993a; Hay and Goldie, 1995). Several years ago, two major ET receptor subtypes were identified and characterized: an ETA receptor, which has a higher affinity for ET-1 or ET-2 than for ET-3, and an ETB receptor, which has equal affinity for the three ET ligands (Arai et al., 1990; Sakurai et al., 1990; Masaki et al., 1992). Recent research suggests the presence of additional ET receptors, including an ETB receptor (selective for ET-3) (Martin et al., 1990; Samson et al., 1990; Masaki et al., 1992; Douglas et al., 1995) and subtypes of ETA (Yonemaya et al., 1995) and ETB receptors (Sokolovsky et al., 1992; Warner et al., 1993), although considerable uncertainty exists in the area of ET receptor classification (Bax and Saxena, 1994).

In airways, both ETA and ETB receptors appear to mediate contractions produced by ET-1 and other ET ligands, and both species and regional differences are apparent in the relative contribution of the two receptor subtypes to the responses (Hay et al., 1993a; b; Henry, 1993; Battistini et al., 1994a; Goldie et al., 1994; Hay and Goldie, 1995; Yonemaya et al., 1995). In human bronchus, for example, responses to
ET-1 and S6c, an ET<sub>B</sub> receptor selective agonist (Williams et al., 1991), appear to be mediated predominantly via stimulation of ET<sub>B</sub> receptors (Hay et al., 1993b), although recent evidence suggests a contribution of ET<sub>A</sub> receptors to the ET<sub>B</sub>-induced contraction (Fukuroda et al., 1996). Similarly, several studies in guinea pig bronchus, based primarily on the activity of S6c and the effects of BQ-123, a selective ET<sub>A</sub> receptor antagonist (Ihara et al., 1992a), indicate that ET<sub>B</sub>-induced responses in this tissue involve mainly the activation of ET<sub>B</sub> receptors (Hay, 1992; Hay et al., 1993b; Battistini et al., 1994a,b; Kizawa et al., 1994). Thus, in the context of ET ligand-induced contractions, the guinea pig bronchus appears to be a useful model system of human airways.

The major goal of the present study was to gain more information, using nonpeptide and peptide receptor antagonists, on the characteristics of the ET<sub>B</sub> receptors mediating contractions elicited by the ET ligands ET-1, ET-3 and the ET<sub>B</sub> receptor selective agonists S6c (Williams et al., 1991) and BQ-3020 (Ihara et al., 1992b) in isolated guinea pig bronchus. The compounds examined were the nonpeptide, combined ET<sub>A</sub> and ET<sub>B</sub> receptor antagonist SB 209670, which has high affinity for the ET<sub>A</sub> receptor and lower but significant affinity for the ET<sub>B</sub> receptor (Ohlstein et al., 1994a,b), the peptide ET<sub>B</sub> receptor selective antagonists BQ-788 (Ishikawa et al., 1994) and RES-701 (Tanaka et al., 1994) and the peptide ET<sub>A</sub> receptor selective antagonist BQ-123 (Ihara et al., 1992a).

Materials and Methods

All experiments were performed in accordance with the guidelines of the Animal Care and Use Committee, SmithKline Beecham Pharmaceuticals.

Tissue preparation. Primary bronchi were removed from male Hartley guinea pigs (Hazelton Research Animals, Denver, PA; 450–650 g b.w.) and placed in modified Krebs-Henseleit solution. A 21-g syringe needle was inserted into the lumen of each bronchus, which was cleaned of adherent fat and connective tissue and cut into 4 to 5 rings of approximately 3 mm wide (o.d.). The epithelium was removed from each tissue by its rotation several times around the syringe needle. The individual rings from each bronchus were put randomly in the different treatment groups.

The tissues were then placed in 10-mL water-jacketed organ baths containing Krebs-Henseleit solution and connected via silk suture to Grass FT03C force-displacement transducers (Grass Instrument Co., Quincy, MA). Mechanical responses were recorded isometrically by MP100WS/Acknowledge data acquisition system (BIOPAC Systems, Goleta, CA) run on Macintosh computers. The composition of the Krebs-Henseleit solution, which was gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub> and maintained at 37°C, was as follows (mM): NaCl 113.0, KCl 4.8, CaCl<sub>2</sub> 2.5, KH<sub>2</sub>PO<sub>4</sub> 1.2, MgSO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25.0 and glucose 5.5. Tissues were equilibrated under approximately 1.5 g resting load for at least 1 hr, and washed every 15 min with fresh Krebs-Henseleit solution, before the start of each experiment.

Concentration-response curves. After the equilibration period, and before construction of agonist concentration-response curves, tissues were exposed to 10 μM carbachol. After plateau of this response, tissues were washed several times over 30 to 60 min until the tension returned to base-line level. The preparations were then left for at least 30 min before the start of the experiment. ET-1, ET-3, S6c and BQ-3020 concentration-response curves were obtained by their cumulative addition to the organ bath in 3-fold increments according to the technique of Van Rossum (1963). Each drug concentration was left in contact with the preparation until the response reached a plateau before the subsequent agonist concentration was added. At the end of the experiment, tissues were exposed again to 10 μM carbachol, which yielded the reference contraction for data analysis. The carbachol-induced contraction was larger at the end than at the beginning of the experiment. The antagonists used in this study had no effect on the ratio between the magnitudes of the carbachol-induced responses at the two different times, which indicates that they do not have nonspecific effects on the contractile response. In experiments examining the effects of antagonists, we added the compound under study to the organ bath 30 min before addition of the contractile agonist. Only one agonist concentration-response curve was generated per tissue. Experiments were conducted in the presence of 1 μM sodium meclofenamate, the cyclooxygenase inhibitor, which was added 45 min before initiation of the curves.

Analysis of data. All data are given as the mean ± S.E.M., and n represents the number of animals studied in a particular group. 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 using nonlinear least-squares regression (Ohlstein et al., 1994a), and geometric mean EC<sub>50</sub> values (pD<sub>2</sub> values) were calculated. Evidence indicates that the ET family of ligands may not interact with their receptors in a classical manner that results in a reversible competitive interaction among agonist, antagonist and receptor (Marsault et al., 1991; Waggoner et al., 1992; Ohlstein et al., 1995). However, for the purposes of comparing the activities of compounds used in this study, as well as in previous reports, we calculated antagonist potencies by assuming a classical competitive interaction and expressed them as pK<sub>IC</sub><sub>50</sub> = −log [agonist]/<sub>x</sub> − 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 (Arunlakshana and Schild, 1959). Results for control and treated-tissues were analyzed for differences in both the pD<sub>2</sub> value (−log EC<sub>50</sub>) and maximal contractile response. Statistical analysis was conducted using ANOVA (Fisher’s protected least-squares difference) or two-tailed Student’s t test for paired samples, where appropriate, with P < .05 regarded as significant.

Drugs. All drug solutions were made daily (from stock solutions or powder) and stored on ice. The following drugs were used: ET-1, ET-3, S6c, and BQ-3020 and BQ-123 (cyclo-D-Trp-D-Asp-Pro-D-Val-Leu) (American Peptide Co., Sunnyvale, CA) and carbachol and dimethylsulphoxide (Sigma Chemical Co. St. Louis, MO). SB 209670 ((+)-(1S,2R,3S)-3-(2-carboxymethoxy-4-methoxyphenyl)-1-(3,4-methyl-52-2,6-dimethylpiperidino-carbonyl-L-Tyr-Trp) and sodium meclofenamate were gifts from Warner Lambert (Ann Arbor, MI) and Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan), respectively.

Results

Contractile effects of ET-1, ET-3, S6c and BQ-3020. ET-1, ET-3, S6c and BQ-3020 produced concentration-dependent contractions of guinea pig bronchus (fig. 1). S6c was about 10-fold more potent than ET-1, ET-3 or BQ-3020 (P < .001); the latter three agonists had similar potencies (table 1; fig. 1). ET-1 produced a greater maximal response than ET-3, S6c and BQ-3020, which had equivalent efficacies (table 1; fig. 1). The effects of SB 209670 (ET<sub>A</sub>/ET<sub>B</sub> receptor antagonist), BQ-788 (ET<sub>B</sub> receptor antagonist), RES-701 (ET<sub>B</sub> receptor antagonist) and BQ-123 (ET<sub>A</sub> receptor antagonist) on contractions induced by ET-1, ET-3, S6c or BQ-3020 were examined.

Effects of antagonists alone against ET-1, ET-3, S6c and BQ-3020-induced contractions. SB 209670 (10 μM)
antagonized concentrations induced by ET-1, a result reflected by a shift to the right in the agonist concentration-response curve, with a pKB value of 6.1 (table 2; fig. 2A). In contrast, BQ-788 (10 μM) or BQ-123 (10 μM) were without significant effect on ET-1 concentration-response curves (table 2; fig. 2B and C).

SB 209670 (10 μM) antagonized S6c concentration-response curves with pKB = 6.6 (fig. 3A). SB 209670 (10 μM) also increased the maximal contraction produced by S6c; maximal contraction (% 10 μM carbachol); control = 54.3 ± 5.0; + SB 209670 = 68.8 ± 4.6, n = 7, P < .05. BQ-788 (10 μM) had a significant inhibitory effect on S6c concentration-response curves with pKB = 5.5 (table 2; fig. 3B). In contrast, both RES-701 (10 μM; fig. 3C) and BQ-123 (10 μM; fig. 3D) were without effect on responses produced by S6c.

ET-3 concentration-response curves were antagonized by SB 209670 (10 μM) with a pKB value of 5.4 (fig. 4A; table 2). BQ-788, RES-701 and BQ-123 (all 10 μM) were all without effect on ET-3-induced contractions (fig. 4 B–D; table 2).

Because the potency of ET receptor antagonists has been shown to be dependent on the ET agonist (Warner et al., 1993; Kizawa et al., 1994; Yoneyama et al., 1995), we compared the effects of BQ-788 on contractions induced by the selective ETB agonists S6c and BQ-3020. BQ-788 (10 μM) antagonized responses produced by the two ligands with similar potencies: pKB = 5.8 vs. S6c and 6.25 vs. BQ-3020 (n = 4) (fig. 5).

**Effects of combinations of antagonists against ET-1-, ET-3- and S6c-induced contractions.** In view of the demonstration of both ETA and ETB receptor populations in guinea pig airways (Hay et al., 1993b; Battistini et al., 1994a), we examined the effects of the combination of BQ-123 and BQ-788 or SB 209670 on ET-1-induced contractions.

Like either antagonist alone, the combination of BQ-788 (10 μM) and BQ-123 (10 μM) had no effect on ET-1 concentration-response curves (fig. 2B). The ability of BQ-788 (10 μM) to antagonize contractions induced by ET-3 or S6c was not influenced by BQ-123 (10 μM; data not shown). Also, SB 209670 had about the same potency against ET-1-induced contractions in the presence and in the absence of BQ-123 (10 μM) (fig. 2A). We obtained similar results when we explored the effect of the combination of SB 209670 and BQ-123 on S6c and ET-3 concentration-response curves (data not shown).

**Discussion**

The present study, utilizing various ET ligands and peptide and nonpeptide ET receptor antagonists, indicates that contractions induced by ET-1, ET-3, S6c and BQ-3020 in guinea pig bronchus are mediated via activation of ETB rather than ETA receptors. However, the limited potencies of the ET receptor antagonists studied suggests that responses produced by the ET ligands in this tissue involve stimulation of an ETB receptor population that is not sensitive to the classical ETA receptor antagonists BQ-788 and RES-701 and thus may be mediated via activation of an atypical ET receptor population. In addition, the data provide further evidence that the potencies of ET receptor antagonists depend on the specific ET agonist under study.

Significant controversy exists in the area of ET receptor classification. Only two mammalian ET receptor subtypes have been cloned to date: a receptor designated ETα, which has higher affinity for ET-1 or ET-2 than for ET-3 or S6c, and another subtype, named ETβ, which does not discriminate among ET ligands (Arai et al., 1990; Sakurai et al., 1990; Masaki et al., 1992). Nevertheless, there are several proposals, based on functional studies, for the existence of additional ET receptor subtypes, including ETA1, ETA2, ETr1 and ETβ2 (Sokolovsky et al., 1992; Warner et al., 1993; Bax and Saxena, 1994; Douglas et al., 1995; Karaki et al., 1994a; Sudjarwo et al., 1994; Yoneyama et al., 1995). A complicating factor associated with ET receptor classification is that some of the ET ligands do not appear to interact with the receptors in a classical fashion that leads to a reversible, competitive interaction among agonist, antagonist and receptor (Mar-
sault et al., 1991; Waggoner et al., 1992; Ohlstein et al., 1995). Nevertheless, the utility of several structurally diverse ET receptor antagonists, as well as further molecular biological studies, should assist in clarifying the actual number, location and function of the ET receptor subtypes.

Based on the present receptor classification, in this study the lack of effect of BQ-123, a selective ETA receptor antagonist (Ihara et al., 1992a), and the potent contractile effects of the selective ETB receptor agonists S6c (Williams et al., 1991) and BQ-3020 (Ihara et al., 1992b), concomitant with the increased potency of S6c vs. ET-1 and ET-3, confirm previous observations and conclusions that responses produced by ET ligands in guinea pig bronchus are mediated predominantly, if not exclusively, via ETB receptor activation (Hay, 1992; Hay et al., 1993b; Battistini et al., 1994a; Kizawa et al., 1994). Further characterization of the ETB receptors in this tissue was explored using the nonpeptide, nonselective receptor antagonist SB 209670, which has a high affinity for ETA (Ki = 0.2 nM vs. cloned human receptor) and also ETB (Ki = 18 nM) receptors (Ohlstein et al., 1994b), and the peptide, ETB receptor selective antagonists BQ-788 (IC50 = 12 nM for inhibition of [125I]-ET-1 binding to ETB receptors

Fig. 2. Effects of SB 209670 (10 μM, panel A) and BQ-788 (10 μM, panel B) in the absence (circles; continuous line) and presence (triangles; dashed lines) of BQ-123 (10 μM). C) Effect of BQ-123 (10 μM) alone on ET-1 concentration-response curves in guinea pig bronchus (epithelium-free). Results are expressed as a percentage of the response to carbachol (10 μM) and are given as the mean ± S.E.M. of 4 to 5 (panel A), 5 (panel B) and 10 (panel C) experiments. In panels (A and B), ○ = control; □ = + antagonist; ▽ = + BQ-123 and antagonist. In panel C, ○ = control; □ = + BQ-123. Experiments were conducted in the presence of 1 μM meclofenamic acid.

Fig. 3. Effects of SB 209670 (10 μM, panel A), BQ-788 (10 μM, panel B), RES-701 (10 μM, panel C) and BQ-123 (10 μM, panel D) on S6c concentration-response curves in guinea pig bronchus (epithelium-free). Results are expressed as a percentage of the response to carbachol (10 μM) and are given as the mean ± S.E.M. of 7 (panel A), 7 (panel B), 3 (panel C) and 7 (panel D) experiments; ○ = control; □ = + antagonist. Experiments were conducted in the presence of 1 μM meclofenamic acid.
on human Girardi heart cells, and IC$_{50}$ > 1 µM for ET receptors in human neuroblastoma cell line (Ishikawa et al., 1994) and RES-701 (IC$_{50}$ = 10 nM and 20 nM for inhibition of $^{125}$I-ET-1 binding to rabbit lung ET$_B$ and human ET$_B$ receptor, respectively) (Tanaka et al., 1994, 1995). Using these tool compounds yielded two notable findings: 1) The antagonists were much less potent than anticipated on the basis of the results of previous binding and functional studies (in many cases BQ-788 and RES-701 were without effect on ET ligand-induced contraction). 2) The antagonist potency was dependent on the specific ET ligand.

Regarding the limited antagonist potencies, given that SB 209670, BQ-788 and RES-701 have been reported to have affinities for human ET$_B$ receptors in the 10 to 20 nM range (Ishikawa et al., 1994; Ohlstein et al., 1994b; Tanaka et al., 1994), one might have anticipated reasonable functional activity (manifest by marked shifts in ET ligand concentration-response curves), especially with the high concentration tested, 10 µM. High functional potency as ET$_B$ receptor antagonists has been reported for SB 209670 ($K_p$ = 199 nM against ET-1-induced contraction in rabbit pulmonary artery) (Ohlstein et al., 1994b) and BQ-788 ($pK_p$ = 8.4 against BQ-3020-induced contraction in rabbit pulmonary artery; and a 100-fold shift to the right in the S6c concentration-response curve with 3 µM in rabbit saphenous vein) (Ishikawa et al., 1994; Karaki et al., 1994a). Nevertheless, in the present study, RES-701 was without effect on ET-3- or S6c-induced contractions in guinea pig bronchus, and BQ-788 had no effect on ET-1 or ET-3 concentration-response curves and had only a minor inhibitory effect on contractions induced by S6c ($pK_p$ = 5.5). SB 209670 was the most potent of the three compounds, inhibiting contractions induced by ET-1, ET-3 and S6c, although its potency was limited ($pK_p$ = 5.4–6.6). The differences between radioligand binding and functional potencies of the antagonists may be related to the pseudoirreversible binding nature of the ET ligands (Mar- sault et al., 1991; Waggoner et al., 1992; Ohlstein et al., 1995). For example, the slow dissociation of endothelin from its receptor may create nonequilibrium conditions that affect the estimation of receptor dissociation constants. Alternatively, the lack of effect or limited potency demonstrated in this study with BQ-788 and RES-701 suggests that ET$_B$-induced responses in this tissue are not so sensitive to these prototypical ET$_B$ receptor antagonists as reported previously in other tissues. Thus there may be differences in the contractile ET$_B$ receptors, and responses elicited by various ET ligands in guinea pig bronchus may be mediated by stimulation of an atypical ET receptor population. Another possible
explanation for the observed findings is that the ET ligand-induced contractions involve activation of multiple ET receptor subtypes, one of which is not sensitive to the antagonists used in this study. The lack of effect of the combination of BQ-788 and BQ-123 against responses suggests that the receptor subtypes are not the classical ET_A and ET_B receptors. Support of this postulate requires molecular biological and pharmacological evidence for additional ET receptor subtypes and determination of their location in guinea pig bronchus.

The dependence of the potencies of the antagonists on the specific ET ligand that was noted in the present study has been demonstrated previously (Warner et al., 1993; Kizawa et al., 1994; Ohlstein et al., 1993; Yonekura et al., 1995). For example, in guinea pig bronchus, Ro 46-2005 had no effect on responses induced by ET-1 and ET-3 but antagonized those elicited by IRL 1620 (pK_B = 6.35) (Kizawa et al., 1994). In this study, SB 209670 was about 10-fold more potent at inhibiting contractions induced by S6c than those produced by ET-3, and BQ-788 antagonized S6c- and BQ-3020-induced responses but not those elicited by ET-1 or ET-3. The explanation for this phenomenon is unclear, but it may indicate the presence of heterogeneous populations of ET receptors for which different ET receptor antagonists have different affinities. Another possibility is that a single population of ET_B receptors may exist in two different conformational states, as postulated 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. Alternatively, ET-1, S6c, ET-3 and BQ-3020 may interact with different binding domains within a single population of ET_B receptors, and receptor antagonists may have differential affinities for these domains (Hiley et al., 1992). The findings of the present study may also reflect the nonclassical interaction between some ET ligands and the ET receptors.

BQ-788 had no effect on ET-1 concentration-response curves in either the absence or the presence of the ETA receptor antagonist BQ-123. This confirms that, unlike the guinea pig trachea, where both ET_A and ET_B receptors are present (Hay et al., 1993b; Battistini et al., 1994a), the guinea pig bronchus has no functionally significant ET_B receptors. RES-701 antagonized the initial depressor response to ET-1 (ET_B1-like mediated) in rats in vivo and has been classified as an ET_B receptor antagonist (Tanaka et al., 1994; Karaki et al., 1994b). In this study, RES-701 did not antagonize responses produced by S6c or ET-3 in guinea pig bronchus. Similar findings have been observed in rabbit trachea (Yoneyama et al., 1995). These data would support the proposal that RES-701 does not antagonize the contractile ET_B receptor.

In conclusion, the present data from experiments utilizing various ET ligands and peptide and nonpeptide ET receptor antagonists indicate that contractions induced by ET-1, ET-3, S6c and BQ-3020 in guinea pig bronchus are mediated predominantly, if not exclusively, via activation of an ET_B rather than an ET_A receptor population. However, the limited potencies, and in some instances the lack of effects, of the ET receptor antagonists suggests that ET ligand-induced contractions in this tissue involve stimulation of an atypical ET_B receptor population that is not sensitive to the classical ET_B receptor antagonists, such as BQ-788 and RES-701. In addition, further evidence is provided that the potencies of the receptor antagonists depend on the ET agonist.

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References


