Typical Endothelin ETA Receptors Mediate Atypical Endothelin-1-Induced Contractions in Sheep Isolated Tracheal Smooth Muscle 1

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ABSTRACT
Contraction of vascular and nonvascular smooth muscle induced by the endothelin/sarafotoxin family of peptides frequently does not readily fit into the current classification criteria for ETA and ETB receptors, raising the possibility of additional atypical receptors. In the current study, isometric tension recording and radioligand binding techniques were used to characterize the ETA receptor population in sheep isolated tracheal smooth muscle. Endothelin-1 and sarafotoxin S6b induced similar concentration-dependent contractions, although endothelin-1 was 2.6-fold more potent (P < .05, n = 15–18). The ETA receptor-selective antagonists BQ-123 and FR139317 caused concentration-dependent inhibition of the contractions induced by endothelin-1 and sarafotoxin S6b, but both antagonists were significantly less potent in inhibiting contractions induced by endothelin-1 than sarafotoxin S6b. For example, 0.03 μM FR139317 shifted the endothelin-1 and sarafotoxin S6b concentration–effect curves to the right by 1.8- and 8.3-fold, respectively (P < .01, n = 6–8). Although the observed agonist dependence of antagonist potency may indicate the presence of atypical ETA receptors, competition binding studies using 125I-endothelin-1 and 125I-sarafotoxin S6b identified only a single population of BQ-123- and sarafotoxin S6b-sensitive ETA receptors. Additional association-, dissociation-, and saturation-binding studies revealed that 125I-endothelin-1 binding to these ETA receptors was pseudoirreversible, whereas 125I-sarafotoxin S6b binding was readily reversible. Thus, marked differences in the kinetic profiles of ETA receptor binding to endothelin-1, sarafotoxin S6b, and BQ-123, rather than the existence of another ETA receptor subtype, may explain the stark agonist dependence of antagonist potency observed in contractile studies.

Endothelin receptors, members of the rhodopsin superfamily of heptahelical receptors, mediate the wide-ranging actions of the endothelin and sarafotoxin families of peptides. Two major types of endothelin receptor, termed ETA and ETB, have been cloned, sequenced, and characterized (Masaki et al., 1994). ETA receptors exhibit a higher affinity for endothelin-1 and endothelin 2 than for endothelin 3, and they are selectively blocked by BQ-123 (Ihara et al., 1991) and FR139317 (Sogabe et al., 1993). In contrast, ETB receptors display equal affinity for each of the endothelin-1, -2, and -3 isoforms, are selectively activated by sarafotoxin S6c (Williams et al., 1991), and are selectively blocked by BQ-788 (Ishikawa et al., 1994). Binding of endothelin-1 to ETA and ETB receptors can also be inhibited by nonselective antagonists including bosentan (Clozel et al., 1993) and SB209670 (Ohlstein et al., 1994).

However, not all responses induced by endothelin-1 and related peptides can be readily classified as having been mediated via these conventional ETA and ETB receptors. For example, endothelin-1-induced contractions in some ETα receptor-containing vascular preparations are less sensitive to inhibition by BQ-123 than are contractions induced by sarafotoxin S6b or ET-3 (Bax et al., 1993; Salom et al., 1993; Clark and Pierre, 1995; Maguire et al., 1996; Devadason and Henry, 1997). One possible explanation for these findings is that endothelin-1 stimulated two types of ETA receptor, only one of which was sensitive to the actions of BQ-123, ET-3, or sarafotoxin S6b. Consistent with this, Maguire and coworkers (1996) reported that endothelin-1 bound to a larger population of binding sites than did sarafotoxin S6b, although competition binding studies failed to identify two subtypes of ETA receptor. Recent studies using rat renal artery also failed to identify atypical ETA receptors in competition binding studies despite strong evidence from functional contractile studies of atypical ETA receptor-mediated responses (Devadason and Henry, 1997). Thus, although functional studies have provided considerable data on the existence of atypical ETA receptor-mediated responses (i.e., agonist dependence of antagonist potency), evidence from radioligand-binding stud-

ABBREVIATION: ET, endothelin.
ies supporting the existence of atypical ET_\text{A} receptors has been less forthcoming.

The existence of atypical ET_\text{A} receptor-mediated responses does not seem to be unique to vascular ET_\text{A} receptors, having been reported in studies using smooth muscle preparations from the rat vas deferens (Eglezos et al., 1993) and rabbit iris (Ishikawa et al., 1996). However, it is not known whether atypical ET_\text{A} receptors or atypical ET_\text{A} receptor-mediated responses exist in airway smooth muscle. This is particularly relevant in light of a recent study by Hay et al. (1998), which reported that contractions induced by endothelin-1, endothelin-3, or sarafotoxin S6c in the ET_\text{B} receptor-containing human isolated bronchus were not sensitive to classical ET_\text{B} receptor antagonists such as BQ-788, and which suggested the presence of a novel ET_\text{B} receptor subtype. Thus, the aim of the current study was to characterize the ET_\text{A} receptor population on airway smooth muscle using isometric tension recording and radioligand-binding techniques and to investigate the possibility that atypical ET_\text{A} receptor-induced responses were mediated via typical ET_\text{A} receptors but reflect kinetic differences in the binding characteristics of the ligands to the ET_\text{A} receptor. These studies were performed on sheep isolated tracheal smooth muscle, a preparation that contains high densities of ET_\text{A} receptors (Goldie et al., 1994).

### Experimental Procedures

**Materials.** ^125I^-Endothelin-1 (2000 Ci/mmol), ^125I^-sarafotoxin S6b (2000 Ci/mmol), endothelin-1, sarafotoxin S6b, sarafotoxin S6c, BQ-123 (cyclo[D-Trp-D-Asp-l-Pro-l-Val-l-Leu]), (Auspep, Melbourne, Australia), phenylmethylsulfonyl fluoride (Calbiochem, La Jolla, CA), and phosphoramidon, indomethacin, and BSA (Sigma Chemical Company, St. Louis, MO) were used. FR139317 was a generous gift from Fujisawa Pharmaceutical Company (Osaka, Japan). Endothelin-1, sarafotoxin S6b, and sarafotoxin S6c were prepared as 50 μM stock solutions in 0.1 M acetic acid and stored at −20°C. Dilutions were made in 0.9% NaCl and kept on ice.

**Preparation of Sheep Isolated Tracheal Smooth Muscle.** Tracheal tissue was obtained from 6-month-old lambs that had been sacrificed with a bolt to the head (Murdoch Veterinary School, Perth, Australia). The trachea was placed in chilled Krebs' bicarbonate solution and cut into rings. The tracheal smooth muscle band was isolated from associated connective tissue and epithelium under a dissecting microscope. Krebs' bicarbonate solution (pH 7.4) contained: 117 mM NaCl, 5.36 mM KCl, 25 mM NaHCO_3, 1.03 mM KH_2PO_4, 7 mM MgSO_4, 0.57 mM H_2O_2, 2.5 mM CaCl_2, 11.1 mM glucose, and 0.0025 mM indomethacin.

**Functional Studies.** Strips of tracheal smooth muscle, approximately 2 mm wide by 4 mm in length, were suspended in 3-ml organ baths containing Krebs' bicarbonate solution at 37°C and bubbled with 5% CO_2 in O_2. Preparations were washed twice at 15-min intervals during a 40-min equilibration period and then exposed to KCl solution at a final bath concentration of 40 mM. Following a further 15-min washout period, all preparations were incubated with a leukotriene receptor antagonist SKF 104353 (1 μM) and with BQ-123 (0.1, 0.5, or 3 μM), FR139317 (0.05 or 0.3 μM), or vehicle (control) for another 20 min. Cumulative concentration-response curves to either ET-1 or Stx-S6b were then constructed using 0.5-log concentration increments. Only one concentration-effect curve was constructed in each preparation.

**Radioligand-Binding Studies.** Frozen blocks were prepared by placing a stack of six tracheal smooth muscle bands (each 1-mm thick and 7-mm square) in Macrodex (6% dextran 70, in 5% glucose solution) and freezing in isopentane cooled by liquid nitrogen. Sections (10-μm thick) were cut on a cryostat at −20°C and mounted on gelatin chrom-alum-coated glass slides. Each slide contained three sections from different blocks (animals) and were stored at −85°C before use. Thawed slide-mounted sections were washed twice for 10 min in binding medium containing 50 mM Tris-HCl, 100 mM NaCl, 0.25% BSA, and 10 μM phenylmethylsulfonyl fluoride at pH 7.4. Typically, tissue sections were incubated with ^125I^-endothelin-1 or ^125I^-sarafotoxin S6b at 22°C in binding medium containing 100 nM sarafotoxin S6c (to block ET_\text{B} receptor binding) for a designated incubation time, washed twice for 15 min in binding medium, and wiped from the slide with glass fiber filter paper; radioactivity was then measured using a gamma counter. Levels of nonspecific binding were determined by incubating with 1 μM BQ-123 and separately with 30 nM endothelin-1. The levels of nonspecific binding obtained with these two methods were not significantly different in any experiment.

In association-binding experiments, slide-mounted tissue sections were incubated with 0.03 nM, 0.2 nM, or 1.0 nM radiolabeled ^125I^-endothelin-1 or 0.2 nM ^125I^-sarafotoxin S6b for 10, 20, 40, 80, 120, and 240 min before washing, wiping, and counting as described above. In dissociation-binding studies, slide-mounted tissue sections were incubated with 0.2 nM ^125I^-endothelin-1 or ^125I^-sarafotoxin S6b for 3 h, washed twice, and then incubated with binding medium containing 30 nM endothelin-1 and 100 nM sarafotoxin S6c to prevent rebinding of the radiolabel during the dissociation phase. At intervals of 0.5, 1, 2, 3, 4, and 5 h, groups of total and nonspecific slides were washed, wiped, and measured for radioactivity. In saturation-binding studies, tissue sections were incubated for 3 h in solutions of 0.15 nM ^125I^-endothelin-1 or ^125I^-sarafotoxin S6b containing endothelin-1 (0.01 nM to 3 μM, 0.5 log concentration increments), BQ-123 (0.03 nM to 3 μM, 0.5 log concentration increments), or sarafotoxin S6b (0.03 nM to 3 μM, 0.5 log concentration increments).

**Data and Statistical Analyses.** In functional studies, contractile responses to endothelin-1 and sarafotoxin S6b were expressed as a percentage of the response induced by 40 mM KCl (100%). Grouped responses were expressed as the arithmetic mean ± S.E.M. The concentration of endothelin-1 or sarafotoxin S6b that produced 40% KCl response (EC_{40}) was estimated from linear interpolation of individual concentration-effect curves and used as an estimate of agonist potency. The 40% level of response (rather than the more conventional 50% level) was selected post hoc because it permitted the calculation of concentration ratios for the lowest concentrations of BQ-123 and FR139317 against the agonist sarafotoxin S6b. The geometric mean of EC_{40} values (and associated 95% confidence limits) was calculated for each agonist in preparations from n animals. Concentration ratios were calculated as the EC_{40} in the presence of antagonist/EC_{40} (in the absence of antagonist). Differences between concentration-ratio values were determined from Student's t test analysis of log (EC_{40}) data.

In radioligand-binding studies, mean total binding and mean nonspecific binding was each determined from triplicate slide radioactivity measurements and levels of specific binding calculated from the difference between mean total binding and mean nonspecific binding. The analysis of association-, dissociation-, and competition-binding data for ^125I^-endothelin-1 and ^125I^-sarafotoxin S6b has been described in detail elsewhere (Devadason and Henry, 1997). Briefly, association-binding data for ^125I^-endothelin-1 was fitted to an equation that describes pseudoinversible binding as follows:

\[
RL_i = B_{\text{max}}(1 - e^{-k_iL_i}),
\]

where RL_i is the amount of ligand bound to a saturable binding site at time i, B_{\text{max}} is the total receptor concentration, L_i is the concen-
tion of free ligand, and \( k_1 \) is the association rate constant (Waggoner et al., 1992). On the other hand, association-binding data for \(^{125}\text{I}-\text{sarafotoxin S6b} \) was fitted to the following equation describing a simple model of reversible bimolecular association:

\[
RL_E = RL_G(1 - e^{-k_{obs}}),
\]

where \( RL_G \) is the bound receptor concentration at equilibrium and \( k_{obs} \) is the pseudo-first order association rate constant (McPherson, 1985). \( RL_G \) and \( k_{obs} \) can be further defined as follows:

\[
RL_G = \frac{B_{max}}{1 + (K_D \cdot L)}
\]

\[
k_{obs} = k_1 \cdot L + k_{-1},
\]

where \( k_{-1} \) is the rate constant for dissociation and \( K_D \) is the equilibrium dissociation constant. \(^{125}\text{I}-\text{sarafotoxin S6b} \) saturation-binding data were fitted to eq. 3, and \( B_{max} \) and \( K_D \) were estimated using nonlinear least-squares regression analysis.

Competition-binding data were fitted to a one-site model based on this logistic equation:

\[
\% \text{Specific Binding} = \frac{100}{1 + (x/IC_{50})^n}
\]

where \( x \) is the concentration of the competing ligand, \( IC_{50} \) is the concentration of competing ligand at which 50% of the specific binding was inhibited, and \( n \) is the slope of the line tangent to \( IC_{50} \). The equation for a two-site model was as follows:

\[
\% \text{Specific Binding} = \frac{100}{1 + (x/IC_{50H})^n} + \frac{100}{1 + (x/IC_{50L})^m}
\]

where \( p \) and \( 1 - p \) are the fractions of the binding attributed to the higher and lower affinity sites, respectively. \( IC_{50H} \) and \( IC_{50L} \) are the \( IC_{50} \) values estimated for the high and low affinity sites, and \( n \) and \( m \) are the slopes of these curves. The F ratio test was used to determine whether the binding data were better described by the two-part model than the one-part model. A P value less than or equal to 0.05 was considered to be statistically significant.

Results

Functional Studies. Endothelin-1 and sarafotoxin S6b induced concentration-dependent contractions of sheep tracheal smooth muscle (Figs. 1 and 2). The mean concentrations of endothelin-1 and sarafotoxin S6b that produced 40% of the contraction induced by 40 mM KCl were 13.1 nM (95% CLs, 11.7–14.8 nM, \( n = 15 \)) and 34.0 nM (31.5–37 nM, \( n = 18 \)), respectively. Thus, endothelin-1 was on average 2.6-fold more potent than sarafotoxin S6b. Both endothelin-1- and sarafotoxin S6b-induced contractions were inhibited by the ET\(_A\) receptor antagonists BQ-123 (Fig. 1) and FR139317 (Fig. 2) in a concentration-dependent manner. However, when determined at the 40% response level, both antagonists were significantly more potent at inhibiting contractions induced by sarafotoxin S6b than by endothelin-1 (Figs. 1 and 2). For example, 0.1 \( \mu \)M BQ-123 shifted the concentration-effect curve to sarafotoxin S6b by 2.6-fold (95% CLs, 1.1–6.2-fold, \( n = 6 \)) but caused no significant shift of the curve to endothelin-1 (1.05-fold; 95% CLs, 0.62–1.8-fold, \( n = 7 \), \( P < .05 \)). Similarly, when determined at the 40% response level, 0.3 \( \mu \)M FR139317 shifted the concentration-effect curve to sarafotoxin S6b by 8.3-fold (95% CLs, 2.9–24-fold, \( n = 8 \)) and to endothelin-1 by only 1.8-fold (95% CLs, 1.1–2.9-fold, \( n = 8 \); \( P < .01 \)).

Radioligand-Binding Studies. Specific binding of \(^{125}\text{I}-\text{endothelin-1} \) to sheep tracheal smooth muscle sections increased in a time-dependent manner (Fig. 3A). The rate of association of specific \(^{125}\text{I}-\text{endothelin-1} \) binding was concentration-dependent, with the higher concentration binding reaching a plateau level of binding more quickly (Fig. 3A). The plateau levels of specific binding obtained at 3 h with 0.2 and 1.0 nM \(^{125}\text{I}-\text{endothelin-1} \) were not significantly different and were not significantly different from the levels obtained with 0.5 nM \(^{125}\text{I}-\text{endothelin-1} \) (100%, data not shown). The levels of specific \(^{125}\text{I}-\text{endothelin-1} \) binding to sheep isolated tracheal smooth muscle sections did not decrease significantly during a 5-h period following the replacement of unbound \(^{125}\text{I}-\text{endothelin-1} \) with an excess of unlabeled endothelin-1 (Fig. 3B). In competition-binding experiments, the binding of \(^{125}\text{I}-\text{endothelin-1} \) was concentration-dependently inhibited by endothelin-1, BQ-123, and sarafotoxin S6b (Fig. 3C). Each of the competing ligands produced a similar extent of inhibition of \(^{125}\text{I}-\text{endothelin-1} \) binding, and each of the competition binding curves was best fitted to a one-site model.

Specific \(^{125}\text{I}-\text{sarafotoxin S6b} \) binding to sheep isolated tracheal smooth muscle sections was time-dependent (Fig. 4A). Moreover, specific \(^{125}\text{I}-\text{sarafotoxin S6b} \) binding was reversible, with only 20% of \(^{125}\text{I}-\text{sarafotoxin S6b} \) binding remaining after a 5-h washout period (Fig. 4B). In saturation-binding studies, the number of specific \(^{125}\text{I}-\text{sarafotoxin S6b} \)-binding
sites was not significantly different from the number of specific binding sites identified using 0.5 nM 125I-endothelin-1 (Fig. 4C). In competition-binding experiments, endothelin-1, BQ-123, and sarafotoxin S6b inhibited 125I-sarafotoxin S6b binding, and the competition-binding curves were well described using a one-site model (Fig. 4D).

Discussion

The principle aim of this study was to pharmacologically characterize the population of ET<sub>A</sub> receptors that mediate endothelin-1-induced contraction in sheep isolated tracheal smooth muscle. In isometric tension-recording studies, the ET<sub>A</sub> receptor antagonists BQ-123 and FR139317 were significantly more potent in inhibiting contractions induced by sarafotoxin S6b than endothelin-1. Although agonist dependence of antagonist potency may be indicative of the existence of multiple ET<sub>A</sub> receptor subtypes, subsequent radioligand binding experiments failed to identify more than a homogeneous population of ET<sub>A</sub> receptors to which endothelin-1, sarafotoxin S6b, and BQ-123 each bound. Additional kinetic studies suggest that this apparent paradox may be explained by consideration of the markedly different kinetic binding profiles of these ligands.

Previous studies have demonstrated that endothelin-1-induced contractions in sheep isolated tracheal smooth muscle were mediated via stimulation of ET<sub>A</sub> receptors, a conclusion based on the findings that endothelin-1-induced contractions were inhibited by an ET<sub>A</sub> receptor antagonist BQ-123 and that the ET<sub>B</sub> receptor-selective agonists sarafotoxin S6c and BQ3020 were inactive as spasmogens (Goldie et al., 1994). The current study has extended these findings by investigating the influence of the ET<sub>A</sub> receptor-selective antagonists BQ-123 and FR139317 on contractions induced by endothelin-1 and sarafotoxin S6b. Of particular interest was the finding that, when determined at the 40% response level, both antagonists were significantly more potent at inhibiting contractions induced by sarafotoxin S6b than by endothel-
lin-1. One possible explanation for the agonist dependence of antagonist potency is the existence of multiple ET<sub>A</sub> receptors. For example, if endothelin-1 could stimulate an atypical ET<sub>A</sub> receptor subtype, in addition to the typical ET<sub>A</sub> receptor stimulated by sarafotoxin S6b and blocked by BQ-123 and FR139317, then it might be expected to be less effectively inhibited by the antagonists than sarafotoxin S6b. Similar mechanisms have been proposed to explain this phenomenon in vascular (Bax et al., 1993; Salom et al., 1993; Clark and Pierre, 1995; Maguire et al., 1996) and nonvascular preparations (Eglezos et al., 1993; Ishikawa et al., 1996). Subtypes of ET<sub>A</sub> receptors, designated ET<sub>A1</sub> (BQ-123 sensitive) and ET<sub>A2</sub> (BQ-123 insensitive), have been proposed to mediate rabbit isolated tracheal smooth muscle contraction (Yoneyama et al., 1995), although these studies were complicated by the existence of a large functional population of ET<sub>B</sub> receptors.

Radioligand-binding studies were used to determine whether endothelin-1 bound to a population of atypical ET<sub>A</sub> receptors in addition to the typical ET<sub>A</sub> receptors bound by BQ-123 or sarafotoxin S6b. If so, 125I-endothelin-1 would be expected to have bound to a significantly larger population of ET<sub>A</sub>-binding sites (typical and atypical ET<sub>A</sub> receptors) than the number of binding sites identified by 125I-sarafotoxin S6b (typical ET<sub>A</sub> receptors only). However, the estimated 3<sub>max</sub> values for 125I-endothelin-1 and 125I-sarafotoxin S6b were not significantly different; furthermore, in competition-binding studies both BQ-123 and sarafotoxin S6b were able to fully compete for specific 125I-endothelin-1-binding sites. Specific 125I-sarafotoxin S6b binding was also monophasically and completely inhibited by increasing concentrations of endothelin-1, sarafotoxin S6b, and BQ-123. Together, these findings suggest that binding of endothelin-1, BQ-123, and sarafotoxin S6b was to a single common site. An important assumption made in these studies is that 125I-endothelin-1 will label all existing ET<sub>A</sub> subtypes and not exclusively a subtype to which BQ-123 and sarafotoxin S6b preferentially bind. At present, we cannot exclude the possibility that the low concentrations of 125I-endothelin-1 used in these binding studies have failed to label a lower affinity ET<sub>A</sub> receptor subtype (atypical receptor) that mediates contraction induced by higher concentrations of endothelin-1 but not sarafotoxin S6b. However, all endothelin receptor investigators to date have exhibited very high affinity for endothelin-1, and it is thus unlikely that 125I-endothelin-1 has failed to label all existing endothelin receptors in the current study. On the balance of evidence, it is improbable that the existence of an atypical ET<sub>A</sub> receptor subtype has contributed significantly to the atypical ET<sub>A</sub> receptor-mediated responses observed in sheep isolated tracheal smooth muscle. These findings concur with several recent studies in rat isolated renal artery (Devadason and Henry, 1997) and rabbit iris (Nosaka et al., 1998).

In the absence of any supporting evidence for the existence of atypical ET<sub>A</sub> receptors in sheep isolated tracheal smooth muscle, alternative mechanisms need to be considered to explain the agonist dependence of antagonist potency. In the current study, specific 125I-endothelin-1 binding to ET<sub>A</sub> receptors was essentially irreversible, consistent with many previously published findings (Hemsen et al., 1991; Waggner et al., 1992; Ihara et al., 1992, 1995; Devadason and Henry, 1997). In contrast, the binding of 125I-sarafotoxin S6b to ET<sub>A</sub> receptors was readily reversible, with about 80% of specific binding having dissociated within 5 h. A similar rate of reversibility for 125I-sarafotoxin S6b binding from ET<sub>A</sub> receptors was observed in rat renal artery (Devadason and Henry, 1997). The binding of BQ-123 is also rapidly reversible (Ihara et al., 1995). Thus, it is possible that these marked
differences in binding characteristics may explain, at least in part, the appearance of atypical responses (i.e., agonist dependence of antagonist potency) despite the presence of typical receptors. It is conceivable that differences in the rates of dissociation of endothelin-1 and sarafotoxin S6b from the ET_A receptor contribute significantly to the potency of BQ-123 in inhibiting contractile responses. Indeed, if endothelin-1 does not dissociate from its receptor, then the ability of a reversibly binding antagonist such as BQ-123 to compete for the receptor will be severely reduced. In support of this postulate, the ability of BQ-123 to inhibit endothelin-1 binding was found to decrease significantly with longer periods of coincubation (Wu-Wong et al., 1994a,b; 1995), presumably because of the greater reversibility of antagonist binding than endothelin-1 binding. Furthermore, Gresser et al. (1996) reported that the agonist dependence of BQ-123 potency is an intrinsic property of ET_A receptors, rather than indicative of the presence of atypical receptors.

In conclusion, radioligand-binding studies have demonstrated significant differences in the binding characteristics of 125I-endothelin-1 and 125I-sarafotoxin S6b to a single subtype of ET_A receptor in sheep isolated tracheal smooth muscle. Thus, the observed greater potency of BQ-123 for inhibiting contractile responses to sarafotoxin S6b than endothelin-1 may be due to the significantly greater reversibility of ET_A receptor binding to sarafotoxin S6b and BQ-123 than to endothelin-1, rather than to the presence of multiple ET_A receptor subtypes.

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References


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