Mutational Analysis of the Endothelin Type A Receptor (ET_A): Interactions and Model of the Selective ET_A Antagonist BMS-182874 with the Putative ET_A Receptor Binding Cavity

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ABSTRACT: Endothelin (ET) receptor antagonism is a potential therapeutic intervention in the treatment of vascular diseases. To elucidate the mechanism of antagonist−ET receptor complex formation, the interactions of four chemically distinct antagonists were investigated using a combination of genetic and biochemical approaches. By site-specific mutagenesis we previously demonstrated that Tyr129 in the second transmembrane domain was critical for high-affinity, subtype-selective binding to the A subtype of ET (ET_A) receptors [Krystek et al. (1994) *J. Biol. Chem. 269*, 12383−12386]. Affinities of the constrained cyclic pentapeptide BQ-123, the pyrimidinylbenzenesulfonamide bosentan, the indancarboxylic acid SB 209670, and the naphthalenesulfonamide BMS-182874 were decreased 20−1000-fold in Tyr129Ala, Tyr129Ser, and Tyr129His ET_A receptor mutants. Substitution of Tyr129 with Phe or Trp did not alter the high-affinity binding of BQ-123, bosentan, or SB 209670. BMS-182874 binding affinity was decreased 10-fold in Tyr129Phe and Tyr129Trp ET receptors. These data indicate a role of aromatic interactions in the binding of these antagonists to ET_A receptors and, in the case of BMS-182874, also suggested a hydrogen bond with the tyrosine hydroxyl. This hypothesis was supported by structure−activity data with analogs of BMS-182874 that varied the C-5 dimethylamino substituent on the naphthalene ring. Mutation of Asp126 and Asp133 also altered binding of BMS-182874 and C-5 analogs. In all cases, naphthalenesulfonamide binding was more severely affected by mutation of Asp133 than by mutation of Asp126. Phosphoinositide hydrolysis and extracellular acidification rate studies demonstrated the importance of Tyr129 to ET_A-mediated signal transduction. On the basis of these data, two plausible models of the docked conformation of BMS-182874 in the ET_A receptor are proposed as a starting point for further delineation of interactions that underlie antagonist−ET_A receptor complex formation.

Endothelin (ET)[1] receptors are members of the family of heterotrimeric guanine nucleotide protein-coupled (GPC) proteins, the hallmark of which is the presence of seven hydrophobic membrane-spanning regions thought to be configured as α-helices (Baldwin, 1993). Our understanding of the structure of the ligand binding sites of GPC receptors in general, and of the ET receptors in particular, is limited by the relative absence of structural data available on these proteins. To date, bacteriorhodopsin, which is not GPC and does not have significant sequence homology to any GPC receptor, is the only membrane-associated protein with seven hydrophobic transmembrane (TM) regions for which there are three-dimensional structural data. Hence, bacteriorhodopsin has been used extensively as the scaffold from which to derive computer models of membrane-associated proteins thought to be helical bundles. On the basis of crystals obtained from purified protein, a 9 Å projection map of bovine rhodopsin was consistent with this GPC light-sensory receptor protein having seven TM helices oriented almost perpendicular to the membrane in a formation similar to that of bacteriorhodopsin (Schertler et al., 1993). Thus, until structural data for mammalian GPC receptors is obtained, bacteriorhodopsin appears to be an appropriate template on which to base model-building efforts aimed at gaining insight to the three-dimensional nature of these important signal proteins. In the absence of structural data on ET receptors, we (Krystek et al., 1994) and others (Lee et al., 1994) have built computer-generated, three-dimensional models of the ET receptors to guide receptor mutagenesis and elucidate the nature of ligand−receptor interactions.

Due to the diverse biological functions of ETs and their potential role in numerous pathobiological situations [see Sakurai and Goto (1993) and Takuwa (1993) for reviews], antagonism of the ET receptors has been viewed as a site of pharmacological intervention. To date, only two mammalian ET receptor subtypes, designated ET_A and ET_B, have been cloned (Arai et al., 1990; Sakurai et al., 1990). The human

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[1] Abbreviations: ET_A, endothelin A receptor; ET, endothelin; GPC, guanine nucleotide protein coupled; TM, transmembrane; PMSF, phenylmethanesulfonyl fluoride; LiCl, lithium chloride; EDTA, ethylenediaminetetraacetic acid; IP, inositol phosphate; DMEM, Dulbecco's modified Eagle's media; EC_{50}, concentration for 50% effect; IC_{50}, concentration for 50% inhibitory effect; QSAR, quantitative structure−activity relationship; E_{max}, maximal effect.

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ET_A and ET_B receptor subtypes are 59% homologous over the entire coding sequences, but this degree of identity is increased to 70% in the TM regions. The primary sequences of the native 21 amino acid ET isopeptides are also conserved with ET-2 and ET-3 diverging from ET-1 in only two and six residues, respectively (ET-2, Trp6 and Leu7 for Leu6 and Met7; ET-3, Thr2, Phe4, Thr5, Tyr6, Lys7, and Tyr14 for Ser2, Ser4, Ser5, Leu6, Met7, and Phe14).

In contrast to the conserved sequences of agonist peptides, several chemically distinct antagonists of ET_A receptors have been identified. The first potent ET receptor antagonists described were the ET_A receptor-selective pentapeptide BQ-123 (Ihara et al., 1991) and acyltripeptide FR 139317 (Sogabe et al., 1993). Several nonpeptide inhibitors of ET receptor binding have since been described. These include a methanol extract of bayberry *Myrica cerifera* (Fujimoto et al., 1992; Mihara et al., 1994) and asterric acid from the culture filtrate of *Aspergillus* sp. (Ohashi et al., 1992). More recently, BMS-182874 (Stein et al., 1994; Webb et al., 1994) and PD 156707 (Doherty et al., 1995) were reported to be ≈1000-fold ET_A-selective. SB 209670, an indancarboxylic acid (Elliott et al., 1994), has 100-fold selectivity for the ET_A subtype. In contrast, Ro 46-2005 (Clozel et al., 1993) and Ro 47-0203 (Clozel et al., 1994), *N*-pyrimidinylbenzenesulfonamides, bind ET_A and ET_B receptors with approximately equivalent affinity and thus are nonselective antagonists of ET. These compounds are useful tools for dissecting interactions that underlie formation of ligand−ET receptor complexes.

In the absence of structural data on ET receptors, a combination of molecular modeling, mutagenesis, and biochemical techniques have been used to identify receptor amino acid residues involved in ligand binding. Chemical modification of Tyr with tetranitromethane (Bousso et al., 1992) or His with diethyl pyrocarbonate (Huggins et al., 1993) has led to the suggestion that these residues participate in forming the ET receptor−ligand binding site. However, such studies are limited by the specificity of the chemical modification reagent and by the inability to precisely localize the modified amino acid. ET receptor chimeras employing a domain switching approach have identified regions spanning TM domains 1 through 3 and TM7 as containing the necessary determinants for ET_A-selective binding and TM domains 4 through 6 for ET_B-selective binding (Sakamoto et al., 1993). Site-specific mutation of Lys140 in the first extracellular loop region of ET_A receptors to Ile resulted in a mutant protein with 10-fold decreased affinity for ET-1 (Adachi et al., 1994). Most recently, Krystek et al. (1994) and Lee et al. (1994) demonstrated that mutation of Tyr129 in the second TM region of ET_A receptors to Ala improved the binding affinity for ET-3 and sarafotoxin S6c by 1−2 orders of magnitude without affecting the affinity for ET-1 or ET-2. These data clearly demonstrated that Tyr129 is critically involved in subtype-selective binding observed for the ET isopeptide agonists.

Less is known of antagonist−ET_A receptor interactions. Adachi et al. (1992) demonstrated that substitution of the second extracellular loop of the ET_A receptor for that of the ET_B receptor resulted in a chimera which retained high affinity for BQ-123. These data are consistent with those of Sakamoto et al. (1993), who demonstrated that TM regions 1−3 and 7 were involved in BQ-123 binding. Subsequently, Krystek et al. (1994) and Lee et al. (1994) reported that Tyr129 in the second TM domain was essential for high-affinity BQ-123 binding to the ET_A receptor subtype. In addition, Krystek et al. (1994) reported that Tyr129 was important for BMS-182874 binding, but not for Ro 46-2005, suggesting that the binding sites for certain peptidic and nonpeptidic antagonists overlap.

Elucidation of interactions underlying formation of specific antagonist−ET_A receptor complexes would aid in a general understanding of the mechanism of action of these potential therapeutically beneficial agents and would facilitate rational design of future small molecule antagonists. In the present study, we demonstrate that four chemically distinct antagonists are largely dependent on aromatic interactions with Tyr129 for maintenance of high-affinity binding to ET_A receptors. Further, we propose models of the ET_A receptor-selective antagonist, BMS-182874, in the putative binding cavity.

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression of ET_A Receptors. Substitutions of the Tyr129 in the second transmembrane region of the wild-type human ET_A receptor were constructed as previously described (Deng & Nickoloff, 1992; Krystek et al., 1994). Mutant receptor DNAs were identified by restriction analysis, and the accuracy of mutation was subsequently verified by sequencing the entire coding region for the ET_A receptor according to standard procedures (Sanger et al., 1977). Wild-type and mutant receptor cDNAs were subcloned into the mammalian expression vector pCDM8 for expression in COS-7 cells. Receptor cDNAs were transfected into COS-7 cells using the polycationic lipid lipofectamine (Life Technologies, Grand Island, NY). Cells were harvested for 48−72 h after transfection in buffer A [Dulbecco's modified Eagle's media (DMEM) containing 20 mM Hepes, pH 7.4, at 37 °C, 0.1 mM PMSF (phenylmethanesulfonyl fluoride), 10 μg/mL soybean trypsin inhibitor], polytron homogenized, and centrifuged at 100000*g* for 1 h at 4 °C. The supernatant was discarded, and the membrane pellet was resuspended in buffer A. Membranes were homogenized and stored in aliquots at −80 °C until used in binding assays. Transfection efficiency, as measured by expression of βgal DNA, varied from 5% to 20%, resulting in corresponding ET_A receptor densities from ≈50 to 200 fmol/mg of protein. This level of receptor expression did not affect binding affinity (data not shown).

Radioligand Binding Assays. [^{125}I]ET-1 (2200 Ci/mmol; NEN, Boston, MA) competition binding assays were conducted as described (Webb et al., 1993). COS-7 cell membranes (5−10 μg of protein) were incubated with 30−50 pM [^{125}I]ET-1 in the presence of increasing concentrations of competitor for 2 h at 37 °C. Nonspecific binding was defined in the presence of 100 nM ET-1. Data were analyzed by iterative curve fitting to a one-site model.

Measurement of Phosphoinositide Turnover. Phosphoinositide turnover was assessed as previously described (Webb et al., 1995). Briefly, cells were cultured in six-well plates and labeled with [^{3}H]myoinositol (8 μCi/mL) for 48 h. Cell monolayers were incubated for 15−30 min in Ham's F12 media containing 10−20 mM LiCl and 1 mg/mL albumin. The cells were then treated with ET-1 for 15 min and the media removed. Boiling EDTA (2 mM) was added to the cell monolayer to disrupt cell integrity and release soluble

inositol phosphate (IP). The solution was applied to a Dowex AG 1-X8 anion-exchange column, and total IP was collected as described by Berridge (1983). Eluant was counted in a Packard Tri-Carb 4640 (Downers Grove, IL) scintillation counter at 40% efficiency.

Measurement of Cell Activation Using the Cytosensor Microphysiometer. The Cytosensor microphysiometer (Molecular Devices, Sunnyvale, CA) measures extracellular acidification rates of cells with silicon pH-sensitive sensors (Owicki et al., 1990). Upon activation of cells with agonists, the extracellular acidification rate increases in a time- and concentration-dependent manner. Cells were serum-starved (16 h) and perfused with DMEM without $NaHCO_3$ and serum. Cells were allowed to stabilize in the Cytosensor chambers for 30−40 min until steady baselines were established. Acidification rates were measured using 90 s cycles (60 s on, 30 s off) and rates plotted continuously against time. Antagonists were incubated with cells for 15 min prior to stimulation with ET-1. ET-1 applications were made for 1 min, and EC_{50} values were calculated using peak height of stimulation after drug application.

Model Building. Individual amino acids corresponding to the transmembrane regions of the ET_A receptor were identified as previously described (Krystek et al., 1994). These sequences were threaded through seven α-helices with idealized ϕ/φ values arranged in an elliptical helix bundle corresponding to the helices in bacteriorhodopsin and consistent with the projection map of bovine rhodopsin (Schertler et al., 1993). The bias imposed by use of bacteriorhodopsin helices for GPC receptor models is removed as the idealized helices do not contain kinks caused by prolines and other sequence-specific effects. Amino acid side chains were generated using the rotamer library of Ponders and Richards (1987) through an iterative placement protocol as implemented in InsightII (Biosym Technologies, San Diego, CA). The geometry of the complete model was further optimized by energy minimization.

Ligand/Receptor Docking. The docking used was a Monte Carlo rigid body docking of the ligand into the rigid minimized receptor model. All docked orientations showed the naphthyl ring of BMS-182874 and side chain of Tyr129 to be consistent with aromatic interactions (Burley & Petsko, 1985). In addition, the docked orientations were evaluated on the basis of the hypothesis that the dimethylamine nitrogen of BMS-182874 hydrogen bonded with the phenolic OH of Tyr129 and that the sulfonamide group of BMS-182874 hydrogen bonded to either the COO^- of Asp133 or the ϵ-amino of Lys166. Orientations that did not fulfill these hypotheses were rejected. The cavity in the receptor model corresponding to the putative binding site was selected by visual inspection of the model. The conformation of BMS-182874 was derived from three-dimensional quantitative structure−activity relationship (QSAR) studies which provided a low-energy, biologically active conformation for this ligand (Krystek et al., 1995). The ligand was placed in several orientations within the binding site. Docking of the ligand into the minimized ET_A receptor model was then conducted using the following process for each initial orientation:

1. Grid-based energy evaluation of the ligand orientation within the receptor using van der Waals potentials (InsightII, Biosym Technologies, Inc.)
2. Random rotation or translation of ligand (by 10° or 0.5 Å)
3. New ligand orientation assessed on the basis of energy evaluation
4. Evaluation of the hydrogen-bonding hypothesis of the dimethyamine N and sulfonamide group; reject orientations which do not meet criteria
5. If the energy is less than the previous orientation, keep the new orientation
6. Repeat

The sampling of ligand orientations was conducted for 1000−10000 iterations for each starting orientation. The resulting ligand−receptor complexes were then subjected to minimization using the full Biosym force field with flexible receptor and ligand.

RESULTS

Binding of Agonists to Tyr129 ET_A Receptor Mutants. The effect of substitution at Tyr129 on binding of ET isopeptides to wild-type and mutant ET_A receptors was examined. Site-specific replacement of Tyr129 by Trp, Phe, Lys, Ile, Thr, Ser, or Ala resulted in a panel of ET_A mutants that differed in bulk, charge, and hydrophobicity from the wild-type ET_A. Specific [^{125}I]ET-1 (50 pM) binding, defined in the presence of 100 nM unlabeled ET-1, to membrane fractions of transiently transfected COS-7 cells expressing each Tyr129 variant was 75−90%, whereas no specific binding was observed in mock transfected cells (data not shown). Competition binding analysis demonstrated that the IC_{50} values for ET-1 to Tyr129 ET_A variants were similar to wild-type affinity with the exception of the Tyr129Ile and Tyr129Thr mutants (Table 1). In these cases, the affinity for ET-1 was 8−10-fold decreased. Binding affinity of ET-3 for all of the Tyr129 ET_A mutants was improved >10-fold with the exception of ET-3 binding to Tyr129Trp which showed a 7-fold improvement. Thus, the affinity of ET-3 for ET_A receptors improved when the residue at position 129 was not a hydroxyl-containing aromatic residue or a large aromatic residue. The finding that the affinity for ET_B-selective ligands is consistently improved even in Tyr129 variants that have reduced affinity for ET-1 suggests that it is unlikely that the structure of these altered ET_A receptors is grossly different from the wild-type receptor.

Binding of Antagonists to Tyr129 ET_A Receptor Mutants. To elucidate the interactions of antagonists with the ET_A receptor, binding of four chemically and pharmacologically distinct compounds to Tyr129 variants of the ET_A receptor subtype was examined. BQ-123 is an ET_A-selective constrained pentapeptide (Ihara et al., 1991), BMS-182874 is an ET_A-selective naphthalenesulfonamide (Stein et al., 1994), bosentan is a benzenesulfonamide that binds ET_A and ET_B receptors with approximately equal affinity (Clozel et al., 1994), and SB 209670 is an indancarboxylic acid with high affinity for both ET_A and ET_B receptors (Elliott et al., 1994) (Figure 1).

The effect of substitution at Tyr129 on binding of these antagonists to wild-type and mutant ET_A receptors was examined. With respect to BQ-123, substitution of Tyr with charged, neutral hydrophilic, or neutral hydrophobic amino acids caused a 100−2000-fold loss in affinity from that for the wild-type ET_A receptor. High-affinity BQ-123 binding was maintained by replacement of the native Tyr with Phe

Table 1: Inhibition of [^{125}I]ET-1 Binding (50 pM) to Tyr129 ET_A Receptor Mutants[a]

agent	wild-type ET_A	Tyr129Phe ET_A	Tyr129Trp ET_A	Tyr129Lys ET_A
ET-1	0.4 ± 0.2	0.6 ± 0.0	0.4 ± 0.1	0.2 ± 0.1
ET-3	820 ± 260	**8 ± 1**	120 ± 40	**5 ± 1**
BQ-123	40 ± 6	30 ± 1	185 ± 40	**>50000**
BMS-182874	260 ± 60	**3200 ± 1260**	**3400 ± 395**	**>50000**
SB 209670	1 ± 0.2	2 ± 0.2	2 ± 0.1	**25** ± 1
bosentan	8 ± 3	60 ± 10	**80 ± 10**	35 ± 9

agent	Tyr129Ile ET_A	Tyr129Thr ET_A	Tyr129Ser ET_A	Tyr129Ala ET_A
ET-1	3 ± 1	4 ± 0.4	0.8 ± 0.1	0.6 ± 0.2
ET-3	**1 ± 0.1**	**4 ± 0.4**	**0.6 ± 0.2**	**4 ± 1**
BQ-123	**25000 ± 1400**	**10000 ± 340**	**>10000**	**>50000**
BMS-182874	**20000 ± 2600**	**>50000**	**>50000**	**>50000**
SB 209670	**10 ± 1**	**15 ± 5**	**26 ± 7**	**20 ± 4**
bosentan	**225 ± 5**	**310 ± 20**	**245 ± 25**	**190 ± 15**

[a] Binding was conducted in membranes from COS-7 cells transiently transfected with wild-type or mutant receptor cDNAs. IC_{50} values in nanomolar are the means ± SEM from three to eight competition curves. Bold typeface denotes ≥10-fold change in affinity from wild-type ET_A binding.

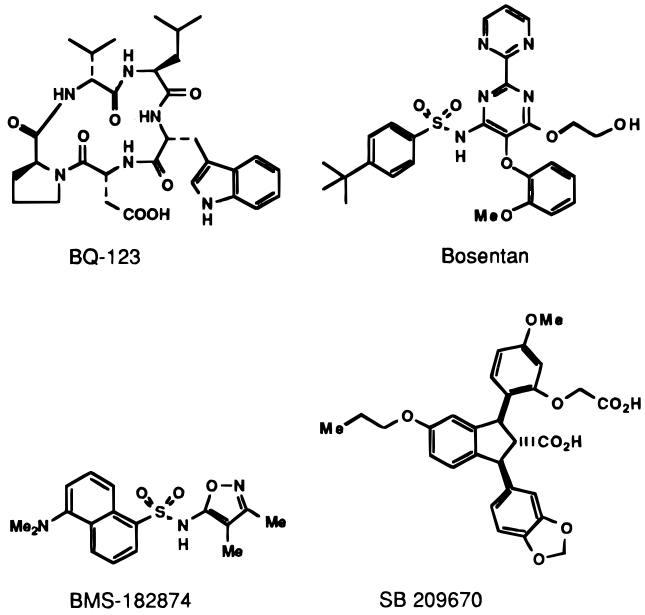

FIGURE 1: Structures of BQ-123, bosentan, BMS-182874, and SB 209670.

or Trp (Table 1). These data are consistent with the interpretation that high-affinity BQ-123 binding is in part dependent on aromatic interactions with Tyr129 in the second transmembrane region of ET_A.

Binding of three nonpeptidic antagonists to this series of ET_A receptor tyrosine variants suggests a similar involvement of aromatic interactions as those observed with the peptidic antagonist BQ-123 (Table 1). Binding affinities of bosentan and SB 209670 were not greatly altered from wild-type when Tyr129 was replaced with Phe or Trp. In contrast, the affinity of BMS-182874 for all Tyr129 receptor mutants was decreased (Table 1). However, substitution with either Phe or Trp attenuated this reduction in affinity, a result consistent with previous observations concerning aromatic involvement between ET_A antagonists and residue 129 of the ET_A receptor. These data show that Phe and Trp were the preferred substitutes for Tyr for the four peptide or nonpeptide antagonists examined. The relationship between the presence of an aromatic group at position 129 of ET_A and antagonist binding affinity emphasizes the importance of aromatic interactions between these four antagonists and ET_A receptors.

A second observation from the BMS-182874 binding profile to Tyr129 ET_A receptor variants was the ≈10-fold loss in affinity to Tyr129Phe and Tyr129Trp. Although substitution with these aromatic amino acids reduced the effect on BMS-182874 affinity compared to that observed for the other ET_A receptor mutants (Table 1), Tyr was preferred at this position. These results are consistent with the suggestion that, in addition to aromatic interactions, the phenolic hydroxyl group of Tyr129 also plays a role in the formation of a BMS-182874−ET_A receptor complex, possibly through formation of a hydrogen bond. On the basis of the relationship between $\Delta G°$ and binding affinity ($\Delta G° = -RT \ln K_{eq}$), a 10-fold loss in affinity is equivalent to ≈1.4 kcal/mol of energy, consistent with the average free energy in the formation or loss of a hydrogen bond.

Binding of Analogs of BMS-182874 to Tyr129 Mutants. The observation that the phenolic hydroxyl group contributes to the BMS-182874 binding affinity for ET_A receptors raised the question of which pharmacophore on this naphthalenesulfonamide was interacting with Tyr129. Structure−activity relationships from this series of naphthalenesulfonamides indicated that the C-5 dimethylamino group in BMS-182874 is responsible for a 10−100-fold increase in binding affinity for ET_A over the unsubstituted naphthalenesulfonamide (BMS-183085) (Stein et al., 1994). We hypothesized that a hydrogen bond between the Tyr hydroxyl and the C-5 dimethylamino of BMS-182874 contributed to the improved affinity. To test this hypothesis, binding of a series of naphthalenesulfonamide analogs varying at C-5 to native or mutant ET_A receptors was examined. The affinities of these analogs for Tyr129Phe and Tyr129Ala mutants were decreased 3−650-fold relative to wild-type receptor (Table 2). The affinity of BMS-182542, which has a primary amine at C-5, was reduced 3.5-fold compared to the Phe mutant and 20-fold compared to the Ala-substituted mutant. In comparison, binding of BMS-182874, containing the more basic C-5 dimethylamino, was much more affected by mutation of Tyr129 to Phe or Ala, consistent with the notion that the more basic substituent leads to the formation of a stronger hydrogen bond. IC_{50} values were increased ≈10-fold and

Table 2: Structure−Activity Relationship of BMS-182874 and Analogs at Wild-Type Tyr129 ET_A Receptor and Tyr129Phe and Tyr129Ala ET_A Receptor Mutants[a]

substituent	wild-type ET_A	Tyr129Phe ET_A	Tyr129Ala ET_A
HR	9.5 ± 1.3	2.6 ± 0.4	167.8 ± 26.7
(BMS-183085)	(1×)	**(0.3×)**	(18×)
H_2NR	1.1 ± 0.3	3.9 ± 0.5	21.4 ± 1.4
(BMS-182542)	(1×)	(4×)	(20×)
Me_2NR	0.3 ± 0.1	3.2 ± 1.3	67.3 ± 8.8
(BMS-182874)	(1×)	(10×)	(225×)
	0.1 ± 0.04	0.5 ± 0.2	22.8 ± 2.4
(BMS-185511)	(1×)	(5×)	(230×)
	0.1 ± 0.03	0.6 ± 0.04	64.7 ± 12.6
(BMS-185008)	(1×)	(1×)	(1×)

[a] Values are mean IC_{50} ± SEM in micromolar ($n = 3-5$). The fold change in IC_{50} relative to wild-type ET_A is shown in parentheses with bold print denoting improved affinity.

225-fold to the Phe- and Ala-substituted mutants. Comparison of a C-5 pyrrolidine (BMS-185511) and C-5 pyrrolidinone (BMS-185008) emphasized the involvement of hydrogen bonding. Although the amide is not basic, it can function as a hydrogen bond acceptor. Thus we reasoned that, if hydrogen bonding was involved, the relative changes in binding affinity for these two compounds would be similar in both the Tyr129Phe and Tyr129Ala mutants since neither can hydrogen bond. The data show that the IC_{50} values of the basic pyrrolidine are 5-fold and 230-fold greater for the Phe- and Ala-substituted mutants. Similarly, the IC_{50} values for the pyrrolidinone are 6-fold and 540-fold greater for the Phe and Ala mutants. These relative changes in binding are consistent with the hypothesis that a hydrogen bond between the C-5 substituent and Tyr129 contributes to high-affinity interaction with ET_A receptors.

Role of Asp126 and Asp133 in Binding of BMS-182874 to ET_A Receptors. We have previously reported that alanine replacement of the aspartate residues that flank Tyr129, Asp126 and Asp133 reduced the binding affinity of BMS-182874 for ET_A receptors (Rose et al., 1995). To further investigate the mechanism by which BMS-182874 interacts with these aspartates, the binding activity of analogs of BMS-182874 varying at C-5 to Asp126Ala and Asp133Ala was examined. As observed previously, binding affinity of BMS-182874 for Asp126Ala or Asp133Ala is 30-fold and 80-fold decreased. Replacement of the C-5 dimethylamine of BMS-182874 with either the pyrrolidine or pyrrolidinone substituent reduced the effect on binding affinity (Table 3). Nevertheless, in all cases, naphthalenesulfonamide binding was more severely affected by mutation of Asp133 than by mutation of Asp126.

Role of Tyr129 in ET-1 Mediated Signaling. Although these and previous data (Krystek et al., 1994; Lee et al., 1994) demonstrate that Tyr129 is critical to nonpeptide antagonist binding to ET_A receptors, it is not clear if this residue is involved in agonist-induced ET_A-mediated signaling processes as is Asp126 (Rose et al., 1995). To determine the role of Tyr129 in signaling, phosphoinositide turnover and alterations in extracellular acidification rates were examined in CHO cell lines that stably express the wild-type ET_A or mutant Tyr129Ala or Asp126Ala ET_A receptors. The latter cell line was included as a negative control as we previously

Table 3: Structure−Activity Relationship of BMS-182874 and Analogs at Wild-Type, Asp126Ala, and Asp133Ala ET_A Receptors[a]

substituent	wild-type ET_A	Asp126Ala ET_A	Asp133Ala ET_A
Me_2NR	0.3 ± 0.1	7.6 ± 0.2	25 ± 2.2
(BMS-182874)	(1×)	(30×)	(80×)
	0.1 ± 0.04	1.8 ± 0.5	2.6 ± 0.2
(BMS-185511)	(1×)	(18×)	(25×)
	0.1 ± 0.03	0.6 ± 0.06	1.1 ± 0.2
(BMS-185008)	(1×)	(6×)	(10×)

[a] Values are mean IC_{50} in micromolar ± SEM ($n = 3-5$). The fold change in IC_{50} relative to wild-type ET_A is shown in parentheses.

demonstrated that phospholipase C activation was abolished in cells transiently expressing Asp126Ala ET_A receptor mutants (Rose et al., 1995). All three cell lines had high affinity for ET-1 (50−170 pM) and expressed similar amounts of ET receptor (in fmol/mg of protein: ET_A, 100 ± 20; Tyr129Ala, 175 ± 20; Asp126Ala, 190 ± 10). In the presence of increasing concentrations of ET-1, cells expressing the wild-type ET_A receptor responded with a concentration-dependent increase in intracellular inositol phosphate accumulation. Cells expressing the variant Tyr129Ala ET_A receptor also responded with a concentration-dependent increase in inositol phosphates; however, the maximal effect (E_{max}) was reduced 50−75% from the wild-type response (Figure 2A). As expected on the basis of previous results, cells expressing Asp126Ala ET_A receptors failed to respond to ET-1 (Figure 2A). Similar results were obtained when changes in ET-1-induced extracellular acidification rates were monitored. Cells expressing wild-type and Tyr129Ala receptors responded with concentration-dependent increases in acidification rates. However, cells expressing the Tyr129Ala ET_A receptor variants had a 50−75% reduced E_{max} compared to wild-type receptor (Figure 2B). As with intracellular inositol phosphate accumulation, cells expressing Asp126Ala ET_A receptors failed to respond to ET-1 (data not shown).

Molecular Modeling of BMS-182874 to the Putative ET_A Receptor Binding Site. In light of these data indicating that the ET_A receptor-selective antagonist BMS-182874 interacts with Tyr129 via aromatic interactions and hydrogen bonding and that Tyr129 appears to be involved in ET receptor-mediated signal transduction, we attempted to dock this small molecule in the putative ET_A binding cavity of our current model of the helical core of the ET_A receptor.

On the basis of data from mutagenesis and SAR experiments, Tyr129 was hypothesized to have direct contact with BMS-182874. Asp133 was designated as the second contact on the basis of the mutagenesis data and proximity in the binding cavity. Asp126, estimated to be ≈8.5 Å from the center of the Tyr129, is thought to be too deep within the transmembrane domain to have direct contact with the ligand. In this model, BMS-182874 inserts into a hydrophobic binding cavity composed of amino acids from TM2, TM3, and TM7. BMS-182874 was positioned in the binding cavity with the naphthalene ring and C-5 dimethylamino hydrogen bonding with the Tyr129 aromatic ring and hydroxyl group. This position of BMS-182874 provides that the naphthyl ring can interact with the phenyl ring of Tyr129. The aromatic interaction between BMS-182874 and Tyr129 suggests a ring

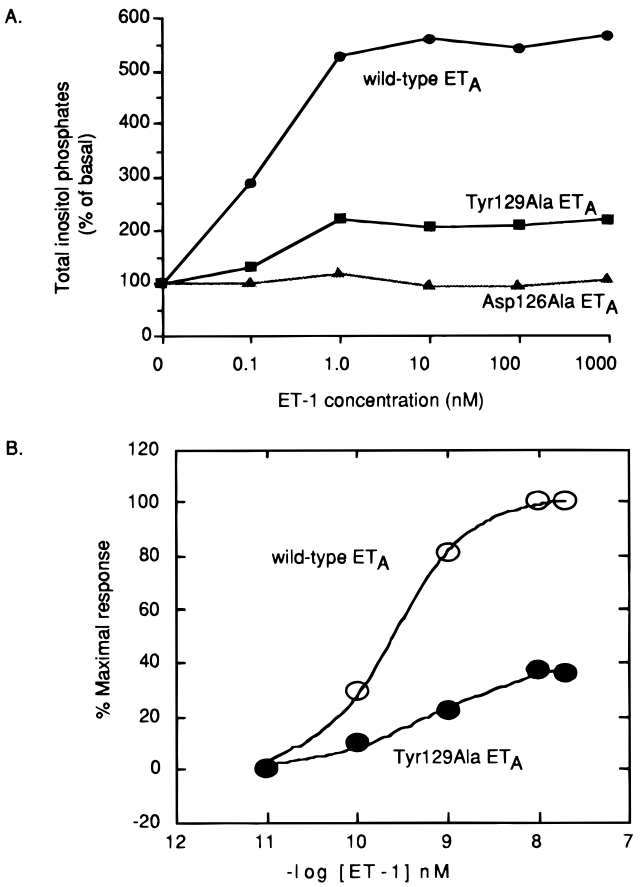

FIGURE 2: Effect of mutation of Tyr129 and Asp126 on ET_A-mediated signal transduction. ET-1-induced inositol phosphate accumulation (A) and extracellular acidification rate change (B) in CHO cells expressing wild-type ET_A, Tyr129Ala ET_A, or Asp126Ala ET_A receptors. This figure is representative of three or more similar experiments.

separation distance of 5.7 Å and a dihedral angle between rings of 77°. Interaction of these two rings is consistent with aromatic−aromatic interactions (Burley & Petsko, 1985) with the preferred separation distance for centroids of the aromatic rings between 4.5 and 7.0 Å and the preferred dihedral angle between interacting aromatic rings ranging from 30° to 90°. In this model, although there would be no direct contact between the carboxylate of Asp133 and the sulfonamide group of BMS-182874, the proximity of these groups could be bridged by a water molecule or positive ion. However, a potential deficiency in this model is the lack of precedent for intramolecular interactions between like-charged residues and the absence of a contact with the highly conserved Lys in TM3.

To account for these potential deficiencies, a second model is proposed in which Lys166 in TM3 of the receptor interacts with BMS-182874. The rationale for a direct interaction of BMS-182874 with Lys166 is based on the involvement this residue has been shown to have in ligand binding in other receptor systems. Specifically, Lys166 corresponds to a conserved Asp in the third transmembrane domains of α- and β-adrenergic receptors that has been shown to be essential for agonist and antagonist binding (Strader et al., 1987, 1988). Second, we and others have noted that this residue plays a critical role in agonist and antagonist binding to ET_B receptors (Zhu et al., 1992; Lee et al., 1994b; Patel et al., unpublished data). Third, replacement of Lys166 in the ET_A receptor with alanine decreases specific binding of [^{125}I]ET-1 (Patel et al., unpublished data), suggesting that it may play a role in agonist if not antagonist binding. Fourth, optimization of the side-chain positions for the lysine suggested it was oriented in the putative ET_A receptor binding cavity (Figure 3A). Thus, in this second model, we hypothesize that the basic amine of Lys166 interacts with the sulfonamide group of BMS-182874 via electrostatic interactions. This proposal is consistent with the estimated distance of ≈3 Å between the sulfonamide group to the terminal amine of Lys166. As before, the naphthalene ring and C-5 dimethylamino hydrogen of BMS-182874 interact with the Tyr129 aromatic ring and hydroxyl group via aromatic interactions and hydrogen bonding, respectively. In addition, a hydrophobic cluster formed by Tyr129 in TM2, and also by Val173 and Leu176 in TM3, and Ile372 in TM7 is envisioned that may act to position the ligand (Figure 3B,C). Therefore, in this model, the isoxazole is near Asp133 and the changes in binding affinity observed when Asp133 is mutated are due to loss of direct interactions between this amino acid and the isoxazole ring of BMS-182874. As in the previous model, BMS-182874 is proposed not to have a direct contact with Asp126 as this residue is ≈8.5 Å deep within the transmembrane domain. Rather, it is likely to interact indirectly by positioning Tyr129 in the binding cavity.

DISCUSSION

Elucidation of structural features in GPC receptors is critical to understanding the basis for binding affinity and selectivity, as well as to facilitating the rational design of therapeutically effective agents. ET receptors are a target for therapeutic intervention in numerous cardiovascular diseases, and several structurally different nonpeptidic antagonists now exist. However, to date little information is available to explain the mechanism of antagonist binding to ET_A receptors.

A major finding from the present study is that high-affinity binding of four chemically distinct peptidic and nonpeptidic antagonists is largely dependent on aromatic interactions with the residue at position 129 in the ET_A receptor since high-affinity binding of BQ-123, SB 209670, and bosentan to ET_A receptors is maintained only when Tyr is replaced with Phe or Trp. That the ET_A variant Tyr129Ile did not retain high-affinity binding indicates that hydrophobic interactions with the residue at position 129 are not sufficient for the high-affinity binding observed in wild-type ET_A receptors. These observations confirm and extend those of Lee et al. (1994a), who examined BQ-123 binding to several Tyr129 mutants including Tyr129Phe but not Tyr129Trp or Tyr129Ile.

Direct interaction between the four antagonists examined here and Tyr129 is of particular interest given the finding that mutation of Tyr129 dampens ET-1 signal transduction. Although we have previously shown that Asp126 is critical to ET_A-mediated signal transduction (Rose et al., 1995), no information on the role of Tyr129 in signaling has been reported. The mechanism underlying the uncoupling of agonist affinity and efficacy in specific mutant receptors such as Tyr129Ala ET_A receptors is unclear. Studies with nonhydrolyzable guanine nucleotides have failed to alter the affinity of ET-1 for wild-type as well as mutant ET_A receptors, suggesting that most of the receptors are already uncoupled from G protein or that ET-1 binding is nucleotide

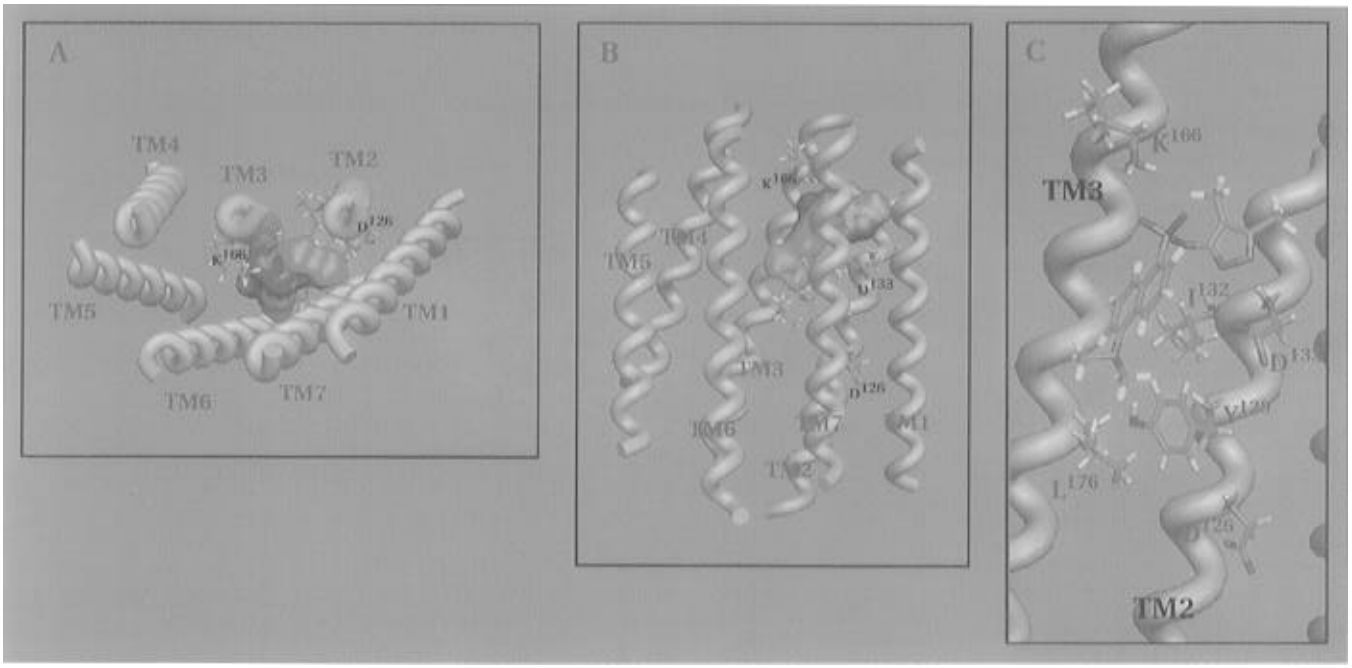

FIGURE 3: Molecular models of BMS-182874 docked in the putative binding cavity of the ET_A receptor. Panel A: Model of ET_A with BMS-182874 docked into the binding cavity as viewed from the extracellular face. The transmembrane domains are displayed with the helices labeled TM1 through TM7 and represented by solid backbone ribbons colored blue. Selected side chains interacting with BMS-182874 (from TM2, TM3, and TM7) are displayed, colored by atom type (see below for atom color code). The molecular surface of the ligand is displayed in order to clearly show its placement in the binding cavity. Panel B: Cross-sectional view of the ET_A receptor showing the placement of BMS-182874 proximal to the extracellular surface of the receptor. Asp126, located deep within TM2, has no direct contacts with the ligand. Panel C: Enhanced view of the putative binding cavity with TM7 removed for clarity. BMS-82874 is displayed in bond representation colored by atom type. The dimethylamine of the ligand is shown interecting with the hydroxyl of Tyr129. Aromatic intereactions between the naphthyl ring of the ligand and Tyr129 are evident. The sulfonamide group of the ligand is hypothesized to interact with Lys166 via electrostatic interactions and Asp133 with the isoxazoyl group of BMS-182874. Atom-type coloring is as follows: green, carbon; blue, nitrogen; red, oxygen; yellow, hydrogens of BMS-182874; pink, hydrogens of receptor; and brown, sulfur.

independent (Webb et al., 1993; Rose et al., 1995). However, the finding that Tyr129 is required for antagonist binding and agonist-induced signal transduction raises the possibility that antagonism may be mediated by preventing agonist interactions with this residue. Recent advances in the β2-receptor field suggest that G protein-coupled receptors exist in equilibrium between inactive and spontaneously active conformations (Bond et al., 1995). Thus, although presently speculative as it pertains to ET_A receptors, one mechanism underlying antagonism of this receptor may be through alteration of such an equilibrium via the formation of ET_A−antagonist complexes through interactions with Tyr129, thereby shifting the conformation of the ET_A receptor to an inactive form.

An interesting point of divergence from the sufficiency of aromatic interactions for ET_A binding affinity is observed with BMS-182874 in that part of the naphthalenesulfonamide association with the ET_A receptor is contributed by a hydrogen bond. Thus, the more basic dimethylamine (BMS-182874) is more affected by loss of Tyr129 than is the less basic primary amine (BMS-182542). The loss of the hydrogen bond upon receptor mutation therefore has a greater effect on the better hydrogen-bonding analogs, i.e., the more basic BMS-182874. Although typically an aromatic amine substituent is not a good hydrogen acceptor due to resonance stabilization of the nitrogen ion pair in the aromatic ring, steric interactions of the dimethylamino group with the C-4 hydrogen result in pyramidalization of the nitrogen, increasing its availability for interaction with the tyrosine hydroxyl group. The importance of hydrogen bonding rather than an ionic interaction is emphasized by comparing BMS-185008, the amide pyrrolidinone, with the pyrrolidine BMS-185511. Whereas both the pyrrolidinone and pyrrolidine are good hydrogen bond acceptors, only the pyrrolidine is basic, consistent with the suggestion that it is the hydrogen-bonding ability which is important, rather than an electrostatic interaction with the protonated amine.

In addition to Tyr129, Asp126 and Asp133 have been shown to play a role in BMS-182874 binding (Rose et al., 1995). Binding affinity of analogs of BMS-182874 with varied C-5 substituents was reduced to both the Asp126Ala (6−30-fold) and Asp133Ala (10−80-fold) mutants. Additional determinants of antagonist binding to ET_A receptors exist in other regions of the molecule. Specifically, data from studies using ET_A and ET_B receptor chimeras have shown the first extracellular loop (Adachi et al., 1992) and transmembrane regions 1, 2, 3, and 7, as well as the intervening loops (Sakamoto et al., 1993), specify the ET_A selectivity of BQ-123. Elucidation of precise determinants of antagonist binding will likely require studies employing receptor mutagenesis in combination with radiolabeled antagonists.

Some insights into the role of Tyr129 in agonist binding can be gained from the present study. The binding affinity for the native peptide ET-1 is similar to wild type, i.e., <1 nM, for all Tyr129 receptor variants with the exception of the Thr and Ile replacements. Although the maintenance of high-affinity ET-1 binding for the Phe, Trp, Lys, Ser, and Ala receptor mutants indicates that the altered receptor has not undergone a large-scale structural change, the 8−10-fold loss in ET-1 affinity for Tyr129Thr and Tyr129Ile mutants may signal that ET-1 either directly contacts Tyr129 or is indirectly influenced by specific substitutions. Lee et

al. (1994a) evaluated the tolerance of substitution for Tyr129 but did not report the effect of Thr or Ile replacement. Given the lack of effect that substitution of Tyr129 with residues other than Thr or Ile, it seems most likely that ET-1 has multiple contacts that compensate for most Tyr substitutions.

In the case of ET-3, the binding affinity improved for all replacements of Tyr with the exception of the conservative Trp mutation. Previous work showed that removal of the phenolic hydroxyl of tyrosine by replacement with phenylalanine improved the affinity of ET-3 for ET_A receptors (Lee et al., 1994a). The present observation that replacement of Tyr with Trp, but not with Phe, maintains the low-affinity interaction of ET-3 for wild-type ET_A receptors suggests that steric interactions may also be involved in the gain in affinity for ET-3. Another possibility is that the hydroxyl group of Tyr129 is involved in specific interactions with Asp126. This view is supported by data from Rose et al. (1995), who showed that binding of ET-3 improved over 100-fold to the Asp126Ala ET_A mutant. In this view, interaction of the Tyr129 hydroxyl group with Asp126 would establish a set of steric constraints that are rearranged by replacement of Asp126 or the tyrosine hydroxyl, thereby resulting in improved ET-3 affinity for ET_A receptors.

Together with previous work (Krystek et al., 1994; Lee et al., 1994a; Rose et al., 1995), the present data demonstrate that the binding site for the native ET peptides at least partially overlaps at Tyr129 with that for BMS-182874, BQ-123, SB 209670, and bosentan. Several precedents exist for overlapping but distinct binding sites for small molecule antagonists and peptidic ligands. Studies with cholecystokinin (Beinborn et al., 1993), tachykinin (Fong et al., 1992, 1993; Gether et al., 1993a,b; Sachais et al., 1993; Zoffmann et al., 1993), and angiotensin (Ji et al., 1994; Hjorth et al., 1994) receptors have elucidated separate determinants for peptidic agonist and nonpeptidic antagonist binding. Rose et al. (1995) showed that mutation of either Asp126 or Asp133 to Ala affected binding affinity for BMS-182874 but not that of ET-1, consistent with the notion that the binding sites for agonists and antagonist are distinct or, at least, are comprised of some disparate elements. However, it is interesting to note that the affinity of ET-3, another peptide agonist, for Tyr129Ala and Asp126Ala mutants improved over 100-fold (Krystek et al., 1994; Rose et al., 1995). Lee et al. (1994b) recently reported that binding of several peptidic agonists and two chemically distinct nonpeptidic antagonists was affected by mutation of Lys182 in the ET_B receptor. Although more evidence is necessary for a broader application to other GPC receptors for peptide or protein ligands, it is presently reasonable to conclude that, with respect to the ET_A and ET_B receptors, the agonist and antagonist binding sites have some determinants in common. Thus, data from ET_A and ET_B receptors present an alternate example and diverge from the view that agonist and antagonist sites are generally different in GPC receptors that bind peptide ligands.

Specific determinants of nonpeptidic antagonist binding are of great interest in developing therapeutic agents that specifically compete with native peptides. In our attempt to develop a working model of an antagonist−receptor complex, the ET_A-selective small molecule antagonist BMS-182874 was used. Common features in the BMS-182874−ET_A receptor complex models proposed herein are the configuration of a putative hydrophobic binding pocket formed by residues in TM2, TM3, and TM7 and a direct contact between the C-5 dimethylamino group and naphthalene ring of BMS-182874 and the Tyr129 hydroxyl group and aromatic ring via a hydrogen bond and aromatic interaction, respectively, as supported by the structure−activity data reported here. Other direct contacts between BMS-182874 and the ET_A receptor are unclear; however, several possibilities were examined. Because Asp126 is estimated to be 8.5 Å from the tyrosine center, it is unlikely that this aspartate directly interacts with BMS-182874. It is more likely that the effect of Asp126 on BMS-182874 binding is through positioning of Tyr129. A direct interaction between Asp133 and the sulfonamide group is possible but involves a water or positively charged ion bridge between two negatively charged moieties. The preferred model involves an interaction between the sulfonamide of BMS-182874 and Lys166. Although it is not presently clear what mutation of Lys166 does to BMS-182874 binding, previous data for the ET_B receptor (Mauzy et al., 1992; Zhu et al., 1992; Lee et al., 1994) and preliminary results from the Lys166Ala mutant of the ET_A receptor (Patel et al., unpublished data) suggest a role for this residue in ET_A receptor binding.

To our knowledge, this is the first model of a nonpeptidic antagonist−ET_A receptor complex. Studies with other antagonists are necessary to refine this model and to determine its broader applicability both within the ET receptor subtypes and to the larger family of GPC receptors for which peptides are the native ligands. For example, within the ET receptor subtypes, it should be possible to determine if a core binding site exists for all chemically distinct antagonists and, as compounds become available with defined selectivity for either the ET_A or ET_B subtype, to develop a structural understanding for the basis of subtype selectivity.

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