Lysine 182 of Endothelin B Receptor Modulates Agonist Selectivity and Antagonist Affinity: Evidence for the Overlap of Peptide and Non-Peptide Ligand Binding Sites

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ABSTRACT: The potent vasoactive peptide hormone endothelin (ET) binds to receptors which belong to the G-protein coupled receptor family. The availability of non-peptide antagonists for ET receptors allows investigation of the relationship among the binding sites for peptide and non-peptide ligands. In this study, a lysine residue, conserved within transmembrane domain 3 (TM3) of the ET_A and ET_B receptor subtypes, is implicated in agonist and antagonist binding by its analogous position within TM3 to a binding site aspartate residue conserved within bioactive amine receptors. Replacement of this lysine within hET_B by arginine, alanine, methionine, aspartate, or glutamate results in hET_B variants with unaltered affinities for agonist peptide ET-1 but which have affinities for peptide agonists ET-2, ET-3, sarafotoxin 6C, and IRL 1736 which are between 1−3 orders of magnitude lower than their corresponding wild-type hET_B values. Significantly, the affinities of non-peptide antagonists, (±)-SB 209670 and its analogs as well as Ro 46-2005, are abrogated. The results suggest that an interaction of K182 of hET_B with the indan 2-carboxyl of (±)-SB 209670 may contribute to the high-affinity binding of the diarylindan antagonists. The results indicate that TM3 of hET_B is a region of overlap among the binding sites of non-peptide antagonists and the affected peptide agonists.

G-protein coupled receptors (GPCRs)[1] are postulated to have a conserved secondary structure composed of seven amphiphilic transmembrane (TM) spanning helices connected by loops of between 15 and >200 residues in length. The tertiary structure of the helices is thought to resemble the barrel-like arrangement observed for bacteriorhodopsin (Henderson *et al.*, 1990) and suggested for rhodopsin (Schertler *et al.*, 1993). While the agonists which bind to various pharmacologically important GPCRs are highly diverse ranging from small molecules such as bioactive amines to proteins such as interleukin-8 (IL-8), receptor mutagenesis studies have consistently implicated the TM regions as important for binding their respective ligands (Strader *et al.*, 1987, 1988; Fraser *et al.*, 1989; Wang *et al.*, 1991, 1993; Ho *et al.*, 1992; Mauzy *et al.*, 1992; Zhu *et al.*, 1992; Fathi *et al.*, 1993; Hebert *et al.*, 1993; Krystek *et al.*, 1994; Lee *et al.*, 1994; Perlman *et al.*, 1994).

The receptors which bind endothelin-1 (ET-1), a potent vasoactive peptide, are GPCRs. Due to the structural diversity of available ligands (Figure 1), ET receptors are well suited for investigating the relationships among their binding sites. ET-related peptides are divided into two groups with high sequence similarity, the endothelins (ET-1, ET-2, and ET-3) found in mammals and the sarafotoxins (S6A, S6B, S6C, and S6D) found in the venom of *Atractaspis engaddensis* (Sokolovsky, 1992). Currently, two major classes of ET receptor subtypes, ET_A and ET_B, are known and defined by their rank order affinity for agonist peptide ligands (Takayanagi *et al.*, 1991). The binding profile of ET_A is selective: ET-1 and ET-2 bind with high and similar affinity, ET-3 with a 70−100-fold lower affinity than ET-1, and S6C with >1000-fold lower affinity than ET-1. The binding profile of ET_B is nonselective: ET-1, ET-2, ET-3, and S6C bind with a high and similar affinity. Peptide ligands which are highly receptor subtype selective have been described: BQ123, a cyclic peptide antagonist for ET_A, and IRL 1620 and IRL 1736, linear agonist peptides for ET_B (Ihara *et al.*, 1991; Takai *et al.*, 1992). Recently, a number of research groups have described non-peptide ET receptor antagonists of varying structure, activity, and selectivity, *e.g.*, SB 209670 (Elliott *et al.*, 1994; Ohlstein *et al.*, 1994), Ro 46-2005 (Clozel *et al.*, 1993), Ro 47-0203 (Roux *et al.*, 1993), Shionogi [50-235] (Mihara *et al.*, 1993), and BMS 182874 (Stein *et al.*, 1994).

We have utilized the potential conservation of functionally important regions among members of the GPCR family to develop a hypothesis concerning the residues of ET receptors which may be important for the binding of agonist and/or

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[1] Abbreviations: AT1, angiotensin receptor type 1; CCK-B, cholecystokinin-B/gastrin; ET-*X*, endothelin-1, -2, or -3; GPCR, G-protein coupled receptor; $hET_{A/B}$, human endothelin receptor subtype A or B; IL-8, interleukin-8; K_i, dissociation constant; NK1, neurokinin 1; S6C, sarafotoxin 6C; TM, transmembrane domain. Single letter abbreviations for amino acids are used.

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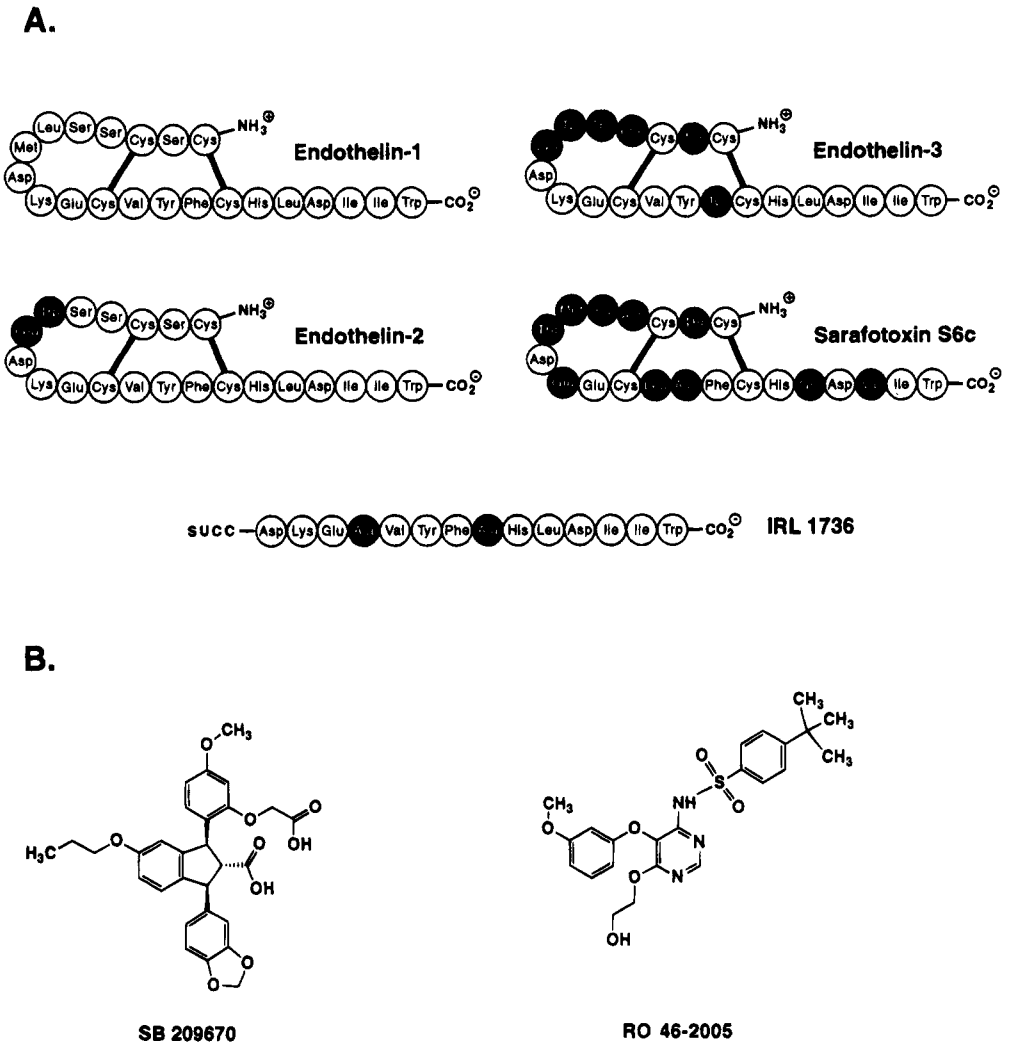


FIGURE 1: Structures of endothelin receptor ligands. (A) Agonist peptides, amino acids that differ from ET-1 (reverse print), and disulfide bonds (bold lines) are indicated. (B) Non-peptide endothelin receptor antagonists.

antagonist ligands. The eight and ninth residues following the highly conserved cysteine at the start of transmembrane domain 3 (TM3) have been shown to be important for the high-affinity binding of small molecule ligands to bioactive amine receptors (Strader *et al.*, 1987, 1988; Fraser *et al.*, 1989; Wang *et al.*, 1991, 1993; Ho *et al.*, 1992) and of peptide/protein ligands to the IL-8 and thyrotropin releasing hormone receptors (Hebert *et al.*, 1993; Perlman *et al.*, 1994). By analogy, a lysine positioned nine residues following the conserved cysteine within TM3 of ET receptors has been implicated in agonist peptide binding (Mauzy *et al.*, 1992; Zhu *et al.*, 1992; Huggins *et al.*, 1993). In our current study, replacements of this lysine residue in hET_B do not affect the affinity of the resulting receptor variants for ET-1 but decrease the binding affinity of ET-2, ET-3, S6C, and IRL 1736 by 1−3 orders of magnitude. Significantly, these replacements eliminate the binding affinity of non-peptide antagonists (±)-SB 209670 and Ro 46-2005. The data suggests that the binding sites for certain peptide agonists and non-peptide antagonists overlap within TM3 of hET_B.

MATERIALS AND METHODS

Chemical Synthesis. All compounds were prepared in the Department of Medicinal Chemistry, SmithKline Beecham. Compounds **1**−**5** (Table 2) were synthesized as previously described (Elliott *et al.*, 1994). The synthesis of compounds **6** and **7** will be described elsewhere.

Mutagenesis and Receptor Expression. Mutations of hET_B/pRCCMW were performed by the Kunkel method (Kunkel *et al.*, 1987) and confirmed by DNA sequencing. Plasmid DNA was prepared by alkaline lysis and purified by Qiagen column chromatography. Human embryonic kidney (HEK) 293 cells (ATCC CRL 1573) were grown and transfected by the calcium phosphate method (Lee *et al.*, 1994).

Radioligand Binding Assays. Membranes from HEK 293 cells transfected with plasmids encoding hET_B variants were prepared and quantitated as described (Lee *et al.*, 1994). Peptide competition binding was initiated by addition of membranes (0.33−56.7 μg/mL) from HEK 293 cells transfected with wild-type or hET_B receptor variants to an assay mixture composed of 0.1% BSA, 0.12 nM [^{125}I]ET-1 (2200 Ci/mmol), and the designated concentration of unlabeled ET-related peptides (American Peptides) in 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$. For antagonist competition binding, DMSO solutions of (±)-SB 209670 and Ro 46-2005 were mixed with [^{125}I]ET-1 and binding reactions initiated by membrane addition. The final DMSO concentration was 3.3%, a noninhibitory concentration of solvent. All binding

experiments were conducted and analyzed as described (Elshourbagy *et al.*, 1992). The apparent K_i values were derived from duplicate to quadruplicate experiments utilizing membrane preparations from two independent transfections. Variation in the expression level of the various ET receptors ranged from 24.4 pmol/mg to 385 fmol/mg; endogenous sites in HEK 293 membranes constituted a maximum of 6% of specific binding. The concentration of nonlabeled endothelin-related peptides was determined by quantitative amino acid analysis; the concentration of [^{125}I]ET-1 was calculated directly by utilizing a constant specific activity (New England Nuclear) due to the catastrophic decay of labeled ligand.

RESULTS

The importance of K182 within TM3 of hET_B to peptide and non-peptide binding was evaluated by replacing this residue with alanine, aspartate, methionine, arginine, or glutamate (K182X). Separately, a neighboring lysine residue, K175, positioned at the extracellular terminus of TM3 and conserved in the ET, angiotensin II, bombesin, neuropeptide Y, cholecystokinin-B/gastrin (CCK-B), and NK1 receptors (Battey *et al.*, 1991; Bergsma *et al.*, 1992; Elshourbagy *et al.*, 1992, 1993; Fong *et al.*, 1992a; Kopin *et al.*, 1992; Larhammar *et al.*, 1992), was replaced with alanine and arginine (K175A, K175R). The resulting hET_B variants were evaluated for their apparent binding affinity for agonist peptides, ET-1, ET-2, ET-3, S6C, and IRL-1736, and non-peptide antagonists, (±)-SB 209670 and its analogs (Elliott *et al.*, 1994; Ohlstein *et al.*, 1994) as well as Ro 46-2005 (Clozel *et al.*, 1993).

Figure 2 shows the relative abilities of peptide ligands to compete for [^{125}I]ET-1 binding to wild-type, K175A, K175R, and K182X hET_B. All peptide ligands compete for [^{125}I]-ET-1 binding to K175A, K175R, and wild-type hET_B with comparable affinities (Table 1). In contrast, replacement of lysine 182 by either arginine, alanine, methionine, aspartate, or glutamate decreases the apparent affinities for ET-2 by 11−48-fold, for ET-3 by 70−276-fold, for S6C by 88−880-fold, and for IRL 1736 by >4900-fold without affecting the apparent affinity for ET-1 (Figure 2; Table 1). Similar results were obtained for ET-1, ET-3, and S6C with rat ET_B receptor when lysine 181, the residue analogous to K182 in hET_B, was replaced with aspartate (Mauzy *et al.*, 1992; Zhu *et al.*, 1992).

Figure 3 depicts the relative abilities of two non-peptide ET antagonists to compete for [^{125}I]ET-1 binding to wild-type, K175A, and K182X hET_B. The apparent K_i of (±)-SB 209670 (15 nM) is 13-fold lower than that of Ro 46-2005 (200 nM) for wildtype hET_B. The affinities of both non-peptide antagonists for K182X hET_Bs are dramatically reduced. The K182R hET_B variant demonstrates the smallest apparent reduction in (±)-SB 209670 affinity (460-fold). These antagonists (up to 100 μM) are unable to complete for [^{125}I]ET-1 binding to the other K182X hET_Bs. Like the peptide agonists, the affinities of (±)-SB 209670 and Ro 46-2005 are not significantly altered for K175A hET_B (Figure 3; Table 1).

Table 2 summarizes the apparent K_i values of (±)-SB 209670 (compound **1**) and various (±)-SB 209670 analogs for binding to wildtype and K182R hET_B. Analogs of (±)-SB 209670 containing an indan 2-carboxyl group (compounds **2**−**5**) have measurable binding affinities for wild-type hET_B (Elliott *et al.*, 1994) but have significantly reduced

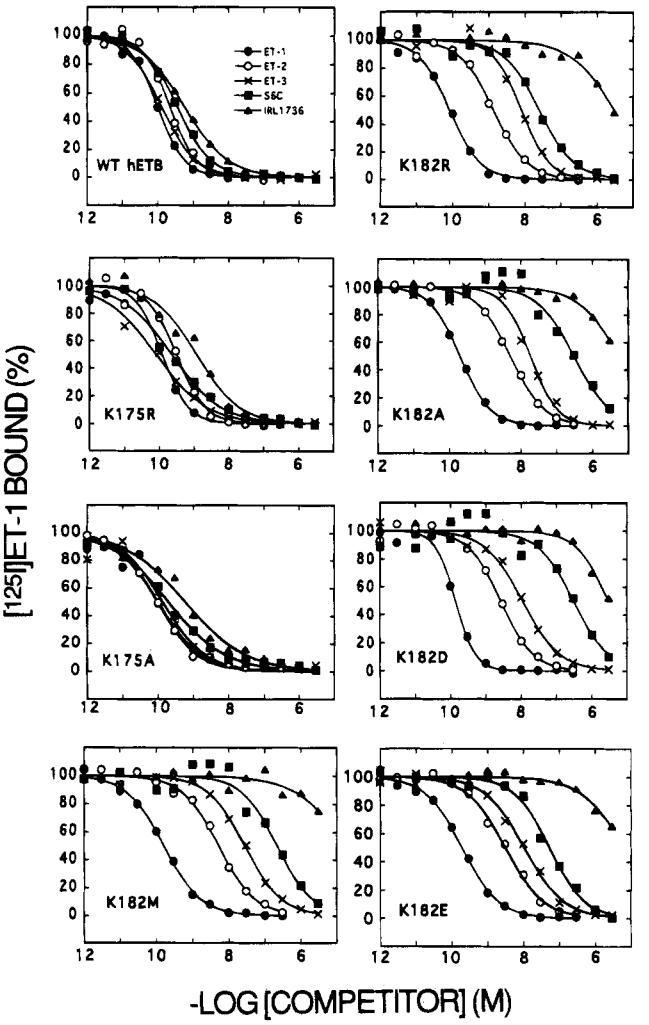


FIGURE 2: Competitive ligand binding of agonist peptides. Competitive binding between [^{125}I]ET-1 and unlabeled ET-1 (solid circles), ET-2 (open circles), ET-3 (crosses), S6C (solid squares), and IRL 1736 (solid triangles) to membranes from HEK 293 cells transfected with wildtype, K175A, K175R, and K182X hET_B. Data points are the averages of duplicate determinations which usually varied by less than 7% of the total specific binding. A representative data set from the two to four data sets for each ligand/hET_B variant combination is shown.

affinities for K182R hET_B (Table 2). Replacement of the diarylindan 2-carboxyl by a hydroxymethyl substituent (R_4, Table 2), as in compound **6**, diminishes binding affinity by 520-fold relative to (±)-SB 209670 for wild-type hET_B. Significantly, the binding affinity of compound **6** is not altered upon replacement of K182 by arginine (Table 2) or alanine (data not shown). Deletion of the diarylindan 2-carboxyl group results in compound **7** which has >1700-fold lower binding affinity for wild-type hETB than (±)-SB 209670 (Table 2).

DISCUSSION

The manner in which GPCRs recognize structurally diverse ligands is important to understanding molecular recognition by GPCRs and for the development of novel therapeutic agents. In order to identify ET receptor regions that are important to agonist and/or antagonist binding, we have hypothesized that these regions may be conserved within distantly related members of the GPCR family. Extensive mutagenesis studies (Strader *et al.*, 1987, 1988; Fraser *et*

Table 1: Ligand Affinities for Wild-Type and Mutant hET_B Receptors[a]

	peptide agonists K_i (nM)					non-peptide antagonists K_i (nM)	
receptor	ET-1	ET-2	ET-3	S6C	IRL 1736	(±)-SB 209670	Ro 46-2005
hETB	0.012	0.018	0.013	0.030	0.063	15	200
K175A	0.011	0.015	0.034	0.039	0.059	17	450
K175R	0.012	0.036	0.010	0.023	0.138	12	nd
K182R	0.014	0.19	0.91	2.64	306	≥7000*	>10 000
K182A	0.042	0.86	2.56	25.1	566	>10 000	>10 000
K182D	0.022	0.29	1.45	26.4	384	>10 000	>10 000
K182E	0.025	0.45	1.46	6.0	960	>10 000	>10 000
K182M	0.037	0.70	3.59	23.9	1900	>10 000	>10 000

[a] Competitive binding between [^{125}I]ET-1 and unlabeled ligands was performed as described in Materials and Methods. The K_i values were calculated from IC_{50} values determined from nonlinear regression of the competition data as described (Elshourbagy *et al.*, 1992). The K_i values listed are the average of between two and four determinations using membranes prepared from two independent HEK 293 transfections. The maximum range of the K_i values was ±30% of the mean values. Lower limits of the K_i values for antagonist binding to K182X hET_B are derived from the maximum concentration of ligand utilized or the estimated IC_{50} value (*). nd, not determined.

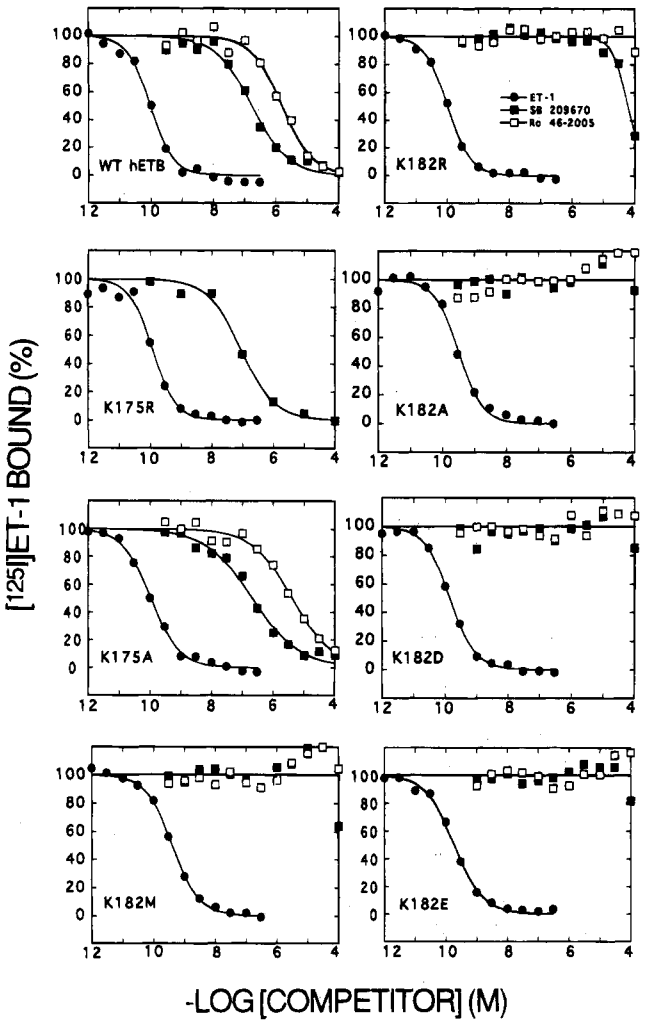


FIGURE 3: Competitive ligand binding of non-peptide antagonists. Competitive binding between [^{125}I]ET-1 and unlabeled ET-1 (solid circles), (±)-SB 209670 (solid squares), and Ro 46-2005 (open squares) to wildtype, K175A, and K182X hET_B. Data points are the averages of duplicate determinations. A representative data set from the two to four data sets for each ligand/hET_B variant combination is shown.

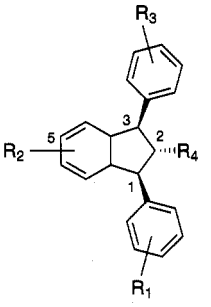
al., 1989; Wang *et al.*, 1991, 1993; Ho *et al.*, 1992) and affinity labeling studies (Kurtenbach *et al.*, 1990) have suggested that an aspartate residue conserved within TM3 of ARs functions as the receptor counterion for the positively charged ligand. Replacement of K182, the hET_B residue which occupies a similar position within TM3 as the binding site aspartate in ARs, results in hET_B variants with wild-type affinities for ET-1 but 1−3 orders of magnitude lower affinities for ET-2, ET-3, S6C, and IRL 1736 and at least a 460-fold lower affinity for the non-peptide antagonist (±)-SB 209670 (Figures 2 and 3; Table 1). These results indicate that K182 has an integral role in the binding of both peptide and non-peptide ligands.

Two lines of evidence suggest that the changes in ligand affinity on mutation of hET_B are due to direct effects on receptor−ligand interactions and not global changes in receptor conformation. Both conservative and nonconservative replacements of K182 alter the affinity of ET-2, ET-3, S6C, and IRL 1736 *without* alteration of the ET-1 affinity (Figure 2; Table 1). Replacement of the residue analogous to K182 in rat ET_B (K181) did not affect maximal stimulation of phosphoinositide hydrolysis (Mauzy *et al.*, 1992).

Effect of K182 Mutations on Agonist Binding to hET_B. Of the two ET receptor subtypes, ET_B displays a nonselective ligand binding profile with agonist peptide ligands. Replacement of K182 within TM3 of hET_B differentially decreases the binding affinity of peptide ligands, thus altering the ligand selectivity profile to resemble that of the selective receptor subtype, ET_A. Previously, replacement of Y129 within TM2 of hET_A was shown to differentially increase agonist peptide affinities and alter the ligand binding profile to resemble the nonselective receptor subtype, ET_B (Krystek *et al.*, 1994; Lee *et al.*, 1994). Models for both ET receptor subtypes in conjunction with TM2 and TM3 mutagenesis studies of ET receptor (Mauzy *et al.*, 1992; Zhu *et al.*, 1992; Krystek *et al.*, 1994; Lee *et al.*, 1994; this work) suggest that a portion of the binding site for agonist ET peptides resides within a receptor cavity bounded by TMs 1−3, 6, and 7 and is likely to encompass residues from multiple TM regions (Lee *et al.*, 1994). The potentially large interaction surface within this cavity could explain the insensitivity of ET-1 and the differential changes in binding affinity for other ET peptides in response to K182(hET_B) or Y129(hET_A) mutations if the receptor−ligand interactions within this general binding site differentially contribute to the binding of each peptide ligand.

The observation that a single amino acid replacement within hET_B or hET_A differentially affects the binding affinity of peptide ligands contrasts with studies of the NK1 receptor where single amino acid replacements in the extracellular regions (Fong *et al.*, 1992b,c) or within TM regions (Huang *et al.*, 1994) results in a general decrease of substance P, neurokinin A, and neurokinin B binding affinities. Strader

Table 2: Binding Affinities of (±)-SB 209670 Analogs[a]



compd	R_1	R_2	R_3	R_4	K_i (μM) hET$_B$	K_i (μM) K182R hET$_B$
1	3,4-methylenedioxy	5-O-*n*-Pr	2-carboxymethoxy 4-OMe	carboxy	0.017	>30
2	4-OMe	H	4-OMe	carboxy	1.13	>30
3	3,4-methylenedioxy	H	4-OMe	carboxy	0.70	>30
4	3,4-methylenedioxy	5-OH	4-OMe	carboxy	0.43	>30
5	3,4-methylenedioxy	5-O-*n*-Pr	4-OMe	carboxy	0.62	>30
6	3,4-methylenedioxy	5-O-*n*-Pr	2-carboxymethoxy 4-OMe	hydroxymethyl	8.8	10.5
7	3,4-methylenedioxy	5-O-*n*-Pr	2-carboxymethoxy 4-OMe	H	>30	>30

[a] Competitive binding between [^{125}I]ET-1 and unlabeled ligands was performed as described in Material and Methods and the legend of Table 1. Lower limits of the K_i values for antagonist binding are derived from the maximum concentration of ligand utilized. All compounds are racemic mixtures.

and co-workers (Huang *et al.*, 1994) suggest that this general decrease of peptide binding affinity upon NK1 receptor mutation may indicate that each peptide ligand presents the same binding features to the receptor but in different conformations. In contrast, the differential changes in agonist affinity upon mutation of K182 within hET$_B$ suggest that various ET peptides, despite their overall similarity in structure and hET$_B$ binding affinity, do not quantitatively use the same set of receptor−ligand interactions when bound to hET$_B$.

Effect of K182 Mutations on Antagonist Binding to hET$_B$. Replacement of K182 by arginine, alanine, methionine, aspartate, or glutamate reduces the relative binding affinity of (±)-SB 209670 and Ro 46-2005 by at least 460- and 50-fold, respectively (Table 1). In contrast, both classes of non-peptide antagonists have wildtype binding affinities for K175A hET$_B$ (Figure 3; Table 1). The data indicate that the binding sites for these two classes of non-peptide antagonists overlap within TM3 of hET$_B$, specifically at K182.

Wild type and K182R hET$_B$ were examined for their ability to bind analogs of (±)-SB 209670 (Table 2). (±)-SB 209670 analogs which vary at substituent positions R_1 and R_2 but maintain the R_4 carboxyl group (compounds **2**−**5**) have similar affinities for wildtype hET$_B$ but significantly reduced affinities for K182R hET$_B$. Analogs **6** and **7** which differ from (±)-SB 209670 only at the R_4 carboxyl group have binding affinities for wildtype hET$_B$ that are significantly reduced with respect to (±)-SB 209670 (520- and >1700-fold, respectively). Unlike the (±)-SB 209670 analogs which have a carboxyl group at the indan 2-position, the binding affinity of compound **6**, which has a hydroxymethyl substituent at this position, is not further decreased when K182 is replaced with arginine or alanine (data not shown). The 3-dimensional similarity of these molecules supports a common receptor binding mode. These results therefore highlight the importance of a carboxyl group at R_4 and suggest that an interaction between K182 of hET$_B$ and the indan 2-carboxyl of (±)-SB 209670 may contribute to the high-affinity binding of the diarylindan antagonists. Although speculative, the reduction of Ro 46-2005 binding affinity upon replacement of K182 (Figure 3; Table 1) may result from the disruption of an interaction analogous to that between K182 and the indan 2-carboxyl in (±)-SB 209670, but in this case involving the acidic sulfonamide moiety.

Relationship among Agonist and Antagonist Binding Sites. Replacement of K182 significantly decreases the affinities of both non-peptide antagonists, (±)-SB 209670 and Ro 46-2005, and agonist peptides, ET-3, S6C, and IRL 1736 (Figures 2 and 3; Table 1). The magnitude of these changes (ranging from >50- to >4900-fold) suggests that at least a portion of the receptor binding site for these two classes of non-peptide antagonists and for several peptide agonists overlaps within TM3 of hET$_B$. This data, in conjunction with previous mutational studies of Y129 within TM2 of hET$_A$ (Krystek *et al.*, 1994; Lee, *et al.*, 1994), indicates that the binding sites for peptide agonists/antagonists and non-peptide antagonists overlap in ET receptors.

This interpretation of the mutant ET receptor data contrasts with conclusions drawn from mutagenesis studies that identify CCK-B, NK1, and AT1 receptor residues which predominantly affect the binding affinity of *either* peptide ligands or non-peptide antagonists (Beinborn *et al.*, 1993; Fong *et al.*, 1993; Gether *et al.*, 1993a,b; Huang *et al.*, 1994; Ji *et al.*, 1994; Schambye *et al.*, 1994). This previous work suggests that peptide GPCRs generally use distinct sets of receptor residues to engage peptide and non-peptide ligands.

The apparent overlap among the binding sites for peptide and non-peptide ligands within hET$_A$ (Krystek *et al.*, 1994; Lee *et al.*, 1994) and hET$_B$ (Figure 3; Table 1) does not conflict with the CCK-B, NK1, and AT1 conclusions (Beinborn *et al.*, 1993; Bihoreau *et al.*, 1993; Fong *et al.*,

1993; Gether *et al.*, 1993a,b; Huang *et al.*, 1994; Ji *et al.*, 1994; Schambye *et al.*, 1994) if it is not assumed that all peptide-binding GPCRs must engage ligands in the same manner. The emerging literature suggests that peptide-binding GPCRs may fall into at least two classes with respect to their relationship among non-peptide and peptide ligand binding sites, one characterized by distinct binding sites and another characterized by overlapping binding sites. Clearly further investigations are needed.

Conserved Ligand Binding Sites within GPCRs. Many GPCRs have a residue(s) important for ligand binding located within the third transmembrane helix. The receptors for bioactive amines, cationic ligands of molecular weight less than 0.5 kDa, contain a conserved aspartate which is important to both agonist and antagonist binding (Strader *et al.*, 1987, 1988; Fraser *et al.*, 1989; Wang *et al.*, 1991, 1993; Ho *et al.*, 1992). In the GPCR for thyrotropin releasing hormone, replacement of Y106 decreases affinity for the three-residue peptide by approximately 80 000-fold (Perlman *et al.*, 1994). Simultaneous replacement of K117 and E118 within the A type (nonpermissive) IL-8 receptor eliminates binding of IL-8, a 72-residue polypeptide (Hebert *et al.*, 1993). In rat AT1 receptor, replacement of V108 by isoleucine, the homologous residue within the *Xenopus* AT1 receptor, reduces the binding affinity of the nonpeptide antagonist Dup 753 by 40-fold without affecting the binding affinity of the peptide ligand, saralasin (Ji *et al.*, 1994). Finally, K182 in hET_B has been shown in the present study to affect the binding of a variety of structurally diverse ligands. These collective observations support the idea that a region of TM3 may be part of a general binding site in GPCRs. It is interesting to note that on the basis of the similar location of important binding residues within TM5 and TM6 of the NK1 and β2-adrenergic receptors, Fong and co-workers have suggested a common binding site for small molecules (Fong *et al.*, 1993, 1994).

CONCLUSION

Peptide agonist and non-peptide antagonist ligands have been shown to utilize the same residue within TM3 of hET_B for binding. These results together with previous observations regarding the interaction of peptide agonists and BQ123, a peptide antagonist, with a residue located in TM2 of hET_A, support the idea that agonist and antagonist binding sites are composed of overlapping regions within ET receptors. Collectively, these data support rational, peptidomimetic approaches for the development of antagonists of ET receptors (Elliott *et al.*, 1994). K182 of hET_B is positioned analogously to residues important to ligand affinity in a variety of receptors. This commonality may reflect an evolutionarily related binding site within distantly related GPCRs.

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