Aspartate mutation distinguishes ET_A but not ET_B receptor subtype-selective ligand binding while abolishing phospholipase C activation in both receptors

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Abstract **The endothelin receptors, ET_A and ET_B, are G protein-coupled receptors (GPCR) that show distinctively different binding profiles for the endothelin peptides and other ligands. We recently reported that Tyr^{129} in the second transmembrane region (TM2) of the ET_A receptor was critical for subtype-specific ligand binding [Krystek, S.R. et al. (1994) J. Biol. Chem. 269, 12383-12386]. Receptor models indicated that aspartic acids located one helical turn above (Asp^{133}) and below (Asp^{126}) Tyr^{129} in ET_A had their side chains directed toward the putative binding cavity. Similarly in ET_B, Asp^{147} and Asp^{154} are located one turn below and above His^{150}, the residue that corresponds to Tyr^{129}. Asp^{126} in ET_A and Asp^{147} in ET_B correspond to the highly conserved aspartate present in TM2 of many GPCR that has frequently been shown to be crucial for agonist efficacy. Mutagenesis of Asp^{126} of the human ET_A receptor to alanine resulted in an unaltered affinity for ET-1, a 160-fold increase in ET-3 affinity and a decrease in affinity for the ET_A selective naphthalenesulfonamide, BMS-182874. ET-1 activation of phospholipase C was abolished. In addition, despite the gain in binding affinity, ET-3 failed to activate phospholipase C, suggesting that Asp^{126} is required for signal transduction. Mutagenesis of Asp^{133} to alanine indicated that it was critical only for the binding of BMS-182874. In the ET_B receptor, mutation of His^{150} to alanine or tyrosine indicated that it plays a minor role in ET_B subtype-selective ligand binding; mutation of the aspartates in TM2 of ET_B did not alter ligand binding. As in the Asp^{126}Ala ET_A variant, ET-1 and ET-3 failed to increase intracellular levels of inositol phosphates in the Asp^{147}Ala ET_B mutant. Taken together, these data support the hypothesis that Asp^{126} and Asp^{133} flanking Tyr^{129} in TM2 of the ET_A receptor play a role in defining ET_A subtype-selective ligand binding but Asp^{147} and Asp^{154} that flank the His^{150} in TM2 of the ET_B receptor do not. Furthermore, these data indicate that Asp^{126} in ET_A and Asp^{147} in ET_B are important for transmembrane signaling via phospholipase C.**

Key words: Endothelin receptor; G protein-coupled receptor; Site-directed mutagenesis; Radioligand binding; Molecular modeling; Phospholipase C

1. Introduction

Endothelins (ETs) are a family of peptides that mediate diverse and potent biological activity in mammals through at least two distinct heptahelical G protein-coupled, cell surface receptors. The ET_A subtype binds ET-1, the cyclic pentapeptide antagonist BQ-123, and the naphthalenesulfonamide antagonist, BMS-182874, preferentially, whereas ET_B binds ET-1, ET-3 and sarafotoxin S6c with approximately equal affinity [1–4]. Ro 46–2005 and Ro 47–0203 are pyrimidinyl naphthalenesulfonamide antagonists that bind both receptor subtypes with equal affinity [5,6]. SB 209670 is an indanecarboxylic acid that binds ET_A and ET_B receptors with high affinity [7]. Due to the potent biological activities of the ET peptides, inhibition of their action through receptor antagonism has been proposed as a point for therapeutic intervention in numerous cardiovascular diseases.

Elucidation of receptor amino acids involved in ligand binding and signal transduction is critical to understanding the mechanism of ligand–receptor–G protein interactions and may facilitate drug design. Evidence from chimeric ET_A/ET_B receptors indicates that transmembrane regions 1, 2, 3, and 7 [8] and the first extracellular loop [9] may contain a binding site for BQ-123. Takasuka et al. [10] reported that a 29 amino acid segment of the ET_B N-terminus adjacent to the first transmembrane region was important for ET-1 binding while Zhu et al. [11] reported that the lysine in TM3 of ET_B was important for ET isopeptide binding and critical for sarafotoxin S6c binding. Finally, Adachi et al. [12] suggested that Lys^{140} of the ET_A receptor is important for ET-1 binding. This residue is thought to be located at or near the C-terminus (extracellular side) of TM2. Recently, we and others reported that Tyr^{129} in TM2 of the ET_A receptor is critically involved in subtype-selective ligand binding [13,14]. Mutation of Tyr^{129} to alanine or to histidine, the corresponding amino acid in ET_B, resulted in a binding profile characteristic of an ET_B receptor. In addition, this represented one of the first indications that amino acids in the transmembrane regions are in contact with ET isopeptides as well as non-peptidic ligands [11,13-14].

Inspection of the sequence of TM2 in the human ET_A receptor indicated that there are two aspartate residues flanking Tyr^{129}; one is a turn above (Asp^{133}) and the other is a turn below (Asp^{126}) in the transmembrane helix. A similar configuration is present in ET_B, with Asp^{147} and Asp^{154} flanking His^{150}. The Asp^{126} in ET_A, or Asp^{147} in ET_B, is conserved in many GPCR [15] and has been shown to be important in agonist binding and/or signal transduction in several receptors [16–20]. According to our computer generated three-dimensional model of the

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Abbreviations: TM2, second transmembrane region; ET, endothelin; IP, inositol phosphate; PLC, phospholipase C.

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ET_A receptor and our previous studies of Tyr^{129} in the ET_A subtype-selective ligand binding, we hypothesized that the side chains of these aspartates are directed toward the putative binding cavity and may be involved in ligand binding and G protein-coupled signaling. To test these hypotheses and to investigate the role of the aspartate-tyrosine-aspartate and aspartate-histidine-aspartate helical turns in subtype-selective ligand binding, Asp^{126}Ala and Asp^{133}Ala ET_A mutants, as well as Asp^{147}Ala and Asp^{154}Ala ET_B mutants, were prepared and the binding affinities of a panel of ET receptor ligands were evaluated. Phosphoinositide turnover was measured in Asp^{126}Ala and Asp^{147}Ala mutants to assess agonist efficacy. We show here that mutation of Asp^{126} to alanine causes an increase in the affinity of the ET_B selective agonist, ET-3, and a decrease in the affinity of the ET_A selective antagonist, BMS-182874. Mutation of Asp^{133} to alanine only causes a decrease in the affinity of BMS-182874. In contrast, mutagenesis of the analogous aspartates in ET_B, Asp^{147} and Asp^{154}, failed to affect the ET_B receptor binding profile, demonstrating that TM2 is important in defining ET_A but not ET_B subtype selective ligand binding. In addition, we show that phospholipase C activation is abolished in the Asp^{126}Ala ET_A and Asp^{147}Ala ET_B mutants, indicating that, despite their differing importance in ligand binding, these residues play a critical role in transmembrane signaling in both receptor subtypes.

2. Materials and methods

2.1. Materials

Restriction enzymes and DNA modifying enzymes were from Promega (Madison, WI). The pACYC184 vector was obtained from New England BioLabs (Beverly, MA); the pCDM8 vector was from Invitrogen (San Diego, CA). The Transformer site-directed mutagenesis system was obtained from Clontech (Palo Alto, CA). The MutaGene Phagemid in vitro Mutagenesis kit was obtained from BioRad (Hercules, CA). COS-7 cells were obtained from ATCC (Rockville, MD); tissue culture reagents and Lipofectamine were from Life Technologies, Gibco/BRL (Gaithersburg, MD). [^{125}I]ET-1 (2,200 Ci/mmol) was obtained from DuPont NEN (Boston, MA), ET-1, ET-3 and sarafotoxin S6c from Peninsula Labs. Other biochemical reagents were obtained from Sigma (St. Louis, MO).

2.2. Model building

The construction of the ET_A and ET_B receptor models was performed as described previously [13]. Briefly, sequences of the receptor subtypes were aligned and the transmembrane regions identified by pairwise comparisons based on hydrophobicity conservation. The putative transmembrane sequences were then threaded through a helix bundle which was derived from the coordinates of bacteriorhodopsin [21]. For this model the kinks found in the bacteriorhodopsin helices were removed and the helices were kinked based on the location of prolines in the ET receptor sequences. Geometry of the new seven helix bundle was refined as described [13]. Molecular modeling was conducted with INSIGHT and DISCOVER (Biosym Technologies, San Diego, CA) performed on a Silicon Graphics 4D/440 work station. Model analysis and display was conducted interactively using GRASP [22].

2.3. Mutagenesis

Site-directed mutagenesis of the human placental ET_A receptor [23] was conducted in our laboratory according to Deng and Nickoloff [24] and has been described elsewhere [13]. Mutagenesis of the human placental ET_B receptor [25] was conducted according to Kunkel [26]. Briefly, the ET_B cDNA in pCDM8 was grown in the dut^-ung^- CJ236 strain of *E. coli*, resulting in DNA carrying a number of uracil containing nucleotides in place of thymine, and single stranded DNA was isolated. A mutagenic oligonucleotide was annealed to the uracil containing single stranded template and the complementary strand was synthesized in vitro. When this double stranded DNA was transformed into dut^+ung^+ MV1190 *E. coli*, the uracil containing strand was inactivated and the non-uracil containing, presumably mutant, strand was replicated. Mutant plasmids were initially identified by restriction analysis and subsequently verified by sequencing the entire cDNA insert using the dideoxy sequencing method of Sanger et al. [27].

2.4. Receptor expression and binding

Wild-type and mutant ET_A and ET_B receptor cDNAs were transfected into COS-7 cells using the polycationic lipid Lipofectamine according to the manufacturer's instructions. COS-7 cells were routinely maintained in a growth medium of Dulbecco's modified essential medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 mg/ml). Transfection of COS-7 cells with a CMV/αgal plasmid indicated a transfection efficiency of 5–20%. Cells were harvested 48–72 h after transfection and membranes were prepared as previously described [13]. [^{125}I]ET-1 (50 pM) binding assays were performed as described previously [2] with specific binding defined in the presence of 100 nM ET-1. Data were analyzed by iterative curve fitting to a one binding site model.

2.5. Measurement of phosphoinositide turnover

COS-7 cells were seeded in 6-well plates ($1–3 \times 10^4$ cells/well) in growth medium, and transfected as described above. Two to six hours following removal of transfection reagents, cells were labeled with [^{3}H]myoinositol (8 μCi/ml) for 48–72 h. Cell monolayers were washed and incubated for 15 min in DMEM containing 20 mM LiCl and 0.1% bovine serum albumin. Cells were 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 resulting mixture was applied to a Dowex AG 1×8 anion exchange column and total IP was collected as described [28]. Eluant was counted in a Packard Tri-Carb 4640 scintillation counter at 40% efficiency. As a control for expression efficiency, specific [^{125}I]ET-1 binding (50 pM) was assessed concomitantly in COS-7 cells transfected with wild-type or mutant cDNA. Following the binding reaction, the cells were washed and transferred in 500 μl of 0.5 N NaOH to a Packard Gamma counter for measurement of radioactivity.

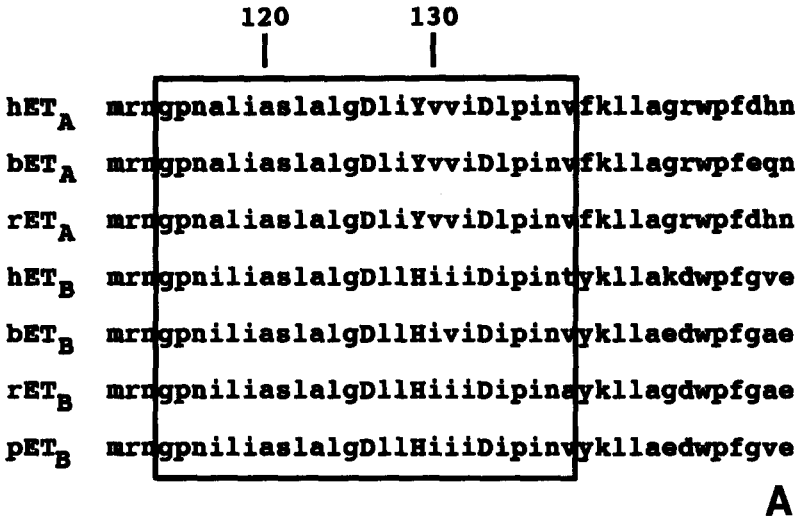
2.6. Statistical analysis

Data are expressed as mean ± standard error (S.E.) from three or more determinations.

3. Results

3.1. Binding to the Asp^{126}Ala and Asp^{133}Ala ET_A receptor mutants

Previous results with Tyr^{129} ET_A receptor mutants [13] indicated that TM2 contributes to 'fine selectivity' of ligand binding to ET receptors. Based on the same ET_A receptor model, two aspartate residues located approximately one helical turn above and below the Tyr^{129} (Fig. 1A), are oriented in a manner similar to the tyrosine with their side chains directed toward the putative ligand binding pocket (Fig. 1B). Asp^{133} and Asp^{126} are postulated to be approximately six and thirteen amino acid residues from the extracellular surface respectively. Alanines were substituted at each position and the resulting ET_A receptor mutants, Asp^{126}Ala and Asp^{133}Ala, were expressed in COS-7 cells. The affinity of peptidic agonists and antagonists and non-peptidic antagonists for the mutant receptors was compared to that for wild-type ET_A and ET_B receptors. Based on energetic criteria, ten-fold or greater alterations in affinity from control were generally considered relevant [13,29]. Inhibition of specific [^{125}I]ET-1 binding in membranes from transiently transfected COS-7 cells indicated that the affinity of ET-1 and ET-2 were not altered by either the Asp^{126}Ala or Asp^{133}Ala mutations (Table 1), suggesting that the mutant receptors have not undergone a general or large scale structural rearrangement. However, the affinity of ET-3 for the Asp^{126}Ala ET_A receptor was



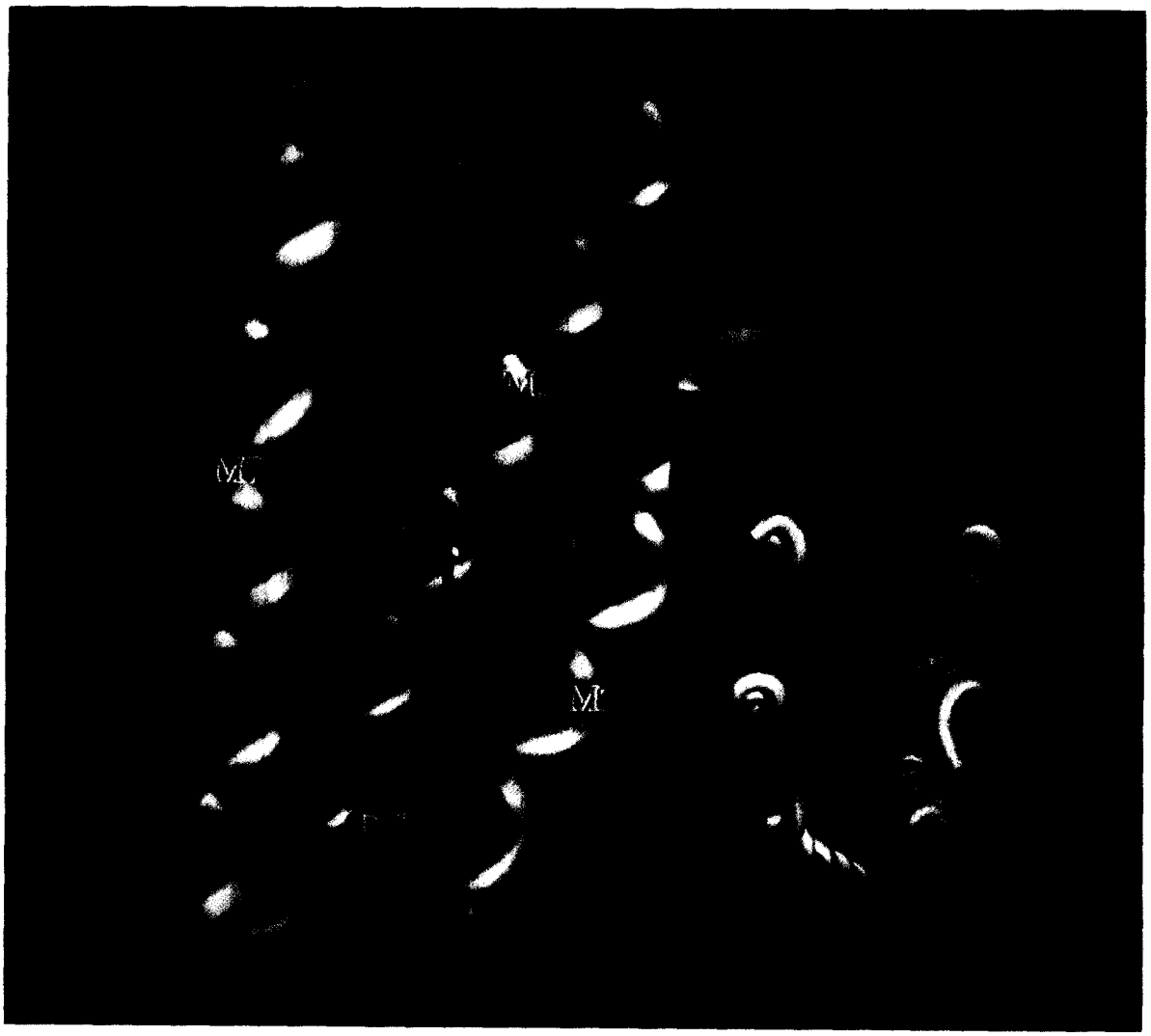


Fig. 1. Human ET_A and ET_B receptor models and corresponding amino acid sequences. Panel A: primary structure of human (h), bovine (b), rat (r) and porcine (p) ET_A and ET_B receptor sequences corresponding to predicted TM2 (indicated by the boxed residues). Numbering is according to the ET_A receptor. Panel B: comparison of the superimposed models of the second TM2 of the ET_A and ET_B receptors. Shown is the least-squares superposition (of backbone atoms) for ET_A and ET_B displayed using ribbon representation for backbone atoms of transmembrane region 7 (TM7), 2 (TM2) and 3 (TM3). The aspartates and tyrosine/ histidine residues in TM2 of ET_A/ET_B are displayed as well as Lys^{166}/Lys (ET_A/ET_B) as a point of reference. The insert to panel B shows an axial view of the helices with side chains directed towards the putative ligand binding cavity.

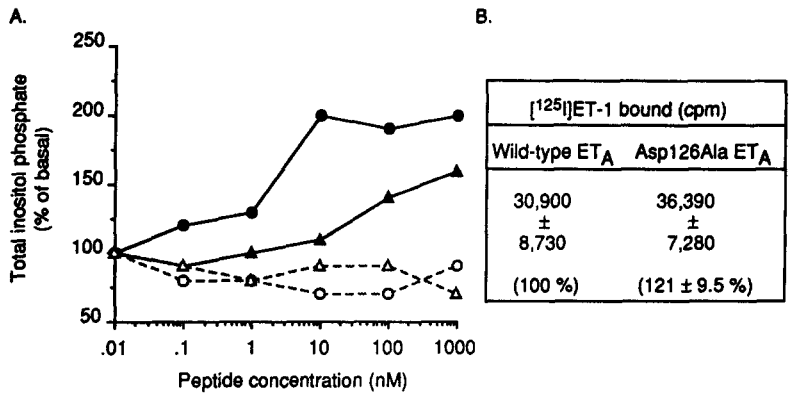


Fig. 2. Effects of ET-1 and ET-3 on inositol phosphate accumulation in COS-7 cells expressing the wild-type and mutant ET_A receptors. Panel A: COS-7 cells expressing the wild-type (solid symbols and lines) or $Asp^{126}Ala$ (open symbols and dashed lines) ET_A receptors were treated with increasing concentrations of ET-1 (circles) and ET-3 (triangles) for 15 min. IP production was measured as described in section 2. Results are representative of three similar experiments and data points are the mean of duplicate determinations. Panel B: comparison of expression levels of wild-type and $Asp^{126}Ala$ ET_A receptors in transiently transfected COS-7 cells. Specific [^{125}I]ET-1 (50 pM) binding was determined in the absence and presence of 100 nM ET-1. Data are expressed as the mean ± S.E.M. of specific [^{125}I]ET-1 binding in three experiments and were normalized to percent of wild-type levels.

improved approximately 160-fold while the affinity of BMS-182874 decreased 20-fold (Table 1). Since ET-3 is approximately 1,000-fold selective for the ET_B receptor subtype and BMS-182874 is approximately 1,000-fold selective for the ET_A receptor subtype [4], these findings support the hypothesis that Asp^{126} plays a role in subtype selective ligand–receptor interactions.

In contrast to the $Asp^{126}Ala$ binding profile, ligand affinities for the $Asp^{133}Ala$ ET_A mutant were largely unchanged from wildtype values with the exception of that for BMS-182874 (Table 1). The binding affinity (IC_{50}) for this antagonist decreased 70-fold from 340 nM to 25 μM.

3.2. Effects of mutation of Asp^{126} on ET_A-mediated phosphoinositide turnover

Recombinant wild-type ET_A receptors stably expressed in CHO cells activate phospholipase C (PLC) as assessed by measurements of intracellular IP accumulation [1,30]. Previous investigations demonstrated that the conserved aspartate residue in TM2 (corresponding to Asp^{126} in ET_A) of the α2-adrenergic [16], β2-adrenergic [19], angiotensin AT1 [18], lutropin [17] and opioid [20] receptors is crucial to signaling by agonist occupied receptors. Thus, the effect of alanine substitution for Asp^{126} on PLC activation was examined. COS-7 cells transfected with the recombinant wild-type receptor or the $Asp^{126}Ala$ variant were treated with increasing concentrations of ET-1. Cells transfected with wild-type ET_A responded to ET-1 treatment with a concentration-dependent increase in total IP accumulation (EC_{50} = 1 ± 0.6 nM). However, the IP response to ET-1 was abolished in $Asp^{126}Ala$ transfected cells (Fig. 2A). The ablation of the IP response was not due to differences in receptor expression because concomitant measurement of specific [^{125}I]ET-1 binding to wild-type and $Asp^{126}Ala$ transfected cells was similar (Fig. 2B).

Given the 160-fold increase in ET-3 binding affinity for the $Asp^{126}Ala$ ET_A receptor, it was of interest to determine if this ET_B-selective agonist could activate PLC. As shown in Fig. 2A, ET-3 produced a concentration-dependent increase in IP accumulation in cells transfected with wild-type ET_A cDNA. The estimated EC_{50} agrees with the IC_{50} value for ET-3 at the wild-type ET_A receptor (EC_{50} = 800 nM; IC_{50} = 820 nM). However, ET-3 concentrations up to 1 μM failed to elicit an increase in intracellular IP levels above basal levels in COS-7 cells expressing the $Asp^{126}Ala$ ET_A variant where the IC_{50} for ET-3 improved to 5 nM. Thus, the gain in ET-3 binding affinity observed in the $Asp^{126}Ala$ mutant did not result in a gain in ET-3-induced functional activity.

3.3. Binding to $Asp^{147}Ala$, $His^{150}Ala$, $His^{150}Tyr$, and $Asp^{154}Ala$ ET_B mutants

As it became clear that TM2 in ET_A played an important role in ligand–receptor–G protein interactions, the question arose as to whether the corresponding region in ET_B played a similar role. Examination of TM2 (Fig. 1A) of all currently cloned ET_A

Table 1
Inhibition of specific [^{125}I]ET-1 binding (50 pM) to $Asp^{126}Ala$ and $Asp^{133}Ala$ ET_A receptor mutants

Agent	Wild-type ET_A	$Asp^{126}Ala$ ET_A	$Asp^{133}Ala$ ET_A	Wild-type ET_B
ET-1	0.4 ± 0.2	0.2 ± 0.06	0.8 ± 0.12	0.2 ± 0.0
ET-2	0.4 ± 0.04	1.8 ± 0.2	1.6 ± 0.2	0.2 ± 0.0
ET-3	820 ± 260	5.0 ± 1.2	600 ± 40	0.4 ± 0.2
S6c	29,000 ± 4,000	12,100 ± 1,700	6,600 ± 320	0.3 ± 0.1
BQ-123	40 ± 6	320 ± 40	40 ± 1	66,000 ± 2,400
BMS-182874	340 ± 60	7,600 ± 200	25,000 ± 2,200	110,000 ± 27,000
Ro 47-0203	20 ± 4	40 ± 5	60 ± 6	160 ± 40

Values are inhibition constants (IC_{50}) in nM ± S.E.M. ($n \geq 3$). Binding to the wild-type ET_A and ET_B receptors is shown for comparison.

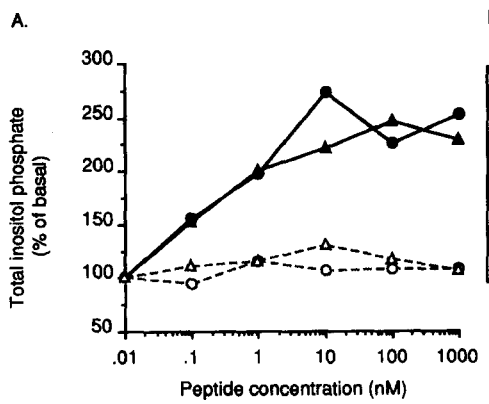


Fig. 3. Effects of ET-1 and ET-3 on inositol phosphate accumulation in COS-7 cells expressing the wild-type and mutant ET_B receptors. Panel A: COS-7 cells expressing the wild-type (solid symbols and lines) or $Asp^{147}Ala$ (open symbols and dashed lines) ET_B receptors were treated with increasing concentrations of ET-1 (circles) and ET-3 (triangles) for 15 min. IP production was measured as described in section 2. Results are representative of three similar experiments and data points are the mean of duplicate determinations. Panel B: comparison of expression levels of wild-type and $Asp^{147}Ala$ ET_B receptors in transiently transfected COS-7 cells. Specific [^{125}I]ET-1 (50 pM) binding was determined in the absence and presence of 100 nM ET-1. Data are expressed as the mean ± S.E.M. of specific [^{125}I]ET-1 binding in three experiments and were normalized to percent of wild-type levels.

and ET_B receptors shows a single major non-conservative amino acid difference: Tyr^{129} in ET_A is His^{150} in ET_B. All other sequence differences are conservative replacements (ET_A/ET_B: Ile^{128}/Leu, Val^{130}/Ile, Val^{131}/Ile, Leu^{134}/Ile) except for Ala^{117}/Ile and Val^{138}/Thr. Val^{138} of human ET_A and Thr^{159} in human ET_B are of similar size while both Ala^{117} and the corresponding ET_B amino acid, Ile^{138}, are neutral, hydrophobic, aliphatic amino acids. Thus, both of these replacements can also be considered conservative. Therefore, except for the tyrosine/histidine divergence one might expect the configuration of side chains in TM2 to be similar in ET_A and ET_B. Interestingly, the aspartic acids in TM2 of ET_A (Asp^{126} and Asp^{133}), hypothesized to be facing the putative binding cavity, are conserved in ET_B (Asp^{147} and Asp^{154}) (Fig. 1B). These considerations suggested that it might be informative to mutate the histidine and aspartate residues in TM2 of ET_B to alanine. In addition, His^{150} was mutated to tyrosine, the amino acid present in the ET_A receptor.

Compared to the wild-type ET_B receptor, ligand affinities to the $Asp^{154}Ala$ and $Asp^{147}Ala$ ET_B mutants did not show greater than 10-fold changes (Table 2). On the other hand, when His^{150} was mutated to alanine, the binding affinity for sarafotoxin S6c decreased 30-fold from 0.3 nM to 8 nM. Moreover, mutation of this histidine residue to tyrosine led to a decreased affinity of 10- to 200-fold for ET-3 and sarafotoxin S6c respectively. Thus, as the amino acid at position 150 of TM2 became more ET_A-like, the affinity for ET_B selective peptides was reduced. Interestingly, binding of the ET_A/ET_B non-selective sulfonamide Ro 47-0203 decreased approximately 10-fold in the $His^{150}Ala$ mutant. These findings are consistent with the suggestion that His^{150} in TM2 of ET_B is involved in high affinity binding of the ET_B selective agonists, ET-3 and sarafotoxin S6c, and provides some interaction for the non-selective antagonist Ro 47-0203.

3.4. Effects of mutation of Asp^{147} on ET_B-mediated phosphoinositide turnover

Despite the lack of effects that mutation of Asp^{147} had on agonist binding affinity, it was of interest to determine if $Asp^{147}Ala$ ET_B receptors had altered agonist efficacy. Similar to ET_A receptors, recombinant ET_B receptors that are stably expressed in CHO cells activate PLC as assessed by measurements of IP accumulation [1,30]. In the present experiments, treatment of COS-7 cells transiently expressing wild-type ET_B receptors with ET-1 and ET-3 led to a concentration-dependent increase intracellular IP (Fig. 3A). The EC_{50} values for the ET-1 and ET-3 induced-responses were 0.2 ± 0.1 nM and 0.6 ± 0.4 nM, respectively. However, both agonists failed to increase IP levels in COS-7 cells expressing $Asp^{147}Ala$ ET_B receptors (Fig. 3A). Concomitant experiments demonstrated that the level of specific [^{125}I]ET-1 binding was similar for COS-7 cells transfected with wild-type or $Asp^{147}Ala$ ET_B receptors, consistent with equivalent receptor expression (Fig. 3B).

4. Discussion

Identification of receptor amino acids involved in ligand binding and signal transduction is a fundamental step in elucidating ligand–receptor–G protein interactions and in under-

Table 2
Inhibition of specific [^{125}I]ET-1 binding (50 pM) to $Asp^{147}Ala$, $His^{150}Ala$, $His^{150}Tyr$ and $Asp^{154}Ala$ ET_B receptor mutants

Agent	Wild-type ET_B	$Asp^{147}Ala$ ET_B	$His^{150}Ala$ ET_B	$His^{150}Tyr$ ET_B	$Asp^{154}Ala$ ET_B
ET-1	0.2 ± 0.0	0.1 ± 0.02	0.2 ± 0.1	1.5 ± 0.1	0.1 ± 0.02
ET-3	0.4 ± 0.2	0.1 ± 0.03	1 ± 0.1	6 ± 0.4	0.2 ± 0.1
S6c	0.3 ± 0.1	1.2 ± 0.6	8 ± 1	50 ± 4	0.1 ± 0.03
BMS-182874	110,000 ± 27,000	> 50,000	> 50,000	> 50,000	> 50,000
Ro 47-0203	160 ± 40	95 ± 5	1,700 ± 400	700 ± 30	190 ± 20

Values are inhibition constants (IC_{50}) in nM ± S.E.M. (n = 3–6). Binding to the wild-type receptor is shown for comparison.

standing the mechanisms by which GPCRs transduce signals from the extracellular to intracellular milieux. Several recent studies have begun to characterize residues in the ET receptors that are involved in agonist and antagonist binding [8-12] as well as in defining the subtype-selective ligand binding profile [13-14]. Less information is presently available on specific residues involved in signal transduction [31]. In the present study we demonstrate that the aspartate residues flanking Tyr^{129} of ET_A are involved in binding and PLC activation (Asp^{126}) and that the corresponding aspartate (Asp^{147}) in the ET_B receptor are involved in signaling.

A significant finding from this study was the altered ligand binding profile of the Asp^{126}Ala ET_A mutant. While we had recently observed a 200-fold improvement in ET-3 affinity for the Tyr^{129}Ala ET_A receptor mutant [13], the observation that mutation of Asp^{126}, a residue proposed to lie 13 amino acids within the TM2, similarly improves ET-3 affinity was unexpected. In the absence of ligand–receptor structure-activity data that would point clearly toward a direct ligand interaction with Asp^{126}, and because our model suggests that Asp^{126} is deep within the transmembrane region, we must consider the possibility that Asp^{126} influences binding by affecting the position of the Tyr^{129} side chain in the binding cavity rather than by directly interacting with a ligand. Recently, it has been shown that mutation of Tyr^{129} to phenylalanine [14] as well as alanine [13,14] causes more than a 100-fold increase in ET-3 binding. Since ET-3 binding improved in the Tyr^{129}Phe mutant, a direct, unfavorable interaction of ET-3 with the phenolic OH of the Tyr^{129} is possible. However, in light of the present Asp^{126}Ala data, another possibility is that the OH group of Tyr^{129} is involved in specific interactions with Asp^{126}. According to this view, the interaction of Asp^{126} with the phenolic OH may constrain the orientation of Tyr^{129}. Removal of the OH group, as in the Tyr^{129}Phe mutant, or mutation of Asp^{126} to alanine may alter these constraints and allow for steric rearrangements that result in improved ET-3 binding.

In contrast to ET-3, sarafotoxin S6c binding affinity to Asp^{126}Ala receptors was not improved (Table 1). Sarafotoxin S6c and ET-3 are 50% homologous, suggesting that one or more of the non-conserved amino acid residues in ET-3 is responsible for the improved affinity for the Asp^{126}Ala mutant receptor. In this regard, it is important to note that for both the Tyr^{129}Ala and Asp^{126}Ala mutants, the side chains of the native receptor were replaced with amino acids containing side chains of considerably less bulk. Correspondingly, ET-3 has amino acids of greater steric bulk at positions 4 and 5 when compared to ET-1, ET-2 and sarafotoxin S6c (Phe4 and Thr^5 in ET-3 v Ser^4 and Ser^5 in ET-1 and ET-2 v Asn^4 and Asp^5 in sarafotoxin S6c). Thus, reduction in the bulk of receptor amino acids may contribute to the changes observed in ligand binding affinities. Additional experiments are necessary to precisely map the binding sites for the ET peptides.

Interaction with Asp^{133} of ET_A receptors appears not to be a general requirement for conferring the well-defined subtype-selectivity profile since only the affinity of BMS- 182874 was altered in this mutant from wild-type ET_A receptor values. Rather, it seems more likely that structural differences between BMS-182874 and BQ-123 lead to disparate contacts in the ET_A receptor and that only BMS-182874 binding is dependent on the presence of Asp^{133}. It is interesting to note that the pyrimidinyl naphthalenesulfonamide, Ro 47–0203, which binds to ET_A and ET_B receptors with approximately equal affinity [6], had similar binding affinity for wild-type and Asp^{133}Ala mutant ET_A receptors. This may suggest that the decreased affinity of BMS-182874 for the Asp^{133}Ala ET_A mutant was specific for the ET_A selective naphthalenesulfonamide series of antagonists.

That Asp^{126} and Tyr^{129} [13] appear to be important for the high affinity binding of ET-3 and BMS-182874 is consistent with the hypothesis that the binding sites for agonists and antagonists are, at least in part, overlapping. On the other hand, the data showing that Asp^{133} is critical only for the high affinity binding of BMS-182874 suggests that the binding site for this antagonist is also, at least in part, distinct from the binding site for the peptides. This is reminiscent of the observation that agonists and antagonists bind differently to the AT1 angiotensin II [32], cholecystokinin-B/gastrin [33], neurokinin-1 [34- 35] and bradykinin receptors [36]. Studies in progress that are aimed at docking ligands into the putative binding cavities of the ET receptors should yield more precise information on agonist and antagonist binding sites.

Asp^{126} in ET_A and Asp^{147} in ET_B receptors correspond to the aspartate residue in TM2 that is conserved in many GPCR [15]. This conserved aspartate has been shown to play a role in agonist binding and transmembrane signaling via G proteins in the α2-adrenergic [16], LH [17] and AT1 angiotensin II [18] receptors and has been implicated in the allosteric modulation of agonist binding in the α2-adrenergic [37], somatostatin type 2 [38] and serotonin receptors [39] by sodium ion. A significant finding from the present study was the requirement of Asp^{126} in the ET_A receptor and Asp^{147} in the ET_B receptor for PLC activation. Thus, agonist efficacy did not correlate with binding affinity in the Asp^{126}Ala ET_A or Asp^{147}Ala ET_B mutants as high affinity ET-1 and ET-3 binding is better than or comparable to that in wild-type receptors yet stimulation of IP accumulation was abolished. Because of the transient nature of receptor expression in these experiments, care was taken to concomitantly assess agonist binding and efficacy. In all experiments, specifically bound [^{125}I]ET-1 to mutated ET_A or ET_B receptors was equal to or slightly greater than that to the corresponding wild-type ET receptors demonstrating that reduced efficacy was not due to reduced receptor expression. At present, it is not clear if the receptor mutations have an effect on G protein coupling or whether this in turn effects agonist binding. Such investigation is best conducted in mammalian cell lines that stably express the mutant receptors rather than the transient expression studied here. However, high concentrations (200 μM) of the non-hydrolyzable guanine nucleotide, GTPγS, did not alter the binding affinity of ET-1 to transiently expressed wild-type or Asp^{126}Ala ET_A receptors (E.C-K. Liu and M.L. Webb, unpublished) suggesting that either most of the ET_A receptors are already uncoupled from G-protein or that ET-1 binding is nucleotide independent.

In addition, the present study has demonstrated that, despite the 160-fold improvement in ET-3 affinity to the Asp^{126}Ala mutant, the efficacy of this agonist remained ET_B-like. Although the precise reason for this finding is unclear, these data are consistent with the suggestion that the removal or re-positioning of residues in TM2 that led to enhanced ET-3 binding, possibly through localized structural changes in the Asp^{126}-Tyr^{129}-Asp^{133} region, are different from the receptor conformational changes that are necessary for signal transduction normally triggered by Asp^{126}.

In summary and in combination with our previous studies [13], the present data demonstrate that the Asp^{126}-Tyr^{129}-Asp^{133} turn in TM2 contributes to ET_A receptor subtype-selective ligand binding, largely defined by Tyr^{129}, and to transmembrane signal transduction. In contrast, whereas His^{150} contributes to high affinity ET-3, sarafotoxin S6c and Ro 47-0203 binding, the corresponding aspartates in TM2 of the ET_B receptor, Asp^{147} and Asp^{154}, are not involved in defining the agonist binding profile. Thus, a region that is critical in defining high affinity ligand binding and transmembrane signaling in ET_A is involved predominantly in signaling in ET_B.

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