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Replacement of Lysine-181 by Aspartic Acid in the Third Transmembrane Region of Endothelin Type B Receptor Reduces Its Affinity to Endothelin Peptides and Sarafotoxin 6c Without Affecting G Protein Coupling

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Abstract A conserved aspartic acid residue in the third transmembrane region of many of the G protein-coupled receptors has been shown to play a role in ligand binding. In the case of endothelin receptors, however, a lysine residue replaces this conserved aspartic acid residue. To access the importance of this residue in ligand binding, we have replaced it with an aspartic acid in the rat endothelin type B (ETb) receptor by PCR mediated mutagenesis. The binding characteristics and functional properties of both the wild type and mutant receptors were determined in COS-7 cells transiently expressing the cloned receptor cDNAs. Using ^{125}I-ET-1 as the radioactive peptide ligand in displacement binding studies, the wild type receptor displayed a typical non-isopeptide-selective binding profile with similar IC_{50} values (0.2–0.6 nM) for all three endothelin peptides (ET-1, ET-2, and ET-3) and sarafotoxin 6c (SRTX 6c). Interestingly, the mutant receptor showed an increase in IC_{50} values for ET-1 (5 nM), ET-2 (27 nM), and ET-3 (127 nM) but displayed a much larger increase in IC_{50} value for SRTX 6c (> 10 uM). The lysine mutant receptor still elicited full inositol phosphate (IP) turnover responses in the presence of saturating concentrations of endothelins (10 nM of ET-1, 100 nM of ET-2, or 1 uM of ET-3), indicating that the mutation (K181D) did not affect the coupling of mutant receptor to the appropriate G protein. These results demonstrate that lysine-181 on the receptor is important for binding ET peptides; however, it is required for binding the ETb selective agonist-SRTX 6c.

Key words: G protein-coupled receptor, site-directed mutagenesis, receptor binding, inositol phosphate, agonist, antagonist

The endothelins and the sarafotoxins are two structurally related families of potent vasoactive peptides which contain 21 amino acids and two intramolecular disulfide bridges. From studies of the structure-activity relationships of endothelin peptides, Nakajima et al. [1989] have shown that both terminal amino and carboxyl groups, carboxyl side chains of Asp-8 and Glu-10, and the aromatic side chain of Phe-14 are important for binding of the ET ligand to the endothelin receptors [Nakajima et al., 1989]. Recently, sarafotoxin 6c (SRTX 6c) was shown to be a highly selective ETb agonist with 300,000-fold higher affinity for ETb than for ETa receptor [Williams Jr. et al., 1991]. Furthermore, structure-activity studies of sarafotoxin S6b and S6c chimeric peptides [Takasaki et al., 1991] suggested that the difference in affinities for ETa receptor between these two peptides is related to the amino acid residue at position 9 (glutamate in SRTX 6c verses lysine in SRTX 6b). These data indicate that ionic interactions are important in determining the binding affinities of ligands such as endothelins and sarafotoxins to ET receptors.

Recently, two types of endothelin receptors, ETa and ETb, have been cloned [Arai et al., 1990; Sakurai et al., 1990; Lin et al., 1991; Sakamorto et al., 1991; Nakamuta et al., 1991; Ogawa et al., 1991]. The two receptors differ in the rank order of their binding affinities for

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ET-1, ET-2, and ET-3. The ETa receptor has the highest selectivity for the peptide ligands ET-1, followed by ET-2 and ET-3. The ETb receptor, however, binds to all three ET peptides with equal affinity. Both ETa and ETb receptors belong to the seven membrane spanning domain G protein-coupled receptor family. In many members of the gene family (e.g., adrenergic, muscarinic, dopamine, and serotonin receptors) which bind biogenic amines, several specific aspartic acid residues in the second and third membrane spanning domains are highly conserved and have been demonstrated to be essential for ligand binding and/or G protein coupling [Strader et al., 1987; Strader et al., 1988; Chung et al., 1988; Fraser et al., 1988]. Interestingly, one of the conserved aspartic acid residues in the third membrane spaning domain is replaced by a lysine residue in both types of endothelin receptors, ETa and ETb. To investigate the possibility that this specific lysine residue (lysine-181) in ETb receptor may be involved in binding to one of the carboxyl groups of the ET ligands, we have constructed a mutant ETb receptor in which the lysine-181 residue has been replaced by an aspartic acid residue and studied its effects on ligand binding and second messenger responses.

EXPERIMENTAL PROCEDURES

Materials

Sprague-Dawley rats were from Charles River Breeding Laboratories, Wilminhton, MA. The Fast Track mRNA Isolation Kit, pCR1,000, and pRC/CMV vectors were from Invitrogen. AMV reverse transcriptase and oligo(dT)$_{15}$ primer were from Promega. ^{125}I-labelled ET-1 (2,000 Ci/mM) and [^{3}H]-inositol (108.4 Ci/mM) were from Amersham. Unlabeled ET-1, ET-2, and ET-3 were from Peninsula Lab. Sarafotoxin 6c was from BACHEM Bioscience.

Isolation of Rat ETb Receptor cDNA

Whole brains were removed from 150 g male Sprague-Dawley rats. Brain mRNA was isolated following the protocols of the Fast Track mRNA Isolation Kit. First strand cDNA was synthesized from brain mRNA using AMV reverse transcriptase and oligo(dT)$_{15}$ primer. The protein coding portion of the rat ETb receptor cDNA was amplified from the first strand cDNA by polymerase chain reaction (PCR) using primers derived from the published DNA sequences [Sakurai et al., 1990] (primer A, 5′-TGC AAC ATG CAA TCG TCC GCA AGC CGG-3′ and primer B, 5′-CCT TCA AGA TGA GCT GTA TTT ATT GCT GGA-3′). The amplified ETb receptor DNA was first cloned into the TA vector then re-inserted into the mammalian expression vector pRC/CMV. The inserted DNA was sequenced and found to be identical to the published rat ETb receptor DNA sequence.

Mutant Construction and Transfection

Lysine-181 of the ETb receptor was replaced by an aspartic acid via PCR-mediated mutagenesis [Higuchi 1990]. In brief, two complimentary mutant primers (5′-TTC ATA CAG GAC GCT TCT GTG GGG ATC-3′) containing the underlined K181D mutation sequences (AAG change to GAC) were used in conjuction with primers A and B to amplify the N- and C-terminal portion of the mutant receptor gene. Full length mutant receptor DNA was amplified in a secondary PCR reaction using A and B primers and the overlapping mutant N- and C-terminal fragments as templates. The amplified mutant receptor DNA was cloned into the pCR1,000 vector then re-inserted into the mammalian expression vector pRC/CMV. The sequence of the mutant ETb receptor gene was confirmed by DNA sequencing. COS-7 cells were propagated in Dulbecco's modified Eagles medium with 10% fetal bovine serum, in 5% CO_2. Wild type and mutant DNAs (20 μg each) were transfected into COS-7 cells (6×10^7 cells/ml) by electroporation techniques. After electroporation, cells were diluted 1:40 and seeded on multiple 12-well plates at 1.5×10^6 cells/well.

Receptor Binding Assay

Two days after transfection, cells were washed twice with 2 ml of assay buffer (EMEM with 20 mM HEPES buffer, pH 7.4, 0.1% BSA, 0.2% bacitracin). Cells were then incubated either with various concentrations of ^{125}I-labeled ET-1 (saturation binding assay) or with 20 pM ^{125}I-labeled ET-1 plus the indicated concentrations of unlabelled peptides (displacement binding assay) for 60 min at 37°C in a 0.5 ml of assay buffer. After incubation, the cells were washed twice with 1 ml ice-cold DPBS and the reaction was terminated by the addition of 0.5 ml 2% SDS. Cell-bound radioactivity was determined in a gamma radioactive counter. The K_d, B_{max}, and IC_{50} values were determined by using the Ligand computer program.

Inositol Phosphate Assay

After transfection, COS-7 cells were seeded on 12-well plates and incubated in medium containing [^{3}H]-inositol (1 Ci/ml). Two days later, the cells were washed twice with 1 ml of assay buffer (EMEM with 10 mM LiCl, 20 mM HEPES, and 1 mg/ml BSA) and then incubated at 37°C for 1 h with 1 ml assay buffer containing the indicated agents. To terminate the incubation, the medium was aspirated and replaced with 0.5 ml of ice-cold 5% trichloroacetic acid. Total [^{3}H]-inositol phosphates were analyzed by applying the trichloroacetic acid extract directly to Dowex anion exchange columns. [^{3}H]-Inositol was removed by washing with 10 ml of 5 mM inositol, and total [^{3}H]-inositol phosphates were eluted with 4 ml of 1 M ammonium formate in 0.1 M formic acid.

RESULTS

As shown in Figure 1A, COS-7 cells transfected with the wild type ETb receptor cDNA displayed specific and saturable binding to ^{125}I-ET-1. No specific binding was observed from nontransfected cells or from cells transfected with the vector DNA alone. Scatchard analysis (Fig. 1B) showed a single high-affinity binding site for ET-1. The K_d and B_{max} were 0.2 nM and 253 fmole per 1×10^6 cells, respectively. The K_d value for ET-1 for the expressed ETb receptor is very similar to the K_d value (0.18 nM) for the cloned bovine ETb receptor [Arai et al., 1990]. In a similar experiment (data not shown), in which the COS-7 cells were transfected with the mutant ETb receptor cDNA, a significant increase (20-fold) in K_d value (4 nM) for ET-1 was observed which suggested that Lys-181 might be involved in ligand binding of the ET-1 peptide. The B_{max} value (274 fmole/1×10^6 cells) for the mutant is similar to the value in the wild type receptor.

To further characterize the mutant receptor, we examined the ability of several endothelin and sarafotoxin peptides to displace ^{125}I-ET-1 binding. As shown in Figure 2A, the wild type receptor bound ET-1, ET-2, ET-3, and SRTX-6c with essentially equal affinities (Table I). Interestingly, the rank order of binding affinities for these peptides in mutant ETb receptor is very different from that of the wild type with the following order: ET-1 > ET-2 > ET-3 ≫ SRTX-6c (Fig. 2B and Table I). Thus the binding profile for these peptides in ETb mutant is close parallel to that of the ETa receptor except

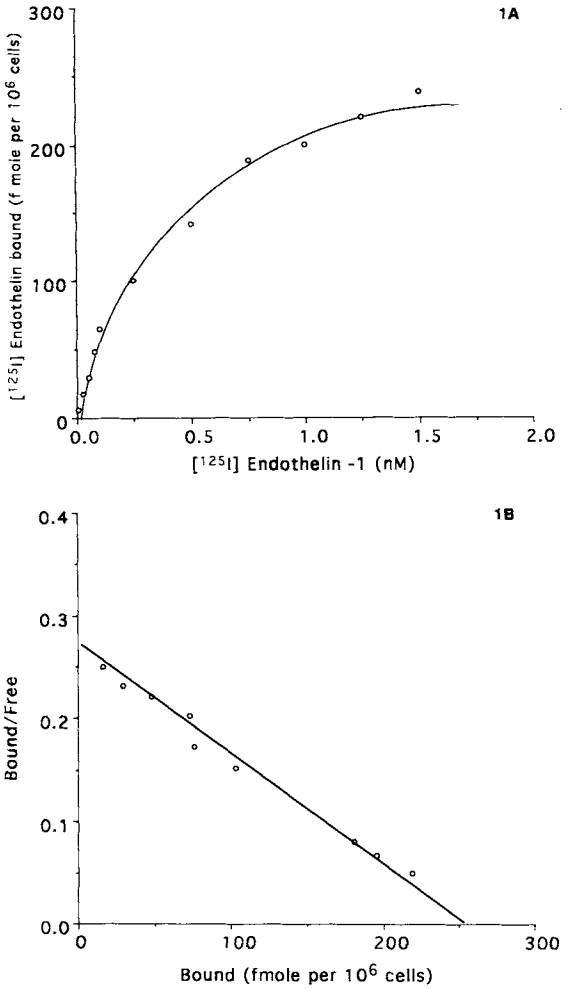

Fig. 1. Saturation binding and Scatchard analysis of ^{125}I-labeled ET-1 to cells transfected with receptor cDNA. (**A**) Saturation binding curve, (**B**) Scatchard plot. Data are representative of two separate experiments each performed in triplicate.

that the binding affinities are much lower in the mutant receptor. It is interesting that substitution of a single amino acid can change the rank order of binding affinities for these peptides. Since the ETa receptor also contains a lysine residue at the corresponding position, it will be interesting to see the effects of this mutation on ETa receptor. It is possible that the lysine residue in ETa receptor may play a different functional role than the same residue in the ETb receptor, since rat ETa and ETb receptors share only 55% identity of protein sequence [Lin et al., 1991]. Our results seems to suggest that lysine 181 in the ETb receptor is critical for high affinity binding to the selective ETb agonist, SRTX 6c.

To investigate whether the mutant receptor was still functionally coupled to its effector system, the ability of ET-1, ET-2, and ET-3 to

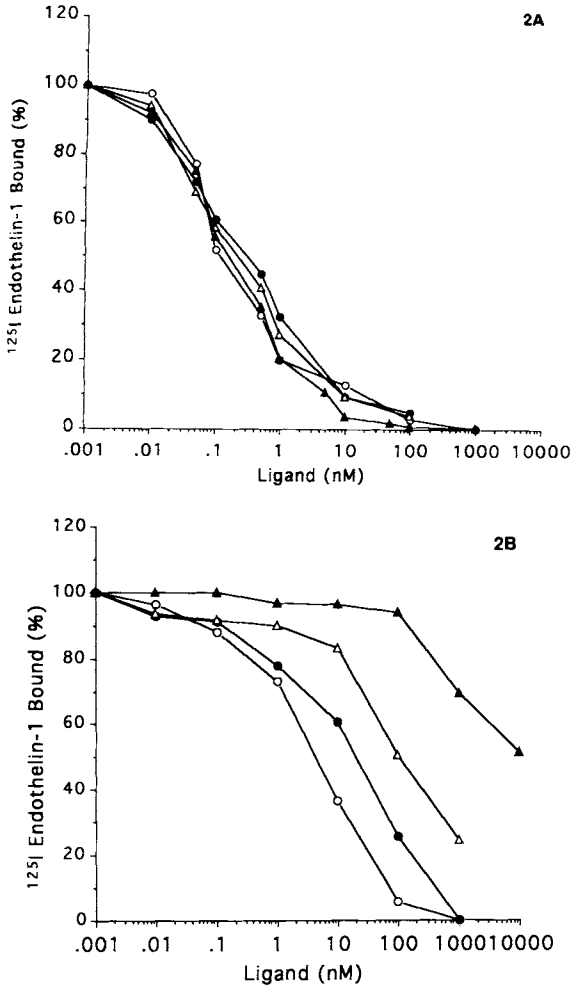

Fig. 2. Competitive inhibition of ^{125}I-ET-1 binding by unlabeled ET-1, ET-2, ET-3, and sarafotoxin 6c in COS-7 cells expressing wild type and mutant receptors. ET-1 (open circles), ET-2 (solid circles), ET-3 (open triangles), and SRTX 6c (solid triangles). Competition binding curves with wild type receptor (**A**), and mutant receptor (**B**). Data are expressed as percent of specific ^{125}I-ET-1 binding and represent two or three experiments performed in triplicate. The values for 100% maximal binding are in the range of 3,000 to 4,000 cpm.

stimulate IP turnover in cells transfected with the ETb mutant receptor gene was examined. As shown in Figure 3, the wild type receptor can elicite full IP turnover responses with the addition of 10 nM of each ET peptide. For the mutant receptor, the peptide concentrations necessary for full responses were higher but closely paralleled the IC_{50} values for these peptides. The magnitude of IP production (data not shown) was also similar between cells expressing wild type and mutant receptors. From these results it was concluded that the K181D substitution did not alter the ability of the receptor to couple to its normal effector system.

TABLE I. IC_{50} Values of ET-1, ET-2, ET-3, and Sarafotoxin-6c in Competition Binding Studies With ^{125}I-ET-1*

Compound	Wild type IC_{50} (nM)	Mutant IC_{50} (nM)
ET-1	0.19 ± 0.14	4.8 ± 2.4
ET-2	0.18 ± 0.15	26.8 ± 12.6
ET-3	0.20 ± 0.12	127.2 ± 51.0
Sarafotoxin 6c	0.63 ± 0.09	≥ 10 μM

*IC_{50} values are calculated from data in Fig. 2 using the Ligand computer program.

DISCUSSION

In this report we have shown that substitution of lysine-181 by an aspartic acid residue in the ETb receptor reduced its affinity for ET peptide ligands and a specific agonist, STRX 6c, without affecting its ability to couple to the appropriate G protein. Since ET-1 and ET-2 peptides bind to the mutant ETb receptor with nanomole affinity and the mutant receptor and G protein coupling was normal, it seems unlikely that the significant reduction in affinities for ET-3 and sarafotoxin 6c is due to altered folding of the mutant receptor caused by the lysine to aspartic acid substitution. A working hypothesis for the functional role of lysine-181 is that this residue may be involved in binding to the C-terminal end of ET peptides and sarafotoxins. Supporting evidence for this hypothesis comes from structural considerations. If it is assumed that the N-terminal portion of the ET peptides and sarafotoxins containing the two disulfide bridges is too bulky to enter the cleft on the receptor, the lysine-181 residue may be located on the surface of the receptor in the correct position to interact with the C-terminal end of these peptides. Since all ET peptides share the same C-terminal sequence but only ET-1 and ET-2 still bind to the mutant receptor with relatively high affinity, the results would suggest that the remaining portions of ET-1 and ET-2 molecules contribute more to the overall binding affinity for the mutant ETb receptor than does the counter part of ET-3. This hypothesis might be tested by studying the binding affinity of small synthetic peptides corresponding to the C-terminal end of ET peptides.

Since all ET peptides still elicite IP responses in cells expressing mutant receptors, binding to lysine-181 is not required for the receptor to couple to its effector systems. These results are consistent with a recent study [Strader et al., 1991] in which the aspartic acid residue-113 in

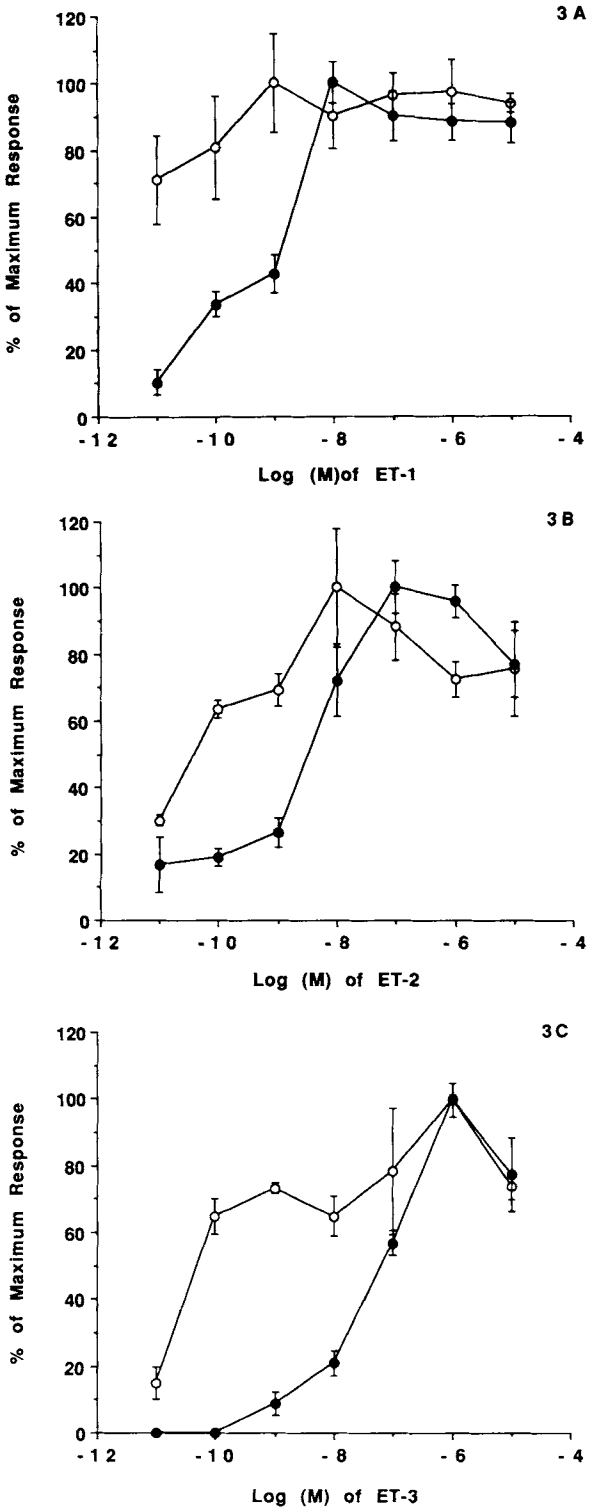

Fig. 3. Stimulation of total inositol phosphate production in COS-7 cells expressing wild type and mutant ETb receptors. Stimulation of wild type receptor (open circles) and mutant receptor (solid circles), with different concentrations of ET-1 (A), ET-2 (B), and ET-3 (C). Data are expressed as percent of maximal IP response with basal substracted. The values for maximal IP response and basal are in the range of 2,600–3,600 cpm and 900–1,000 cpm, respectively. Each data point represents the mean ± standard error of triplicate samples.

β-adrenergic receptor (corresponding to the lysine-118 in ETb receptor) was replaced by a serine residue. This substitution altered the ligand binding specificity of the receptor but the mutant receptor could still be fully activated. If lysine-181 of ETb receptor is important for binding to selective ligand as our results suggest, it may have important implications for identifying specific agonists and antagonists. Though a selective antagonist for ETa receptor has been recently reported [Ihara et al., 1991], no selective antagonist for ETb receptor has been reported. By comparing the amino acid sequences surrounding the lysine residue of the ETa and ETb receptors, it may be possible to design an ETb antagonist with greater affinity and/or selectivity.

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