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ACCELERATED COMMUNICATION

Subtype Selectivity of a Novel Endothelin Antagonist, FR139317, for the Two Endothelin Receptors in Transfected Chinese Hamster Ovary Cells

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SUMMARY

We investigated the receptor-binding properties and the antagonist activities of FR139317, a novel endothelin (ET) antagonist, in transfected Chinese hamster ovary cells permanently expressing the two ET receptor subtypes (ET_A and ET_B). In displacement analysis using membrane preparations derived from the receptor-expressing cells, FR139317 showed a high affinity for ET_A (K_i = 1 nM) and a lower affinity for ET_B (K_i = 7.3 μM). FR139317 inhibited ET_A-mediated phosphatidylinositol hydrolysis and arachidonic acid release and produced a parallel shift in the dose-response curve for ET-1, with respective pA_2 values of 8.2 and 7.7. In contrast, FR139317 had no inhibitory effects on these ET-1-induced responses in ET_B-expressing cells. FR139317 itself showed no stimulatory effects on phosphatidylinositol hydrolysis and arachidonic acid release in ET_A- and ET_B-expressing cells. Thus, FR139317 is a potent, competitive, and highly selective antagonist for ET_A. This compound should be a powerful tool for investigation of the physiological properties of ET_A and exploration of its role in diseases.

ETs are members of a family of three different peptides, ET-1, ET-2, and ET-3, which consist of 21 amino acid residues with two interconnecting disulfide linkages (1, 2). ET-1 was first identified as a potent vasoconstrictor produced by vascular endothelial cells (1). Subsequently, the members of the ET family were found to have a variety of biological activities in both vascular and nonvascular tissues, including hemodynamic, cardiac, pulmonary, and renal effects (1, 3–7), the modulation of neural functions (8), and cell mitogenesis (9). Recent molecular cloning studies demonstrated the existence of a family of two ETR subtypes, termed ET_A and ET_B (10–13). The two ETRs have seven hydrophobic segments and share a significant sequence similarity with guanyl nucleotide-binding protein-coupled receptors. ET_A exhibited a rank order of binding affinities of ET-1 > ET-2 ≫ ET-3, whereas ET_B showed a binding selectivity with comparably high affinities for the three ET peptides. *In situ* and blot hybridization analyses revealed that the mRNAs for rat ET_A and ET_B show specialized expression patterns in cell types in both brain and peripheral tissues (14). ET_A mRNA is predominantly expressed in vascular smooth muscle cells of a variety of tissues, bronchial smooth muscle cells, myocardium, and the pituitary gland. ET_B mRNA is more widely distributed in various cell types of many tissues but is not significantly expressed in vascular smooth muscle cells (14). Our transfection experiments using CHO cells indicated that both ET_A and ET_B mediated the activation of PI hydrolysis and arachidonic acid release but exhibited distinct effects on the cAMP cascade when expressed in the same cell type (15). Thus, the varied physiological responses of ETs occur as a result of the selectivity, different distribution, and distinct signal transduction pathways of the two receptors.

The involvement of ETs in the pathology of human diseases, including essential hypertension, acute myocardial infarction, renal failure, and subarachnoid hemorrhage, has been suggested by studies of animal models and humans (16–19). To assess the disparate roles of the multiple receptor subtypes in each disease, it is important to study the precise pharmacological properties of the two individual ETRs. Recently, several ET antagonists were reported (20, 21). However, the accurate determination of the potencies of antagonists for individual receptors was hampered by the possible existence of multiple receptor subtypes in tissues or cell preparations. The functional expression of the cDNA clone for each ETR subtype in the same cell type can provide a useful system to study the pharmacological profiles

ABBREVIATIONS: ET, endothelin; ETR, endothelin receptor; FR139317, (*R*)2-[(*R*)-2-[(*S*)-2-[[1-(hexahydro-1*H*-azepinyl)]carbonyl]amino-4-methylpentanoyl]amino-3-[3-(1-methyl-1*H*-indolyl)]propionyl]amino-3-(2-pyridyl)propionic acid; PI, phosphatidylinositol; CHO, Chinese hamster ovary; BSA, bovine serum albumin; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

of antagonists for a single receptor subtype without any ambiguity resulting from the presence of multiple receptor subtypes (15). FR139317 (Fig. 1) is a novel ET antagonist that is effective on ET-induced vasoconstriction and pressor responses (22). In this investigation, we extended the pharmacological characterization of FR139317 and examined the potencies and selectivities of FR139317 for the two ETR subtypes in transfected CHO cells.

Experimental Procedures

Materials. Materials were obtained from the following sources: α-minimal essential medium lacking ribonucleosides and deoxyribonucleosides from Flow (Irvine, Scotland); Dulbecco's modified Eagle medium from Nissui (Tokyo, Japan); dialyzed fetal bovine serum from Cell Culture Laboratories (Cleveland, OH); ^{125}I-ET-1 (2000 Ci/mmol) and *myo*-[2-^{3}H]inositol (18.8 Ci/mmol) from Amersham (Des Plaines, IL); [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (181.2 Ci/mmol) from DuPont/New England Nuclear (Boston, MA); and ET-1, ET-2, and ET-3 from Peptide Institute (Osaka, Japan). FR139317 was prepared by Exploratory Research Laboratories of Fujisawa Pharmaceutical Co., Ltd. (Tsukuba, Japan).

Cell culture. CHO (*dhfr*$^-$) cells that were transfected with and stably expressed bovine ET_A and rat ET_B have been described previously (15). Cells were maintained in α-minimal essential medium lacking ribonucleosides and deoxyribonucleosides, supplemented with 10% dialyzed fetal bovine serum.

Ligand binding of ETRs. For the determination of the ligand-binding selectivities of ETRs expressed in clonal cells, the isolation of crude membranes and ligand binding assays were performed as described previously (10, 15); cell membranes (0.8–20 μg) were incubated with 50 pM ^{125}I-ET-1 in 0.25 ml of the binding solution. Each experiment was carried out in duplicate. The nonspecific binding was identified as the binding activity in the presence of 1 μM unlabeled ET-1 and was subtracted from the total binding activity for determination of the specific binding. The specific binding activity amounted to 90–92% of the total binding activity.

Measurements of PI hydrolysis. PI hydrolysis was measured essentially as described previously (15). CHO (*dhfr*$^-$) cells expressing individual ETRs were seeded in 12-well plates at a density of 1×10^5 cells/well and were cultured for 1 day. The cells were labeled with [^{3}H] inositol (1 μCi/ml) for 24 hr. The cells were washed twice with PBS containing 0.2% BSA and were incubated with the same solution for 30 min and then with PBS containing 0.2% BSA and 10 mM LiCl for 30 min at 37°. Agonist stimulation was started by replacing the medium with fresh PBS containing 0.2% BSA, 10 mM LiCl, and test reagents. The reaction was terminated with 5% (w/v) trichloroacetic acid after incubation for 10 min at 37°. Separation of [^{3}H]inositol phosphates was carried out by Bio-Rad AG1 × 8 chromatography essentially as described (23). Inositol monophosphate, inositol bisphosphate, and inositol trisphosphate were serially eluted with 5 mM disodium tetraborate/180 mM sodium formate, 0.1 M formic acid/0.4 M ammonium formate, and 0.1 M formic acid/1.0 M ammonium formate, respectively. The radioactivity in the eluates was determined in a liquid scintillation counter.

Measurements of arachidonic acid release. [^{3}H]Arachidonic acid release was measured essentially according to the procedure described (15). Receptor-expressing cells were seeded in 24-well plates at a density of 1×10^5 cells/well and were cultured for 1 day. The cells were labeled with [^{3}H]arachidonic acid (0.25 μCi/ml) for 24 hr, washed three times with α-minimal essential medium supplemented with 20 mM HEPES (pH 7.4) and 0.2% BSA, and incubated in the same medium for 30 min at 37°. The reaction was started by replacing the medium with fresh α-minimal essential medium containing 20 mM HEPES (pH 7.4), 0.2% BSA, and test reagents. After incubation for 30 min at 37°, the [^{3}H]arachidonic acid released in the incubation medium was measured with a liquid scintillation counter.

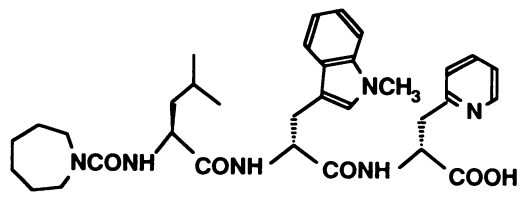

Fig. 1. Structure of FR139317.

Data analysis. In the radioligand binding experiments, displacement data were fitted to the equation $\%B = 100/\{1 + (x/IC_{50})\}$, where $\%B$ is percentage of bound radioligand relative to the total specific binding, x is the concentration of competing ligand, and IC_{50} values are the half-maximal concentrations to inhibit specific binding. Equilibrium dissociation constants (K_i) were derived using the Cheng and Prusoff correction (24), $K_i = IC_{50}/(1 + L/K_d)$, where L and K_d are the concentration and the equilibrium dissociation constant of ^{125}I-ET-1, respectively. Schild plots (25) were produced from dose ratios calculated from the EC_{50} values (the effective concentrations for half-maximal response) estimated from the dose-response curves for ET-1 obtained in the absence and presence of antagonists. The slopes were determined by linear regression by the method of least squares.

Results

Radioligand binding studies. The transfection and expression of cDNA clones for single ETR subtypes in the same cell type are useful for the accurate characterization of ligand-receptor interactions because uncertainties arising from the presence of multiple receptor subtypes can be successfully eliminated. We determined the potencies and selectivities of FR139317 in inhibiting specific radioligand binding to membranes prepared from cells expressing individual ETR subtypes. Curves for ^{125}I-ET-1 binding displacement by FR139317 and the three ET peptides are presented in Fig. 2. The two ETR subtypes expressed in clonal cells showed distinguishable rank orders of binding affinities for these ligands. FR139317 was a potent inhibitor of ^{125}I-ET-1 binding to ET_A, and this was in marked contrast to weak inhibition of binding to ET_B. The K_i values of ET_A and ET_B for FR139317 calculated from the corresponding IC_{50} values are 1 nM and 7.3 μM, respectively. Thus, FR139317 is a selective ligand for ET_A, and its affinity for ET_A is 7300-fold higher than that for ET_B.

Effects of FR139317 on ET-1-induced PI hydrolysis and arachidonic acid release in clonal cells expressing ETR subtypes. Clonal cells permanently expressing either ET_A or ET_B both showed a marked stimulation of PI hydrolysis and arachidonic acid release in response to agonist interaction (15). These functional assays of ETR activation allow the quantitative measurement of the potencies of antagonists for a single receptor subtype. FR139317 was tested by measuring the inhibitory activity on ET-stimulated PI hydrolysis in clonal cells stably expressing individual receptors. In this experiment, receptor-expressing cells were preincubated with different concentrations of FR139317 for 30 min. The amount of total inositol phosphates (inositol mono-, bis-, and trisphosphates) was determined after the activation of ETRs with various concentrations of ET-1 for 10 min in the presence of FR139317. In ET_A-expressing cells, FR139317 produced a concentration-related parallel rightward shift in the dose-response curve of ET-1 (Fig. 3A). In contrast, FR139317 exhibited no inhibitory

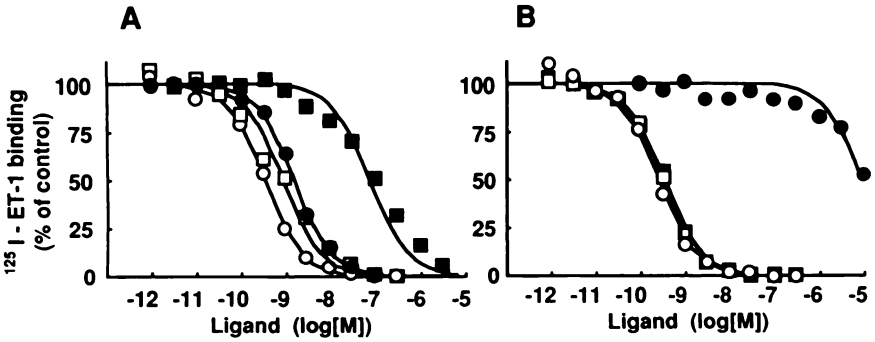

Fig. 2. Displacements of specific ^{125}I-ET-1 binding to membranes of clonal cells expressing the ETR subtypes. Experimental details are described in Experimental Procedures. The unlabeled ligands added to the binding assays of ET_A (A) and ET_B (B) are as follows: ○, ET-1; □, ET-2; ■, ET-3; ●, FR139317.

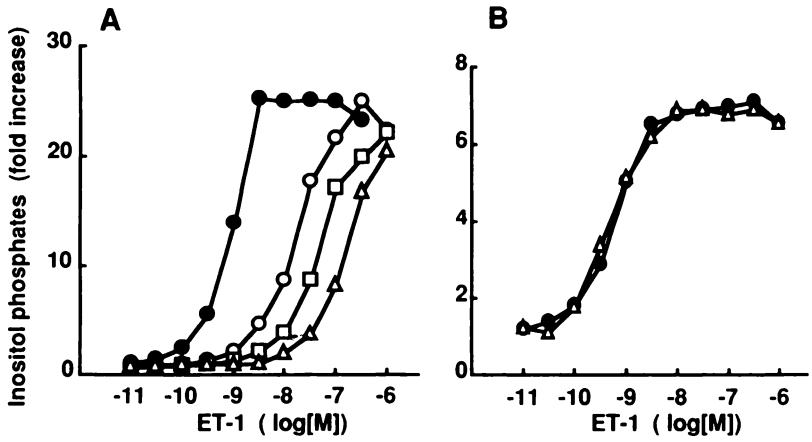

Fig. 3. Effect of FR139317 on ET-1 induced inositol phosphate formation in clonal cells expressing the ETR subtypes. Experimental details are described in Experimental Procedures. Cell lines expressing ET_A (A) and ET_B (B) were preincubated in the absence (●) and presence of FR139317 at 100 nM (○), 320 nM (□), and 1 μM (△) for 30 min. The cells were incubated with the indicated concentrations of ET-1 for 10 min in the absence and presence of FR139317, and then total inositol phosphate formation was measured. The inositol phosphate formation is expressed as the fold increase in inositol phosphate levels, compared with cells not treated with ET-1. Data were taken from a representative experiment done in duplicate.

effect on PI hydrolysis in ET_B-expressing cells at 1 μM (Fig. 3B).

The selectivity of FR139317 for ET_A was further examined by measuring the ET-induced arachidonic acid release in clonal cells expressing individual receptors. ET_A- and ET_B-expressing cells were preincubated with different concentrations of FR139317 for 30 min, and then the release of arachidonic acid was measured after incubation of the cells with various concentrations of ET-1 for 30 min in the presence of FR139317. Compared with control, increasing concentrations of FR139317 produced a parallel shift in the dose-response curve of ET-1 in ET_A-expressing cells (Fig. 4A). However, in ET_B-expressing cells FR139317 showed no inhibitory effect at 1 μM (Fig. 4B). Schild analyses (25) of the antagonism of ET-1-induced PI hydrolysis and arachidonic acid release in ET_A-expressing cells yielded comparable pA_2 values of 8.2 and 7.7, respectively (Fig. 5). The slopes of the regression lines for the antagonism of PI hydrolysis and arachidonic acid release were 1.1 and 0.96, respectively. These results indicated that FR139317 selectively interacted with ET_A and produced competitive inhibitory effects on both ET-induced PI hydrolysis and arachidonic acid release. FR139317 itself showed no stimulatory effects on PI hydrolysis and arachidonic acid release in ET_A- and ET_B-expressing cells at the concentration of 10 μM (data not shown). Thus, the results obtained demonstrate that FR139317 is a potent, competitive, and highly selective antagonist of ET_A.

Discussion

In this study, we investigated the receptor-binding properties and antagonist activities of FR139317 for the two ETR subtypes in transfected CHO cells. The radioligand binding analysis indicated that FR139317 is a highly selective and potent ligand for ET_A. Its affinity for ET_A was 7300-fold higher than

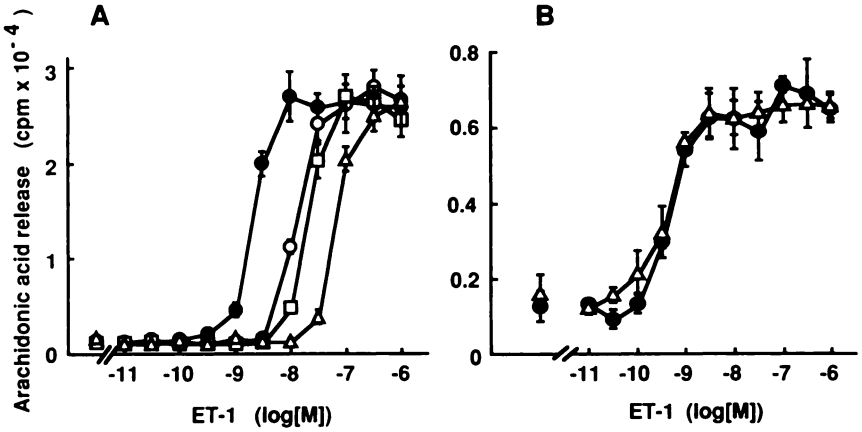

Fig. 4. Effect of FR139317 on ET-1-induced arachidonic acid release in clonal cells expressing the ETR subtypes. Experimental details are described in Experimental Procedures. Cell lines expressing ET_A (A) and ET_B (B) were preincubated in the absence (●) and presence of FR139317 at 100 nM (○), 320 nM (□), and 1 μM (△) for 30 min. The cells were incubated with the indicated concentrations of ET-1 for 30 min in the absence and presence of FR139317, and then the release of arachidonic acid was measured. The values are means ± standard deviations of triplicate determinations.

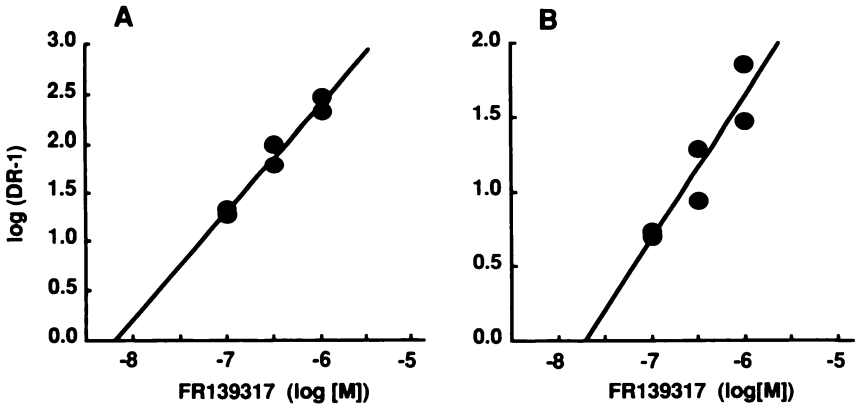

Fig. 5. Schild plots for the antagonism by FR139317 of ET-1-induced inositol phosphate formation (A) and arachidonic acid release (B) in ET_A-expressing cells. Dose ratios (*DR*) were calculated from the EC_{50} values estimated from the dose-response curves for ET-1 obtained in the absence and presence of FR139317. The values were obtained from separate experiments performed in duplicate (A) or triplicate (B), and the lines were fitted by linear regression.

that for ET_B. FR139317 also potently inhibited ET-induced PI hydrolysis and arachidonic acid release in ET_A-expressing cells. In ET_B-expressing cells, FR139317 had no inhibitory effects on ET_B-mediated signal transduction pathways. FR139317 itself showed no stimulatory effects on PI hydrolysis and arachidonic acid release in ET_A- and ET_B-expressing cells. Transfection and functional expression of the cDNA for a single receptor subtype in the same cell thus serves as a powerful system to characterize accurately and effectively the properties of an antagonist for closely related but different subtypes of receptors. In previous reports, ET-related peptides (26–28), fermentation products (20), and their derivatives (21) have been examined as agonists and antagonists for ETRs. In these investigations, functional studies were performed by measuring the vasoconstrictor activities, vasorelaxant activities, or inhibitory effects on ET-induced vascular responses. Radioligand binding studies were also conducted in various tissue preparations in an attempt to characterize the receptor binding properties for the two ETRs. However, determination of receptor selectivities of these compounds may have been complicated by the possible existence of multiple receptor subtypes of this receptor family in tissue or cell preparations. The functional and quantitative assay of the two ETRs described in this investigation should provide a useful tool to examine and develop selective agonists and antagonists for the two ETR subtypes.

FR139317 will facilitate the elucidation of the physiological properties of ET_A and its role in diseases, because it is a highly selective antagonist for ET_A and lacks agonist activity. In our separate investigation, we have shown that FR139317 inhibits the ET-1-induced contraction of rabbit aorta with a pA_2 value of 7.2 (22). This pA_2 value is comparable to those determined in this paper for the antagonism of PI hydrolysis and arachidonic acid release in ET_A-expressing cells. We have also observed that FR139317 inhibits the ET-1-induced pressor response in conscious normotensive rats, in a dose-dependent manner (22), but has no effect on the initial depressor response that is thought to be mediated by ET_B on vascular endothelium (4, 29). Consistent with these observations, previous *in situ* and blot hybridization analyses have indicated that the ET_A mRNA is predominantly expressed in vascular smooth muscle cells of a variety of tissues, including aorta, whereas no significant expression of ET_B is observed in vascular smooth muscle cells (14). In agreement with these functional studies and the hybridization analyses, FR139317 has been shown to compete potently with the specific binding of ^{125}I-ET-1 to membranes from porcine aorta but only weakly with that to membranes from porcine brain (22), where ET_B is highly expressed (14). The rank orders of binding affinities of ETs and FR139317 for these two membrane preparations are characteristic of those determined for ET_A and ET_B in this investigation. All of these observations indicate that ET_A is responsible for evoking the potent vasoconstriction caused by ET-1 and plays a key role in controlling blood supply in many tissues.

We have also demonstrated that the intracisternal administration of FR139317 significantly inhibits the vasoconstriction of the basilar artery in an experimental model of subarachnoid hemorrhage in dogs (22), indicating that ET_A may play an important role in the pathogenesis of cerebral vasospasm after subarachnoid hemorrhage. In contrast to the restricted expression of ET_A mRNA in smooth muscle cells of blood vessels, ET_B mRNA is widely distributed at high levels in many regions of the brain including epithelial cells of the choroid plexus and ependymal cells of the ventricle (14). Therefore, highly selective ET_A antagonists, such as FR139317, would be useful as therapeutic agents in the treatment of vasospasm after subarachnoid hemorrhage. In addition, availability of our expression system that is specific for a single ETR subtype should facilitate the development of new ET agonists or antagonists that will be valuable as human therapeutic agents.

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