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An endothelin B receptor-selective antagonist: IRL 1038, [Cys[11]-Cys[15]]-endothelin-1(11-21)

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In the inhibition of specific binding of [^{125}I]endothelins (ETs) to membranes from various tissues of rats, guinea pigs, pigs and humans, [Cys[11]-Cys[15]]-ET-1(11-21), IRL 1038, has a much higher affinity for ET_B receptors (K_i = 6–11 nM) than for ET_A receptors (K_i = 0.4–0.7 μM). In contraction assays, with ET-3 as a stimulant, 3 μM IRL 1038 antagonized the ET_B receptor-mediated contraction of guinea pig ileal and tracheal smooth muscle without any significant agonistic activity, but did not effect the ET_A receptor-mediated contraction of rat aortic smooth muscle. IRL 1038 is, therefore, considered to be the first antagonist selective to the ET_B receptor.

Endothelin; Receptor; Antagonist; Smooth muscle; Contraction

1. INTRODUCTION

Endothelin (ET) was discovered as a potent vasoactive peptide produced by cultured endothelial cells [1]. The peptide, now known as ET-1, is a member of a family of three distinct isopeptides, ET-1, ET-2, and ET-3 [2]. Sarafotoxins, isolated from the venom of the snake, *Atractaspis engaddensis*, also possess structural features and pharmacological activities similar to those of ETs [3]. ETs and sarafotoxins exert various pharmacological actions, such as transient vasodilation and prolonged contraction of bronchial, intestinal and uterine smooth muscle (see [4] for review). These pharmacological actions are mediated by two distinct subtypes of ET receptor, i.e. the ET_A receptor (ET-1- and ET-2-selective) and the ET_B receptor (ET isopeptide non-selective) (see [5] for review). However, a distinction between ET_A- and ET_B-mediated functions still remains unestablished, mainly because of the lack of antagonists specific for each subtype of the receptor. Several specific antagonists for the ET_A receptor have recently been isolated from *Streptomyces* fermentation products [6,7] and bayberry extracts [8] and prepared by chemical modification of ET-1 [9], but no antagonists selective to the ET_B receptor have as yet been reported.

In an attempt to develop specific antagonists for the ET_B receptor, we noticed the fact that this receptor recognizes the structure common to all the three ET isopeptides. We therefore synthesized a peptide corresponding to the C-terminal half (residues 11–21) of ET-1, because this segment is conserved in all the three ET isopeptides. The structure of the peptide thus synthesized (called IRL 1038) is: [Cys[11]-Cys[15]]-ET-1(11-21). It has a Cys[11]-Cys[15] disulfide bond instead of the two disulfide bonds at Cys[1]-Cys[15] and Cys[3]-Cys[11] in ETs. Here we report that IRL 1038 is in fact a specific antagonist for the ET_B receptor. By using this antagonist, we also show that contraction of guinea pig ileal and tracheal smooth muscle is, at least in part, mediated by the ET_B receptor.

2. MATERIALS AND METHODS

2.1. *Materials*

[^{125}I]ET-1 and [^{125}I]ET-3 (~74 TBq/mmol) were purchased from Amersham (Buckinghamshire, UK). Authentic ET-1 and ET-3 (Peptide Institute Inc., Osaka, Japan), protected amino acids, reagents for peptide synthesis (Applied Biosystems, Foster City, CA), and disuccinimidyl suberate (DSS) (Pierce Chemical, Rockford, IL) were obtained commercially. All other chemicals used were of reagent grade.

2.2. *Peptide synthesis*

IRL 1038 (Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile-Ile-Trp) was synthesized by a solid-phase procedure on an Applied Biosystems Model 431A automated peptide synthesizer. The peptide was constructed on 0.5 mmol of Boc-Trp(CHO)-phenylacetoamidomethyl resin and elongated by in situ prepared 1-hydroxybenzotriazole esters using a 4-fold excess of Boc-amino acid derivatives. The completed peptide resin was

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Abbreviations: ET, endothelin; DSS, disuccinimidyl suberate; HPLC, high performance liquid chromatography; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; K_i, apparent binding inhibition constant; EC_{50}, the concentration inducing half maximum contraction.

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treated with hydrogen fluoride in the presence of anisole and 1,2-ethanedithiol to remove all the protecting groups and the resin. The intramolecular disulfide bond was formed by the ferricyanide procedure. The peptide having the intramolecular disulfide bond was purified by preparative reverse-phase high performance liquid chromatography (HPLC) on a column of TSKgel ODS 120-T (21.5 mm i.d. × 300 mm) (Tosoh, Tokyo, Japan) using a linear acetonitrile gradient (20–60% over 1 h) in 0.1% trifluoroacetic acid at a flow rate of 10 ml/min. The purity of the peptide was confirmed by analytical reverse-phase HPLC and amino acid analysis. Fast atom bombardment mass spectrometry and 600 MHz ^{1}H NMR indicated that the synthetic IRL 1038 did not contain polymers or aggregates.

2.3. *Membrane preparation*

Tissues (rat aorta, lung and cerebellum; guinea pig heart, lung, trachea, ileum and cerebellum; porcine aorta and lung; and human umbilical vein and placenta) were homogenized with 9 vols. of ice-cold 0.25 M sucrose containing 20 mM Tris-HCl (pH 7.4), 0.2 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin, 0.1 mM EDTA, and 0.5 mM EGTA in a Polytron homogenizer (Kinematica, Lucerne, Switzerland). The homogenate was centrifuged at 1,000 × g for 10 min at 4°C, and the resultant supernatant was centrifuged again at 48,000 × g for 20 min. The sedimented membranes were washed three times with the same buffer and stored in aliquots at −80°C until use.

2.4. *ET binding assays*

[^{125}I]ET-1 was used as the ligand to assess binding to the ET_A receptor in the presence of 1 nM unlabeled ET-3 to mask the ET_B receptor, whereas the binding to the ET_B receptor was measured with [^{125}I]ET-3 as the ligand. The binding assay was performed as described previously [10]. In brief, the membranes (1–30 μg of protein) were incubated at 37°C for 1 h with [^{125}I]ET-1 or [^{125}I]ET-3 in 20 mM HEPES (pH 7.4), 145 mM NaCl, 4 mM KCl, 1.2 mM $MgCl_2$, 1.0 mM EGTA, 0.1% bovine serum albumin, and 0.02% bacitracin (final vol. 1.0 ml). The incubation was terminated by centrifugation of the mixture at 20,000 × g for 20 min at 4°C. The membrane-associated radioactivity was then measured in a gamma counter. Specific binding was defined as total binding *minus* non-specific binding obtained in the presence of 100 nM unlabeled ET-1 or ET-3. Total binding was always <10% of the total radioactivity added. Protein was determined by using a BCA assay kit (Pierce).

2.5. *Covalent labeling of ET receptors*

Membranes were incubated with [^{125}I]ET-1 as described above except that bovine serum albumin and bacitracin were omitted from the buffer. After the incubation, 1 mM DSS, a covalent cross-linking reagent, was added and incubation was carried out further at 25°C for 30 min. The cross-linking reaction was then quenched by adding 100 mM ammonium acetate. The membranes were recovered and washed by centrifugation, solubilized in 1% sodium dodecyl sulfate (SDS) by heating at 100°C for 5 min, and subjected to SDS-PAGE. After drying the gel, the radiolabeled ET receptors were visualized with the aid of a Bioimage Analyzer BAS-2000 (Fuji Photo Film, Tokyo, Japan).

2.6. *Contraction assays*

Rat aortic smooth muscle denuded of the endothelium, guinea pig trachea without the epithelium, and guinea pig ileal longitudinal muscle were excised and placed in an oxygenated (95% O_2, 5% CO_2) normal physiological salt solution (136.9 mM NaCl, 5.4 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 23.8 mM $NaHCO_3$, 0.01 mM EDTA, and 5.5 mM glucose) at 37°C. The tissue was placed in a 20-ml organ bath containing the oxygenated salt solution. The contractile activity was measured isometrically via a strain gauge transducer (Nihon-Koden, Tokyo, Japan), maintaining the preparation under a resting tension of 0.1 g for the aorta and trachea and of 0.5 g for the ileum [11]. The high K^+ (65.4 mM) solution was made by substituting NaCl of 60 mM with equimolar KCl and used to obtain a control value for contraction of each segment. Student's t-test was used for the statistical analysis of the results.

3. RESULTS AND DISCUSSION

The affinities of IRL 1038, ET-1, and ET-3 for the ET_A and ET_B receptors in membranes were assessed by measuring the inhibition of specific binding of [^{125}I]ETs by the unlabeled ligands. Inhibition curves obtained for [^{125}I]ET-3 binding to the ET_B receptor in guinea pig ileal membranes and those for [^{125}I]ET-1 binding to the ET_A receptor in rat aortic membranes are shown in Fig. 1A and B, respectively. Table I summarizes the apparent binding inhibition constant (K_i) values thus obtained for the two receptors in membranes from various mammalian tissues. As can be seen, IRL 1038 showed K_i values of 6–11 nM for the ET_B receptor in the membranes tested. These values are 350- to 1,800-fold higher than those determined for the binding of ET-1 and ET-3 to the same receptor (5–26 pM). On the other hand, the K_i values of IRL 1038 for the ET_A receptor were 0.4–0.7 μM, which are 7,000- to 15,000-fold higher than the K_i values of ET-1 (30–96 pM) and about 20- to 60-fold higher than those of ET-3 (15–32 nM). These results indicate that IRL 1038 has a much higher affinity for the ET_B receptor (K_i = 6–11 nM) than for the ET_A receptor (K_i = 0.4–0.7 μM), although the affinity of IRL 1038 for the ET_B receptor is considerably lower than that of ET-3. It can, therefore, be said that IRL 1038 acts as a specific antagonist for the ET_B receptor at a suitable concentration range.

The selectivity of IRL 1038 to the ET_B receptor was confirmed by cross-linking experiments. Guinea pig ileal and rat aortic membranes, which contain predominantly the ET_B and ET_A receptors, respectively, were incubated with 50 pM [^{125}I]ET-1 and then cross-linked with DSS. Upon SDS-PAGE, the ileal membranes gave two radioactive bands of M_r 77,000 and 38,000 (Fig. 2A). The M_r of the larger band coincides with that of the ET_B receptor, whereas the smaller band seems to be a proteolytic product of the ET_B receptor, as has been reported [12]. The radioactivity of both bands decreased to about 50% when incubated in the presence of 100 nM IRL 1038, and disappeared in the presence of 1 μM IRL 1038. On the other hand, after the cross-linking, the ET_A receptor in the aortic membranes was recovered as a radioactive band of M_r 77,000, but its intensity was almost unchanged when incubated in the presence of 1 μM IRL 1038 (Fig. 2B).

As shown in Fig. 3, ET-3 as an agonist relatively specific for the ET_B receptors induced dose-dependent contractions of rat aortic smooth muscle, guinea pig ileal longitudinal muscle, and guinea pig tracheal ring. Maximum responses were induced by 300 nM ET-3 in the ileum and trachea, which were about 41% and 96% of those induced by the high K^+ solution, respectively. On the other hand, in the aorta, the muscle tension did

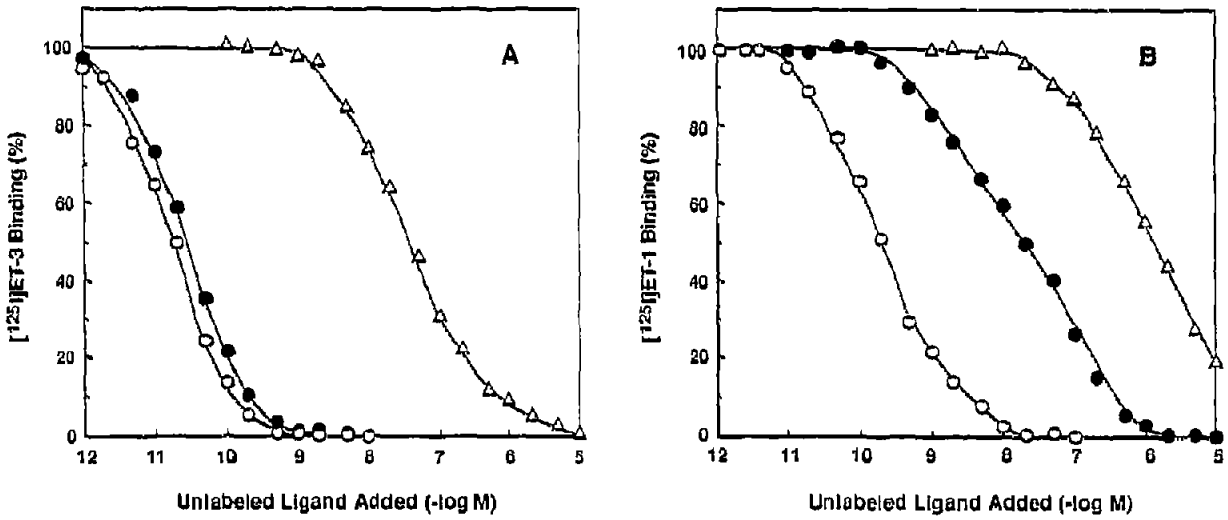

Fig. 1. Inhibition of binding of [^{125}I]ET-3 to the ET_B receptor in guinea pig ileal membranes (A) and of [^{125}I]ET-1 to the ET_A receptor in rat aortic membranes (B) by unlabeled ET-1 (○), ET-3 (●), and IRL 1038 (△). The ileal membrane (2 μg protein) was incubated with 10 pM [^{125}I]ET-3 and the aortic membrane (30 μg protein) with 40 pM [^{125}I]ET-1, in the presence of various concentrations of unlabeled ligands as described in Materials and Methods. Each value is expressed as a percentage of the specific binding.

not reach a maximum even at a concentration of 300 nM ET-3. The ET-3 concentration inducing half maximal contraction (EC_{50}) was about 30 nM for both the ileum and trachea. The dose-response curve for the aorta was unaffected by the presence of 3 μM IRL 1038 (Fig. 3A), even though the affinities of ET-3 and IRL 1038 for the ET_A receptor are not much different (K_i of IRL 1038/K_i of ET-3 = 59). In contrast, even though the affinity of IRL 1038 for the ET_B receptor is markedly lower than that of ET-3 (K_i of IRL 1038/K_i of ET-3 = 1,100), the ET-3-induced contraction of the ileal and tracheal muscles was attenuated by 3 μM IRL 1038 (Fig. 3B and C), the maximum tension being decreased by 52% and 38%, respectively. IRL 1038 at 3 μM did

Table I

K_i values of ET-1, ET-3 and IRL 1038 on binding of [^{125}I]ETs to ET_B and ET_A receptors in membranes of various mammalian tissues

Species and tissues	Receptor subtypes	K_d* (pM)	B_{max}* (fmol/mg prot.)	K_i values		
				ET-1 (pM)	ET-3 (pM)	IRL 1038 (nM)
Rat						
Lung	ET_B	19	3,700	26	18	9
Cerebellum	ET_B	15	1,700	17	11	6
Aorta	ET_A	46	100	96	12,000	700
Guinea pig						
Lung	ET_B	16	2,300	10	10	11
Trachea	ET_B	11	360	8	8	9
Ileum	ET_B	3	520	5	8	9
Cerebellum	ET_B	5	340	11	11	11
Heart	ET_A	28	860	41	32,000	630
Pig						
Lung	ET_B	8	360	8	10	10
Aorta	ET_A	32	260	53	15,000	700
Human						
Placenta	ET_B	12	490	12	11	9
Umbilical vein	ET_A	37	370	30	16,000	430

*The K_d and B_{max} for ET_B receptors were determined with [^{125}I]ET-3, and those for ET_A receptors were with [^{125}I]ET-1, in the presence of 1 nM unlabeled ET-3 to mask the binding sites for ET_B receptors (also see Fig. 1).

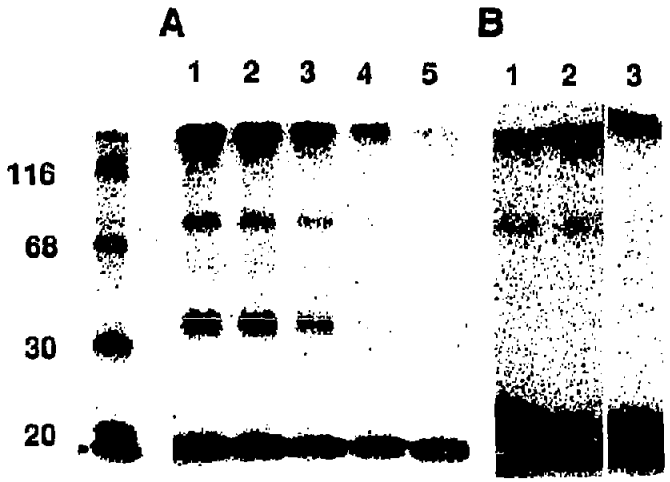

Fig. 2. Autoradiograms of SDS-PAGE of [^{125}I]ET-1 which was covalently linked to the ET_B receptor in guinea pig ileal membranes (A) and the ET_A receptor in rat aortic membranes (B). The membranes were incubated with 50 pM [^{125}I]ET-1 in the absence (lane 1 in A and B), or the presence of 10 nM (lane 2 in A), 100 nM (lane 3 in A), and 1 μM (lane 4 in A and lane 2 in B) IRL 1038. Labeling of these bands was inhibited effectively by an excess concentration (100 nM) of unlabeled ET-1 (lane 5 in A and lane 3 in B). The same bands were also observed under reducing conditions. The positions of ^{125}I-labeled marker proteins (DuPont New England Nuclear) and their M_r values in thousands are indicated on the left: β-galactosidase (M_r=116,000), bovine serum albumin (68,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100).

not affect the contraction induced by the high K^+ solution in all the three tissues (data not shown). IRL 1038 itself at 10 μM exerted little contractile effect in the three tissues, indicating that IRL 1038 has practically no agonistic activity in these tissues.

Taken together, these results indicate that IRL 1038 is an antagonist specific for the ET_B receptor in various mammalian tissues, and demonstrate that contraction of guinea pig ileal and tracheal smooth muscle is, at least in part, mediated by the ET_B receptor.

Previously we synthesized a series of linear peptides corresponding to the C-terminal portion of ET-1, and their N^{α}-succinyl derivatives, and found that Suc-[Glu^9,Ala^{11,15}]-ET-1(8-21) (termed IRL 1620) was the most potent and specific ligand for the ET_B receptor [10]. Its K_i value for the ET_B receptor in the porcine lung membranes is 16 pM and it is 60-fold more specific for the ET_B receptor than ET-3 (the ratio of K_i for ET_A/K_i for ET_B being 120,000 for IRL 1620 and 1,900 for ET-3). IRL 1620 is actually an agonist and induces contraction of guinea pig tracheal muscle with an efficiency comparable to that of ET-3 [10]. Since IRL 1620, but not IRL 1038, has a cluster of charged residues in the N-terminal portion, it seems that the cluster is involved in the agonist activity. The cluster also seems to contribute to the high selectivity for the ET_B receptor, in view of the fact that the selectivity of IRL 1038 to the ET_B receptor (K_i for ET_A/K_i for ET_B = 50–120) is markedly lower than that of IRL 1620. Nevertheless it should be pointed out that IRL 1038, when used at appropriate concentrations, is a specific antagonist for the ET_B receptor. When the disulfide bond of IRL 1038 was removed by Cys-Ala substitution (IRL 1443 in [10]), the affinity and selectivity for the ET_B receptor decreased markedly in porcine lung (K_i = 140 nM, K_i for ET_A/K_i for ET_B = 11) [10] and in various tissues of rats, guinea pigs, and humans (K_i = 800 nM–1.2 μM, K_i for ET_A/K_i for ET_B = 2–8), indicating that the disulfide bond in IRL 1038 is important to maintain its high affinity and selectivity for the ET_B receptor.

The ET_B receptor was recently deduced to be significantly involved in positive inotropy [13], inflammation [14], renal functions [15], asthma [16], ocular functions [17], venous constriction [18], vasodilation [19] and also in several central nervous functions [20]. Thus, IRL 1620 and IRL 1038, an ET_B receptor-selective agonist and antagonist, respectively, will be useful to clarify the role of ET_B-mediated responses in these biological functions.

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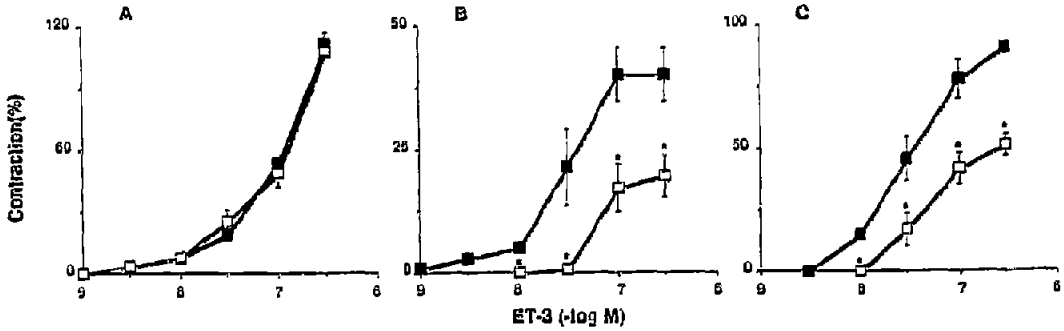

Fig. 3. Effects of IRL 1038 on ET-3-induced contractions in the rat aorta (A), guinea pig ileum (B) and guinea pig trachea (C). ET-3 was applied cumulatively in the absence (■) or presence of 3 μM IRL 1038 (□). IRL 1038 was added 30 min before addition of ET-3. The contractile tension is represented as a percentage of the contraction induced by the high K^+ solution in each tissue. *Significantly different from the point in the absence of IRL 1038 with P<0.01. Each point represents the mean ±S.E.M. of 4 experiments.

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