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RES-701-1, a Novel, Potent, Endothelin Type B Receptor-Selective Antagonist of Microbial Origin

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SUMMARY

The unique cyclic peptide designated RES-701-1 blocked the binding of ^{125}I-labeled endothelin (ET)-1 to bovine cerebellar membranes. ET_B receptors are predominant in bovine cerebellum. However, in bovine lung membranes, where both ET_A and ET_B receptors are expressed, RES-701-1 inhibited ^{125}I-ET-1 binding by up to 70%; RES-701-1, in the presence of the ET_A-selective antagonist BQ-123 at 1 μM, displaced ^{125}I-ET-1 binding completely. With membranes from transfected Chinese hamster ovary cells expressing the human ET_A or ET_B receptors, RES-701-1 inhibited ^{125}I-ET-1 binding to the ET_B receptor with an IC_{50} value of 10 nM but had no effect on ^{125}I-ET-1 binding to the ET_A receptor. Thus, RES-701-1 is highly specific for the ET_B receptor; it has no effect on a number of other receptors. RES-701-1 selectively inhibited the ET-1-induced increase in intracellular Ca^{2+} concentration in COS-7 cells expressing the ET_B receptor but did not inhibit the Ca^{2+} transient in ET_A-expressing cells. When injected intravenously (250 nmol/kg) into anesthetized rats, RES-701-1 abolished the initial depressor response to ET-1 but enhanced the subsequent pressor response. These results suggest that RES-701-1 is a potent and specific antagonist for the ET_B receptor and that RES-701-1 will be a powerful tool for understanding the physiological roles of this receptor.

The ETs are a family of potent vasoactive peptides termed ET-1, -2, and -3. The first member of the family, ET-1, was isolated from the culture supernatant of porcine endothelial cells and found to possess exceptionally potent and long-lasting vasoconstriction activity (1). The ETs all consist of 21 amino acid residues with two intramolecular disulfide bonds, one between Cys^1 and Cys^{15} and another between Cys^3 and Cys^{11}. The amino acid sequence of each member of the family is highly conserved among mammalian species (2). The sequence of the carboxyl-terminal linear portion is identical in all members of the ET family.

Since the discovery of the ETs, many of their pharmacological and physiological actions, such as vasoconstriction, release of endothelium-derived relaxing factors (1), mitogenesis (3), and bronchoconstriction (4), have been investigated. These actions of the ETs are mediated through their receptors, which have been divided into two major subtypes, ET_A and ET_B, based on their differential sensitivity to ET-1 and ET-3 (5); both subtypes have been shown, in many different cell types, to activate phospholipase C (6) and to increase $[Ca^{2+}]_i$. The ET_A receptor shows a difference in affinity for ET-1 and ET-3, with the affinity for ET-1 being 2 orders of magnitude greater than that for ET-3. On the other hand, ET_B receptors bind ET-1 and ET-3 nonselectively. Both ET_A and ET_B receptors have been cloned (7, 8) and the tissue distribution of the receptor subtypes has been elucidated by means of Northern blotting and *in situ* hybridization. ET_B receptor mRNA is distributed in many tissues including brain, lung, kidney, and liver. ET_A receptors are located in vascular smooth muscle, heart, and intestine (9). Thus, the ET_A and ET_B receptors have distinct cell/tissue distributions and, accordingly, different physiological roles. In many blood vessels, for example, ET_A receptors reside generally on smooth muscle cells and mediate vasoconstrictor responses, whereas the endothelial cells express ET_B receptors, which mediate vasodilator effects via the ET-induced release of nitric oxide (10). However, the true physiological roles of ETs and their receptors are still somewhat uncertain. Although changes in the ETs have been suggested to play a role in cardiovascular diseases (11), asthma (12), and other diseases, the pathophysiological roles of ETs in these disease states are also still obscure, due, at least in part, to the lack of adequate antagonists.

To elucidate the physiological and pathophysiological significance of ETs and the responses mediated through each receptor subtype, selective antagonists would be very useful. Although several selective antagonists for ET_A receptors have

ABBREVIATIONS: ET, endothelin; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; BSS, basal salt solution; MOPS, 3-(*N*-morpholino)propanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; 5-HT, 5-hydroxytryptamine; CHO, Chinese hamster ovary.

recently been isolated, e.g., BE-18257B and WS009A and -B from microbial culture broth (13) and cafeoyl ester(50-235) (14) from bayberry extracts, or prepared by chemical modification of the microbial products, e.g., BQ-123 and FR139317 (13), only a limited number of ET_B receptor-selective ET analogues have been reported.

During the course of screening to obtain ET_B receptor antagonists of microbial origin, we isolated a novel compound, RES-701-1, from the culture broth of *Streptomyces* sp. RE-701. In this paper, we report its antagonistic profile for the ET_B receptor *in vitro*, in intact cells, and in whole animals. Some of the results presented in this paper have been published in abstract form (15).

Experimental Procedures

Materials. RES-701-1 was purified in our laboratories from the culture broth of *Streptomyces* sp. RE-701; isolation and purification procedures will be described elsewhere.[1] All radioligands used for receptor binding experiments were purchased from Du Pont-New England Nuclear and Amersham. ET-1 and -3 were products of the Peptide Institute, Inc. (Osaka, Japan). BQ-123 was purchased from Peninsula Laboratories, Inc. Bio-Rad protein assay used in the measurement of proteins was obtained from Bio-Rad Laboratories. The human ET_B receptor gene was the generous gift of Dr. Hirose (Department of Biological Science, Tokyo Institute of Technology, Tokyo, Japan). All other chemicals were of analytical grade.

Receptor binding assay. Bovine tissues were obtained from a local slaughterhouse. Lung parenchyma and cerebellum were dissected. Tissues were homogenized in 5 volumes of buffer A (1 mM $NaHCO_3$, 5 mM EDTA, pH 8.3, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, 40 μM phenylmethylsulfonyl fluoride), with a Polytron homogenizer (setting 8, for 2 × 30 sec) at 4°. The homogenates were centrifuged at 8000 × *g* for 10 min. The supernatants were then centrifuged at 40,000 × *g* for 1 hr at 4°. The pellets were homogenized in buffer A and recentrifuged at 40,000 × *g* for 1 hr. The resulting pellets were homogenized in buffer A supplemented with 130 mM NaCl, 5 mM Na_2HPO_4, and 1.5 mM KH_2PO_4 and were used for receptor binding assays. For preparation of CHO membranes, after washing with phosphate-buffered saline transfected cells were removed from the culture dish with a rubber policeman and collected by centrifugation. The resulting cell pellets were used to obtain the membrane fraction as described above. To perform the ^{125}I-ET-1 binding assay, reaction mixtures (1 ml) containing 0.74 kBq/ml ^{125}I-ET-1, 50 mM Tris·HCl buffer, pH 7.6, 1 mM EDTA, 0.2% bovine serum albumin, 0.02% bacitracin, 14 μg of lung membrane protein or 14 μg of cerebellar membrane protein, and various concentrations of drugs (RES-701-1 and BQ-123 were dissolved in dimethylsulfoxide) were incubated at room temperature for 2 hr and then filtered through GF/B glass filters. The glass filters were washed three times with cold 50 mM Tris·HCl buffer, pH 7.6, containing 1 mM EDTA, using a Brandel M-24R cell harvester. The radioactivity on the washed filters was estimated using a Packard γ counter. Nonspecific binding was measured in the presence of 0.1 μM unlabeled ET-1. [^{3}H]WB4101, [^{3}H]clonidine, [^{3}H]dihydroalprenolol, [^{3}H]SCH23390, [^{3}H]spiperone, [^{3}H]pyrilamine, [^{3}H]tiotidine, [^{3}H]quinuclidinyl benzilate, [^{3}H]8-hydroxy-2-(di-*n*-propylamino)tetralin, and [^{3}H]ketanserin binding to adrenaline α_1, α_2, and β_1, dopamine D_1 and D_2, histamine H_1 and H_2, acetylcholine M_1, and serotonin 5-HT_{1A} and 5-HT_2 receptors, respectively, was measured as described (16-24). ^{125}I-Atrial natriuretic peptide and ^{125}I-angiotensin II binding was performed by published methods (25, 26).

Expression of ET_A and ET_B receptors in COS-7 and CHO cells. Human ET_A receptor genes were obtained from human placenta mRNA by reverse transcription and polymerase chain reaction methods (27). Human ET_A and ET_B receptor genes were cloned into the pcDNA-I vector (Invitrogen) for transient expression in COS-7 cells or were cloned into pAGE107 whose promoter was replaced by the Moloney murine leukemia virus promoter for stable expression in CHO cells, as described (28). The resulting ET_A or ET_B receptor gene was introduced into COS-7 cells as described (8), and cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. After 3 days, the COS-7 cells were used for studies on $[Ca^{2+}]_i$. For stable expression of ET_A and ET_B receptors, the genes were introduced into CHO cells, and ET_A or ET_B receptor-expressing cells were selected as described (28). After selection, the cells were cultured in α-minimal essential medium supplemented with 10% dialyzed bovine calf serum, but without ribonucleosides and deoxyribonucleosides. Binding experiments were performed with membranes prepared from these transfected CHO cells.

Measurement of $[Ca^{2+}]_i$. The transfected COS-7 cells were plated on a glass coverslip with a silicon rubber wall (Flexiperm-Disc, Heraeus Instrument GmbH). The culture was maintained for 3 days in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, in a humidified atmosphere of 5% CO_2 in air at 37°. After cultivation, the culture medium was removed, and the cells on the coverslip were washed at least three times with BSS (140 mM NaCl, 4 mM KCl, 1.25 mM $CaCl_2$, 11 mM D-glucose, 1 mM $MgCl_2 \cdot 6H_2O$, 1 mM $Na_2HPO_4 \cdot 12H_2O$, 1 mg/ml bovine serum albumin, 5 mM HEPES-NaOH, pH adjusted to 7.4). Fura-2/acetoxymethyl ester (10 μM) in BSS was then incubated with the cells for 60 min at 37°, and the cells were then washed extensively with BSS. The coverslip with transfected COS-7 cells that had been loaded with fura-2 was filled with 1 ml of BSS, and ET-1 and/or RES-701-1 was added. Fluorescence measurements were carried out at 37° using an ARUGAS 2000 system (Hamamatsu Photonics). Excitation was at 340 or 380 nm, and emission intensity was measured at 510 nm. The concentration of Ca^{2+} was estimated by comparison with the fluorescence intensity ratios of Ca^{2+}/EGTA mixtures in MOPS buffer that were added to 10 μM fura-2 and excited at the two wavelengths.

Antagonism of blood pressure responses to exogenously administrated ET in anesthetized rats. Male Sprague-Dawley rats (Japan SLC Inc., Shizuoka, Japan) weighing 300-470 g were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). The carotid vein was catheterized for the injection of experimental compounds. Catheters were also inserted in the carotid artery to monitor the mean arterial pressure. The trachea was also cannulated, and artificial respiration with room air was started using a rodent respirator at a rate of 600 ml/kg/min. The animals received constant inhalational anesthesia with 0.5% halothane throughout the experiment.

Statistical analysis. In both the $[Ca^{2+}]_i$ measurement study and the *in vivo* study, data were expressed as mean ± standard error. The data were evaluated with Student's unpaired *t* test. Differences were considered significant at $p < 0.05$.

Results

Structure of RES-701-1. RES-701-1, a novel microbial cyclic peptide found to act as an ET_B receptor antagonist, was isolated from the culture broth of *Streptomyces* sp. RE-701 (Fig. 1). RES-701-1 consists of 16 amino acids, Gly-Asn-Trp-

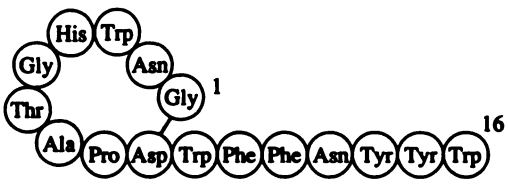

Fig. 1. Structure of RES-701-1.

[1] Y. Morishita, S. Chiba, E. Tsukuda, T. Tanaka, T. Ogawa, M. Yamasaki, M. Yoshida, I. Kawamoto, and Y. Matsuda. RES-701-1, a novel and selective endothelin type B receptor antagonist produced by *Streptomyces* sp. RE-701. I. Characterization of producing strain, fermentation, isolation, physico-chemical and biological properties. Submitted for publication.

His-Gly-Thr-Ala-Pro-Asp-Trp-Phe-Phe-Asn-Tyr-Tyr-Trp, with the α-amino group of the amino-terminal Gly[1] forming an amide bond with the β-carboxyl group of Asp[9]. All of the 16 amino acids are in the L-configuration. There was no amino acid sequence homology between RES-701-1 and the ETs except for the existence of a carboxyl-terminal tryptophan. Details of structural elucidation studies are described in a separate paper.[2]

Effect of RES-701-1 on the binding of ET. Binding experiments using ^{125}I-ET-1 as a ligand were carried out. When receptors prepared from bovine cerebellum were used, ET-1 and -3 both inhibited the binding of ^{125}I-ET-1 to the receptor, with IC_{50} values of 0.06 nM and 0.04 nM, respectively. The similar affinities of ET-1 and ET-3 for the ET receptor are characteristic of the ET_B-type receptor, as described before (5). RES-701-1 inhibited ^{125}I-ET-1 binding to bovine cerebellar membranes in a dose-dependent manner, with an IC_{50} value of 10 nM; RES-701-1 at 1.7 μM inhibited ^{125}I-ET-1 binding completely. BQ-123, an ET_A receptor-selective antagonist, did not show any inhibition of ^{125}I-ET-1 binding to cerebellar membranes when added to the reaction mixture at concentrations up to 1 μM (Fig. 2A).

On the other hand, ET-3 showed a biphasic pattern of inhibition of ^{125}I-ET-1 binding to bovine lung membranes, whereas ET-1 showed a monophasic inhibition curve with an IC_{50} value of approximately 0.09 nM, which suggests the existence of multiple sites with different affinities for ET-3. RES-701-1 showed dose-dependent inhibition of ^{125}I-ET-1 binding to bovine lung membranes with an IC_{50} value of 30 nM, although complete inhibition of ^{125}I-ET-1 was not observed. Approximately 30% of the specific binding of ^{125}I-ET-1 binding to lung membranes remained even at a concentration of 5 μM RES-701-1. BQ-123 showed inhibition of about 30% of ^{125}I-ET-1 binding to lung membranes at a maximum concentration of 1 μM (Fig. 2B).

To investigate the receptor subtype selectivity of RES-701-1, 1 μM BQ-123 was used to block the ET_A-type receptor. In the presence of 1 μM BQ-123, ET-1 and -3 showed monophasic inhibition of ^{125}I-ET-1 binding to bovine lung membranes, with almost equal potencies. This pattern of inhibition of ^{125}I-ET-1 binding by both ET-1 and -3 is characteristic of the ET_B receptor. Under the same conditions, RES-701-1 also showed a monophasic inhibition curve, with an IC_{50} value of 10 nM, and the binding of ^{125}I-ET-1 was completely inhibited at a concentration of 1.7 μM (Fig. 3A). Conversely, in the presence of 5 μM RES-701-1, a concentration that was sufficient to mask a RES-701-1-sensitive binding site, ET-1 and -3 showed monophasic patterns of inhibition of the binding of ^{125}I-ET-1 to bovine lung membranes. The inhibition curve of ET-3 was shifted 2 orders of magnitude to the right, compared with the displacement curve seen in the presence of BQ-123, as described above, and IC_{50} values for ET-1 and -3 were calculated to be 0.1 nM and 10 nM, respectively, values that agree with previous observations for the ET_A receptor (29). In the presence of 5 μM RES-701-1, BQ-123 showed a monophasic inhibition curve, with an IC_{50} value of 25 nM, and the binding of ^{125}I-ET-1 to lung membranes was completely inhibited at a concentration of 1 μM (Fig. 3B). Taken together, these results suggest that RES-701-1 selectively recognizes the ET_B receptor.

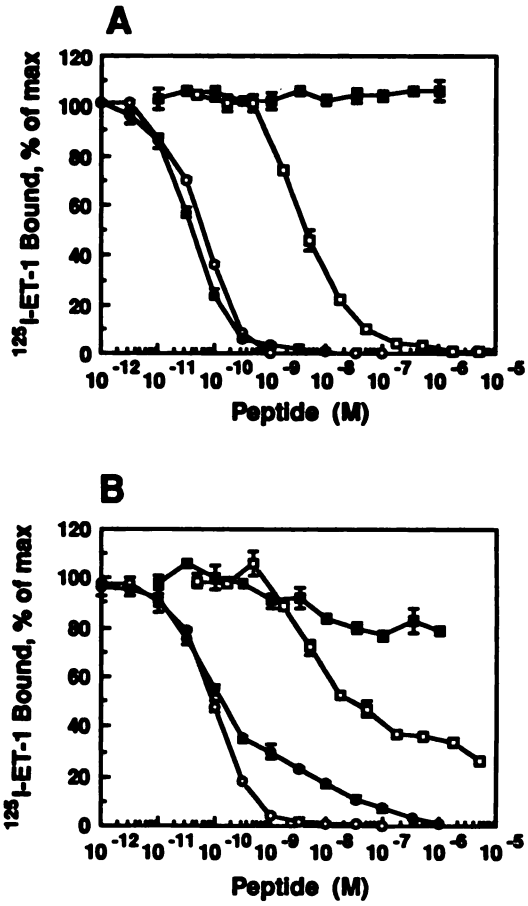

Fig. 2. Inhibition of ^{125}I-ET-1 binding to bovine cerebellum (A) or lung (B) membranes by RES-701–1, BQ-123, ET-1, and ET-3. Binding studies were carried out as described in Experimental Procedures. RES-701–1 and BQ-123 were dissolved in dimethylsulfoxide at various concentrations, and 10 μl were added to the reaction mixture. A, Specific binding of ^{125}I-ET-1 to bovine cerebellar membranes; B, specific binding of ^{125}I-ET-1 to bovine lung membranes. One of the following unlabeled ligands was added to the reaction mixture: RES-701–1 (□), BQ-123 (■), ET-1 (○), or ET-3 (●). All measurements were performed in triplicate.

To confirm that RES-701-1 specifically recognizes the ET_B receptor, membranes prepared from transfected CHO cells stably expressing ET_A and ET_B receptors were used as receptor sources. Expression of ET_A and ET_B receptors was established by the inhibition by ET-1 and ET-3 of ^{125}I-ET-1 binding (data not shown). RES-701-1 inhibited the binding of ^{125}I-ET-1 to human ET_B receptors with an IC_{50} value of 10 nM but did not alter the binding to the human ET_A receptor at concentrations up to 5 μM. In contrast, BQ-123 showed ET_A receptor-selective inhibition of the binding of ^{125}I-ET-1; the IC_{50} value was calculated to be 3 nM (Fig. 4).

Specificity of RES-701-1 receptor binding. RES-701-1 failed to affect the binding of ^{125}I-angiotensin II or ^{125}I-atrial natriuretic peptide. Also, no effect was observed on the adrenaline α_1, α_2, and β_1, dopamine D_1 and D_2, histamine H_1 and H_2, muscarine M_1, or serotonin 5-HT_{1A} and 5-HT_2 receptors at concentrations up to 1 μM (Table 1).

Effect of RES-701-1 on intracellular Ca^{2+} transients induced by ET-1. The significance of RES-701-1 as an antagonist was assessed by measuring its effects on the increase

[2] M. Yamasaki, K. Yano, M. Yoshida, Y. Matsuda, and K. Yamaguchi. RES-701-1, a novel and selective endothelin type B receptor antagonist produced by *Streptomyces* sp. RE-701. II. Detemination of the primary sequence. Submitted for publication.

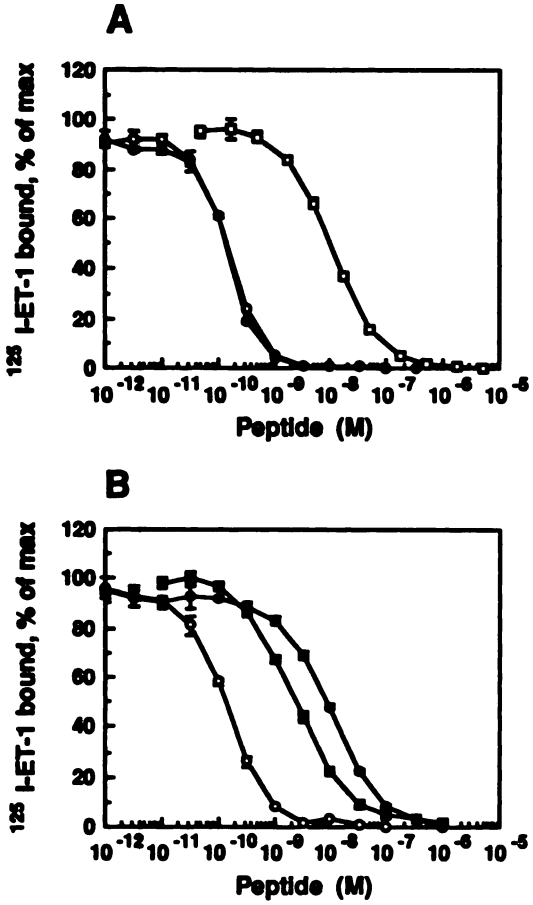

Fig. 3. Inhibition of ^{125}I-ET-1 binding to lung membranes by RES-701-1, BQ-123, ET-1, and ET-3 in the presence of 1 μM BQ-123 (A) or 5 μM RES-701-1 (B). Binding studies were carried out as described in Experimental Procedures. RES-701-1 and BQ-123 were dissolved in dimethylsulfoxide at various concentrations, and 10 μl were added to the reaction mixture. A, Specific binding of ^{125}I-ET-1 to bovine lung membranes measured in the presence of 1 μM BQ-123; B, specific binding of ^{125}I-ET-1 to bovine lung membranes measured in the presence of 5 μM RES-701-1. One of the following unlabeled ligands was added to the reaction mixture: RES-701-1 (□), BQ-123 (■), ET-1 (○), or ET-3 (●). All measurements were performed in triplicate.

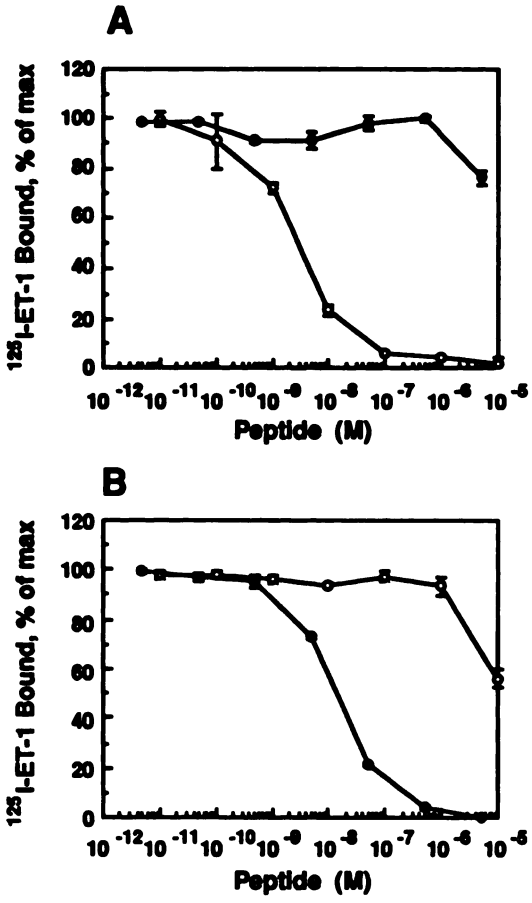

Fig. 4. Inhibition of ^{125}I-ET-1 binding to membranes of ET_A (A) or ET_B (B) receptor-expressing cells by RES-701-1 or BQ-123. Binding studies were carried out as described in Experimental Procedures. RES-701-1 and BQ-123 were dissolved in dimethylsulfoxide at various concentrations, and 10 μl were added to the reaction mixture. A, Specific binding of ^{125}I-ET-1 to membranes of ET_A-expressing CHO cells; B, specific binding of ^{125}I-ET-1 to membranes of ET_B-expressing CHO cells. RES-701-1 (●) or BQ-123 (○) was added to the reaction mixture. All measurements were performed in triplicate.

in $[Ca^{2+}]_i$ elicited by 1 nM ET-1 in COS-7 cells expressing human ET_A or ET_B receptors. An ET-1-induced elevation of $[Ca^{2+}]_i$ was observed in both human ET_A and ET_B receptor-expressing COS-7 cells, but not in mock-transfected COS-7 cells (data not shown). RES-701-1 blocked the ET-1-induced increase in $[Ca^{2+}]_i$ in ET_B receptor-expressing COS-7 cells, in a dose-dependent manner, whereas it did not inhibit the $[Ca^{2+}]_i$ increase in ET_A receptor-expressing COS-7 cells. The inhibition of the ET-1-induced $[Ca^{2+}]_i$ increase in ET_B receptor-expressing COS-7 by RES-701-1 at concentrations of 50, 500, and 5000 nM was 28, 60, and 94%, respectively (Fig. 5). RES-701-1 alone did not influence $[Ca^{2+}]_i$ in either ET_A or ET_B receptor-expressing COS-7 cells (data not shown).

Inhibition of blood pressure responses to exogenously administrated ET in anesthetized rats. The *in vivo* effects of RES-701-1 as an ET_B receptor antagonist were examined by administering it intravenously to anesthetized rats. An intravenous bolus injection of ET-1 at a dose of 0.4 nmol/kg produced early transient depressor responses followed by sustained pressor responses. When RES-701-1 at a dose of 100 nmol/kg or 250 nmol/kg was administrated intravenously 5 min before ET-1, early transient depressor responses to ET-1 were attenuated in a dose-dependent manner and sustained pressor responses to ET-1 were potentiated (Fig. 6). An intravenous bolus injection of RES-701-1 alone did not influence blood pressure significantly (data not shown). Although a transient decrease in blood pressure was observed at the time of drug pretreatment, this was found to be due to the solvent used for the administration of the drug.

Discussion

RES-701-1, which is the first compound isolated from a microbial source that acts selectively on the ET_B receptor, has a novel structure. In the binding study, RES-701-1 inhibited ^{125}I-ET-1 binding to ET_B receptors in bovine cerebellar and lung membranes in a dose-dependent manner but did not influence ^{125}I-ET-1 binding to ET_A receptors in bovine lung membranes.

To confirm the selectivity of RES-701-1, human ET_A and ET_B receptors were stably expressed in CHO cells and the interaction of RES-701-1 with each receptor subtype was assessed. Both ET_A and ET_B receptor-transfected CHO cells expressed ^{125}I-ET-1 binding activity, but RES-701-1 inhibited

TABLE 1
Binding specificity of RES-701-1 for the ET_B receptor

Receptor	Subtype	Radioligand	Receptor source	IC_{50} value	Methods described by
				μM	
ET	ET_B	^{125}I-ET-1	Human placenta	0.01	Experimental Procedures
	ET_A	^{125}I-ET-1	Human placenta	>5	Experimental Procedures
Adrenaline	α_1	[^{3}H]WB4101	Rat forebrain	>1	Greenberg *et al.* (16)
	α_2	[^{3}H]Clonidine	Rat cerebral cortex	>1	Greenberg *et al.* (16)
	β_1	[^{3}H]Dihydroalprenolol	Calf cerebral cortex	>1	U'Prichard *et al.* (17)
Dopamine	D_1	[^{3}H]SCH23390	Rat striatum	>1	Billard *et al.* (18)
	D_2	[^{3}H]Spiperone	Rat striatum	>1	Leysen and Gommeren (19)
Histamine	H_1	[^{3}H]Pyrilamine	Guinea pig cerebellum	>1	Chang *et al.* (20)
	H_2	[^{3}H]Tiotidine	Guinea pig cerebral cortex	>1	Gajtkowski *et al.* (21)
Acetylcholine	M_1	[^{3}H]QNB[a]	Rat cerebral cortex	>1	Bloom *et al.* (22)
Serotonin	5-HT_{1A}	[^{3}H]8-OH-DPAT	Rat hippocampus	>1	Gozlan *et al.* (23)
	5-HT_2	[^{3}H]Ketanserin	Rat prefrontal cortex	>1	Leysen *et al.* (24)
ANP	NPR-A or -B	^{125}I-rANP	Rabbit kidney cortex	>1	Napier *et al.* (25)
Angiotensin II	AT_1	^{125}I-Angiotensin II	Bovine adrenal cortex	>1	Glossmann *et al.* (26)

[a] QNB, quinuclidinyl benzilate; 8-OH-DPAT, 8-hydroxy-*N,N*-di-*n*-propyl-2-aminotetralin; ANP, atrial natriuretic peptide; NPR, natriuretic peptide receptor; AT_1, angiotensin type 1.

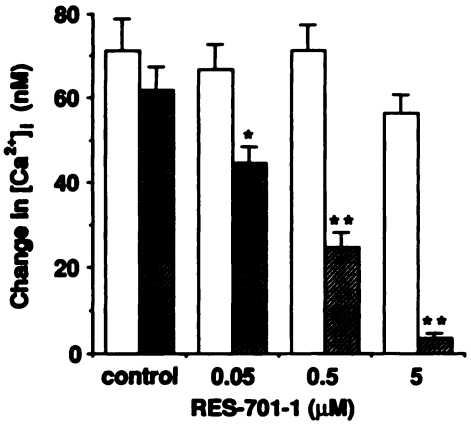

Fig. 5. Effect of RES-701-1 on the increase in $[Ca^{2+}]_i$ induced by ET-1. The effects of various concentrations of RES-701-1 on the ET-1 (1 nM)-evoked increase in $[Ca^{2+}]_i$ values above resting levels in COS-7 cells expressing ET_A (□) and ET_B (▨) receptors were examined. *control*, ET-1-induced $[Ca^{2+}]_i$ elevation seen in the absence of RES-701-1. The values are means ± standard errors of determinations on 11–31 cells. Statistically significant differences are indicated as follows: *, $p < 0.05$; **, $p < 0.01$, compared with each control.

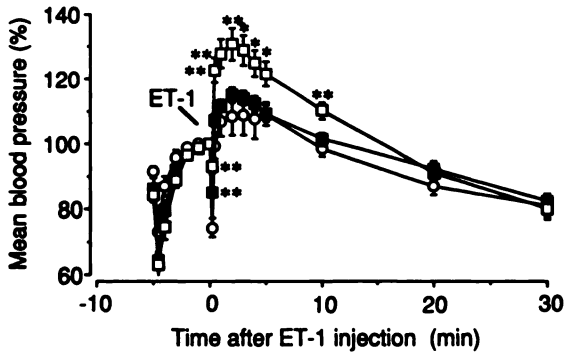

Fig. 6. Effect of RES-701-1 on the changes in blood pressure induced by ET-1 in anesthetized rats. The effects of RES-701-1 on the changes in blood pressure induced by ET-1 in anesthetized rats were examined. RES-701-1 at a concentration of 100 nmol/kg (▨) or 250 nmol/kg (□) was administered intravenously 5 min before ET-1 administration (0.2 nmol/kg, intravenously). Control animals received 0.1 ml/kg dimethylsulfoxide (○) instead of RES-701-1. Each value represents the mean ± standard error for six animals. *, $p < 0.05$; **, $p < 0.01$, significantly different from the control group (dimethylsulfoxide).

^{125}I-ET-1 binding only to ET_B receptors; its affinity for ET_B receptors was >500-fold higher than that for ET_A receptors. These binding data also indicate that RES-701-1 selectively interacts with human ET_B receptors.

In $[Ca^{2+}]_i$ measurements, ET-1-induced $[Ca^{2+}]_i$ increases in human ET_A or ET_B receptor-expressing CHO cells were sought. $[Ca^{2+}]_i$ increases in ET_A receptor-expressing CHO cells were observed. However, it was not possible to detect a $[Ca^{2+}]_i$ increase induced by ET-1 in ET_B receptor-expressing CHO cells, for unknown reasons (data not shown), although detection of a transient $[Ca^{2+}]_i$ increase in CHO cells expressing rat ET_B receptors has been reported (30). We therefore used COS-7 cells transiently expressing human ET_A or ET_B receptors for the measurement of ET-1-induced $[Ca^{2+}]_i$ increases in the present study. ET-1 induced a $[Ca^{2+}]_i$ increase in both human ET_A and ET_B receptor-transfected COS-7 cells. RES-701-1 alone did not influence $[Ca^{2+}]_i$ in ET_A or ET_B receptor-expressing COS-7 cells. Upon application of RES-701-1, the $[Ca^{2+}]_i$ increase induced by 1 nM ET-1 was suppressed in ET_B receptor-transfected COS-7 cells but not in ET_A receptor-transfected COS-7 cells. These results support the hypothesis that RES-701-1 is a selective antagonist for ET_B receptors.

Upon intravenous administration of the ET_B receptor antagonist RES-701-1, before ET-1 administration, RES-701-1 attenuated the ET-1-induced transient depressor response and potentiated the sustained pressor response. Because the initial transient depressor response has been reported to be mediated by ET_B receptors, mainly via the release of relaxing factor from vascular endothelium (10), the inhibition of the depressor response must be the result of selective antagonism of ET_B receptors *in vivo*. Because enhancement of the sustained pressor response to ET-1 by indomethacin via inhibition of prostaglandin release has been reported (10), enhancement of the sustained pressor response to ET-1 by RES-701-1 must be the result of direct or indirect inhibition of prostaglandin release mediated by the ET_B receptor. These results might suggest the involvement of the ET_B receptor in systemic depressor action.

Several ET-related peptides that selectively recognize the ET_B receptor have been reported; sarafotoxin S6c (31) and 4-Ala-ET-1 (32) are selective ET_B receptor agonists, and ET-1(16-21) (33) and [Cys^{11},Cys^{15}]-ET-1(11-21) (IRL 1038) (34) are selective ET_B antagonists. The structure of RES-701-1, a

cyclic peptide, is different from those of the ET isopeptides and those of known ET_B receptor agonists and antagonists. RES-701-1 is highly stable and resistant to hydrolysis by several proteases (data not shown). One structural feature of RES-701-1, the absence of a free amino terminus, must be beneficial in increasing the stability of the compound to the proteases in plasma. Although there is no amino acid sequence homology between RES-701-1 and ET-1, they share the following properties: a carboxyl-terminal tryptophan that is reported to be important for activity (35), a hydrophobic region near the carboxyl terminus, and a cyclic structure. Some of these structural features must be important for binding to the ET receptor. Because conservation of the charged amino acids, such as Asp^8, Glu^{10}, His^{16}, and Asp^{18}, in ET-1 has been reported to be necessary for activity (36), the absence of charged amino acids in comparable positions must be a reason for the loss of agonistic properties in RES-701-1.

In guinea pig trachea, bronchoconstriction induced by ET-1 was not inhibited by BQ-123 (37), suggesting the possible participation of ET_B receptors. Also, a high density of ET_B receptors in human renal cortex and medulla (38), an increased number of ET receptors in cyclosporin A-induced nephrotoxicity (39), an increased affinity of ET receptors in ischemia-induced acute renal failure (40), and a protective effect of anti-ET antibody in an ischemia-induced acute renal failure model (41) suggest the possible involvement of ET_B receptors in renal failure. The expression of ET_B receptors is also reported in a variety of other tissues. However, the true role of ET_B receptors remains obscure, due to the lack of a specific antagonist. RES-701-1 could be a most useful tool in this regard. RES-701-1 showed no acute toxicity in mice at doses up to 300 mg/kg when given intravenously (data not shown).

In summary, the present studies indicate that the novel compound RES-701-1 is a selective and potent antagonist for the ET_B receptor. RES-701-1 is effective in blocking ET_B receptor-mediated responses such as increases in $[Ca^{2+}]_i$ and transient decreases in blood pressure. RES-701-1, then, provides a powerful new tool with which to elucidate the physiological and pathophysiological roles of the ET_B receptor.

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