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Identification of a domain of ET_A receptor required for ligand binding

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Various chimeric ET_A and ET_B receptors were produced in CHO cells for the elucidation of a specific domain which influences the affinity of the receptor toward BQ-123, a selective ET_A antagonist. Replacement of the first extracellular loop domain (B-loop) of the ET_A receptor with the corresponding domain of the ET_B receptor, reduced the inhibition by BQ-123 drastically, while the replacements of other extracellular domains of ET_A did not. By contrast, the introduction of the B-loop of ET_A in place of the corresponding domain of the ET_B receptor endowed the ET_B-based chimeric receptor with a sensitivity to BQ-123. These observations suggest that the B-loop domain of the ET_A receptor is involved in ligand binding.

Endothelin (ET); Receptor; Binding site; Ca^{2+} mobilization; Expression; Antagonist

1. INTRODUCTION

Endothelin (ET), a 21-amino acid peptide, induces strong and long acting vasoconstriction in a wide variety of vascular beds, and serves as an important modulator of vascular tonus [1]. There are three isoforms of endothelin: ET-1, ET-2 and ET-3 [2]. Concerning its receptor, there are at least two types of endothelin receptors, ET_A and ET_B [3]. The ET_A receptor is predominantly located in vascular smooth muscle cells [4,5], where it plays a major role in vasoconstriction; the ET_B receptor is predominantly located in vascular endothelium cells [5,6] and is linked to vasodilation through the release of endothelium-derived relaxation factor and prostacycline [7,8]. We have cloned cDNAs coding for human ET_A and ET_B receptors [9,10]. The cloned cDNA sequences indicated that both ET_A and ET_B are members of the seven transmembrane-spanning and G-protein-coupled receptor family [4,9,11]. Both of the cloned cDNA have recently been expressed in the transfected *COS* cells [9,10]. The expressed ET_A receptor represented different affinities to three endothelin isoforms in the order of ET-1 > ET-2 ≫ ET-3 [5,9], while the ET_B binds to ET-1, 2 and 3 with an almost equal affinity [6,11].

Several receptor antagonists have been reported, which compete with endothelins for binding to receptor and block the G-protein-mediated signal transduction and calcium channeling. The antagonist that inhibits the ET_A or ET_B receptor specifically or inhibits a certain step in signal transduction, provides a powerful tool for the elucidation of the functional domains of the receptor structure. BQ-123 is a novel cyclic pentapeptide, cyclo(-D-Asp-L-Pro-D-Val-L-Leu-D-Trp-), and binds to the ET-1 selective ET_A receptor, but not to the ligand non-selective ET_B receptor [12]. In this paper, the structure and function of two ET receptor species were investigated by construction and production in CHO cells of various chimeric receptors, and by inhibition of their activities by the ET_A-specific antagonist BQ-123.

2. MATERIALS AND METHODS

2.1. *Materials*

ET-1 was purchased from Peptide Institute Inc. (Osaka, Japan) and the ^{125}I-labeled ET-1 (81.4 TBq/mmol) was from New England Nuclear. The site-directed mutagenesis system, Mutan-K, was from Takara Co. Ltd. (Osaka, Japan). 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and Fura-2 (Fura-2-penta-acetoxymethylester) was from Dojin Chemical Institute Co. (Kumamoto, Japan). BQ-123 was chemically synthesized and purified to homogeneity. Details of the procedure will be described elsewhere.

2.2. *Cells and transfection*

CHO cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco Laboratories, New York, USA) supplemented with heat-inactivated 10% fetal bovine serum. The cells were grown at ca. 3 to 4 × 10^6 cells per 90 mm-diameter dish 1 day before transfection and the medium was replaced with 6 ml of DMEM supplemented with heat-inactivated 10% Nu-serum. About 8 μg of pCDM8-based chimeric plasmids in 100 μl of distilled water and 60 μl of 40 mg/ml of DEAE-dextran in phosphate-buffered saline were mixed with vortexing and added to the cell monolayer. After the further addition of 60 μl of 10 mM Chloroquine (Sigma Co., St. Louis, USA), the mixture

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Abbreviations: ET, endothelin; $[Ca^{2+}]_i$, intracellular calcium concentration; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DMEM, Dulbecco Modified Eagle Medium; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; CHO cells, chinese hamster ovary cells; Fura-2, Fura-2-penta-acetoxymethyl ester.

was incubated for 3 h. Cells were treated with 3 ml of 10% dimethylsulfoxide for 3 min, and were fed with fresh medium again and incubated for 72 h prior to ligand binding or Ca^{2+} induction assays.

2.3. *Binding assay*

CHO cells were removed from the monolayer plates and collected by centrifugation. After sonication of CHO cells, the crude membrane was prepared from the cell homogenate by centrifugation at 50,000 rpm for 20 min, and the pellet was suspended in 200 μl of assay buffer (50 mM sodium phosphate buffer, pH 7.4, and 0.1% CHAPS). The assay mixture (50 μl) consists of 30 μl of assay buffer, 15 μl of crude membrane from transfected CHO cells, 2.5 fmoles of ^{125}I-labeled ET-1 and 10^{-10}–10^{-5} M BQ-123. It was incubated for 2 h at room temperature. The receptor–[^{125}I]ET-1 complex was separated from free [^{125}I]ET-1 and determined the radioactivity of the bound ET-1 as described [13]. Non-specific binding was determined in the presence of 1 μM nonradioactive ET-1.

2.4. *Measurement of intracellular calcium concentration ($[Ca^{2+}]_i$)*

Three days after transfection, the CHO cells were treated with trypsin, washed twice with solution A (140 mM NaCl, 4 mM KCl, 1 mM Na_2HPO_4, 1 mM $MgCl_2$, 1.25 mM $CaCl_2$, 11 mM glucose, 5 mM HEPES buffer, pH 7.4, 0.2% bovine serum albumin) and incubated in solution A containing 10 μM Fura-2 for 1 h at 20°C. The Fura-2-loaded cells were washed twice and resuspended in 4.5 ml of fresh solution A without the dye. After transfer of one ml aliquot of the cell suspension to the chamber of the intracellular ion analyzer (Japan Spectroscopic Co., Tokyo, Japan). ET-1 was added at a final concentration of 1 nM and the fluorescence of the cells was measured with excitation at 340 nm and 380 nm, and emission at 500 nm. Next, BQ-123 was added to another one ml aliquot of the Fura-2-loaded cell suspension in the chamber at the final concentration of 1 μM, 1 min and 20 s before the addition of 10^{-9} M ET-1, and ET-1 induced $[Ca^{2+}]_i$ was measured. The total intracellular calcium concentrations of transfected CHO cells before and after the addition of ET-1 were determined as described [14,15].

3. RESULTS

3.1. *Expression of chimeric ET receptors*

We have constructed pCDM8-based expression plasmids to synthesize various chimeric ET receptors, in which each extracellular domain, A, B, C and D of ET_A receptor, was replaced with the corresponding domain of the ET_B receptor. List of these chimeric receptors is shown in Fig. 1. After transfection of CHO cells with these plasmids, the binding of ^{125}I-labeled ET-1 to the receptor was determined as described in section 2. The parental and chimeric ET receptors bound 0.6 to 2.2 fmol range of ^{125}I-labeled ET-1 in the assay condition described in section 2.

3.2. *Differential effect of BQ-123 on the ligand binding activity of various chimeric ET receptors*

BQ-123 was reported to be a ET_A-specific antagonist [12]. We have synthesized BQ-123 to investigate its inhibitory effect on the ligand binding activity of chimeric ET receptors. The synthesized BQ-123 indeed inhibited the [^{125}I]ET-1 binding in an ET_A-specific manner: the binding IC_{50} was 6 nM for the ET_A and more than 10 μM for the ET_B receptor (Fig. 2(I)). When the N-terminal half of ET_A receptor was substituted with the corresponding half of ET_B receptor (Fig. 1K), the resulting

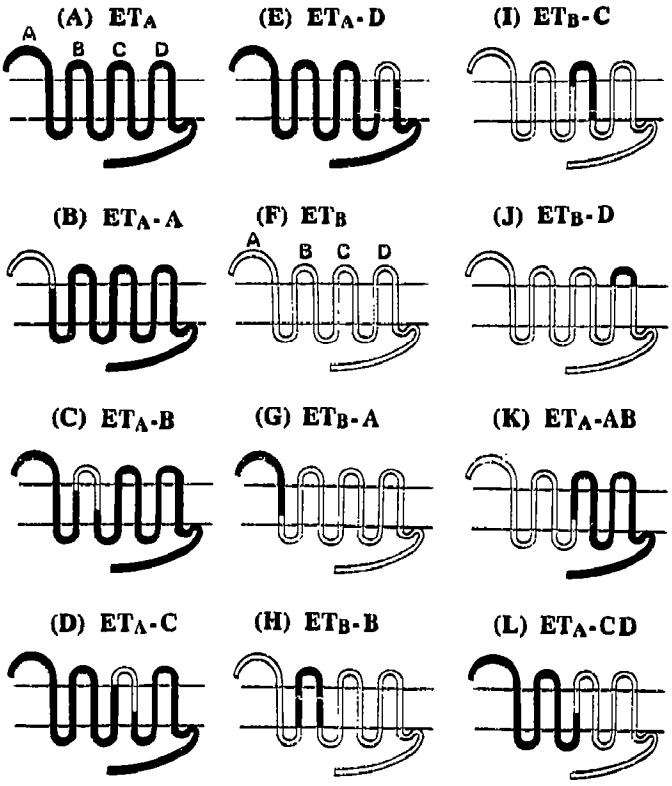

Fig. 1. Structure of chimeric ET receptors. (A) ET_A receptor. (F) ET_B receptor. (K and L) Half of ET_A receptor was replaced by that of ET_B receptor and vice versa. Numbers on the chimeric receptors represent the regions of changed amino acid sequences of the parental ET receptor: ET_A-CD (212–427 of C-terminus), ET_A-AB (1–210). (B–D and G–I) Each of the extracellular A, B and C domains of ET_A receptor was replaced by the same domain of ET_B receptor and vice versa. To construct the expression plasmids for various chimeric ET another ET receptor through several ligation steps. The following amino acid sequences of ET_A receptor were substituted with the corresponding sequences of the ET_B receptor and vice versa. Numbers on the chimeric receptors represent the regions of changed amino acid sequences of the parental ET receptor: ET_A-A (1–79), ET_A-B (139–175), ET_A-C (229–270), ET_B-A (1–120), ET_B-B (138–197), ET_B-C (241–291). (E and J) D domains of the ET_A and ET_B receptors were mutually exchanged. ET_A and ET_B cDNAs were subcloned in the *Xba*I site of M13-mp19 plasmid vector and were subjected to site-directed mutagenesis using the Kunkel procedure [16]. The nucleotide sequence encoding the D-loop domain of ET_A receptor was changed to that of the ET_B receptor. The same approach was done to form the chimeric ET_B receptor that contains the D-loop of the ET_A. The mutated cDNAs were inserted into *Xba*I site of pCDM8 vector at correct orientation. The resultant plasmids were designated as ET_A-D and ET_B-D. The closed bar and open bar represent the sequences of human ET_A and ET_B receptor, respectively. The extracellular A, B, C and D domains are indicated.

chimeric receptor, surprisingly, showed a strong resistance to inhibition by BQ-123. The concentration of BQ-123 required for 50% inhibition (IC_{50}) went up from 6 nM (to ET_A receptor) to over 10 μM for this chimeric receptor (Fig. 2(I)). By contrast, the substitution of the C-terminal half of ET_A with that of ET_B receptor (Fig. 1L) showed only a subtle change in the IC_{50} of BQ-123

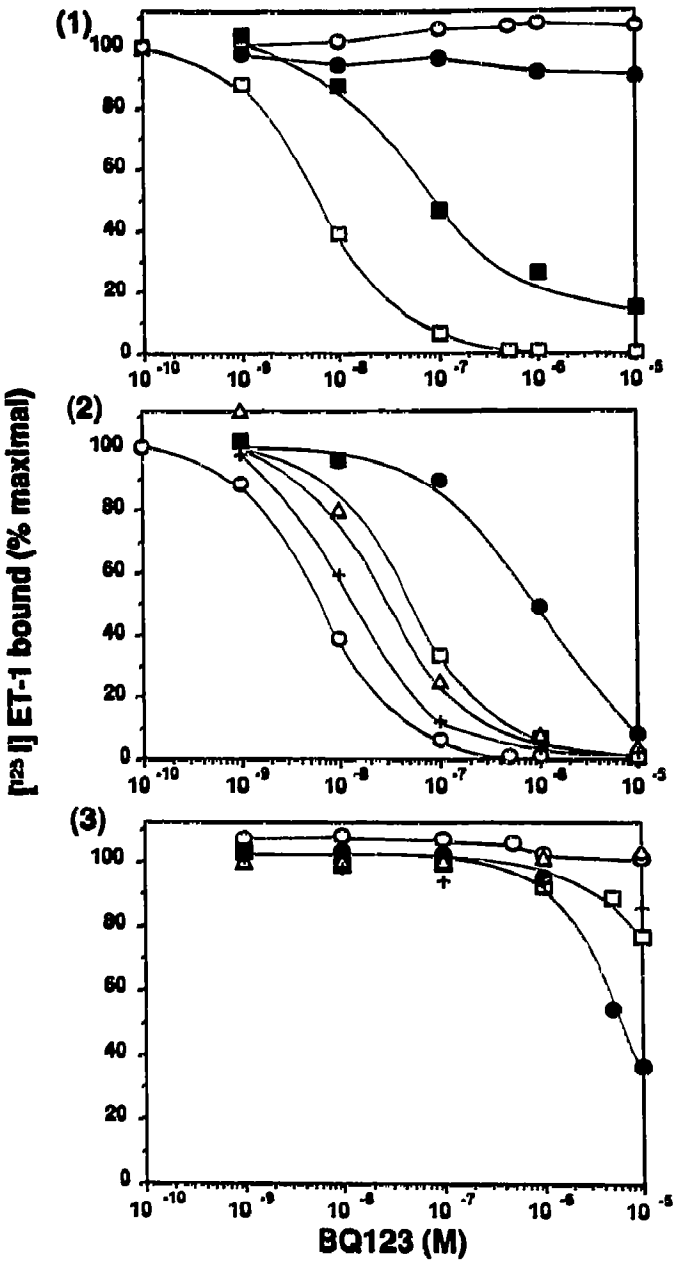

Fig. 2. Inhibitory effects of BQ-123 on the ligand binding of parental and chimeric ET receptors transiently expressed in CHO cells. (1) human ET_A (□) and ET_B (○) receptors, ET_A-CD (■) and ET_A-AB (●) chimeric receptors; (2) human ET_A receptor (○) and the chimeric ET_A-A (□), ET_A-B (●), ET_A-C (△) and ET_A-D (+) receptors; (3) human ET_B (○), ET_B-A (□), ET_B-B (●), ET_B-C (△) and ET_B-D (+) receptors.

(Fig. 2(1)). These results suggest that the N-terminal half which includes A and B-domains of ET_A is responsible for the specific inhibition by BQ-123.

Next, we have constructed a series of recombinant chimeric receptors to investigate in detail the regions that influence the inhibition profile of BQ-123. Each individual domain of ET_A, the A, B, C and D domains, was replaced with the corresponding region of ET_B receptor. Furthermore, the construction of various chimeric receptors with the same replacements were carried out for ET_B receptor. The substitution of each of A, C and D domain of the ET_A receptor with each of the corresponding region of the ET_B receptor (Fig. 1B,D,E) caused only a marginal decrease in the inhibitory activity of BQ-123, to less than one-tenth-fold of the original inhibitory activity (Fig. 2(2)). However, when the B-loop domain of the human ET_A receptor was substituted with the same domain of the ET_B receptor (Fig. 1C), a drastic decrease in antagonistic activity (more than 1/100-fold) of BQ-123 was observed (Fig. 2(2)). Conversely, when the B-loop domain of ET_B receptor was substituted with that of the ET_A receptor (Fig. 1H), the relative antagonistic activity of BQ-123 was increased by more than one order of magnitude (Fig. 2(3)). Thus, the B-loop domain of ET_A receptor must be involved in the interaction with BQ-123.

3.3. *Effect of BQ-123 on the ET induced increase of the* $[Ca^{2+}]_i$

The $[Ca^{2+}]_i$ increased from the 27–77 nM range to the 73–290 nM range, by the addition of 10^{-9} M ET-1 to CHO cells which were transfected with expression plasmids pCDM8-$ET_{(A,B)}$ and chimeric plasmids. As expected, BQ-123 inhibited the ET-induced transient increase of $[Ca^{2+}]_i$ in CHO cells transfected with pCDM8-ET_A, but it did not inhibit the increase in CHO cells transfected with pCDM8-ET_B (Table I). Therefore, BQ-123 inhibited ET-1 binding without any effect on the downstream pathway of signal transduction by which the transient increase of $[Ca^{2+}]_i$ is induced.

The effect of BQ-123 on transient increase of $[Ca^{2+}]_i$ in the transfected CHO cells was examined in detail. When the B-loop region of the human ET_A receptor was substituted with the corresponding region of the ET_B receptor, the inhibition percentage of the ET-1-induced transient increase of $[Ca^{2+}]_i$ by BQ-123 decreased from 98% to 70% (Table I). When the crude membranes were prepared from both recombinant CHO cells, BQ-123 inhibited the binding of ^{125}I-labeled ET-1 to the ET_A and ET_A-B receptors by 99% and 51% at the concentration of 1 μM, respectively (Fig. 2). On the contrary, when the B-loop region of ET_B receptor was substituted with the corresponding region of ET_A receptor, the inhibition percentage by BQ-123 increased from 24% to 89% under the same experimental conditions (Table I). The exchange of A, C and D domains between ET_A and ET_B receptors, however, did not significantly affect the extent of inhibition by BQ-123 for the transient increase of $[Ca^{2+}]_i$ (Table I). Again, these results indicate that the B-loop domain of the ET_A receptor is a site which interacts with BQ-123. The same domain of ET_B receptor, for yet unknown reasons, does not interact with BQ-123.

Table 1

Inhibition by BQ-123 of ET-1 induced transient increase of $[Ca^{2+}]_i$ in transfected CHO cells

ET receptors	Intracellular Ca^{2+} concentration				Inhibition (%) by BQ123
	Basal	10^{-9} M ET-1	Basal	10^{-6} M BQ123 and 10^{-9} M ET-1	
ET_A	76 nM	220 nM	26 nM	29 nM	98%
ET_A-A	48 nM	120 nM	47 nM	47 nM	100%
ET_A-B	34 nM	95 nM	39 nM	57 nM	70%
ET_A-C	63 nM	160 nM	63 nM	63 nM	100%
ET_A-D	44 nM	290 nM	45 nM	45 nM	100%
ET_B	56 nM	140 nM	36 nM	100 nM	24%
ET_B-A	69 nM	250 nM	72 nM	250 nM	2%
ET_B-B	64 nM	240 nM	67 nM	87 nM	89%
ET_B-C	55 nM	220 nM	41 nM	200 nM	4%
ET_B-D	77 nM	250 nM	84 nM	250 nM	4%
ET_A-CD	27 nM	86 nM	39 nM	42 nM	95%
ET_A-AB	29 nM	73 nM	39 nM	91 nM	0%

Increase of intracellular calcium concentration by the addition of 10^{-9} M ET-1, and the inhibition by 1 μM BQ-123 was determined as described in section 2. Inhibitory effect of BQ-123 on the ET-induced increase of $[Ca^{2+}]_i$ was determined and represented by percentages.

4. DISCUSSION

It has been reported that the C-terminal domain of ETs, especially the tryptophan residue at the C-terminus, plays an important role in the binding of ETs to the receptor [17,18]. Supporting this hypothesis, the peptidic endothelin antagonists, BQ-123 and FR-139317 (1-hexahydroazepino-CO-Leu-D-Trp(CH_3)-D-(2-pyridyl)alanine), and the agonist, IRL-1620, Suc-[Glu^9,$Ala^{11,15}$]-ET-1(8–21) [19], that contain C-terminal tryptophan residue, have been found to be specific ET antagonists or agonists. The two former antagonists are ET_A-specific and the latter agonist is ET_B-specific. All these peptidic compounds apparently bind to the endothelin receptor through the structural resemblance of the -Asp(Leu)-Pro(Asp)-Val(Ile)-Leu(Ile)-Trp-sequence to the C-terminal sequence (-Leu-Asp-Ile-Ile-Trp) of endothelins. Although the processing of preprobigET and the second messenger pathways of ET have been extensively investigated, little is known about the function of each ET receptor domain. First of all, it is not clear why the two distinct receptor species that bind ET-1 with an almost identical affinity exist in the different cells and tissues. The two types of receptor differ in binding to ET-3, while the overall structure of receptor molecule and the biological activity, as measured in vitro by ligand binding and induction of $[Ca^{2+}]_i$ increase, are quite similar. The significant similarity of the two ET receptors makes it difficult to define the regions required for ligand selection and binding. BQ-123, a strong ET_A specific inhibitor, is thus one of few valuable reagents that can distinguish the binding site of the ET_A receptor from ET_B receptor.

To assess the structural alterations of chimeric receptors that result in the changes in the profile of ligand binding, we used BQ-123 as a diagnostic reagent. The substitution of the B-loop of the ET_A receptor with the same region of ET_B receptor remarkably reduced the affinity by BQ-123, indicating that BQ-123 perhaps binds to the B-loop of the ET_A receptor, but not, or weakly, to the B-loop of ET_B receptor. As shown in the experiments (Table I) in which the ET-dependent $[Ca^{2+}]_i$ was monitored in CHO cells that express chimeric receptors, BQ-123 inhibited the ET-1-induced increase of $[Ca^{2+}]_i$ significantly when the B-loop of ET_A was present in the chimeric receptor, in accordance with the increased inhibition in ligand binding. The results indicated that BQ-123 is not an agonist and is a useful reagent that competes with ET-1 at the ligand binding step only.

Recently, we have found that part of the N-terminal extracellular domain (amino acid residues 50–76 of the predicted human ET_A sequence), in the vicinity of the first transmembrane, is required for ET-1 binding [20]. The data indicates that part of the N-terminal domain is involved in the tertiary structure formation of ligand binding site, together with the B-loop domain of the ET_A receptor.

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