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RES-701-1/ENDOTHELIN-1 HYBRID PEPTIDE HAVING A POTENT BINDING ACTIVITY FOR TYPE B RECEPTOR

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Abstract : N-Terminal cyclic peptide of RES-701-1, an endothelin (ET) type B receptor selective antagonist, was chemically combined to C-terminal peptide of ET family. Among a series of the hybrid peptide synthesized, RES-701-1(1-10)/[A^{15}]ET-1(12-21) hybrid peptide possessed both a potent binding activity with an IC_{50} value of 0.24nM and a selectivity for type B receptor. © 1997 Elsevier Science Ltd.

Introduction :

In recent years many efforts have been done to exploit more potent antagonist for both types of endothelin (ET) receptors, ET_A and ET_B[1-7], since ET receptor antagonist may have therapeutic potential in cardiovascular disease[8, 9], renal disease[8] and asthma[10]. Additionally these two receptors have been reported to have distinct cell/tissue distributions[11] and, accordingly, their different physiological roles have been suggested[12]. Therefore, selective ligands of these individual receptors will be useful tools for elucidating the physiological and pathophysiological functions of ETs and for the treatment of individual diseases above mentioned.

RES-701-1 has been identified as an ET_B receptor selective antagonist with a binding potency (IC_{50}) of 10nM and its structure has been determined as a unique cyclic peptide consisting of 16 amino acid residues[13, 14] (Figure 1). The cyclic portion of RES-701-1, RES-701-1(1-9), was constructed from nine amino acids, in which N-terminal Gly and beta-carboxyl group of Asp^9 was combined via amide linkage[14].

On the other hand, endothelins (ETs) and sarafotoxins (SRTXs) are a family of potent vasoactive peptides having a conserved bicyclic motif of disulfide bonds[15-17]. Amino acid compositions of these peptides are similar and C-terminal hydrophobic amino acids are known to be important for their biological activities. Substitution and derivatization of amino acids in ETs have been investigated to develop compounds that possess high binding affinity to both of these receptor subtypes[18, 19]. It has been suggested that cyclic structure of ETs is essential for vasoconstrictor activity and substitution of hydrophobic amino acids such as Phe and Tyr and deletion of C-terminal Trp cause a drastic decrease of their binding activity[20, 21].

Taken together, it is suggested that in the case of RES-701-1 similarity of cyclic structure and C-terminal hydrophobicity to ET family may be important to retain its specific activity such as receptor specificity and receptor binding potency. In this report C-terminal peptide of RES-701-1 was chemically substituted for the C-

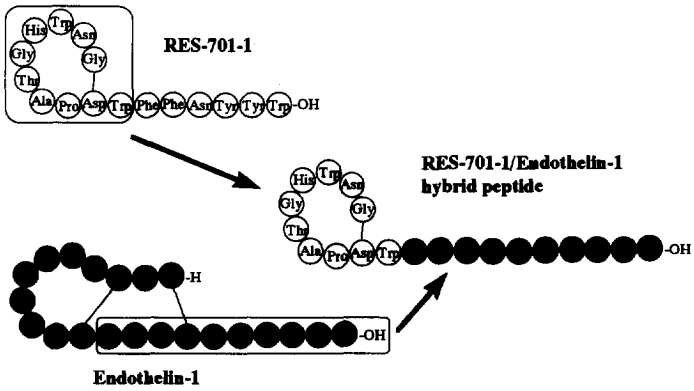

Figure 1. Structure of RES-701-1(1-10)/[A^{15}]ET-1(12-21) hybrid peptide.

terminal of other vasoactive peptides derived from ETs and SRTXs to give RES-701-1/ET family hybrid peptide having a unique cyclic structure of RES-701-1 (Figure 1). It was found that among a series of hybrid peptides composed of cyclic portion of RES-701-1 and other vasoconstriction peptides RES-701-1(1-10)/[A^{15}]ET-1(12-21) hybrid had both a potent binding activity and an ET_B receptor selectivity. Here we show the significance of cyclic peptide of RES-701-1 to retain receptor binding and the binding character of several RES-701-1/ET family hybrid peptides.

Experimental :

Synthesis N-Terminal cyclic peptide portion of RES-701-1 and RES-701-1/ET hybrid peptides were synthesized according to the similar procedure described before[22] with several modifications. Nine-fluorenylmethoxycarbonyl (Fmoc) -protected linear peptide of RES-701-1(1-8) was initially synthesized by using a Shimadzu PSSM-8 peptide synthesizer based on Fmoc-based solid phase synthetic strategy[23], and then dipeptide, β-tert-butyl aspartyl tryptophane benzyl ester, was condensed to the C-terminal of RES-701-1(1-8) in N,N-dimethylformamide (DMF) containing diethyl phosphorocyanidate (DEPC) (1.5 equiv.) and triethylamine (TEA) (2.5 equiv.). The resulting peptide was subjected to deprotection reaction by using trifluoroacetic acid (TFA)-based cocktail[22] and 20% piperidine solution to give a C-terminal protected linear peptide of RES-701-1(1-10). Cyclization of RES-701-1(1-10) was carried out with benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) (2 equiv.)/N-hydroxybenzotriazole (HOBt) (2 equiv.)/N-methylmorpholine (NMM) (3 equiv.) system[22] in DMF and then C-terminal protecting group was eliminated by hydrogenation using palladium-carbon catalyst under the hydrogen stream to give a cyclic peptide of RES-701-1(1-10)(yield 30-50%). Cyclic portion of RES-701-1(1-9) was also prepared in a similar way by using β-tert -butyl aspartic acid benzyl ester for the preparation of C-terminal protected linear peptide of RES-701-1(1-9). C-terminal protected peptides of individual ETs were separately prepared by condensation of Fmoc-protected linear peptide and tryptophane benzyl ester followed by deprotection of N-terminal protecting group. Hybrid

peptide was then obtained by condensation of cyclic portion of RES-701-1(1-10) and C-terminal protected peptide followed by deprotection of C-terminal protecting group. Condensation reactions were carried out with PyBOP (2 equiv.)/HOBt (2 equiv.)/NMM (3 equiv.) system[22].

Cyclization of linear peptide of RES-701-1(1-16) via amide linkage between N-terminal Gly^1 and C-terminal Trp^{16} was carried out to obtain N-C cyclized RES-701-1 by condensation reaction of non-protected liner peptide of RES-701-1 under the conditions of PyBOP (3 equiv.)/HOBt (3 equiv.)/NMM (2 equiv.)[22] (yield 50%).

In above procedure initial peptides and final products were purified to homogeneity by semi-preparative reversed-phase HPLC. Structures of peptides obtained were individually determined by fast atom bombardment mass spectrometry (FAB-MS) and amino acid analysis[22].

Receptor Binding Assay Binding activities for ET_A and for ET_B receptors of synthesized peptides were examined independently by the method as previously described[24]. Bovine lung membrane, in which ET_B receptor was masked by RES-701-1(5 μ M), and bovine cerebellum membrane were used as receptor sources for ET_A and for ET_B respectively. As a control, authentic RES-701-1 obtained from the culture broth by isolation procedure described in a previous paper[13] was used for receptor binding assay.

Results and Discussion :

In order to clarify the essential moiety of RES-701-1 for receptor binding, initially a series of peptide fragments and derivatives of RES-701-1 and ET family were synthesized and examined their binding affinity for ET_B receptor as shown in Table 1, several data from which are agreed with those reported elsewhere[25]. Linear peptide of RES-701-1[26] and N-C cyclized RES-701-1 in which N-terminal Gly^1 and C-terminal Trp^{16} was combined via amide linkage had very weak or no binding activity. From these results importance of cyclic structure of RES-701-1 via Gly^1-Asp^9 linkage was suggested. In addition, although C-terminal peptide of RES-701-1 had weak binding activity for ET_B receptor with an IC_{50} value of 3 μ M, cyclic portion of RES-701-1, RES-701-1(1-9), had not, indicating that in authentic RES-701-1 cyclic structure may enhance and stabilize the binding potency of C-terminal "tail" peptide. Considering from the structural similarity between RES-701-1[13],

Table 1. Binding activity of synthetic peptide fragments of RES-701-1 and ET family for ET_B receptor

Compound	Structure	IC_{50} for ET_B (nM)
RES-701-1 (authentic)	cyclo(GNWHGTAPD)WFFNYYW-OH	10
linear peptide of RES-701-1	H-GNWHGTAPDWFFNYYW-OH	5,000
N-C cyclized RES-701-1	cyclo(GNWHGTAPDWFFNYYW)	>5,000
RES-701-1(1-9)	cyclo(GNWHGTAPD)-OH	>1,000
RES-701-1(10-16)	H-WFFNYYW-OH	3,000
ET-1(12-21)	H-VYFCHLDIIW-OH	1,000
[S^{15}]ET-1(12-21)	H-VYFSHLDIIW-OH	1,000
ET-3(12-21)	H-VYYCHLDIIW-OH	3,000
SRTX6b(12-21)	H-LYFCHQDVIW-OH	3,000

[14] and ETs[15-17], e.g. cyclic structure and C-terminal hydrophobic amino acids, C-terminal peptide of ET-1 being important for receptor binding and having a little higher potency than that of RES-701-1 (Table 1) was combined to a cyclic portion of RES-701-1 to give RES-701-1/ET-1 hybrid peptide as shown in Figure 1. To elucidate the significance of the length and sequence of C-terminal peptide, a series of hybrid peptides having different length of C-terminal portion of ET-1 were prepared according to the procedure described in Experimental. The results are summarized in Figure 2. The hybrid peptide composed of cyclic portion of RES-701-1 and C-terminal three hydrophobic peptide of ET-1, Ile-Ile-Trp, which was conserved among ET family[15-17], did not compete the binding of ^{125}I-ET-1 up to 6 μ M. The hybrid peptide composed of RES-701-1(1-10) and [Ala^{15}]ET-1(15-21) in which Cys^{15} was substituted for Ala was also had a very weak binding potency (data not shown). Among the hybrid peptides synthesized RES-701-1(1-10)/[Ala^{15}]ET-1(12-21) possessed the most potent binding activity for ET_B receptor indicating that length of tail peptide must be important for retaining the receptor binding activity.

IC_{50} of the RES-701-1(1-10)/[Ala^{15}]ET-1(12-21) hybrid peptide was 0.24nM for ET_B receptor. This affinity is 42-fold higher than that of RES-701-1 (IC_{50}=10nM) and close to that of ET-1 (IC_{50}=0.09nM). Considering from the fact that binding potency of C-terminal of ET-1(12-21) was 1 μ M of IC_{50} value (Table 1), it is possible that cyclic portion of RES-701-1, which has little binding potency with the IC_{50} value of >1 μ M for ET_B receptor, may play a role as an enhancer of the binding of C-terminal peptide as in the case of RES-701-1.

Among the ET family C-terminal peptides are well conserved[15-17]. In C-terminal 12-21 peptides of ET family, ET-1, ET-2 and ET-3 were identical except for 14th amino acid, and SRTX-6a, SRTX-6b and SRTX-6c were identical except for 13th amino acid[15-17]. Then these vasoactive peptides consisting of a different sequence were used for constructing hybrid peptides to examine binding potency. The results are shown in Figure 3. The

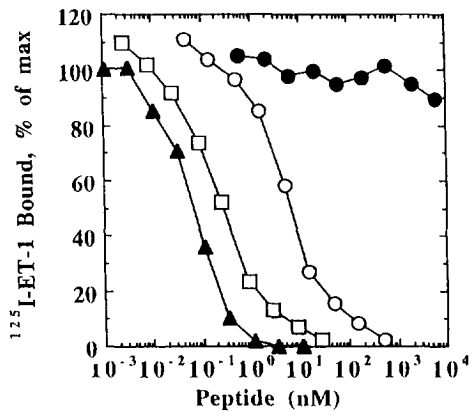

Figure 2. Inhibition of ^{125}I-ET-1 binding to bovine cerebellum membrane by RES-701-1/ET-1 hybrid peptides. Following peptides were used for competition assay described in Experimental. Authentic RES-701-1(-○-), RES-701-1(1-10)/ET-1(19-21)(-●-), RES-701-1(1-10)/[A^{15}]ET-1(12-21)(-□-) and ET-1(-▲-). All measurements were performed in triplicate.

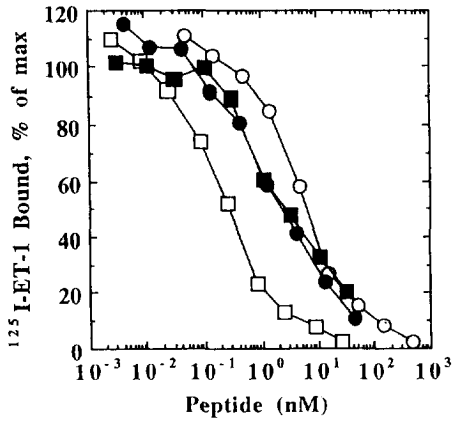

Figure 3. Inhibition of ^{125}I-ET-1 binding to bovine cerebellum membrane by RES-701-1/ET-1, RES-701-1/ET-3 and RES-701-1/STX6b hybrid peptides. Following peptides were used for competition assay described in Experimental. Authentic RES-701-1(-○-), RES-701-1(1-10)/[A^{15}]ET-1(12-21)(-□-), RES-701-1(1-10)/[A^{15}]ET-3(12-21)(-●-), RES-701-1(1-10)/[A^{15}]SRTX6b(12-21)(-■-). All measurements were performed in triplicate.

Table 2. Binding selectivity of RES-701-1/ET hybrid peptides

Compound	Structure	IC_{50} (nM)	
		ET_A	ET_B
RES-701-1 (authentic)	cyclo(GNWHGTAPD)WFFNYYW-OH	>5,000	10
RES-701-1(1-10)/[A^{15}]ET-1(12-21)	cyclo(GNWHGTAPD)WVYFAHLDIIW-OH	>100	0.24
RES-701-1(1-10)/[A^{15}]ET-3(12-21)	cyclo(GNWHGTAPD)WVYYAHLDIIW-OH	>100	2.5
RES-701-1(1-10)/[A^{15}]SRTX6b(12-21)	cyclo(GNWHGTAPD)WLYFAHQDVIW-OH	>100	3.8

hybrid peptides composed of cyclic portion of RES-701-1(1-10) and ET-related peptides showed ET_B receptor binding profile although the binding potencies were not higher than that of RES-701-1(1-10)/[A^{15}]ET-1(12-21) hybrid. IC_{50} values of RES-701-1(1-10)/[A^{15}]ET-3(12-21) and RES-701-1(1-10)/[A^{15}]SRTX6b(12-21) were 2.5nM and 3.8nM respectively. Thus, in the case of other ET-related peptides binding potencies were also enhanced by attaching the cyclic portion of RES-701-1 to their C-terminal peptides, whose own binding potencies were comparable to C-terminal of RES-701-1 (Table 1).

It is well-known that there are several types of ET receptor, typically ET_A and ET_B[4-7]. Binding selectivity of the potent hybrid peptides was also investigated by using receptor source for ET_A[24]. The results are summarized in Table 2. The binding potency for ET_A receptor was above 100nM of IC_{50} value in every hybrid peptides. Thus the hybrid peptides were demonstrated to be ET_B receptor specific ligands. Interestingly, this receptor selectivity was similar to that of RES-701-1 itself, binding potency of which was 10nM (IC_{50}) for ET_B and > 5 μ M (IC_{50}) for ET_A. It is suggested from these data that cyclic portion of RES-701-1 may one of the factors which decide receptor selectivity.

Conclusions :

This is the first report on the hybrid peptides composed of RES-701-1 and ET family. A series of hybrid peptides having a cyclic peptide of RES-701-1 and C-terminal peptide of ETs were chemically synthesized. Although significant binding potencies were not observed in cyclic portion of RES-701-1 and C-terminal peptide fragments of ETs by themselves, both receptor specificity of RES-701-1 and potent binding activity of ET family were synergistically functioned in the hybrid form. Among the hybrid peptides obtained RES-701-1(1-10)/[A^{15}]ET-1(12-21) possessed a most potent binding profile with ET_B receptor selectivity of RES-701-1 maintained. It is suggested that cyclic portion of RES-701-1 enhance and stabilize receptor binding activity of tail peptide and decide receptor specificity. Hybrid peptide described here will be useful tool for the investigation of the function of ET receptors and the pathological significance of ETs and for treatments in putative ET-related diseases.

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