Pergamon

Bioorganic & Medicinal Chemistry 6 (1998) 2459–2467

BIOORGANIC & MEDICINAL CHEMISTRY

Hybrid Peptides Constructed from RES-701-1, an Endothelin B Receptor Antagonist, and Endothelin; Binding Selectivity for Endothelin Receptors and their Pharmacological Activity

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Received 9 June 1998; accepted 2 September 1998

Abstract—Hybrid peptides were constructed from endothelin B receptor (ET_B) selective antagonist RES-701-1 (**1**) and endothelin (ET-1). They have N-terminal 10 amino acids derived from **1** and C-terminal 10 amino acids derived from ET-1. RES-701-1(1-10)-[Ala15]ET-1(12-21) and its analogues substituted or truncated at the residues derived from RES-701-1 had proved to possess high receptor binding activity selective for ET_B as well as **1**. Substitutions at the residues derived from ET-1 had produced some analogues that possessed high affinity not only for ET_B but for ET_A. Although all analogues had antagonistic effects on ET_A, some analogues had proved to function as agonist on ET_B confirmed by the changes in intracellular calcium concentrations of ET receptor-transfected COS-7 cells. We have found four types of ET receptor-binding peptides: (1) ET_B-selective agonist with weak ET_A antagonism (**3**, KT7421); (2) ET_B-selective antagonist with weak ET_A antagonism (**29**, KT7539); (3) ET_B agonist with potent ET_A antagonism (**27**, KT7538); and (4) non-selective ET_A/ET_B antagonist (**26**, KT7540). © 1998 Elsevier Science Ltd. All rights reserved.

Introduction

Endothelins are the family of potent vasoactive peptides,[1,2] of which the first member, ET-1,[3] has been shown to be one of the most potent vasocontractors known. Their versatile physiological actions, such as, for example, vasoconstriction, release of endothelium-derived relaxing factors, mitogenesis and bronchoconstriction, are mediated through their receptors, ET_A and ET_B, which have been distinguished based on their differential sensitivity for ET-1 and ET-3.[4,5] Endothelins have been suggested to play a possible role in many disease states, such as cardiovascular diseases, asthma, myocardial infarction and acute renal failure. Therefore compounds which specifically block the effects of ETs have been thought to have some therapeutic potential in endothelin-related diseases.[4,6,7]

RES-701-1 (**1**, Fig. 1) is an ET_B-selective potent antagonist of microbial origin that consists of 16 amino acids with 'side chain-to-head' cyclic structure by an amide bond between Gly1 α-NH_2 and Asp9 β-COOH.[8–10] We have found that a hybrid peptide constructed from the N-terminal cyclic portion of **1**, RES-701-1(1-10), and the C-terminal fragment of ET-1, [Ala15]ET-1(12-21) (**2**, Fig. 1), has much higher ET_B binding activity than **1** with ET_B-selectivity.[11] The C-terminal fragment of ET-1, [Ala15]ET-1(12-21) (IRL 1442), has been reported to have low affinity, K_i values of micromolar order, for both ET_A and ET_B independently, whereas succinylation at N-terminal of IRL 1442 lead to produce ET_B-selective binding peptide, IRL 1545, K_i values of 810 nM and 4 nM for ET_A and ET_B, respectively.[12,13] Compared to the structure–activity relationships of IRL 1442 and IRL 1545, it is considered that such high affinity and selectivity of **2** for ET_B derives from the

Key words: Receptors; agonists; antagonists; peptides and polypeptides.
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0968-0896/98/$ - see front matter
PII: S0968-0896(98)00183-7

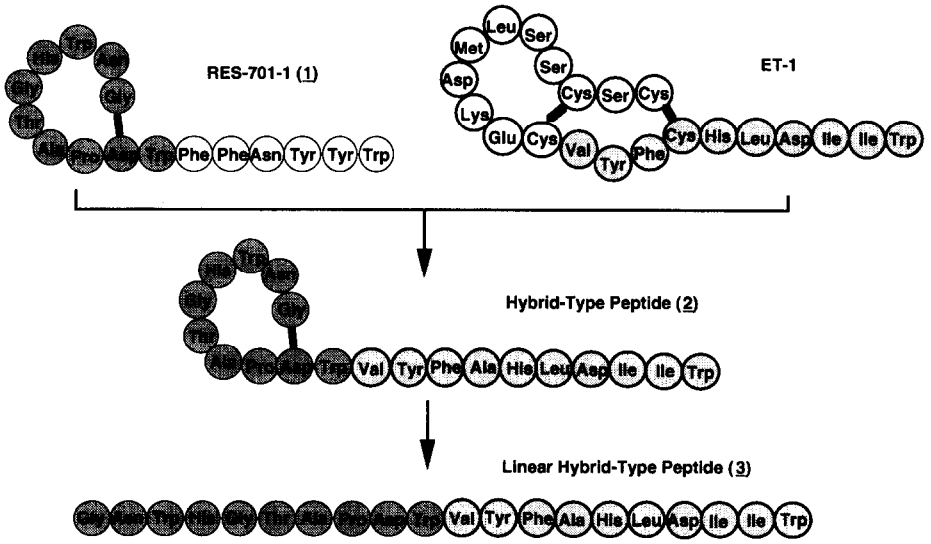

Figure 1. Structures of RES-701-1, ET-1 and their hybrid peptides.

character of N-terminal cyclic portion of **1**, RES-701-1(1-10), which has no negative charge corresponding to the succinyl group of IRL 1545.

Distinct from the linear peptide of **1**, which has significantly reduced receptor binding activity compared to **1**,[11,14] the linear analogue of **2** (**3**, Fig. 1) has proved to have slightly higher affinity for ET_B than **2**. Its affinity and selectivity for ET_B is comparable to that of the linear peptide analogue of ET-1, [Ala1, 3, 11, 15]ET-1,[15] which is an ET_B-selective agonist. Our hybrid peptide **3** also has proved to act as an agonist confirmed by the ability to induce the increase of intracellular calcium concentration ($[Ca^{2+}]_i$) of ET_B-transfected cells, as well as [Ala1, 3, 11, 15]ET-1 or IRL 1620,[12,13] which has four negative charges of a succinyl group, an Asp and two Glu residues added at the N-terminus of IRL 1442. It has also been reported that the substitutions at positions 18 and 19 of ET-1 for Thr and γ-MeLeu, respectively, have led to produce a non-selective antagonist for ET_A and ET_B.[16] We have chemically synthesized some linear hybrid-type analogues of **3** and investigated their receptor binding activity on ET receptor-containing membrane preparations and their agonist/antagonist activity on human ET receptor-transfected cells to find some analogues of various receptor subtype selectivity and functional activity on ET_A and ET_B.

Results and Discussion

Receptor binding and functional activities of hybrid peptides

RES-701-1 almost loses its ET_B receptor binding activity if its amide bond between Gly1 α-NH_2 and Asp9 β-COOH is cleaved. The IC_{50} value increases from 10 nM to 8 μM.[11,14] Conversely, the linear peptide of the hybrid-type analogue **3** has proved to have slightly higher affinity for ET_B than **2** (Table 1). We have investigated the receptor binding activity of some peptides analogous to **3**, which were mainly substituted or deleted in N-terminal portion derived from RES-701-1. Results are shown in Table 1. All of the N-terminal substituted analogues (N-terminal NH_2 and positions 2, 4, 5, 6, 7 and 10) and deleted ones up to three residues retained high affinity and selectivity for ET_B (**4–13**, IC_{50} for ET_B of sub-nanomolar range and for ET_A/ET_B > 100). Succinylation on N-terminal exhibited little influence on the binding activity (**4**), distinct from the results on the C-terminal fragments of ET-1, those have been improved the receptor binding activity by N-terminal succinylation, such as IRL 1442 to IRL 1545.[12] Substitutions at positions 5 and 6, those which have been suggested to form a turn structure in **1** by our NMR study,[17] resulted in elevation of the ET_B binding activities (**8** and **9**), however, the affinity for ET_A also slightly elevated. Trp10 was able to be substituted with a little influences on the receptor binding activity and selectivity (**10**). Deletions of N-terminal residues also exhibited little influences on the binding activity (**11–13**). Some residues derived from ET-1 have proved to be much responsible for the receptor binding activity. A substitution of Val11 to Pro or a simultaneous deletion of Val11 and Tyr12 caused to reduce their activities (**14** and **16**), however, a deletion of only Val11 less influenced on the activity (**15**). Because of the remarkable reduction of the binding activity by the substitution of C-terminal Trp for Phe (**17**), C-terminal Trp residue of the hybrid-type peptides have been proved to be

Table 1. Receptor binding activity of RES-701-1/ET-1 hybrid peptide **3** and its analogues

Compd no.	Structure[a]	ET_A[b]	ET_B[c]
1 (RES-701-1)	Figure 1	> 1000	10
2	Figure 1	> 100	0.24
3 (KT7421)	Figure 1	250	0.083
4	N^{α}-Succinyl	> 400	0.14
5 (KT7420)	[Asp2]	> 100	0.63
6 (KT7419)	[Lys4]	> 100	0.29
7	[Asp2, Lys4, Ser7]	250	0.11
8	[Thr5]	120	0.061
9	[Gly6]	85	0.064
10	[Ala10]	175	0.22
11	[des-Gly1]	> 300	0.10
12	[des-Gly1, Asn2]	220	0.076
13	[des-Gly1, Asn2, Trp3]	370	0.088
14	[Pro11]	> 420	4.2
15	[des-Val11]	260	0.57
16	[des-Val11, Tyr12]	> 470	6.1
17	[Phe20]	> 420	11
18	[Tyr13]	> 23[d]	0.83
19	[Leu11, Gln16, Val18]	> 37[e]	0.33

[a]Represented are the substituted positions of KT7421 (**3**) except compounds **1–3**.
[b]Bovine lung membrane in the presence of 5 μM of RES-701-1. IC_{50} values in nM.
[c]Bovine cerebellum membrane. IC_{50} values in nM.
[d]2% inhibition at 23 nM.
[e]20% inhibition at 37 nM.

significantly important for receptor binding as reported for ET-1.[18] It is distinct from the case of C-terminal substituted analogues of **1**, that the C-terminal Trp residue has been able to be substituted for Phe, Tyr or D-Trp retaining relatively high affinity for ET_B.[19] Hybrid peptides of **1** and ET-3 (**18**) or sarafotoxin 6b (**19**) also exhibited the ET_B-selective binding activity.

To define the functional activity of our hybrid peptides, we assayed the effects of some representative peptides on the $[Ca^{2+}]_i$ transient of human ET receptor-transfected COS-7 cells. As shown in Figure 2, peptides **3**, **5** and **6** inhibited the increase of $[Ca^{2+}]_i$ induced by ET-1 in ET_A-expressing cells at relatively high concentration (3.2 μM), while they independently induced the increase of $[Ca^{2+}]_i$ in ET_B-expressing cells at much lower concentrations (32 or 16 nM). It is proved to be that these compounds functioned as selective agonist on ET_B with weak antagonism on ET_A. It is considered that the agonistic activity of these analogues on ET_B derived from the C-terminal half of ET-1. Some peptide ligands, those having the C-terminal fragment of ET-1, as IRL 1620 or [Ala1, 3, 11, 15]ET-1, are reported to be agonists on ET_B.[12,13,15] The weak but certain antagonism on ET_A with strong ET_B agonism, which has never been reported about the ET_B agonist above, is considered to

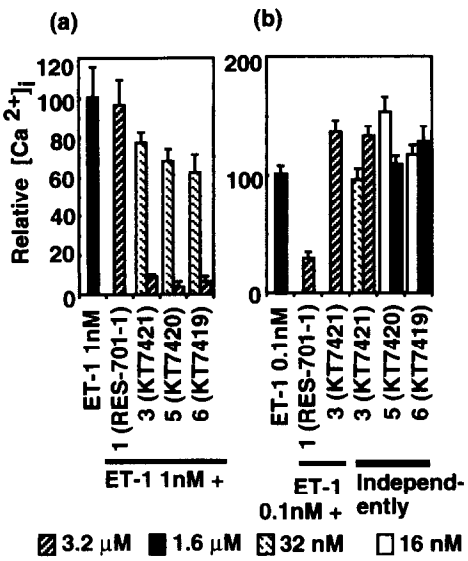

Figure 2. Effects of peptides on elevated $[Ca^{2+}]_i$ by adding ET-1 or independent elevating effects of peptides in ET receptor transfected COS-7 cells. Values show relative changes of $[Ca^{2+}]_i$ elevated by adding ET-1 in % (means ± SEM). (a) ET_A-Transfected cells. ET-1 was added in 1 nM. (b) ET_B-Transfected cells. ET-1 was added in 0.1 nM.

be a unique character of our hybrid peptides. In addition, a known ET_B-selective antagonist IRL 1038, [Cys11-Cys15]ET-1, has been reported to show no antagonism on ET_A.[20] This character, in a sense, seems to mean a low subtype selectivity, however, the mixed antagonism/agonism for ET_A and ET_B, respectively, is considered to be useful to investigate the role of ET receptors.

Substitutions at positions 17 and 18 to produce analogues of various selectivity and function

It has been reported that some analogues of ET-1 substituted at the positions 18 and 19 exhibited antagonistic activity on both ET_A and ET_B.[16] To produce the selective antagonist on ET_B and nonselective ligands for both receptor subtypes, we demonstrated the substitutions at positions 17 and 18 of **3**. Results of the receptor binding assay are shown in Table 2. We have succeeded in producing some potent ET_A/ET_B non-selective analogues (**21–23, 25–27**; IC_{50} for $ET_A/ET_B < 10$) other than ET_B selective analogues (**29, 32** and **35**). Although substitution of Asp17 to Glu resulted only in the reduction of ET_B binding activity (**20**), substitution of Ile18 to Leu resulted in the increase of the affinity for ET_A with retaining moderate affinity for ET_B (**21**). Substitution of Asp17 to Thr accompanied by Leu18 substitution resulted in a little improvement of the ET_B binding activity (**22**). Substitution for Met and Tyr at position 18 were also allowed (**23** and **24**), of which the binding

Table 2. Receptor binding activity of RES-701-1/ET-1 hybrid peptide **3** and its analogues substituted in C-terminal region

Compd no.	Structure[a]	ET_A[b]	ET_B[c]
3 (KT7421)	Figure 1	250	0.083
20	[Glu17]	290	17
21	[Leu 18]	21	5.4
22	[Thr17, Leu 18]	21	2.9
23	[Thr17, Met18]	14	1.7
24	[Thr17, Tyr18]	53	1.2
25	[Asn17, Tyr18]	53	1.2
26 (KT7540)	[Ser17, Met18]	5.3	0.96
27 (KT7538)	[Thr17, Cha18]	5.4	12
28	[Thr17, Abu18]	72	0.11
29 (KT7539)	[Thr17, Thi 18]	30	0.12
30	[Thr17, Phg18]	37	0.58
31	[Thr17, Nle18]	17	0.63
32	[Asp2, Lys4, Ser7, Thr17, Thi18]	49	0.12
33	[Nva16, Thr17, Thi18]	83	0.37
34	[D-Ala14, Thr17, Thi18]	370	4.9
35	[Thr17, Thi18, D-Ile19]	>410	210
36	[Thr17, Thi18, D-Trp20]	>410	210
37	[D-Phe13, Ser17, Met18]	63	3.3

[a]Represented are the substituted positions of KT7421 (**3**) except compounds **3**.
[b]Bovine lung membrane in the presence of 5 μM of RES-701-1. IC_{50} values in nM.
[c]Bovine cerebellum membrane. IC_{50} values in nM.

activity for both ET_A and ET_B is comparable to **22**. In the series of these analogues, Ser17-Met18 (**26**) and Thr17-Cha18 (**27**) substituted ones are proved to have highest affinity for ET_A while Thr17-Thi18 type (**29**) to have highest affinity for ET_B. Among the analogues substituted at position 18 together with Thr17, those which substituted for Phg, Abu and Nle were allowed to possess non-selective character (**28**, **30** and **31**). All of the non-selective analogues are substituted at position 17 from charged residue (Asp) to non-charged ones (Ser, Thr or Asn), however, the selectivity seems to depend upon the residues at position 18. Although many types of substitutions have been carried out at position 18, no apparent relationships have been found between structures and receptor selectivity. Among analogues substituted at other than positions 17 and 18 for D-amino acids (**34**–**37**), Ile19 and Trp20 are proved to be the most critical residues for receptor binding because of remarkable reductions in their binding activity. These results are suitable for some reports on ET-1 analogues as discussed above.

The functional activity of these analogues substituted at positions 17 and 18 are presented in Table 3. As peptide **3** (KT7421), peptides **21**, **27** and **28** exhibited some agonistic activities while peptides **22**, **23**, **26** and **29** inhibited the increase of $[Ca^{2+}]_i$ induced by ET-1 on ET_B-transfected cells. On ET_A-transfected cells, all of

Table 3. Effects of the hybrid peptides on ET receptor transfected COS-7 cells in intracellular Ca^{2+} concentrations in the presence or absence of ET-1

Compds.	ET_A/COS-7[a]		ET_B/COS-7[b]		
	Conc.[c]	$[Ca^{2+}]_i$[d]	Conc.[c]	ET-1	$[Ca^{2+}]_i$[d]
—	—	100.0 ± 6.39	—	+	100.0 ± 4.59
1	3200	91.7 ± 6.33	3200	+	1.4 ± 0.11
3	3200	9.1 ± 1.40	32	+	134.4 ± 10.3
3	—	—	32	—	96.7 ± 8.62
21	32	62.3 ± 10.0	32	—	102.6 ± 6.56
22	32	24.6 ± 7.32	32	+	34.0 ± 4.11
22	320	6.4 ± 1.46	320	+	10.1 ± 1.65
23	320	4.6 ± 0.79	32	+	35.4 ± 4.27
26	320	2.7 ± 0.24	3200	+	9.3 ± 3.33
27	32	21.3 ± 4.28	3.2	–	77.1 ± 4.33
28	32	77.5 ± 11.0	3.2	–	87.0 ± 6.62
29	3.2	66.8 ± 5.91	0.32	+	90.9 ± 5.52
29	320	5.7 ± 2.18	32	+	15.3 ± 2.42

[a]In the presence of 1 nM of ET-1.
[b]In the presence (ET-1 +) or absence (ET-1 –) of ET-1.
[c]In nM.
[d]Relative changes of intracellular Ca^{2+} concentration compared to that caused by ET-1 in percent.

the analogues act as antagonist. Among those having agonistic activity on ET_B, **21** and **27** have some improved activity on ET_A compared to **28**. Thus **21** and **27** are proved to be agonists on ET_B with potent antagonism on ET_A. Among those which have antagonistic activity on ET_B, **22**, **23** and **26** showed nearly the same inhibiting activity on ET_A and ET_B while **29** has selective antagonism on ET_B. That is to say, **22**, **23** and **26** are non-selective ET_A/ET_B antagonist while **29** is ET_B-selective antagonist. These results are also well explained by receptor binding selectivity. Peptides which showed ET_B-selective binding activity have relatively strong effect, either agonistic or antagonistic, on ET_B-transfected cells, and those which showed non-selective binding activity act as agonists or antagonists on ET_A- as well as ET_B-transfected cells. In general, with some exceptions like **26** (KT7540) and **27** (KT7538), simultaneous substitutions of Asp17 for Ser, Thr or Asn and Ile18 for amino acids with γ-branched side chain caused conversions from ET_B agonist to antagonist (**22** and **29** (KT7539), for example). These types of agonist/antagonist conversions were reported about analogues of ET-1 on ET_A receptor.[16] Tyr residue at position 18, which is correspondent to the third residue from the C-terminal of RES-701-1, causes conversion from ET_B agonist to antagonist. Together with the result of Thi18 substitution in **29** (KT7539, ET_B-selective antagonist) and Cha18 substitution in **27** (KT7538, ET_B-selective agonist), it is considered that the aromatic side chains at this position are also important for their antagonistic activity on ET_B receptor.

Effects of ET_B-agonists KT7421 (3) and KT7538 (27) on rat superior mesenteric arteries in vitro

Stimulation of endothelial ET_B receptors leads to the relaxation through the release of endothelium-derived relaxing factor (EDRF). In this study, ET_B-agonism of KT7421 and KT7538 was investigated using superior mesenteric arteries from rats. KT7421 concentration-dependently relaxed the arterial strips contracted with L-phenylephrine (Fig. 3a). KT7538, another ET_B agonist, less active than KT7421, also caused vasorelaxation, but the effect was weak and not significant (Fig. 3a). Contrary to ET_B agonist, ET_B antagonist, both KT7539 (**29**) and KT7540 (**26**), did not show the vasorelaxant effects (Fig. 3b). These results suggest that the vasorelaxant effects of KT7421 and KT7538 are due to the ET_B-agonistic activity. Furthermore, IRL 1620, a known ET_B agonist, reported to cause the relaxation of precontracted rat aorta through EDRF release by the action of endothelial ET_B receptors.[13]

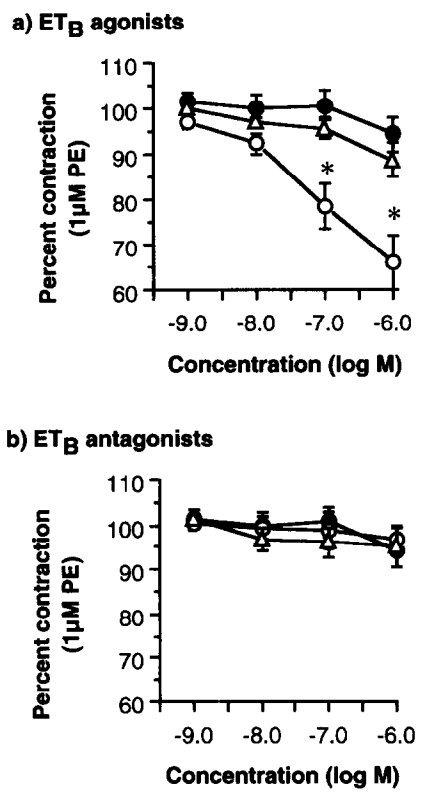

Figure 3. Dose-dependent relaxing effects of ET_B agonist, KT7421 (open circles) and KT7538 (open triangles) (a), or antagonist, KT7539 (open circles) and KT7540 (open triangles) (b) on rat superior mesenteric arteries precontracted by 1 μM of L-phenylephrine. Values show the relative percentage of tensions compared to that of 1 μM of L-phenylephrine in means ± SEM (n = 5 or 6). *; $p < 0.01$ versus control (closed circles) by Wilcoxon's test.

Effects of some hybrid-peptides on rat thoracic aorta contracted by ET-1 in vitro

ET-1 causes vasocontraction in rat or guinea-pig thoracic aortic rings in a concentration-dependent manner. This effect is considered to be mainly due to ET_A-mediated contraction of smooth muscle cells in aorta. The treatments of the rat aorta with 1 μM of the four types of hybrid peptides, KT7421 (**3**), KT7538 (**27**), KT7539 (**29**) and KT7540 (**26**), showed slight inhibition of ET-1-induced contraction (Fig. 4), indicating that they exhibited the effects by ET_A-antagonism. Their effects, especially those of KT7538 or KT7539, seem to be weaker than expected from the effects on human ET_A receptor transfected in CHO-cells. It is not clear that these results can be explained from the different effects of these peptides on human and rat ET_A receptor.

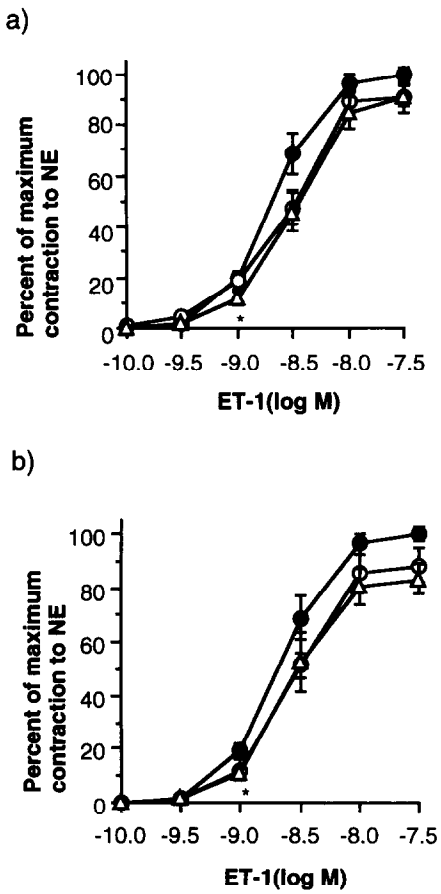

Figure 4. Effects of KT7539 (open circles) or KT7540 (open triangles) (a), and KT7421 (open circles) or KT7538 (open triangles) (b) on rat thoracic aorta contracted by ET-1. Values show the relative percentage of tensions compared to that of 1 μM of L-phenylephrine in means ± SEM (n = 7). *; $p < 0.05$ versus control (closed circles) by Wilcoxon's test.

Effects on the blood pressure of anaesthetized rat in vivo

We investigated the effects of the four types of hybrid peptides on the pressor and depressor responses induced by ET-1 in anaesthetized rats. A bolus iv injection of ET-1 induced the early transient depressor response followed by the sustained pressor response. Among the four types of hybrid peptides, only KT7421 inhibited the early transient depressor response (Figs 5c and d), which was considered to be a ET_B-mediated effect of ET-1. The sustained pressor responses were enhanced significantly by KT7540 and KT7421 (Fig. 5). The effect was also shown by KT7538, but was weak. The profile of KT7421 is to resemble that of RES-701-1, an ET_B-selective antagonist.[21] It is reported that the enhancement of sustained pressor responses by RES-701-1 is suggested to be induced by the inhibition of ET_B-mediated prostaglandin release.[21] Although KT7421 independently acts as an agonist on human ET_B receptors and in rat superior mesenteric arteries, it may act like an ET_B antagonist in ET-1-administered anaesthetized rats. Results obtained here have been considered to be

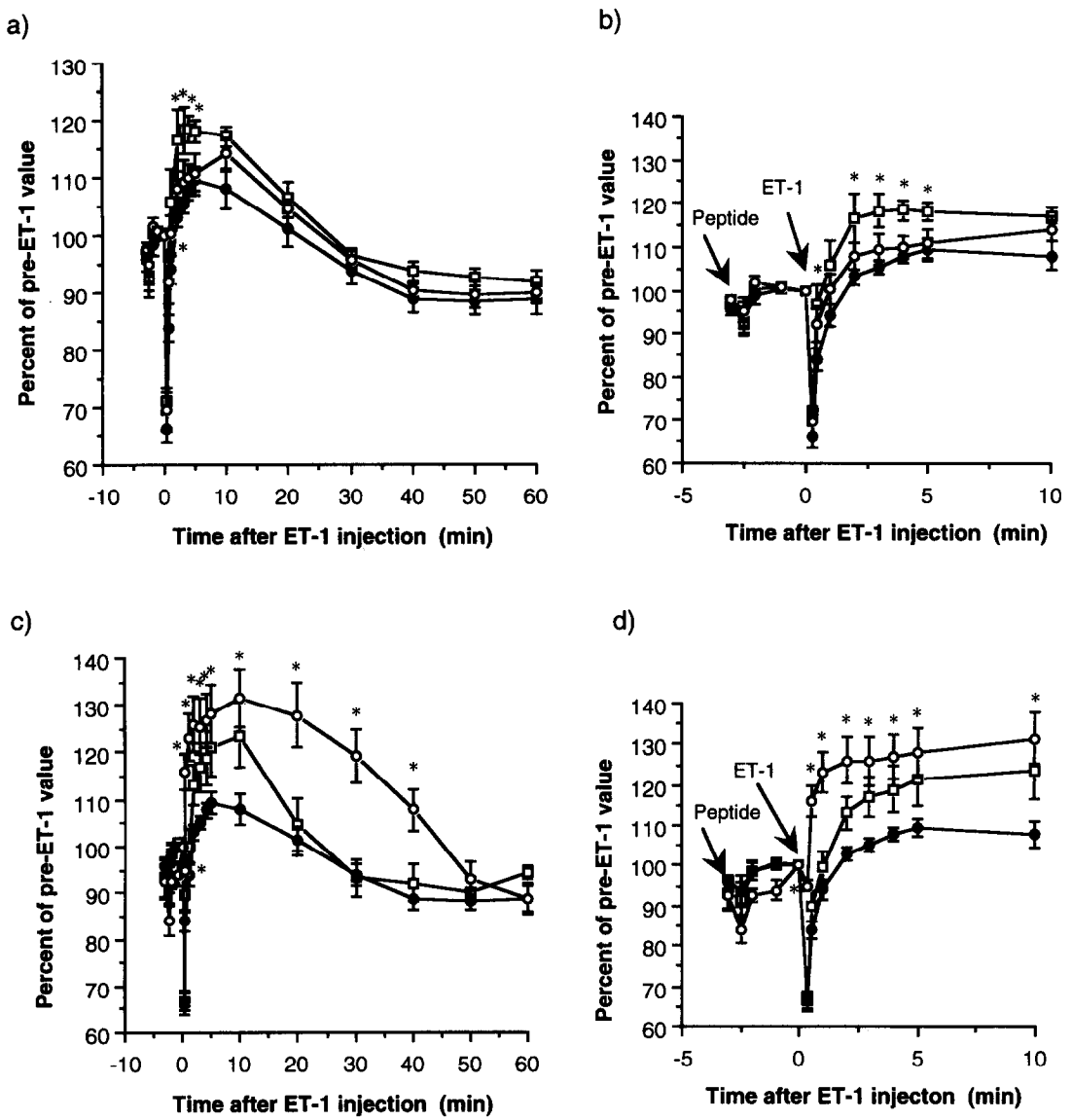

Figure 5. Effects of KT7539 (open circles) or KT7540 (open boxes) (a and b), and KT7421 (open circles) or KT7538 (open boxes) (c and d) on the changes of blood pressure of anaesthetized rat induced by the administration of ET-1. Values show the relative percentage of mean blood pressure compared to that before the administration of ET-1 in means ± SEM (n = 5). *; $p < 0.05$ versus control (closed circles) by Wilcoxon's test.

incompatible to our expectation from the receptor selectivity and functional activity of four types of peptides shown by the in vitro experiment. In order to explain this discrepancy, precise investigations are needed.

Conclusion

From these results, we have found four types of ET receptor binding peptides: (1) ET_B-selective agonist with weak ET_A antagonism (**3**, KT7421); (2) ET_B-selective antagonist with weak ET_A antagonism (**29**, KT7539); (3) ET_B agonist with potent ET_A antagonism (**27**, KT7538) and (4) non-selective ET_A/ET_B antagonist (**26**, KT7540). Recently, ET receptors have been classified more closely as ET_{A1}, ET_{A2}, ET_{B1} and ET_{B2}.[22] ET receptor binding peptides, analogues of sarafotoxin 6b, that function as agonist or antagonist depending upon the receptor subtypes are also recently reported.[23] Hybrid-type peptides obtained here were thought to be useful tools to investigate the roles of ET receptors and to be drug candidates for diseases in which endothelins are involved in the pathogenesis.

Experimental

Abbreviations used follow IUPAC-IUB nomenclature.[24] Additional abbreviations are Cha, 2-cyclohexyl-L-alanine; Phg, L-phenylglycine; Nle, L-norleucine; Abu, 2-aminobutylic acid; Thi, 2-thienyl-L-alanine; Nva, L-norvaline.

Peptide synthesis

All hybrid-type peptides ware prepared using standard 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase synthetic method[25] on a Shimadzu PSSM-8 peptide synthesizer (Shimadzu, Japan). Syntheses were initiated from Fmoc-amino acid-Wang resins (Novabiochem, USA) containing 30 μmol of amino acids. Some of the Fmoc-amino acids were sidechain protected by *tert*-butyl (*t*-Bu) for Asp, Glu, Ser and Thr, by trityl (Trt) for His, Asn and Gln, by *tert*-butoxycarbonyl (Boc) for Lys and by 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for Arg. Ten equivalent of Fmoc-amino acids to the amino group on resin, those were activated by (benzotriazol-1-yloxy)-tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP)/*N*-hydroxybenzotriazole (HOBt)/*N*-methylmorpholine (NMM) in *N*,*N*-dimethylformamide (DMF), were coupled to the growing peptide resin whose N^{α}-Fmoc groups were deprotected by 30% piperidine in DMF. After last coupling and N^{α}-Fmoc deprotection, sidechain-protected peptide resin was treated with trifluoroacetic acid (TFA):1,2-ethandithiol:thioanisole (90:5:5) containing 5 mg/mL of 2-methylindole to cleave from the resin and deprotect the sidechain protections. Only the peptide **4** has been obtained by the reaction of **3** with succinic anhydride. Obtained crude peptides were purified by preparative reversed-phase HPLC eluted by the linear gradient of acetonitrile in 0.1% TFA/water and lyophilized. All peptides were analyzed for homogeneity by analytical HPLC, fast atom bombardment mass spectrometry (FABMS, JMS-HX110A; Jeol, Japan) and amino acid analysis (AAA, Pico-Tag; Waters, USA). The analytical data for all peptides are summarized in Table 4.

Biological activity

The binding activity, functional activity on ET receptor transfected cells and the effects on the blood pressure in vivo were investigated as reported previously.[21]

Rat aortic rings

Thoracic aortas were isolated from male Sprague–Dawley rats and cut into 3 to 4 mm rings. The aortic rings were mounted on metal holders and suspended vertically in an organ bath (20 mL) filled with Krebs–Henseleit solution (composition in mM: NaCl, 133.5; KCl, 4.96; $CaCl_2$, 2.5; NaH_2PO_4, 1.35; $NaHCO_3$, 16.3; $MgSO_4$, 0.61; glucose, 7.77) maintained at 35 °C, gassed with 95% O_2–5% CO_2. The upper metal holder was connected to a force transducer (TB-611T, Nihon Kohden, Japan) coupled to a pen recorder (TYPE3066, Yokeogawa, Japan). Isometric contraction was measured under the resting tension of 1.5 g. After equilibration, the rings were contracted with 0.1–20 μM (±)-norepinephrine·HCl (NE, Sigma Chemical Co., USA), and then washed and allowed to equilibrate to baseline again. In the presence of a peptide or solvent, increasing concentrations (0.1–30 nM) of human ET-1 (Bachem, Switzerland) were added cumulatively to the bath. Increases in tension by ET-1 were expressed as percent of the maximal response to NE.

Rat superior mesenteric arterial rings

The superior mesenteric arteries were isolated from male Sprague–Dawley rats and cut into rings (about 2 mm-length). The rings were tied to two metal holders with thin silk suture and suspended horizontally in an organ bath (10 mL) filled with Krebs–Henseleit solution (the same as described above). One holder was fixed to the bath and the other connected to a force transducer coupled to a pen recorder. Isometric contraction was measured under the resting tension of 200 mg. After equilibration, the rings were contracted with 1 μM L-phenylephrine·HCl (PE, Wako Pure Chem. Ind., Japan). The contraction reached a stable plateau, and then cumulatively increasing concentrations of a peptide,

Table 4. Analytical data of hybrid peptides

No.	FABMS (calcd)[a]	AAA (calcd)[b]
3	2398.89 (2398.60)	G2.4(2), A2.0(2), Asx2.9(3), H1.9(2), I1.0(2), L0.9(1), F0.9(1), P1.1(1), T1.1(1), Y0.9(1), V0.7(1)
4	2498.68 (2430.73)	Not determined.
5	2400.15 (2399.59)	G2.1(2), A2.0(2), Asx2.1(3), H1.4(2), I2.5(2), L1.3(1), P1.0(1), T1.1(1), F0.9(1), Y0.9(1), V1.3(1)
6	2390.18 (2389.63)	G2.6(2), A2.0(2), Asx2.8(3), H1.0(1), I2.4(2), L1.1(1), P1.2(1), T0.9(1), Y1.0(1), V1.1(1), F1.0(1), K0.9(1)
7	2406.90 (2406.62)	G2.2(2), A1.0(1), Asx2.3(3), H1.0(1), I0.9(2), L1.0(1), P1.5(1), T1.0(1), Y0.8(1), V0.8(1), F0.9(1), S1.2(1) K1.3(1)
8	2443.16 (2442.65)	G1.2(1), A2.0(2), Asx2.0(3), H2.0(2), I1.0(2), L1.0(1), P1.3(1), T2.0(2), Y0.9(1), V0.8(1), F1.0(1)
9	2355.08 (2354.55)	G3.8(3), A2.0(2), Asx2.3(3), H2.1(2), I0.8(2), L0.8(1), P1.4(1), Y0.7(1), V0.6(1), F0.8(1)
10	2284.09 (2283.47)	G2.4(2), A3.0(3), Asx2.5(3), H2.2(2), I1.0(2), L0.9(1), P1.3(1), T1.2(1), Y0.8(1), V0.8(1), F0.9(1)
11	2341.90 (2341.55)	G1.1(1), A2.0(2), Asx2.4(3), H2.0(2), I0.8(2), L0.8(1), P1.3(1), T1.2(1), Y0.7(1), V0.7(1), F0.8(1)
12	2227.80 (2227.45)	G1.0(1), A2.0(2), Asx1.2(2), H2.1(2), I0.8(2), L0.8(1), P1.1(1), T1.1(1), Y0.8(1), V0.7(1), F0.8(1)
13	2041.90 (2041.24)	G1.1(1), A2.0(2), Asx1.2(2), H2.0(2), I0.7(2), L0.8(1), P1.2(1), T1.2(1), Y0.7(1), V0.6(1), F0.8(1)
14	2397.00 (2396.59)	G2.1(2), A2.0(2), Asx2.1(3), H2.1(2), I0.9(2), L1.0(1), P2.1(2), T1.0(1), Y1.0(1), F1.0(1)
15	2300.19 (2299.47)	G2.1(2), A2.0(2), Asx1.6(3), H2.1(2), I1.0(2), L0.9(1), P1.1(1), T1.1(1), Y0.9(1), F0.9(1)
16	2137.09 (2136.30)	G2.1(2), A2.0(2), Asx2.0(3), H2.1(2), I0.9(2), L0.9(1), P1.2(1), T1.0(1), F0.9(1)
17	2360.07 (2359.56)	G2.8(2), A3.0(3), Asx2.5(3), H2.1(2), I0.7(2), L0.7(1), P1.5(1), T1.4(1), Y0.6(1), V0.5(1), F1.3(2)
18	2414.80 (2414.60)	G2.3(2), A2.0(2), Asx2.6(3), H2.0(2), I1.6(2), L0.9(1), P1.1(1), T1.1(1), Y1.6(2), V0.7(1)
19	2413.70 (2413.57)	G2.2(2), A2.0(2), Asx2.7(3), H2.0(2), I0.6(1), L1.1(1), P1.1(1), T1.0(1), Y1.0(1), V0.6(1), Glx1.2(1), F1.1(1)
20	2413.10 (2412.63)	G2.1(2), A1.9(2), Asx1.1(2), Glx1.1(1), H2.1(2), T0.9(1), I1.0(2), L1.0(1), P1.1(1), Y0.9(1), V0.8(1), F1.0(1)
21	2399.10 (2398.60)	G2.1(2), A2.0(2), Asx2.2(3), H2.1(2), I0.9(1), L1.9(2), P1.3(1), T1.2(1), Y0.8(1), F1.0(1)
22	2385.20 (2384.62)	G2.0(2), A2.0(2), Asx1.3(2), H2.2(2), I1.1(1), L2.3(2), P1.2(1), T2.0(2), Y0.8(2), V0.8(1), F0.9(1)
23	2403.08 (2402.66)	G2.0(2), A2.0(2), Asx1.3(2), H2.2(2), I1.2(1), L1.1(1), P1.2(1), T2.2(2), Y1.0(1), V0.9(1), F1.0(1), M1.0(1)
24	2435.10 (2434.63)	G1.9(2), A2.0(2), Asx1.1(2), H2.0(2), I0.9(1), L1.0(1), P1.0(1), T1.9(2), Y1.8(2), V0.8(1), F0.9(1)
25	2448.00 (2447.63)	G1.9(2), A2.0(2), Asx2.2(3), H2.0(2), I0.9(1), L1.0(1), P1.2(1), T1.0(1), Y1.7(2), V0.9(1), F0.9(1)
26	2388.90 (2388.63)	G2.0(2), A2.0(2), Asx1.2(2), H2.1(2), I1.0(1), L1.0(1), P1.1(1), T1.0(1), Y0.9(1), V0.9(1), F1.0(1), S1.0(1)
27	2425.00 (2424.68)	G2.1(2), A2.0(2), Asx1.2(2), H2.1(2), I1.1(1), L1.1(1), P1.2(1), T2.0(2), Y0.9(1), V0.8(1), F1.0(1)
28	2357.20 (2356.56)	G2.1(2), A2.0(2), Asx1.3(2), H2.2(2), I1.1(1), L1.2(1), P1.2(1), T2.2(2), Y1.0(1), V1.0(1), F1.1(1)
29	2425.10 (2424.66)	G1.9(2), A2.0(2), Asx1.2(2), H2.1(2), I1.0(1), L1.0(1), P1.1(1), T2.0(2), Y1.0(1), V0.9(1), F1.0(1)
30	2404.94 (2404.61)	G2.1(1), A2.0(2), Asx1.0(2), H2.1(2), I1.7(1), L0.9(1), P1.0(1), T2.0(1), Y0.9(1), V0.8(1), F0.9(1)
31	2385.17 (2384.62)	G2.3(2), A2.0(2), Asx1.3(2), H2.2(2), I1.0(1), L1.0(1), P1.2(1), T2.2(2), Y0.8(1), V0.8(1), F0.9(1)
32	2432.90 (2432.69)	G2.1(2), A1.0(1), Asx1.2(2), H1.0(1), I0.8(1), L1.1(1), P1.3(1), T2.0(2), Y0.8(1), V0.7(1), F1.0(1), Ser1.0(1), Lys1.1(1)
33	2410.99 (2410.64)	G2.1(2), A2.0(2), Asx1.2(2), H2.1(2), I0.9(1), P1.1(1), T1.9(2), Y0.9(1), V0.7(1), F1.0(1)
34	2424.97 (2424.66)	G2.1(2), A2.0(2), Asx1.2(2), H2.1(2), I0.9(1), P1.1(1), T1.9(2), Y0.9(1), V0.8(1), F1.0(1)
35	2424.90 (2424.66)	G2.1(2), A2.0(2), Asx1.2(2), H2.0(2), I0.8(1), L1.0(1), P1.1(1), T2.1(2), Y0.9(1), V0.8(1), F1.0(1)
36	2424.99 (2424.66)	G2.1(2), A2.0(2), Asx1.2(2), H2.1(2), I0.8(1), L1.0(1), P1.1(1), T1.9(2), Y0.9(1), V0.8(1), F1.0(1)
37	2388.93 (2388.63)	G2.0(2), A2.0(2), Asx1.0(2), H2.2(2), I0.9(1), L1.0(1), P1.2(1), T1.0(1), Y0.9(1), V0.8(1), F1.0(1), S1.0(1), M1.0(1)

[a]Presented values are the observed mass ($M+H^+$) and calculated average mass.

[b]Each amino acid is presented in a single letter code except Asx (Asp or Asn) and Glx (Glu or Gln). Trp, Cha, Thi and Abu have not been analyzed.

dissolved in distilled water, were added to the bath. Decreases in tension caused by peptides were measured as the vasorelaxant effects. The values of relaxation were expressed as a percentage of the preconstricted tension with 1 μM PE.

Anaesthetized rats

Male Sprague–Dawley rats (460–570 g, Slc, Japan) were used. Anaesthesia was introduced with pentobarbital sodium (35 mg/kg iv) and maintained with the mixed gas (0.5% halothane in air) by ventilation through an endotracheal tube (60 breaths/min with a tidal volume of 10 mL/kg). The left jugular vein was cannulated with a polyethylene tube for the administration of the peptides. To measure blood pressure, a heparin-filled polyethylene catheter was inserted into the left carotid artery, coupled to a pressure transducer (DX-300, Nihon Kohden, Japan). The blood pressure was recorded on a polygraph (RM-6000, Nihon Kohden) and mean blood pressure (MBP) was calculated. At 3 min after intravenous administration of 100 nmol/kg of the peptides, 0.2 nmol/kg of ET-1 was injected intravenously by a bolus injection. The blood pressure was recorded for 63 min after administration of the peptides.

Statistics

Results are expressed as the mean ± standard error of mean (SEM). Differences between control and peptides were statistically analyzed using Wilcoxon's test and considered significant at $p < 0.05$.

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