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Structure–activity relationship in vasoconstrictor effects of sarafotoxins and endothelin-1

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Sarafotoxins (SRTa, SRTb and SRTc) as well as endothelin-1 (ET-1) produced vasoconstrictions in rat thoracic aorta, rat isolated perfused mesentery and pithed rat in various of extents. The potency was ET-1 > SRTb > SRTa > SRTc at lower doses, but SRTb > ET-1 > SRTa > SRTc at higher doses. [Nitrophenylsulfenylated Trp^{21}]SRTb and SRTb(1–19) caused no vasoconstriction. Either the reduction and carboxymethylation of Cys residues, the destruction of the intramolecular loop or the production of the non-natural disulfide bond, eliminated the constrictor activity. These results indicate that Trp^{21} and the intramolecular loop structure are essential, and Lys^{9} and Tyr^{13} may play some important roles for the vasoconstrictor activity of these peptides.

Sarafotoxin; Endothelin-1; Vasoconstriction; (Rat thoracic aorta, Rat mesenteric artery, Pithed rat)

1. INTRODUCTION

Sarafotoxin S6a (SRTa), sarafotoxin S6b (SRTb) and sarafotoxin S6c (SRTc) are new cardiotoxic isotoxins isolated from the venom of the burrowing asp *Atractaspis engaddensis* [1,2]. It has been reported that SRTb provides powerful coronary vasoconstriction, positive inotropic effect and severe atrioventricular block in mice, rats and humans [3]. Sarafotoxins consist of 21 amino acid residues with two sets of intrachain disulfide linkages [4] and show a high sequence homology with endothelin-1 (ET-1) [5], a potent vasoconstrictor peptide obtained from porcine aortic endothelial cells [6]. In the present study, the vasoconstrictor activity of sarafotoxins was studied using three animal models in order to characterize the structural moiety of sarafotoxins which is essential for vasoconstrictor activity in comparison with a variety of synthetic SRTb analogs.

2. MATERIALS AND METHODS

2.1. *Rat aortic ring*

Male Sprague–Dawley rats (Charles River, Japan, 300–350 g) were sacrificed by cervical dislocation and the thoracic aorta was excised and cleaned of the surrounding tissues. The endothelium was mechanically removed by rolling a small forceps on the luminal surface of the aortic rings. Ring preparations (approximately 4 mm in length) were mounted in 10 ml organ bath filled with Krebs–Henseleit solution of the following composition (mM): NaCl 119.0, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.0, $NaHCO_3$ 25.0, dextrose 11.1, maintained at 37°C and continuously bubbled with a mixture of 95% O_2 and 5% CO_2. Contractile response was measured isometrically using a force displacement transducer (AE875, AME). The resting tension was adjusted to 1.0 g. After 2 h equilibration, contractile response to 40 mM KCl was repeated at intervals of 30 min until a steady response was obtained. The concentration–response relationships for sarafotoxins and ET-1 were determined by means of cumulative administrations. The response to these peptides was expressed as a percent of the contractile response due to 40 mM KCl which was taken as 100%.

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2.2. *Rat isolated perfused mesentery*

Mesenteric arteries of male Sprague–Dawley rats (Charles River, Japan, 300–350 g) were isolated and perfused as described by McGregor [7]. The rats were anesthetized with sodium pentobarbital (35 mg/kg i.p.) and treated with heparin (1300 U/kg i.v.). The superior mesenteric artery was cannulated at its origin, flushed with 15 ml of Krebs–Ringer solution, and isolated by cutting along the intestinal border of the mesentery. Mesenteric vasculature preparations were perfused with Krebs–Ringer solution using a Perista pump (SJ-1215, Atto) at a rate of 5 ml/min. The Krebs–Ringer solution, maintained at 37°C and continuously bubbled with a mixture of 95% O_2 and 5% CO_2, had the following composition (mM): NaCl 130.0, KCl 4.8, $CaCl_2$ 2.2, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, dextrose 5.5. The isolated mesenteric artery was initially perfused for a 30 min conditioning period. The perfusion pressure was measured by means of a pressure transducer (MPU-0.5, Tohyo and 1258, NEC San-ei). The peptides were injected into the arterial cannula using a microsyringe (Kloehn) in a volume of 3–30 μl.

2.3. *Pressor activity in pithed rats*

Male Sprague–Dawley rats (Charles River, Japan, 250–350 g) were anesthetized with diethyl ether and pretreated with atropine sulfate (1 mg/kg i.p.) The vagi were cut, and both the jugular veins and the left common carotid artery were occluded. After the trachea was cannulated, the rats were pithed by passing a steel rod (1.5 mm in

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diameter) through the orbit and down into the spinal canal via the foramen magnum. Immediately after pithing, the tracheal cannula was connected to a respirator (SN-480-7, Shinano) and the rats were ventilated artificially. Systemic arterial blood pressure was recorded from the right common carotid artery using a pressure transducer (MPU-0.5, Tohyo and 1258, NEC San-ei). Heart rate was measured by a pressure pulse-triggered cardiotachometer (1321, NEC San-ei). The caudal vein was cannulated for the administration of drug in a fixed volume of 1 ml/kg. The preparation was rested to reach the stabilized blood presure for at least 30 min before drug administration. Under these conditions, mean blood pressure was 53.8 ± 0.9 mmHg (n = 49, mean ± SE).

2.4. *Drugs*

SRTa, SRTb and SRTc were isolated and purified from the venom of the snake *Atractaspis engaddensis*, as described previously [4]. The venom was provided by Dr. E. Kochva (Tel Aviv University). SRTb (1–19) and SRTb (1–18) were prepared by the digestion of SRTb with carboxypeptidase Y (Oriental Yeast Co.), and [Des 5-9]SRTb and SRTb(5–9) were prepared by the digestion with lysyl endopeptidase (Wako Pure Chemical). [Homoserine^6]SRTb ([Hse^6]SRTb) was converted from SRTb with cyanogen bromide treatment using 100 mol eq. of BrCN in 70% formic acid at 37°C for 6 h. At above conditions, the methionine residue at position 6 of SRTb was converted into a homoserine residue but the resulting Hse^6-Thr^7 bond was not cleaved. These peptides were purified by means of reverse-phase HPLC using a Pep RPC HR5/5 column (Pharmacia Fine Chemicals). [Cys$^{1-11}$,Cys$^{3-15}$]SRTb and SRTb(8–21) were synthesized by the solid-phase method. The reduced and carboxymethylated (RCM), iodinated (Iod) or nitrophenylsulfenylated (NPS) products of SRTb were separated by reverse-phase HPLC. ET-1 was obtained from Peptide Institute (Osaka). Peptides were dissolved to 10^{-5} M in PBS containing 0.1% bovine serum albumin (Sigma) and stored in aliquots at −20°C until use. The peptide stock solution was serially diluted with the same buffer immediately before use.

2.5. *Statistical analysis*

Data were expressed as means ± SE. Statistical analysis of data was conducted by Student's t-test. $P<0.05$ indicated statistical significance.

3. RESULTS

In endothelium denuded rat aorta, SRTb and ET-1 caused slowly developing and concentration-dependent vasoconstrictions, with the EC_{50} values of 4.4×10^{-9} M and 1.3×10^{-9} M, respectively (fig.1). The threshold concentration of SRTb was 3 times larger than that of ET-1, while the response induced by SRTb at 10^{-7} M (150.7 ± 5.3%, n = 4) was greater than that of ET-1 (129.7 ± 7.5%, n = 5). The vasoconstrictor activity of SRTa was less than that of SRTb by approximately one order of magnitude. SRTc caused no vasoconstriction even at concentrations of 10^{-7} M.

In isolated perfused mesentery of rat, the mean perfusion pressure was 13.7 ± 0.3 mmHg (n = 60). Intraarterial injections of SRTb and ET-1 caused dose-dependent increase in perfusion pressure at doses ranging from 30 to 300 pmol and from 10 to 300 pmol, respectively (fig.2). The vasoconstrictor activity of SRTb was less remarkable than that of ET-1 at doses lower than 100 pmol, while at a dose of 300 pmol the activity of SRTb was greater than that of ET-1. The time required for the recovery of perfusion pressure to baselines after a bolus injection of 300 pmol SRTb was 14.9 ± 1.7 min (n = 3) which is shorter than that of ET-1 (>30 min, n = 3). The threshold vasoconstrictor dose of SRTa was 3 times larger than that of SRTb. At a dose of 300 pmol, the rise in perfusion pressue due to SRTa was 8 times smaller than that of SRTb. SRTc exhibited a feeble vasoconstriction producing a rise in perfusion pressure of 1.0 ± 0.2 mmHg (n = 3) even at a

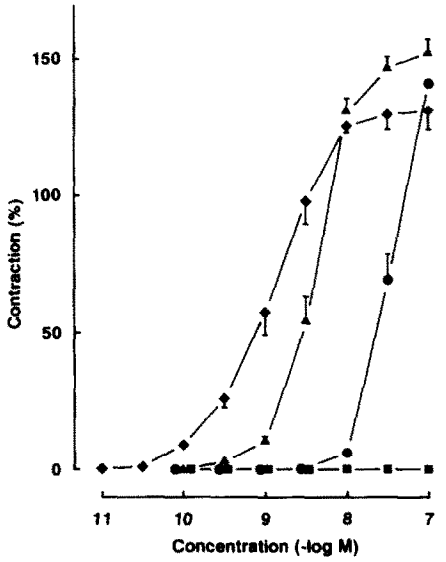

Fig.1. Cumulative concentration-response curves for SRTa(●)-, SRTb(▲)-, SRTc(■)- and ET-1(◆)-induced contractions of denuded rat aortic rings. Contraction is expressed as the percentage of 40 mM KCl-induced contraction. Each point represents mean ± SE (n = 4–5).

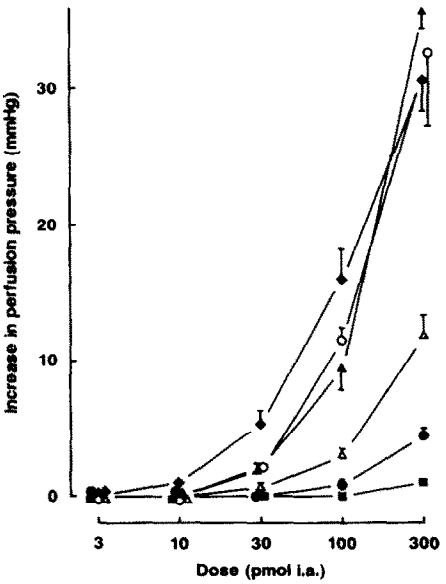

Fig.2. Dose-response curves for the vasoconstrictor effects of SRTa(●), SRTb(▲), SRTc(■), ET-1(◆), Iod-SRTb(○) and [Hse^6]SRTb(△) in rat isolated perfused mesenteries. Each point represents mean ± SE (n = 3–5).

Sarafotoxins and Endothelin-1	Amino-acid sequences	Vasoconstrictor activity (%)
SRTa	CSCKDM$^{7}_{8}$DKECLNFCHQDVIW	12.4
SRTb	CSCKDMTDKECLYFCHQDVIW	100
SRTc	CTCNDMTDEECLNFCHQDVIW	2.8
ET-1	CSCSSLMDKECVYFCHLDIIW	85.9
SRTb analogs		
[Cys$^{1-11}$,Cys$^{3-15}$]SRTb	CSCKDMTDKECLYFCHQDVIW	0
RCM-SRTb	CSCKDMTDKECLYFCHQDVIW	0
NPS-SRTb	CSCKDMTDKECLYFCHQDVIW	0
Iod-SRTb	CSCKDMTDKECLYFCHQDVIW	91.3
[Hse^{6}]SRTb	CSCKDHseTDKECLYFCHQDVIW	33.2
[Des 5-9]SRTb	CSCK ECLYFCHQDVIW	0
SRTb(5-9)	DMTDK	0
SRTb(1-19)	CSCKDMTDKECLYFCHQDV	0
SRTb(1-18)	CSCKDMTDKECLYFCHQD	0
SRTb(8-21)	DKECLYFCHQDVIW	0

Fig.3. Structure-activity relationships in vasoconstrictor effects of sarafotoxins in rat isolated perfused mesenteries. Vasoconstrictor activity at a dose of 300 pmol i.a. is expressed as the percentage of SRTb-induced increase in perfusion pressure. C, cystine; D, aspartic acid; E, glutamic acid; F, phenylalanine; H, histidine; Hse, homoserine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; Q, glutamine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

dose of 300 pmol. The vasoconstrictor activity of [iodinated Tyr^{13}]SRTb (Iod-SRTb) was equipotent to that of SRTb. On a molar basis, the potency of [Hse^{6}]SRTb was 3 times less effective than that of SRTb. In contrast, none of [Cys$^{1-11}$,Cys$^{3-15}$]SRTb, reduced and carboxymethylated SRTb (RCM-SRTb), [nitrophenylsulfenylated Trp^{21}]SRTb (NPS-SRTb), [Des 5-9]SRTb, SRTb(5-9), SRTb(1-19), SRTb(1-18) and SRTb(8-21) induced any rise in perfusion pressure up to a dose of 300 pmol (fig.3).

In pithed hypotensive rat, either of SRTa, SRTb, SRTc and ET-1 produced a dose-dependent and long-lasting increase in blood pressure (fig.4). The duration of the hypertensive response to 1 nmol/kg of SRTb

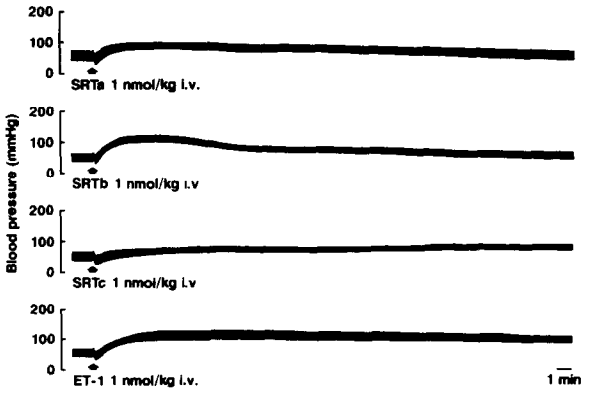

Fig.4. Typical recordings for pressor responses of pithed hypotensive rats to i.v. bolus injections of sarafotoxins and ET-1.

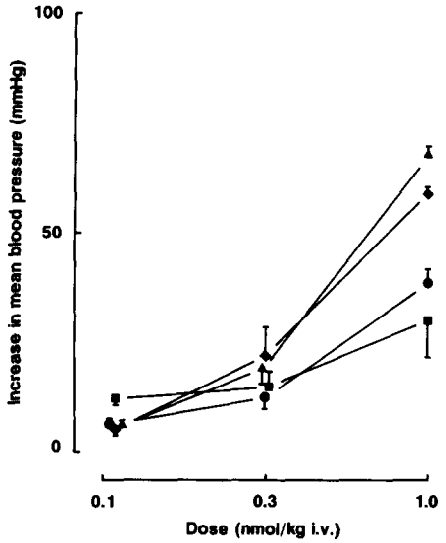

Fig.5. Dose-response curves for the hypertensive effects of SRTa(●), SRTb(▲), SRTc(■) and ET-1(◆) in pithed rats. Each point represents mean ± SE (n = 4–5).

(37.7 ± 1.9 min, n = 5) was significantly shorter than that of ET-1 (48.5 ± 2.5 min, n = 4), while the magnitude of the sustained rise was more prominent. The hypertensive effect of SRTa (1 nmol/kg i.v.) was similar to that of SRTb, while its potency was less than that of SRTb. The hypertensive response due to SRTc was appreciably different from those of other peptides. The increase in blood pressure induced by SRTc 1 nmol/kg reached the highest level about 25 min after the bolus injection and declined very slowly thereafter. On a molar basis, the increase in blood pressure elicited by SRTc was least in these peptides. On the other hand, SRTa, SRTb, SRTc and ET-1 at a dose of 1 nmol/kg caused no remarkable changes in heart rate of pithed rats. Fig. 5 shows the dose-response curves for the effects of sarafotoxins and ET-1 measured at the peak of the sustained rise in pithed rat. At doses ranging from 0.1 to 1.0 nmol/kg i.v., the rank order of the peak pressor activity was SRTb ≧ ET-1 > SRTa ≧ SRTc.

4. DISCUSSION

In the present study it was demonstrated that all three isopeptides of sarafotoxin and ET-1 showed various vasoconstrictor activities in the experiments carried out using rat thoracic aorta, rat isolated perfused mesentery and pithed rat, and that the characteristics of the pharmacological activities were quantitatively different among these isopeptides. The vasoconstrictor effect of ET-1 was in accordance with the results reported by Godfraind et al. [8], Warner et al. [9,10] and Walder et al. [9,11]. In rat thoracic aorta, the contractile activity was ET-1 > SRTb > SRTa > SRTc at lower concentrations, but SRTb > ET-1 > SRTa > SRTc at higher concentrations. The rank order of potency in rat

thoracic aorta closely paralleled the order in rat isolated perfused mesentery and was similar to the order of peak pressor effect in pithed rat. Yanagisawa et al. have reported that the cluster of alternating charges, Asp^{8}-Lys^{9}-Glu^{10}, is highly conserved among the endothelin family, suggesting the importance of these regions for biological activity [12]. The low reactive potency of SRTc indicates that the lysine residue at position 9 (replaced by glutamic acid in SRTc) is practically important for the vasoconstrictor activities of sarafotoxins and endothelin family. Furthermore, SRTb was more potent than SRTa, indicating that the tyrosine residue at position 13 (replaced by asparagine in SRTa1) is also contributable to vasoconstrictive actions of endothelin family and SRTb. NPS-SRTb with nitrophenylsulfenylated Trp^{21}, SRTb(1–19) and SRTb(1–18) caused no vasoconstriction in the mesentery of rat. The destruction of two sets of intrachain disulfide linkages by reduction and carboxymethylation (RCM-SRTb), the deformation of the intramolecular loop ([Des 5–9]SRTb) or the production of the two non-natural disulfide bonds at positions of Cys(1–11) and Cys(3–15) ([Cys^{1-11},Cys^{3-15}]SRTb) all caused disappearance of the constrictor activity. These results are compatible with the structure-activity relationships determined in ET-1 and its analogs [13]. This may suggest that the C-terminal Trp^{21} and the proper intramolecular loop structure are essential for the expression of the constrictor activity.

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