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Sarafotoxin, a Novel Vasoconstrictor Peptide: Phosphoinositide Hydrolysis in Rat Heart and Brain

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Sarafotoxins, a group of 21-residue cardiotoxic peptides from snake venom that induce coronary vasoconstriction, show high-affinity binding to rat atrial and brain membranes and activate the hydrolysis of phosphoinositides. Neither their binding nor their activity is affected by blockers or activators of known receptors and ion channels, suggesting that sarafotoxins act either directly on the phosphoinositide phosphodiesterase system or on a novel receptor. Their amino acid sequence shows a high degree of homology with that of endothelin, a recently described 21-residue vasoconstrictor peptide found in porcine aortic endothelium. This is remarkable, since endothelin is a natural compound of the mammalian vascular system while sarafotoxins are highly toxic components of snake venom.

SARAFOTOXINS S6 (SRTs A, B, AND C) are a group of 21-residue isocardiotoxic peptides, isolated from the venom of the snake *Atractaspis engaddensis*, which are rich in cysteine (four residues per molecule) and show a high sequence homology (*1*). Two of these, a and b, are highly lethal and cause cardiac arrest and death in mice within minutes of intravenous administration. [The median lethal dose (LD_{50}) is ~15 μg per kilogram of body weight (*2*).]

Although the mechanism of action of the sarafotoxins is not fully understood, it has been shown that they have three independent effects on the mouse and rat hearts: a rapid and marked vasoconstriction of the coronary vessels, a severe atrioventricular block, and a slower but very strong positive inotropic effect that is not blocked by either α- or β-adrenergic blockers (*2*). The venom of *Atractaspis* also causes a transient hypertension, which is followed by fluctuation of arterial blood pressure (*2*) and reversible contraction of the ileum in rats. These powerful effects of sarafotoxins could be explained by an increase in intracellular Ca^{2+} (smooth muscle contraction and disturbances in the atrioventricular conducting system), suggesting that the mechanism of their action may involve binding to and activation of a specific protein associated with Ca^{2+} mobilization. In this report we show that sarafotoxins bind specifically and with high affinity to rat atrial and brain membranes and induce the hydrolysis of phosphoinositides in these tissues.

Sarafotoxin S6b (SRT-b), which contains a tyrosyl residue (*1*), was labeled with ^{125}I and used for the binding studies. The toxicity of the ^{125}I-labeled SRT-b was similar to that of the noniodinated toxin. The toxin binds specifically and rapidly to rat atrial membranes (for example, 1600 cpm was recorded with 2.5 n*M* ^{125}I-SRT-b, whereas only 200 cpm was recorded in the presence of 1 μ*M* SRT-b, and the association half-time was 2.5 min with 4 n*M* toxin). Binding activity was not detected in the cytosolic fraction of atrial homogenates. Binding of ^{125}I-SRT-b to the atrial membranes was reversible (dissociation half-time, 10 to 12.5 min) and saturable (Fig. 1). Scatchard analysis (Fig. 1, inset) indicated that the toxin binds to a homogeneous population of sites [maximal binding capacity, 110 fmol per milligram of protein; dissociation constant (K_d), 3 to 5 n*M*]. Ligands of various receptors and ion channels as well as a variety of biologically active peptides (see legend to Fig. 2) failed to inhibit the binding of ^{125}I-SRT-b to the atrial membranes. However, the native toxins SRT-a (Fig. 2), SRT-b, and SRT-c were potent binding inhibitors, with median inhibitory values (I_{50}) of 30, 25, and 100 n*M*, respectively. Evidently, at least one disulfide bond in the sarafotoxins is important for their function and binding, since reduction and carboxymethylation of SRT-b resulted in a loss of toxicity and a marked decrease in affinity (I_{50} > 5000

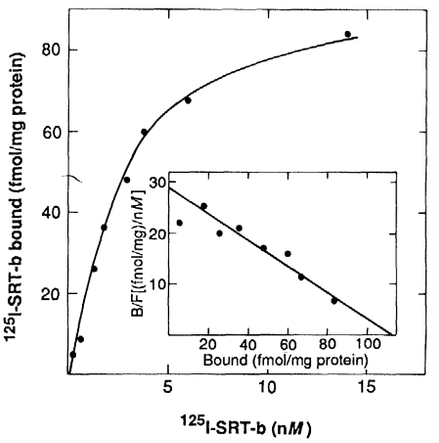

Fig. 1. Binding of ^{125}I-SRT-b to rat atrial membranes, as a function of its concentration. Rat atrial membranes were prepared in 25 m*M* tris-HCl buffer (*p*H 7.4) containing antiproteases [aprotinin (5 U/ml), pepstatin A (5 μg/ml), 0.1 m*M* phenylmethylsulfonylfluoride, 3 m*M* EDTA, and 1 m*M* EGTA) as detailed elsewhere (*9*). Aliquots (50 μl) of membrane preparations (200 μg of protein) were incubated at 25°C for 60 min with various concentrations of ^{125}I-SRT-b (specific activity, 4.7×10^{16} cpm/mol) in a total volume of 200 μl of tris-HCl buffer. Reactions were terminated by the addition of 3 ml of ice-cold tris-HCl buffer and filtration through GF/C filters over vacuum. The filters were washed twice with 3 ml of the buffer, and their radioactivity was estimated by liquid spectrometry. Separate kinetic experiments showed that the bound toxin did not dissociate during ~10-s washings at 4°C (dissociation half-time at 25°C was ~10 min). Assays were performed in triplicate (total binding) together with triplicate samples containing 1 μ*M* unlabeled SRT-b (nonspecific binding). Data are expressed as specific binding per milligram of protein (SEMs are less than 7% of the plotted values). Inset: Scatchard plot.

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nM). Additional experiments showed that specific binding sites for ^{125}I-SRT-b, with properties similar to those of the atrial sites, exist also in the rat brain. In the cerebellum, for example, the density of such sites was 600 fmol per milligram of protein, and the K_d of the toxin was 4 nM.

The above findings led us to conclude that membranes of mammalian heart and brain contain sites that bind sarafotoxins with high affinity; these sites might be associated with the biological actions of the toxins. Since these actions appear to involve Ca^{2+} mobilization and since blockers of receptors and ion channels known to affect intracellular Ca^{2+} levels (see legend to Fig. 2) did not interfere with ^{125}I-SRT-b binding, we investigated a possible association between sarafotoxins and phosphoinositide hydrolysis. The latter reaction, which is catalyzed by phosphoinositide phosphodiesterase (PDE, phospholipase C), leads to the formation of inositol trisphosphate (IP_3), which functions as a second messenger for mobilization of intracellular Ca^{2+} (*3*).

Initial experiments indicated that the sarafotoxins are indeed potent activators of phosphoinositide hydrolysis. In a typical experiment with rat atrial slices prelabeled with [^{3}H]inositol (*4*), addition of SRT-b (10^{-7}M) (in the presence of 10 mM LiCl) resulted within 5 min in the accumulation of IP_3, inositol bisphosphate (IP_2), and inositol-1-phosphate (IP_1). When measured as percentages of total [^{3}H]inositol-labeled lipids, these three [^{3}H]inositol phosphates amounted to 1.7%, 2.9%, and 3.6%, respectively, as compared to their corresponding basal values of 0.3%, 0.5%, and 0.84%. The accumulation of IP_3 and IP_2 leveled off within 15 min, whereas that of IP_1 increased linearly (up to at least 30 min). In further examining the effects of the sarafotoxins on phosphoinositide hydrolysis, we therefore focused on the formation of IP_1 and measured its accumulation in 30 min. Like SRT-b (Fig. 2), SRT-a and SRT-c induced phosphoinositide hydrolysis in rat atrial slices; for the three sarafotoxins, the respective concentrations resulting in 50% of the maximal effect (A_{50}) were 60 ± 20, 100 ± 30, and 300 ± 60 nM (mean ± SD, n = 3). Although these values are about three times as high as the respective I_{50} values recorded in the binding experiments, it is clear that SRT-c is less potent than the other two with regard to both binding and phosphoinositide hydrolysis, a finding that is in accord with its relatively lower toxic potency (*1, 2*). The loss of toxicity and decrease of affinity found in the reduced and carboxymethylated SRT-b was accompanied by failure of this polypeptide to induce phosphoinositide hydrolysis (no response at 10 μM). Results similar to those observed with the rat atrial slices were obtained with slices of rat cerebellum as well as with primary cultures of rat heart myocytes [see (*5*) for preparation]. In both of the latter, for example, SRT-a or SRT-b (10^{-7}M) induced a four- to eightfold increase in IP_1 over basal levels within 30 min.

In additional experiments with rat atrial slices, we examined the pharmacology of the SRT-b–induced phosphoinositide hydrolysis. Antagonists of muscarinic, nicotinic, dopaminergic, opiate, β-adrenergic, α_1- and α_2-adrenergic, and H_1-histaminergic receptors (see legend to Fig. 3) failed to inhibit phosphoinositide hydrolysis induced by SRT-b. These receptors are therefore not involved in the toxin's action. Furthermore, the effects of SRT-b and of either carbamylcholine or norepinephrine were not additive (Fig. 3A). It thus seems that the sarafotoxins share a common pathway with muscarinic and α_1-adrenergic agonists, which are known to activate the PDE system (*6*). This pathway does not involve voltage-dependent Na^+, K^+, or Ca^{2+} channels, since

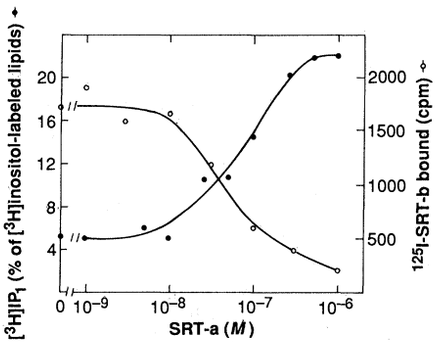

Fig. 2. Concentration-dependent inhibition of ^{125}I-SRT-b binding and induction of phosphoinositide hydrolysis by SRT-a. Binding assays were as detailed in Fig. 1, with 2.5 nM ^{125}I-SRT-b and various concentrations of SRT-a. Data are expressed as the amount of ^{125}I-SRT-b specifically bound as a function of SRT-a concentrations. In these conditions the following substances did not inhibit ^{125}I-SRT-b binding: carbamylcholine (10 μM), norepinephrine (100 μM), atropine (10 μM), curare (10 μM), haloperidol (10 μM), naloxone (10 μM), propranolol (10 μM), yohimbine (10 μM), prazosin (0.1 μM), allersan (10 μM), tetrodotoxin (1 μM), verapamil (1 μM), nimodipine (1 μM), tetraethylammonium (1 mM), 5′-guanylyl imidophosphate (GppNHp) (100 μM), IP_3 (10 μM), cholecystokinin-8 (1 μM), angiotensin II (1 μM), bradykinin (1 μM), arginine vasopressin (1 μM), β-endorphin (1 μM), atriopeptin III (1 μM), somatostatin (1 μM), substance P (10 μM), melanin-concentrating hormone (1 μM), and ouabain (10 μM). The formation of [^{3}H]IP_1 in rat atrial slices was measured by the method of Berridge *et al.* (*10*) essentially as detailed elsewhere (*4*). The slices were prelabeled (60 minutes) with 100 μCi/ml of [^{3}H]inositol (specific activity, 18.7 Ci/mmol) in Krebs medium, then washed three times with 5 mM inositol in the same medium. Packed slices (50 μl) were then incubated in the Krebs medium (total volume 250 μl) containing 10 mM LiCl, in the presence and absence of SRT-b, and the reaction was terminated after 30 min by the addition of 1 ml of a chloroform-methanol mixture (1:2), which was followed by the addition of 0.35 ml of chloroform and 0.35 ml of H_2O. Water-soluble products were separated chromatographically on Dowex columns (*10*) and counted with corrections for quenching. A sample from the lipid extract was counted as well. Assays were performed in triplicate. Data are presented in terms of [^{3}H]IP_1 formed as a percentage of total [^{3}H]-labeled inositol lipids (SEMs are less than 10% of the presented values). Zero time blanks were subtracted. The maximal effects of SRT-b and SRT-c were similar to these induced by SRT-a.

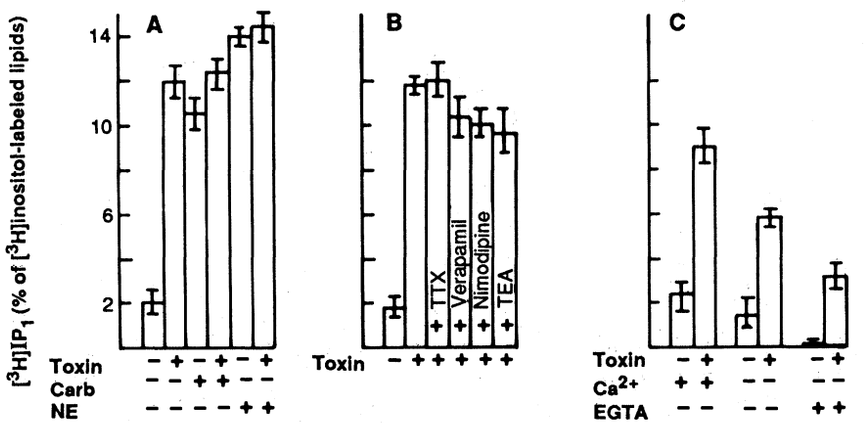

Fig. 3. Pharmacological characterization of phosphoinositide hydrolysis induced by SRT-b in rat atrial membranes. The formation of IP_1 was measured with and without 10^{-7}M SRT-b and the indicated substance for 30 min, as detailed in Fig. 2. Assays were performed in triplicate. Vertical bars denote SD. Carb, carbamylcholine (10^{-3}M); NE, norepinephrine (10^{-4}M); TTX, tetrodotoxin (1 μM); and TEA, tetraethylammonium (10^{-3}M). Concentrations of verapamil, nimodipine, and EGTA were, respectively, 1 μM, 1 μM, and 1 mM. Concentration of Ca^{2+}, where present, was 0.8 mM. The substances failed to inhibit induction of phosphoinositide hydrolysis by SRT-b (10^{-7}M): atropine (10 μM), curare (10 μM), haloperidol (10 μM), naloxone (10 μM), propranolol (10 μM), yohimbine (10 μM), prazosin (0.1 μM), and allersan (10 μM).

blockers of these channels did not block the action of SRT-b (Fig. 3B). Other experiments showed that phosphoinositide hydrolysis could be induced by SRT-b in a calcium-free medium, with and without 1 m*M* EGTA (Fig. 3C). Under the two latter conditions the efficacy of the toxin was lower than that observed in the presence of Ca^{2+}; however, since the basal hydrolysis was also reduced, the ratio of the induced to the basal hydrolysis remained unchanged. Similar effects were observed with the nonproteinaceous maitotoxin, a potent marine toxin found in tropical dinoflagellates (*7*).

Our results suggest that the sarafotoxins are novel and specific activators of the PDE system. They may exert their activity either by direct activation or via an unidentified class of membrane-bound receptors. While this work was in progress, Yanagisawa *et al.* (*8*) isolated a vasoconstrictor polypeptide from cultured porcine aortic endothelial cells (endothelin), which was proposed as a modulator of voltage-dependent ion channels. The following comparison between the structures of SRT-b and endothelin shows that the two polypeptides have very similar sequences:

SRT-b CSCKDMTDKECLYFCHQDVIW

Endothelin CSCS S L MDKECVYFCHL DI IW

This sequence similarity, together with the similar vasoconstrictor activities of the two polypeptides, suggests that they share a common binding site. It is of course possible (although less likely) that the small differences between endothelin and the sarafotoxins are enough to bring into play different mechanisms of action for their respective vasoconstrictor activities. Whatever their mechanisms of action, it is quite unusual to find such remarkable resemblances in sequence and biological action between an endogenous polypeptide, which exists as a natural component of mammalian endothelium, and one which constitutes a lethal component of snake venom.

Two hypotheses are suggested: (i) that it is the small difference in amino acid sequence between endothelin and sarafotoxins that accounts for the high toxicity of sarafotoxins; and (ii) that endothelin might be as toxic as the sarafotoxins, but its tissue content and activity are well regulated by synthetic and degradative mechanisms. This might imply that the pathology of hypertension and other vasoconstrictor diseases could be associated with an anomalous increase in the concentration of endothelin or related peptides.

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SRT-a CSCKDMSDKECLNFCHQDVIW

SRT-b CSCKDMTDKECLYFCHQDVIW

SRT-c CTCNDMTDEECLNFCHQDVIW

Abbreviations for the amino acid residues are: C, Cys; D, Asp; E, Glu; F, Phe; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; Q, Gln; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Heterogeneity of Glycine Receptors and Their Messenger RNAs in Rat Brain and Spinal Cord

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Messenger RNAs isolated from adult or newborn rat spinal cord were fractionated in a sucrose gradient. The fractions were injected into *Xenopus* oocytes to determine their potencies for expression of glycine receptors (GlyRs), which were then examined electrophysiologically. The sedimentation profiles disclosed two classes of GlyR mRNAs, one heavy and the other light. The adult spinal cord was rich in heavy GlyR mRNA, whereas the light GlyR mRNA was more abundant in neonatal spinal cord and in adult cerebral cortex. Glycine receptors encoded by heavy and light mRNAs of adult spinal cord showed some electrophysiological differences. Thus there are two types of GlyRs encoded by mRNAs of different sizes, and the expression of these mRNAs is developmentally regulated. A tissue- and age-dependent distribution of heterogeneous GlyR mRNAs may imply diverse roles of the GlyRs in neuronal function in the central nervous system.

GLYCINE AND γ-AMINOBUTYRIC ACID (GABA) are the major inhibitory neurotransmitters in the central nervous system of vertebrates; GABA operates mainly in the brain, whereas glycine acts mainly in the spinal cord (*1*). In the

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Table 1. Peak amplitudes of the currents elicited by amino acids in oocytes injected with fractionated mRNAs from the rat. The fraction number of each source corresponds to that shown in Fig. 1. Each value represents the average peak amplitude of the currents (with SEM, in nanoamperes) in oocytes (numbers in parentheses) obtained from one to four different donors. The glycine/β-alanine ratios given are the averages (with SEM) of the ratio obtained in individual oocytes.

Class	Source, fraction no.	GABA (1 m*M*)	Glycine (1 m*M*)	β-Alanine (1 m*M*)	Glycine/ β-alanine ratio
H	Adult cord, Fr. 6	<2.0 (8)	585 ± 97 (16)	253 ± 42 (16)	2.7 ± 0.3
H	Neonatal cord, Fr. 3	<2.0 (6)	203 ± 59 (6)	113 ± 33 (6)	2.2 ± 0.2
H	Adult cortex, Fr. 4	<2.0 (3)	55 ± 11 (3)	37 ± 12 (3)	1.7 ± 0.3
L	Adult cord, Fr. 15*	7.3 ± 1.7 (11)	431 ± 56 (19)	34 ± 6 (19)	13.0 ± 0.9
L	Neonatal cord, Fr. 13	16.0 ± 2.7 (6)	2302 ± 205 (6)	147 ± 14 (6)	15.3 ± 1.2
L	Adult cortex, Fr. 14	114 ± 18 (10)	595 ± 122 (10)	44 ± 9 (10)	15.0 ± 1.9

*For concentration of the mRNA, see (*12*).