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SARAFOTOXIN S6c IS A RELATIVELY WEAK DISPLACER OF SPECIFICALLY BOUND ^{125}I-ENDOTHELIN

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Sarafotoxin S6a, S6b and S6c are chemically related vasoconstrictor polypeptides obtained from the venom of the snake, **Atractaspis engaddensis**. Each contains twenty one amino acid residues, two intrachain cysteine linkages and a long hydrophobic tail. Structurally these polypeptides resemble endothelin. Binding studies with ^{125}I-endothelin showed that ^{125}I-endothelin bound to rat ventricular membranes is totally displaceable by sarafotoxin S6b and endothelin, with IC_{50} values of 0.21 and 0.16 nM, respectively. Sarafotoxin S6c, which differs from sarafotoxin S6b in containing threonine instead of serine at residue 2, arginine instead of lysine at residue 4, and glutamic acid instead of lysine at residue 9, only weakly displaced bound ^{125}I-endothelin (IC_{50}, 854 nM). These results indicate that the ability of the sarafotoxins to interact with the endothelin binding site is not solely dependent on the long hydrophobic tail or the cysteine linkages.

The venom of the burrowing asp, Atractaspis engaddensis, contains three cardiotoxic isotoxins - sarafotoxin S6a, S6b and S6c (1). The chemical structure of these toxins is remarkably similar to that of the newly identified vasoconstrictor polypeptide, endothelin (ET) (2-4). Each contains twenty one amino acid residues, two intrachain disulphide bonds and a hydrophobic tail (residues 16-21) with a terminal L-tryptophan (Fig. 1). In addition (Fig. 1) there is a cluster of charged residues at positions 8-10. Sarafotoxin S6c, however, (Fig. 1) differs from ET, or sarafotoxin S6a and S6b, in containing a glutamic acid instead of a lysine residue at position 9 (1,4). There are other differences - for example, residue 2 is threonine in S6c but serine in S6a, S6b and ET, and residue 4

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Abbreviations: ET: endothelin.

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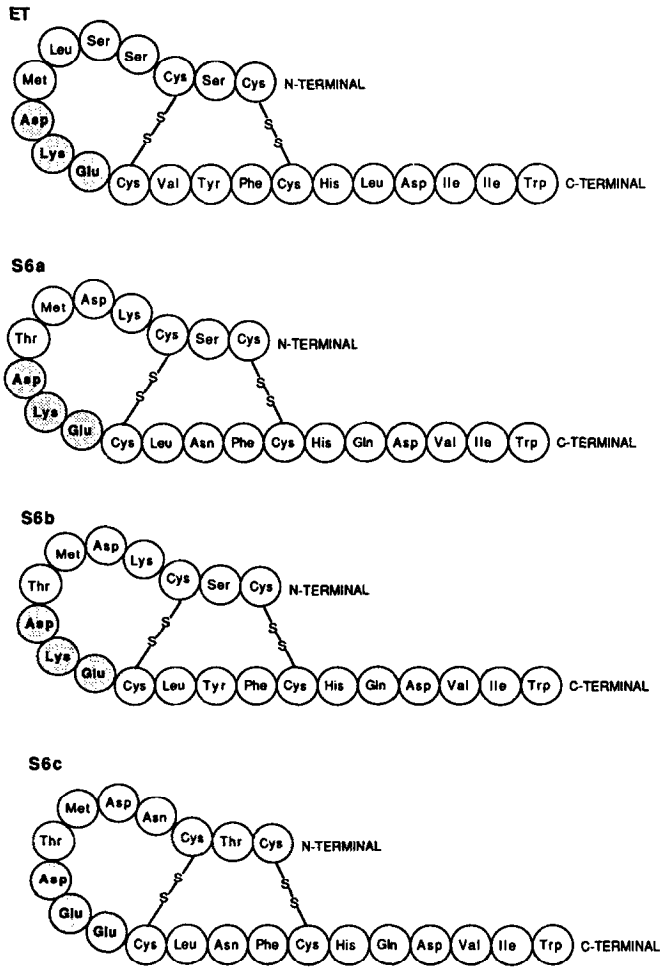

Figure 1 Chemical structure of ET, and the sarafotoxins S6a, S6b and S6c. (Data from ref. 1 and 4). The shaded residues refer to positions 8-10.

is serine in ET, lysine in S6a and S6b, and asparagine in S6c (1,4). Because S6c lacks lysine and arginine residues it is more acidic than the other sarafotoxins (1) and by implication, more acidic than ET.

Kimura et al (5) found that removing the terminal L-tryptophan residue, destroying the intrachain disulphide bonds and progressive depletion of the amino acids from the terminal hydrophobic tail all reduce the vasoconstrictor activity of ET. These same investigators found that the cluster of negative and positive charges (asp^{8}-lys^{9}-glu^{10}) within the loop structure of ET contributes to its biological activity.

We have already shown that ET (as ^{125}I-ET) binds to a single population of high affinity binding sites in rat ventricular membranes (6). The bound ^{125}I-ET is not displacebale by dihydropyridine-based and

other Ca^{2+} antagonists (6), but is completely displaced by cold ET and by sarafotoxin S6b (Fig. 2), with IC_{50} values (concentration required to displace 50 percent of the bound ligand) of 0.162 ± 0.053, and 0.212 ± 0.142 nM respectively, mean ± SEM, 3 experiments (Table I). These results are in agreement with those described in the literature for rat atrial and aortic smooth muscle preparations (7-9) and supports the hypothesis (2,3,10) that ET and the sarafotoxins interact with a common receptor.

The molecular requirements for specific ET and S6b binding are now being explored (9), and already it is assumed that the long hydrophobic tail and intrachain cysteine linkages are of major importance (2). The availability of a small quantity of sarafotoxin S6c which, in common with ET, S6a and S6b, contains the long hydrophobic tail with a terminal tryptophan residue, and the intrachain cysteine linkages, has allowed us to investigate whether apparently minor amino acid substitutions alters the ability of the sarafotoxins to interact with their receptor.

METHOD

Sarafotoxins and endothelin Sarafotoxin S6b and S6c were donated by Dr. Takasaki (Tohoku University, Japan). Whereas the LD_{50} for S6b in mice was 5.6 µg/kg, S6c was not lethal at 970 µg/kg (Takasaki, personal communication). Synthetic porcine ET was purchased from Protein Research Foundation, Osaka, Japan) and iodinated as previously described (6) to a specific activity of approximately 1600 Ci/mmol.

^{125}I-ET binding assay Ventricular membranes were harvested from the hearts of adult female Sprague-Dawley rats, as previously described (11), using a homogenizing medium containing 20 mM $NaHCO_3$ and 0.1 mM phenylmethylsulphonylfluoride (PMSF), pH 7.4. Protein was assayed by the Lowry method (12), with bovine serum albumin as standard. ^{125}I-ET binding was monitored as previously described (6) with a final protein concentration of 0.16 ± 0.3 mg/ml in a volume of 0.25 ml. Non-specific binding was defined in the presence of 10^{-8}M ET. The reaction mixture contained 50 mM Tris and 0.1 mM PMSF (pH 7.4). Incubation was at 37°C for 60 min. Bound and free ^{125}I-ET were separated after dilution with 3.5 ml ice-cold 10 mM Tris buffer containing 6.6% polyethyleneglycol 6000 (pH 7.4). Separation was by rapid vacuum filtration across Whatman GF/C filters. After two additional washes the radioactivity of the filters was counted in a LKB multiwell γ counter (80% efficiency). Binding to the filters was negligible.

Binding selectivity was established by using 10^{-13}-10^{-8}M ET, 10^{-12}-10^{-6}M (+)PN200-110 (isopropyl-4-(2,1,3-benzoxadiazol-4- γ 1)-1,4-dihydro-2,6-dimethyl-5-methoxycarbonylpyridine-3-carboxylate) (protected from the light) and sarafotoxin S6b (10^{-13}-10^{-8}M) and S6c (10^{-12}-10^{-6}M) to displace specifically bound ^{125}I-ET (0.1 - 0.2 nM).

Data analysis Initial estimates of equilibrium binding parameters (K_D and B_{max}) were obtained from Scatchard, Hill and Hofstee analysis, using the "EBDA" programme (11). K_D is the concentration of ligand (^{125}I-ET) required to occupy 50% of the binding sites, B_{max} the density of the binding sites, K_i the inhibition constant of the competing ligand, and IC_{50} the concentration of competing ligand needed to displace 50% of the specifically bound ligand (^{125}I-ET).

RESULTS

^{125}I-ET bound to the ventricular membranes with a K_D of 0.20 ± 0.03 nM, a B_{max} of 93.5 ± 6.4 fmol/mg protein and a Hill coefficient of 0.993 ± 0.003 (mean ± SEM, 6 experiments). Figure 2 shows that the specifically bound ^{125}I-ET was completely displaceable by unlabelled ET and sarafotoxin S6b, with (Table I) K_i and IC_{50} values of 0.049 ± 0.009, and 0.162 ± 0.053 nM respectively for ET, and 0.086 ± 0.014 and 0.212 ± 0.042 nM for S6b. Under these same conditions (+)PN200-110 (10^{-12}-10^{-6}M) failed to displace the bound ^{125}I-ET (Fig. 2). Figure 2 and Table I show that relative to sarafotoxin S6b, sarafotoxin S6c is a remarkably weak displacer of bound ^{125}I-ET.

DISCUSSION

The present study shows that sarafotoxin S6b and S6c, both of which (Fig. 1) resemble ET in containing two intrachain cysteine linkages and a long hydrophobic tail with a terminal tryptophan residue, differ in their ability to displace specifically bound ^{125}I-ET, with S6b being more potent than S6c. Presumably, therefore, some other part of the 21 amino acid

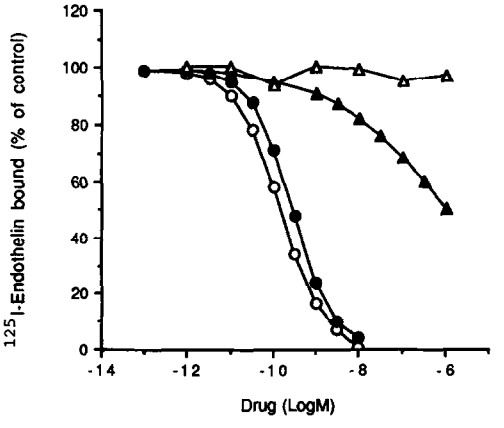

Figure 2 Concentration-dependent inhibition of ^{125}I-ET binding by ET (○), S6b (●), S6c (▲) and (+)PN200-110 (△). Binding assays were as described in Methods, using membranes prepared from rat ventricles. The calculated Hill coefficients centred around unity. (+)PN200-110 was protected from the light. The results of a typical experiment are shown. Similar results were obtained from three other experiments.

Table I INHIBITION OF ^{125}I-ET BINDING TO RAT VENTRICULAR MEMBRANES BY ET AND SARAFOTOXIN S6b AND S6c

Polypeptide	K_i (nM)	IC_{50} (nM)
ET (n=3)	0.049 ± 0.009	0.162 ± 0.053
S6b (n=3)	0.086 ± 0.014	0.212 ± 0.042
$Sig._i$	N.S.	N.S.
S6c (n=3)	333 ± 59.7	854 ± 153
$Sig._{ii}$	$p < 0.001$	$p < 0.001$
$Sig._{iii}$	$p < 0.001$	$p < 0.001$

Results are presented as mean ± SEM of 3 separate experiments. Tests of significance (calculated by analysis of variance with Bonferroni adjustment for multiple comparisons (11)) relates to the differences relative to the values obtained for ET. K_i and IC_{50} are as defined in the text. Sig_i and Sig_{ii} refer to the significance of the differences between the K_i and IC_{50} values obtained for the ability of ET and S6b (Sig_i) and S6c (Sig_{ii}) to displace specifically bound ^{125}I-ET. Sig_{iii} relates to the significance of the difference between the K_i and IC_{50} values obtained for the ability of S6b and S6c to displace bound ^{125}I-ET. Tests of variance calculated by analysis of variance, with correction for multiple comparisons (see text). N.S. = not significant, at $p = 0.05$. n refers to number of experiments.

sequence, in addition to the intrachain cysteine linkages and the hydrophobic tail with its terminal tryptophan residue, influences the ability of these polypeptides to compete with ^{125}I-ET at its binding sites. Our findings are compatible with those of Kloog et al (1988), who found that sarafotoxin S6b and S6a are approximately equipotent in selectively reducing ^{125}I-S6b binding to rat atrial membranes, whereas S6c is relatively weak (IC_{50} values of 30, 25 and 100 nM for S6a, S6b and S6c respectively).

Sarafotoxin S6a and S6b, like ET (Fig. 1) both contain a lysine residue in position 9, whereas S6c contains glutamic acid. It may be this difference which is of importance. Such an idea is supported by the fact that cleavage of the lysine^9 residue of ET markedly reduces its biological activity (6). The lysine residue in position 4 of S6b (and S6a) is unlikely to be important here because ET contains serine in that position but it binds with a similar affinity and density as S6b (9). Another difference between ET and S6b on the one hand, and sarafotoxin S6c on the other, is the serine in position 2 in ET and S6b, and threonine in S6c. As

yet there is not data to show whether the presence of this threonine residue influences the binding activity of S6c, nor is there any data to show whether the acidic nature of S6c, relative to S6b (1), contributes to its relative weakness as a displacer of bound ^{125}I-ET.

In conclusion, our results show that the binding of the sarafotoxins to the ET receptor is not determined solely by the two intrachain cysteine linkages and the long hydrophobic tail. Other relatively minor differences in their amino acid composition must also be taken into account.

ACKNOWLEDGMENTS

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