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INTERACTION OF SYNTHETIC SARAFOTOXIN WITH RAT VASCULAR ENDOTHELIN RECEPTORS

Y. Hirata[1]*, H. Yoshimi[2], F. Marumo[1], T.X. Watanabe[3], S. Kumagaye[3], K. Nakajima[3], T. Kimura[3], and S. Sakakibara[3]

[1]Department of Medicine, Tokyo Medical and Dental University, Yushima, Eunkyo-ku, Tokyo 113 , Japan

[2]Department of Medicine, National Cardiovascular Center, Osaka 565, Japan

[3]Peptide Institute, Inc., Protein Research Foundation, Osaka 562, Japan

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SUMMARY: The effects of synthetic analogs of sarafotoxin (STX) S6b, a snake venom peptide with a high sequence homology to the endothelium-derived vasoconstrictor endothelin (ET), on ET receptor binding activity and cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) were studied in cultured rat vascular smooth muscle cells. Binding studies revealed that [Cys^{1-15}, Cys^{3-11}]STX competed with ^{125}I-ET for the binding to its vascular receptors with lower affinity than that of ET, but was far more effective than [Cys^{1-11}, Cys^{3-15}]STX in inhibiting the binding. [Cys^{1-15}, Cys^{3-11}]STX had a less potent effect on increasing $[Ca^{2+}]_i$ than ET, whereas [Cys^{1-11}, Cys^{3-15}]STX was inactive. These data suggest that there may exist heterogenous subpopulations of the vascular ET/STX receptors, and that the proper double cyclic structure of STX is essential for interacting with its putative receptors to induce the $[Ca^{2+}]_i$ response.

Endothelin (ET) is a potent vasoconstrictor peptide from the supernatants of cultured porcine aortic endothelial cells that has been recently isolated and sequenced (1). ET-induced vasoconstriction is dependent on extracellular Ca^{2+} and can be inhibited by voltage-dependent Ca^{2+}-channel blockers. We have recently demonstrated the presence of high-affinity binding sites for ET in cultured rat vascular smooth muscle cells

*All correspondence should be addressed to: Yukio Hirata, M.D., Endocrine Division, The Second Department of Internal Medicine, Tokyo Medical and Dental University, Yushima 1-5-45, Bunkyo-ku, Tokyo 113, Japan.

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(VSMC) through which ET induces a profound increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$)(2).

Sarafotoxins (STX), on the other hand, are potent cardiotoxic peptides isolated and sequenced from the venom of burrowing asp, Atractaspis engaddensis (3). Surprisingly, it has recently been reported that the endothelium-derived ET and the snake venom peptide STX S6b have a high degree of sequence homology and share common biological actions including vasoconstrictive and cardiotoxic effects (4-6). Both peptides consist of 21 amino acid residues with two pairs of half-cystine residues in identical positions, suggesting that the peptides may have derived from a common ancestral gene. Recent binding studies have shown that high-affinity binding sites for STX are present in the membranes of the rat atrium and various regions of the rat brain (6,7), and that ET competitively displaced the binding of ^{125}I-STX to these membranes (8). Taken together, these data suggest that ET and STX may share common receptor sites in the target tissues.

These recent findings prompted us to examine the effects of synthetic STX and its disulfide analog on the interaction with the vascular ET receptors as well as $[Ca^{2+}]_i$ response in cultured rat VSMC.

MATERIALS AND METHODS

Peptides

STX S6b analogs, [Cys^{1-15}, Cys^{3-11}]STX and [Cys^{1-11}, Cys^{3-15}]STX, were synthezied by the solid-phase method and purified by ion-exchange chromatography on DEAE-cellulose and reverse-phase HPLC as described (9). The homogeneity of the final products was confirmed by analytical reverse-phase HPLC and amino acid analysis. Porcine (p) ET was purchased from Peptide Institute, Inc. (Osaka, Japan).

Binding experiments

Binding studies were performed essentially in the same manner as recently reported (2). In brief, confluent rat VSMCs ($5x10^5$ cells) were incubated with $2.5x10^{-11}$ M ^{125}I-labeled-pET (specific activity: 2000 Ci/mmol, Amersham Japan, Tokyo) at 37°C for 60 min in the absence or the presence of

unlabeled pET or STX analogs. After completion, the cell-bound radioactivity was determined; specific binding was defined as the total binding minus the nonspecific binding in the presence of excess (4×10^{-7} M) unlabeled pET.

Measurement of $[Ca^{2+}]_i$

The fluorescence of fura-2-loaded cell suspensions was spectrofluorimetrically measured as described (2). $[Ca^{2+}]_i$ values were calculated by the formulas reported by Grykiewicz et al. (10).

RESULTS

As shown in Fig. 1, unlabeled pET competitively inhibited the binding of ^{125}I-pET to cultured rat VSMC. The apparent K_d and B_{max} for the ET receptors calculated from the Scatchard analysis were 6×10^{-11} M and 5,400 sites/cell, respectively. Synthetic STX S6b also displaced ^{125}I-pET binding from its binding sites, although it was less potent than pET. The apparent inhibition constant (K_i) for STX was 8×10^{-10} M as calculated from the equation: $K_i = IC_{50}/(1+L/Kd)$, where L is the concentration of ^{125}I-pET and K_d its dissociation constant.

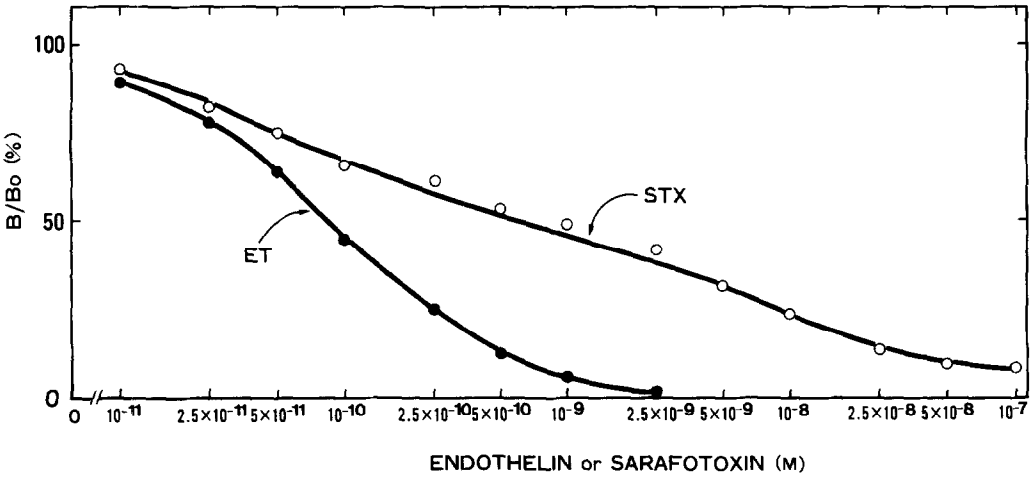

Fig. 1. Competitive binding of ^{125}I-endothelin (ET) by unlabeled ET and sarafotoxin (STX). Confluent rat VSMCs were incubated with 2.5×10^{-11} M ^{125}I-ET in the absence or the presence of porcine ET (●) and STX S6b (o) in the indicated concentrations. Specific binding in the absence of peptide was 95% of the total binding. Each point is the mean of two experiments.

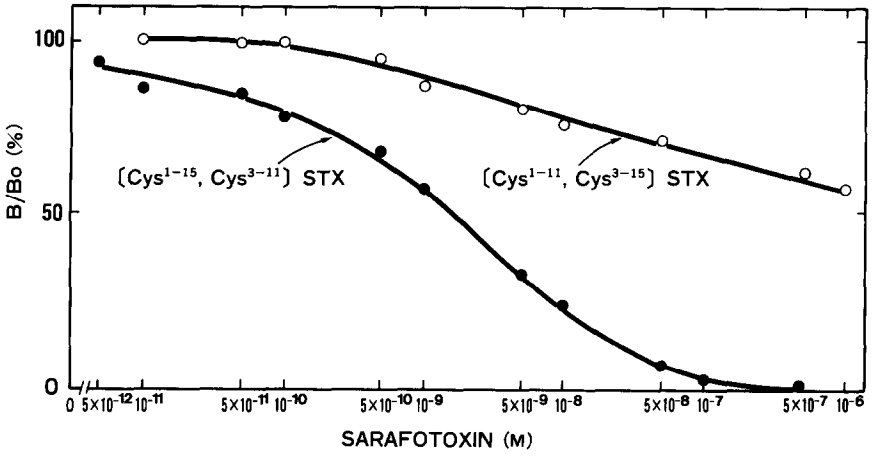

Fig. 2. Competitive binding of ^{125}I-endothelin (ET) by disulfide analogs of sarafotoxin (STX). Confluent rat VSMCs were incubated with 2.5×10^{-11} M ^{125}I-ET in the absence or the presence of [Cys$^{1-15}$, Cys$^{3-11}$]STX (●) and [Cys$^{1-11}$, Cys$^{3-15}$]STX (○) in the indicated concentrations. Specific binding in the absence of peptide was 90% of the total binding. Each point is the mean of two experiments.

The competition binding experiment with ^{125}I-pET by two different disulfide analogs of STX S6b is shown in Fig. 2. [Cys$^{1-15}$, Cys$^{3-11}$]STX was far more potent than [Cys$^{1-11}$, Cys$^{3-15}$]STX in competing with ^{125}I-pET for the binding to rat VSMC with the apparent K_i values of 1.5×10^{-9} M and $\geq 7.6 \times 10^{-8}$ M, respectively.

The effects of pET and STX analogs on the $[Ca^{2+}]_i$ response are compared as illustrated in Fig. 3. pET (10^{-7} M) induced a

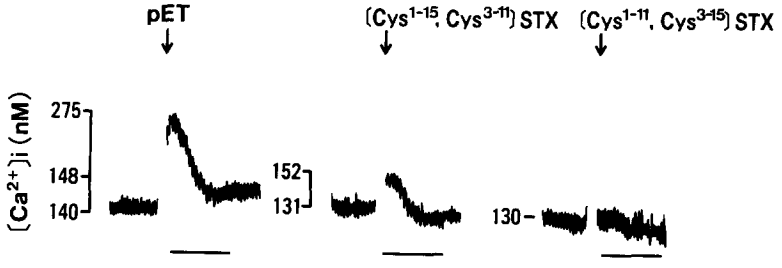

Fig. 3. Typical tracings of the changes in Ca^{2+}-fura-2 fluorescence by porcine endothelin (pET) and sarafotoxin (STX) analogs in cultured rat VSMC. The suspended rat VSMC loaded with fura-2 were challenged with 10^{-7} M each of pET, [Cys$^{1-15}$, Cys$^{3-11}$]STX and [Cys$^{1-11}$, Cys$^{3-15}$]STX, as indicated by the arrows. Calculated values for $[Ca^{2+}]_i$ are shown on the ordinates, and a one-min interval is underlined.

profound (about 2-fold) increase in $[Ca^{2+}]_i$ of the fura-2-loaded rat VSMC. In contrast, [Cys^{1-15}, Cys^{3-11}]STX (10^{-7} M) caused only a slight (about 1.2-fold) increase in $[Ca^{2+}]_i$, whereas [Cys^{1-11}, Cys^{3-15}]STX (10^{-7} M) was ineffective.

DISCUSSION

The present binding study clearly demonstrates that synthetic STX S6b, a snake venom peptide toxin with a high sequence homology to ET, interacts with the ET receptors in cultured rat VSMC. It has recently been reported that natural STX S6b and synthetic pET almost equipotently inhibited the binding of ^{125}I-STX S6b to the membranes of various regions of rat brain as well as cardiac atrium (8), suggesting that the two homologous peptides derived from different sources may share common binding sites in these tissues.

From the present results, however, the K_i value for STX is apparently greater than the apparent K_d value for the vascular ET receptors. Based on the marked difference in the affinities of ^{125}I-STX S6b to its binding sites in various tissues of the rat, the possible existence of multiple subtypes of the putative STX receptors has been suggested (7). Taken together, these data suggest that there exist heterogenous subpopulations of the ET/STX receptors in different target tissues.

The present binding study also shows that [Cys^{1-15}, Cys^{3-11}]STX is far more potent than [Cys^{1-11}, Cys^{3-15}]STX in inhibiting the binding of ^{125}I-pET to its receptors. These data are consistent with our recent results showing that the binding affinity of [Cys^{1-15}, Cys^{3-11}]pET to vascular ET receptors is much higher than that of [Cys^{1-11}, Cys^{3-15}]pET (11). Natural STX S6b and pET with 21 amino acid residues have two

pairs of half-cystine residues in identical positions (4-6). Therefore, our data indicate that the proper double cyclic structure formed by disulfide bonds at the positions of (1-15) and (3-11) within ET and STX molecules is critical for interation with the ET/STX receptors.

Consistent with these binding data are the lower stimulatory effect of [Cys^{1-15}, Cys^{3-11}]STX compared to that of pET and the failure of [Cys^{1-11}, Cys^{3-15}]STX to affect the $[Ca^{2+}]_i$ response in fura-2-loaded cells. These data are also comparable to their biological activities as determined by our in vitro bioassay (9); [Cys^{1-15}, Cys^{3-11}]STX and [Cys^{1-11}, Cys^{3-15}]STX are about three-fold and a thousand-fold less potent than pET in constricting rat pulmonary artery, respectively.

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