Vol. 158, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 16, 1989 Pages 195-201

COMPETITIVE INTERACTION BETWEEN ENDOTHELIN AND SARAFOTOXIN: BINDING AND PHOSPHOINOSITIDES HYDROLYSIS IN RAT ATRIA AND BRAIN

I. Ambar, Y. Kloog, I. Schvartz*, E. Hazum* and M. Sokolovsky#

Laboratory of Neurobiochemistry, Department of Biochemistry,
The George S. Wise Faculty of Life Sciences, Tel Aviv University,
Tel Aviv 69978, Israel

*Department of Biochemistry, Galaxo Research Laboratories,
5 Moore Drive, Research Triangle Park, N.C. 27709

Received November 25, 1988

Binding studies with the structurally similar vasoconstrictor peptides ^{125}I-endothelin and ^{125}I-sarafotoxin b, the former of mammalian origin and the latter derived from snake venom, reveal their mutually exclusive binding to rat atrium and various regions of the rat brain. In these tissues endothelin, like sarafotoxin, induces phosphoinositide hydrolysis which is in part Ca^{2+}-independent. It is suggested that endothelins and sarafotoxins share common binding sites and mechanisms of action.

Two independent studies, one of the mammalian endothelial vasoconstrictor peptide, endothelin (1-3), and the other of the snake venom vasoconstrictor peptides, the sarafotoxins (4-6), have demonstrated the natural occurrence of vasoconstrictors which though diverse, have similar biological effects and a high degree of sequence homology. Porcine (1) and human (2) endothelins (Et) are identical and are highly homologous to rat endothelin (3); all three are 21-residue peptides containing two sets of disulfide bonds. The three homologous isocardiotoxic snake venom peptides, the sarafotoxins (SRTX), are also 21-residue peptides with two sets of disulfide bonds (5), and show a high degree of homology to Ets (6). These similarities, together with the demonstrated high affinity and selectivity of SRTX binding to rat atrial and brain membranes and their induction of phosphoinositide (PI) hydrolysis, led us to propose that SRTX and Et may share a common receptor which is coupled to the PI system (6-8). Nonetheless, the structural differences between Et and SRTX, though small, could obviously be enough to bring about different mechanisms of action of their respective vasoconstrictor activities. Moreover, while SRTX was shown to induce PI hydrolysis in all tissues that showed high-affinity binding of ^{125}I-SRTX (6-8), Et

To whom reprint requests should be addressed.

0006-291X/89 $1.50

did not activate PI hydrolysis in cultured endothelial cells even though ^{125}I-Et bound to these cells with high affinity (9). These findings, and the possible existence of SRTX-receptor subtypes (7), prompted us to examine the relationship between SRTX and Et binding sites in the rat brain and atrium as well as the possibility that Et induces PI hydrolysis in these tissues.

METHODS

Sarafotoxins and endothelin: Sarafotoxins from the venom of the snake Atractapsis engadensis (SRTXa and SRTXb) were donated by Drs E. Kochva and A. Bdolah. ^{125}I-SRTXb ($1.5 \cdot 10^{16}$ cpm/mole) was prepared as described elsewhere (6, 7). Synthetic porcine endothelin was purchased from Peptides International, Louisville, KY. The endothelin was iodinated by the lactoperoxidase method and purified on a VYDAC P-P C-18 column (buffer A, 0.1% TFA; buffer B, 60% acetonitrile in 0.1% TFA). Specific activity of the labeled peptide was approximately 2,200 Ci/mmol.

Assays of ^{125}I-SRTX binding: Adult male Charles River derived (CD) rats were decapitated and their brains and atria removed and dissected as described previously (6,7). Tissues were homogenized in 25 mM Tris-HCl buffer, pH 7.4 (0.05 g tissue/ml buffer), containing antiproteases (5 units/ml aprotinin, 5 µg/ml pepstatin A, 0.1 mM phenylmethylsulfonylfluoride, 3 mM EDTA and 1 mM EGTA). Samples of the whole homogenate were used for binding assays. Aliquots (50 µl) of tissue homogenates (containing 150 µg protein) were incubated at 25°C for 60 min with 5-6 nM ^{125}I-SRTXb and various concentrations of Et or SRTXb in 25 mM Tris-HCl buffer, pH 7.4 (total volume 200 µl). Reactions were terminated by the addition of 3 ml of ice-cold Tris-HCl buffer and filtration under vacuum through GF/C filters (adsorption of ^{125}I-SRTXb to the filters under these assay conditions was negligible). The filters were then washed twice with 3 ml of buffer and their radioactivity was estimated by liquid spectrometry. Assays were performed in triplicate (total binding) together with assays of triplicate samples containing 1 µM unlabeled SRTXa (nonspecific binding).

Assays of ^{125}I-Et binding: Brain membranes were prepared from adult Sprague-Dawley male rats. Following decapitation the entire brain was removed and homogenized by polytron for 1 min in 10 volumes (w/v) of ice-cold sucrose buffer (0.32 M sucrose in 5 mM Tris/HCl, pH 7.5). Following centrifugation of the homogenate (15 min at 4,300 x g) the resulting supernatant was centrifuged (30 min at 30,000 x g); the pellet was resuspended in 5 volumes of the sucrose buffer, kept on ice for 30 min and polytroned. The two centrifugations were then repeated, but this time only the upper, looser pellet was resuspended in 2 volumes of 50 mM Tris-HCl and stored at -20°C. Binding was assayed following incubation of the labeled peptides (30,000 cpm), in the absence or in the presence of increasing concentrations of unlabeled Et or SRTXb, with whole brain membranes (100-150 µg protein) in 0.25 ml of 10 mM Tris-HCl, pH 7.5, containing 1 mM $CaCl_2$ for 90 min at 22°C. The reaction was terminated by filtration under vacuum through Whatman GF/C filters, and the filters were counted in a γ-counter. Specific binding represents the bound radioactivity in the presence of 0.1 µM unlabeled endothelin. Each value is the mean of triplicate incubations from at least two separate experiments, which varied by less than 10%.

Assays of PI hydrolysis: Rat brain and atrial tissue slices were prepared as previously described in detail (8, 10). Briefly, adult male CD rats were decapitated and their brains removed, dissected and sliced with a Sorvall TC-2 tissue sectioner (200×200 µm). The formation of [^{3}H]IP_1 in the tissue slices was assayed (10) by the method of Berridge (11). Slices were prelabeled (60 min) with 60 µCi/ml of [^{3}H]inositol (18.7 Ci/mmol, Amersham) in Krebs medium and washed 3 times with 5 mM inositol in the same medium. Packed slices (50 µl) were then incubated in Krebs medium (total volume 250

µl) containing 10 mM LiCl with and without Et or SRTXb. The reaction was terminated after 30 min by the addition of 1 ml chloroform: methanol (1:2), followed by 0.35 ml chloroform and 0.35 ml H_2O. The water-soluble products were separated chromatographically on Dowex columns (11) and counted, with corrections for quenching. A sample from the lipid extract was also counted. All assays were performed in triplicate. Data are expressed in terms of [^{3}H]inositol phosphate formed as a percentage of the total labeled [^{3}H]-inositol lipids. Zero time blanks were subtracted.

RESULTS

In order to determine whether Et and SRTXb bind to the same population of receptor sites we performed two sets of competition experiments, the first with ^{125}I-SRTXb and Et or SRTXb and the second with ^{125}I-Et and Et or STRXb. The results of the first set of experiments clearly showed that Et competes with ^{125}I-SRTXb for binding to rat cortical or atrial preparations (Fig. 1); the respective IC_{50} values for Et were 7.5 and 12 nM. The IC_{50} values for SRTXb recorded in these experiments were 16 nM (cortex) and 19 nM (atria). Figure 2 demonstrates that both SRTXb and Et compete for ^{125}I-Et binding to rat brain membranes: the recorded IC_{50} values for SRTXb and Et were 10 and 4.5 nM respectively. In all these competition experiments the calculated Hill coefficients were close to unity (see legends to Figs 1 and 2). Additional competition experiments with ^{125}I-SRTXb and Et were performed with preparations from the cerebellum, the hypothalamus and the caudate putamen. In all cases Et displaced ^{125}I-SRTXb binding. The corresponding

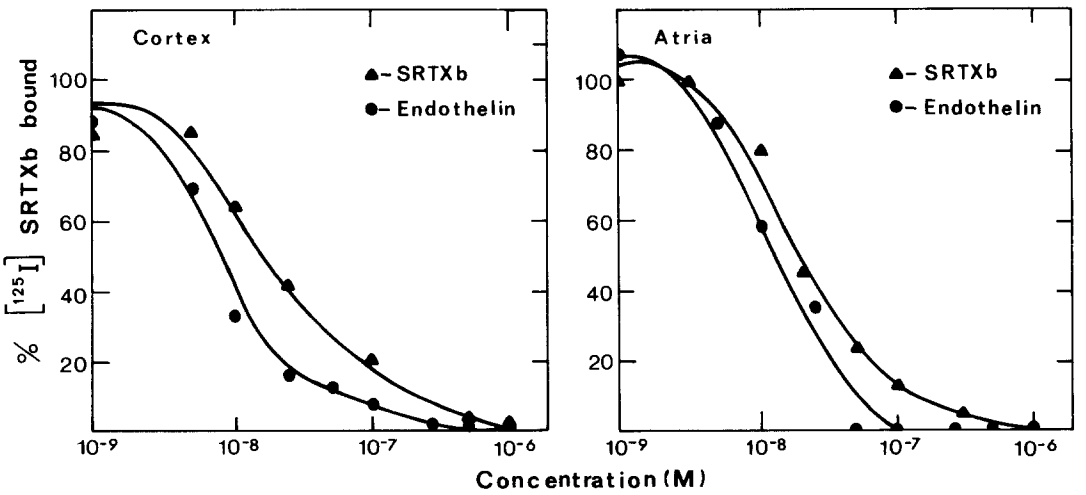

Figure 1
Concentration-dependent inhibition of ^{125}I-SRTXb binding by Et and by SRTXb. Binding assays were as described in Methods, using rat brain cortical (left) or rat atrial (right) preparations, 5 nM ^{125}I-SRTXb and various concentrations of Et or SRTXb. The calculated Hill coefficients for Et were 0.97 (cortex) and 1.3 (atria), and for SRTXb 1.09 (cortex) and 1.3 (atria).

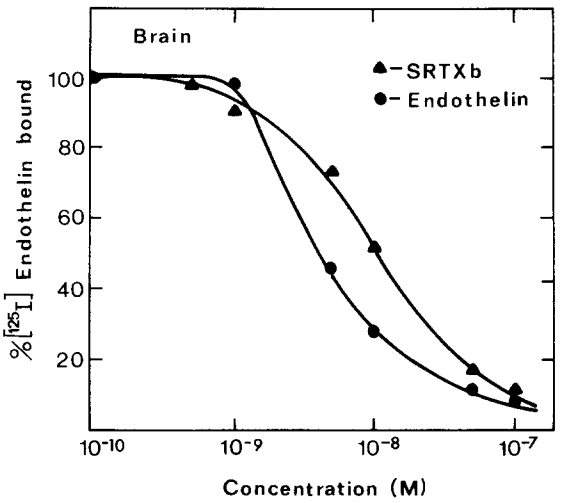

Figure 2
Concentration-dependent inhibition of ^{125}I-Et binding by SRTXb and by Et. Binding assays were as described in Methods using membrane preparations from whole rat brain, 30 pM ^{125}I-Et and various concentrations of SRTX or Et. Calculated Hill coefficients were 0.96 for SRTXb and 0.95 for Et.

IC_{50} values and the calculated Ki values for Et are summarized in Table I; for comparison, the previously determined Kd values of ^{125}I-SRTXb (7) are also shown. Note that both Et and SRTX bind with much higher affinity to the cortical and to the caudate receptors than to those of the cerebellum or the atria.

In order to determine whether Et can activate the hydrolysis of PI, we examined rat cerebellar and atrial tissue slices that were prelabeled with

Table I: Comparison between Ki values of endothelin determined in competition experiments with ^{125}I-SRTXb and Kd values of ^{125}I-SRTXb

Tissue	Endothelin		^{125}I-SRTXb
	IC_{50} (nM)	Ki (nM)	Kd (nM)
Cerebellum	45	19.0	3.5
Hypothalamus	10	1.80	1.50
Cortex	6	0.49	0.30
Caudate putamen	5	0.28	0.33
Atria	15	7.0	4.0

IC_{50} values were determined in competition binding experiments as described in Figure 1 (legend). The Ki (inhibition constants for Et were calculated according to the equation $Ki=IC_{50}/(1+L/Kd)$, where L is the concentration of ^{125}I-SRTXb and Kd its dissociation binding constant determined in direct binding experiments. The Kd values for ^{125}I-SRTXb were taken from ref. 7. Values are means of two to three separate experiments, each performed in triplicate.

Table II: Endothelin-induced PI hydrolysis in rat cerebellar and atrial tissue slices

	+$CaCl_2$ (1 mM)		-$CaCl_2$		-$CaCl_2$ + 1 mM EGTA	
	% [^{3}H]IP_1	Induced / Basal	% [^{3}H]IP_1	Induced / Basal	% [^{3}H]IP_1	Induced / Basal
Atria						
Basal	4.2±1.0	1.0	2.4±0.5	1.0	1.7±0.5	1.0
10^{-6} M Et	25.3±2.0	6.3	14.5±1.0	6.0	6.7±1.0	3.9
10^{-7} M SRTXb	16.1±2.0	4.0	7.7±0.5	3.2	5.5±1.0	3.2
10^{-6} M Et + 10^{-7} M SRTX	23.2±2.0	6.0	12.0±0.5	5.0	7.0±1.0	4.1
Cerebellum						
Basal	2.7±0.1	1.0	0.5±0.1	1.0	0.5±0.1	1.0
10^{-6} M Et	13.7±0.5	5.0	4.1±1.0	8.0	3.0±0.5	6.0
10^{-7} M SRTX b	7.4±0.5	2.7	1.0±0.1	2.0	1.0±0.1	2.0
10^{-6} M Et + 10^{-7} M SRTX	17.8±1.0	6.5	5.2±1.0	10.0	2.5±0.3	5.0

The amount of [^{3}H]IP_1 formed during incubation for 30 min was determined as described in Methods and is expressed as a percentage of total [^{3}H]inositol lipids. Values are means ± S.D. of three separate determinations.

[^{3}H]inositol, then stimulated with 10^{-6} M Et in the presence of 10 mM LiCl (see Methods). These experiments are similar to those performed previously with SRTXb (6,8). Et, like SRTXb, activated PI hydrolysis both in the atrial and in the cerebellar tissue slices (see Table II). As in the case of SRTXb (6, 8) we found that the extent of Et-induced PI hydrolysis was higher in the atria than in the cerebellum: under the assay conditions used, 10^{-6} M Et and 10^{-7} M SRTXb induced the maximal attainable response in the atrial tissue but only 50-70% of the maximal response in the cerebellum. However, the responses of the toxin and of Et in the cerebellum appear to be additive (Table II). Additional experiments revealed that Et can induce PI hydrolysis in a Ca^{2+}-free medium, with or without 1 mM EGTA (Table II). Even though the efficacy of Et was reduced under these conditions, the ratio of basal to Et-induced hydrolysis remained unchanged (Table II). These results closely resemble those previously described for SRTXb (6, 8).

DISCUSSION

The present study demonstrates that the two homologous vasoconstrictor peptides, endothelin (1-3) and sarafotoxin b (4-8), bind with high affinity to the same population of sites in the rat atrium and brain, as indicated by the mutually exclusive binding of the two peptides to these sites (Fig. 1 and Fig. 2). It is further shown that Et, like SRTXb (6, 8), can induce PI

hydrolysis that is in part Ca^{2+}-independent (Table II). It thus appears that the mammalian peptide and the snake venom peptide not only resemble one another in structure and biological activity but also share common binding sites and display similar mechanisms of action.

Of all the drugs (including channel blockers, receptor antagonists, neurotransmitters, and peptide hormones) tested thus far (6, 7), Et is the only substance capable of inhibiting the binding of ^{125}I-SRTXb to rat atrial and brain tissues. Similarly, it was shown that the binding of ^{125}I-Et to cultured epithelial cells could be blocked only by Et (9). These data strongly support the previously raised possibility (6, 7) that both Et and SRTX bind to hitherto undetected populations of receptors. There is already some evidence for the possible existence of SRTX receptor subtypes, based on the marked differences in the affinities of SRTXb towards receptors in the cerebellum and atrium as compared to those in the cortex and caudate putamen (7). Et shows exactly the same pattern of differences in affinities towards these receptors (Table I). It is therefore not unlikely that there are indeed several types of Et/SRTX receptors. If so, one cannot rule out the possibility that more than one second-messenger system is associated with Et/SRTX receptors. It is important to note here that in contrast to the rat atrium and brain, where Et or SRTXb induce PI hydrolysis (Table II), no such response is evoked by Et in cultured epithelial cells (9); in the latter preparation Et did however evoke a transient increase in free Ca^{2+} levels which was not blocked by Ca^{2+} channel blockers (9). Thus the Et/SRTX receptor(s) may be coupled in the endothelial cell to a second messenger system which is distinct from the well-known phosphoinositide-Ca^{2+} pathway (12, 13).

The present findings on Et-induced PI hydrolysis in the rat atrium and brain point however to the likelihood that at least one type of Et/SRTX receptor in these tissues is coupled to the phosphoinositide cycle, since the induced PI hydrolysis is in part independent of extracellular Ca^{2+} (Table II). We have also observed an SRTXb- (6, 8) or Et- (Table II) induced PI hydrolysis that is Ca^{2+}-dependent. Since the reported vasopressor and vasoconstrictor effects of Et depend on the presence of extracellular Ca^{2+} (1-3) as well as on the sustained Et-induced rise in Ca^{2+} levels in epithelial cells (9), one can envisage two alternative mechanisms of Et or SRTX action: (a) a dual mechanism in which the peptides simultaneously activate the Et/SRTX receptor-PI system (6) and the Ca^{2+} channels (1); (b) a sequential mechanism in which the peptides first activate the PI system, leading to the release of Ca^{2+} from intracellular stores (12, 13), which in turn facilitates the opening of Ca^{2+} channels. These possibilities are now under investigation in our laboratory.

ACKNOWLEDGEMENTS

We thank Drs E. Kochva and A. Bdolah for the generous gift of sarafotoxins. We thank Mrs. Shirley Smith for excellent editorial assistance.

REFERENCES

1. Yanagisawa, M., Kurihara, H., Kimura, S., Tomboe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. & Masaki, T. (1988) Nature 332, 411-415.
2. Itoh, Y., Yanagisawa, M., Ohkubo, S., Kimura, C., Kosaka, T., Inoue, A., Ishida, N., Mitsai, Y., Onda, H., Fujino, M. and Masaki, T. (1988) FEBS Lett. 231, 440-444.
3. Yanagisawa, M., Inoue, A., Ishikawa, T., Kasuya, Y., Kimura, S., Kumagaye, S-I, Nakajima, K., Watanabe, T.X., Sakakibara, S., Gotto, K. and Masaki, T. (1988) Proc. Natl. Acad. Sci. USA 85, 6964-6967.
4. Kochva, E., Viljonen, C.C. & Botes, D.P. (1982) Toxicon 20, 581-592.
5. Takasaki, C., Tamiya, N., Bdolah, A., Wollberg, Z. & Kochva, E. (1988) Toxicon 26, 543-548.
6. Kloog, Y., Ambar, I., Sokolovsky, M., Kochva, E., Wollberg, Z. & Bdolah, A. (1988) Science, 242, 268-270.
7. Ambar, I., Kloog, Y., Kochva, E., Wollberg, Z., Bdolah, A., Oron, U. & Sokolovsky, M., (1988) Biochem. Biophys. Res. Commun. In press.
8. Kloog, Y., Ambar, I., Kochva, E., Wollberg, Z., Bdloah, A. and Sokolovsky, M. (1988) FEBS Lett. In press.
9. Hirata, Y., Yoshimi, H., Takata, S., Watanabe, T.X., Kumagai, S., Nakajima, K. & Sakakibara, S. (1988) Biochem. Biophys. Res. Commun. 154, 868-875.
10. Gurwitz, D. & Sokolovsky, M. (1987) Biochemistry 26, 633-638.
11. Berridge, M.J. (1983) Biochem. J. 212, 849-858.
12. Berridge, M.J. & Irvine, R.F. (1984) Nature 312, 315-321.
13. Nishizuka, Y. (1988) Nature 344, 661-665.