High-Affinity Binding of *Clostridium perfringens* Epsilon-Toxin to Rat Brain

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[125]I-epsilon-toxin showed high affinity to rat brain homogenates and synaptosomal membrane fractions, having single binding phases with dissociation constants (K_ds) of 2.5 and 3.3 nM, respectively. Treatment of synaptosomal membrane fractions with pronase and neuraminidase lowered the binding of the labeled toxin, whereas treatment with trypsin and phospholipase C did not. Heating of the fractions resulted in a decrease in the binding of the toxin. These data suggest that interaction of epsilon-toxin with cell membranes in the brain is facilitated by a sialoglycoprotein. On the other hand, treatment of the membrane fractions with lipase resulted in complete loss of binding, suggesting that the interaction may require an appropriate lipid environment. These data suggest the presence of specific binding sites in brain tissue for epsilon-toxin.

Epsilon-toxin produced by *Clostridium perfringens* type D is thought to be closely correlated with enterotoxemia of sheep (2, 3, 13). The toxin has been reported to increase vascular permeability in the brain (7). The major pathological changes caused by enterotoxemia appear to occur in the brain, as reported by Hartley (9) and Griner and Carlson (8). We have reported that the toxin is specifically accumulated in the brain of mice after intravenous injection of the toxin (14). Furthermore, we have reported that lethal and pressor activities of epsilon-toxin in mice and rats, respectively (17), and contractile responses of rat-isolated ileum to the toxin (23) were significantly prevented by pretreatment with epsilon-toxin inactivated by modification of carboxyl groups, amino groups, or histidine residues with various agents (15). In the present work, the binding of the toxin to brain tissue of rats and the effects of agent-inactivated epsilon-toxins and presynaptic neurotoxins on the binding of epsilon-toxin to synaptosomal membrane fractions were investigated.

Epsilon-prototoxin and -toxin were purified by chromatographic techniques from a fluid culture of *C. perfringens* type D (NCTC 8346) as previously described (22). The toxin was inactivated by treatment with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in the presence of glycine methyl ester (20), 2,4,6-trinitrobenzenesulfonic acid (TNBS) (19), succinic anhydride (SA) (19), tetranitromethane (TNM) (21), *N*-acetylimidazole (NAI) (21), or *N*-bromosuccinimide (NBS) (17, 18) as described previously. Protein estimations were determined by the method of Lowry et al. (12) by using bovine serum albumin (BSA) as a standard.

Brain homogenates were prepared from rat brains as follows. The brains were collected in ice-cold 0.01 M phosphate buffer (pH 7.0) containing 0.9% NaCl. Homogenization was carried out in a Teflon-glass apparatus at 4°C. After centrifugation (10,000 × *g*, 15 min) of the homogenates, the pellets were rehomogenized and centrifuged at 1,000 × *g* for 5 min to remove large debris. The supernatant was centrifuged again (10,000 × *g*, 15 min), and the resulting pellet was centrifuged in the same way three more times and finally resuspended in a small volume of 0.01 M phosphate buffer (pH 7.0). Synaptosomal membrane fractions from whole rat

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brain were prepared by the method of Cotman (5). ^{125}I-labeled epsilon-toxin was prepared according to the method of Bolton and Hunter as described previously (1, 14). The labeled toxin retained at least 80% of the lethal activity of the intact toxin.

Properties of ^{125}I-labeled epsilon-toxin binding to rat brain homogenates and synaptosomal membrane fractions were investigated (Fig. 1). When ^{125}I-epsilon-toxin was incubated with the homogenates or the membrane fractions, the bound radioactivity was proportional to the amount of ^{125}I-epsilon-toxin, and the amount of radioactivity bound to the membrane fractions was about three times more than that bound to the brain homogenates. The binding of ^{125}I-epsilon-toxin to the brain homogenates and the membrane fractions was saturable, with half-maximal binding occurring at 0.25 and 0.63 nM toxin, respectively, as shown in Fig. 1. Scatchard analysis for the homogenates indicated only a single phase with a K_d of 2.5 nM and a B_{max} (maximal binding capacity per membrane protein) of 9 pmol/mg (Fig. 1A), and the analysis for the membrane fractions showed a single phase with a K_d of 3.3 nM and a B_{max} of 25.5 pmol/mg (Fig. 1B).

To clarify the chemical nature of the toxin receptor(s), the synaptosomal membrane fractions were pretreated with hydrolytic enzymes and subsequently assayed for toxin binding (Table 1). Membrane fractions (12.5 µg) in 500 ml of 20 mM Tris-HCl buffer (pH 7.5) containing 5 mM $CaCl_2$ were treated with various concentrations of trypsin (porcine pancreas; Sigma), pronase (*Streptomyces griseus*; Sigma), or neuraminidase (*C. perfringens*; Sigma) at 37°C for 30 min. Synaptosomal membrane fractions (12.5 µg) were treated with phospholipase C purified by the method of Fujii et al. (6) in 500 ml of 20 mM Tris-HCl buffer (pH 7.5) containing 0.9% NaCl and 20 mM $CaCl_2$ for 30 min or with lipase (porcine pancreas; Sigma) in 500 ml of 10 mM phosphate buffer (pH 8.0) at 37°C for 30 min. After incubation, the membrane fractions were washed twice with 20 mM Tris-HCl buffer (pH 7.5) containing 0.5% BSA and resuspended in 500 ml of the buffer.

At concentrations as high as 100 µg/ml, trypsin and phospholipase C failed to alter toxin binding. Pronase reduced specific binding by 60% at 60 µg/ml. Neuraminidase also lowered binding by 80 and 51% at 40 and 80 µg/ml, respectively. On the other hand, treatment of the toxin with
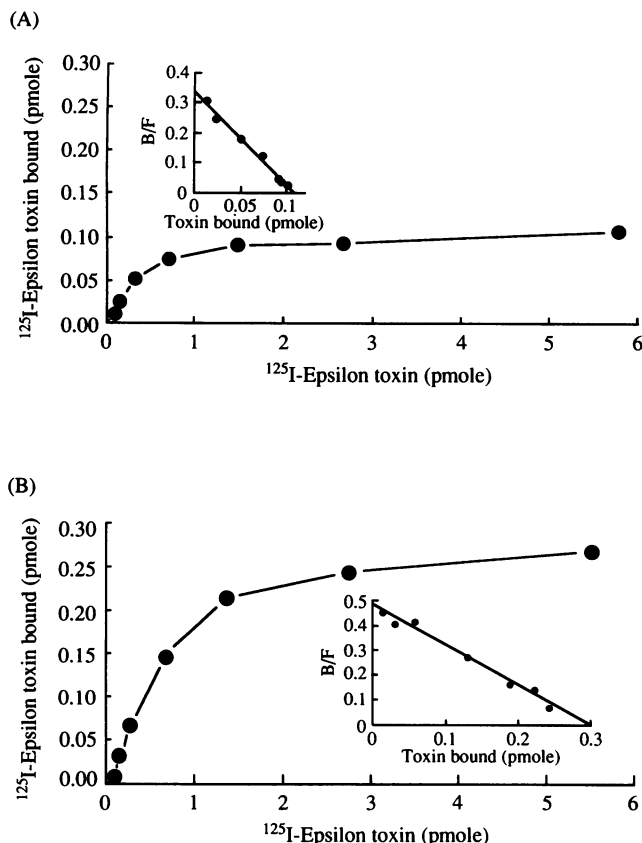
(A)

(B)

FIG. 1. Binding of ^{125}I-labeled epsilon-toxin to brain homogenates and synaptosomal membrane fractions from rats. Rat brain homogenates (A) and synaptosomal membrane fractions (B) (12.5 μg of protein) were incubated in 0.01 M phosphate buffer (pH 7.0) containing 0.5% BSA with increasing concentrations of ^{125}I-labeled toxin in a final volume of 200 μl at 37°C for 10 min. The suspensions were centrifuged at 10,000 × g for 5 min. The pellets were washed twice with 3 ml of the incubation buffer (4°C) and were centrifuged again. After aspiration of the supernatant, the radioactivity bound to the pellets was measured as described previously (14). To determine the level of specific binding, the homogenates and the membrane fractions were preincubated with unlabeled epsilon-toxin for 30 min prior to the addition of the labeled toxin. The insets show the data recalculated to fit a Scatchard plot, according to the method of Klotz and Hunston (11). B/F, bound toxin/free toxin.

proteolytic enzymes under these conditions resulted in no effect on the lethal activity of the toxin and no change of mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lipase decreased binding in a dose-dependent manner to about 5% of that of the control at 64 U/ml. Binding of the toxin to heated membrane fractions was investigated. Heating at 60°C for 10 min resulted in no effect on toxin binding, heating at 70°C caused about a 50% decrease in binding, and heating at 80°C resulted in a complete loss of binding.

Binding of ^{125}I-epsilon-toxin to the membrane fractions preincubated with various amounts of epsilon-toxin inactivated by various agents was investigated. As shown in Fig. 2, TNBS-, EDC-, and SA-inactivated toxins inhibited binding of the labeled toxin to the fractions in a dose-dependent manner, but SA-inactivated toxin did not do so strongly. From these data, the inhibitory concentrations which reduced the binding of the labeled toxin to 50% of control

TABLE 1. Binding of ^{125}I-labeled epsilon-toxin to synaptosomal membrane fractions treated with various enzymes[a]

Enzyme	Concn[b]	Specific binding (cpm/ 12.5 μg of protein)	Inhibition (%)
None (control)		6,050	0
Trypsin	100	6,030	0.3
Pronase	20	5,747	5.0
	40	4,780	21.0
	60	3,633	40.0
Phospholipase C	100	6,036	0.2
Neuraminidase	40	4,840	20.0
	80	3,086	49.0
Lipase	4	5,022	17.0
	32	1,912	68.4
	64	296	95.1

[a] Synaptosomal membrane fractions were treated as described in the text and were assayed for ^{125}I-labeled-toxin binding activity as described in the legend to Fig. 1. To determine the level of specific binding, the membrane fractions treated with enzymes were preincubated with unlabeled epsilon-toxin for 30 min prior to the addition of the labeled toxin. Each value was obtained from the mean of triplicates, with a variation of 10%.

[b] Values for lipase are in units per milliliter; all others are in micrograms per milliliter.

binding (IC$_{50}$s) of these inactivated toxins were determined. The IC$_{50}$ of prototoxin was approximately 8 pmol. The IC$_{50}$s of TNBS- and EDC-inactivated toxins were approximately 32 and 36 pmol, respectively. The IC$_{50}$ of SA-inactivated toxin was approximately 170 pmol. As shown in Fig. 2, 260 pmol of NBS-, NAI-, or TNM-inactivated toxin did not inhibit the binding of the labeled toxin. We have reported that epsilon-toxin causes the release of acetylcholine from cholinergic nerve endings (23). The effect of presynaptic neurotoxins such as β-bungarotoxin or botulinum toxin on the binding of epsilon-toxin to the fractions was investigated (Fig. 3). β-Bungarotoxin blocked the binding of epsilon-toxin to the fractions in a dose-dependent manner, and maximal inhibition was approximately 60% of that of the control under the same conditions. The IC$_{50}$ of β-bungarotoxin was estimated to be approximately 185 pmol. On the other hand, the IC$_{50}$ of prototoxin was approximately 8 pmol, showing that the binding of prototoxin is approximately 24-fold higher than that of β-bungarotoxin. Botulinum toxin did not inhibit binding.

Buxton (4) and Worthington et al. (26) have reported that prior administration of formalinized epsilon-toxin inhibited both the increase in vascular permeability caused by the toxin in the brain and the lethal activity of the toxin. We have reported that lethal activity, pressor activity, and contractile responses of rat-isolated ileum to the toxin are prevented by pretreatment with TNBS-, EDC-, SA-, or ethoxyformic anhydride-inactivated toxin but not by pretreatment with NBS-, NAI-, or TNM-inactivated toxin (15). In the present work, TNBS-, EDC-, and SA-inactivated toxins inhibited binding of the labeled toxin to the membrane fractions of rat brain in a dose-dependent manner, but NBS-, NAI-, and TNM-inactivated toxins did not, indicating that the inhibitory effect of these inactivated toxins on biological activities caused by epsilon-toxin is the same as the effect of these inactivated toxins on the binding of epsilon-toxin to the fractions. We have reported that the pretreatment with
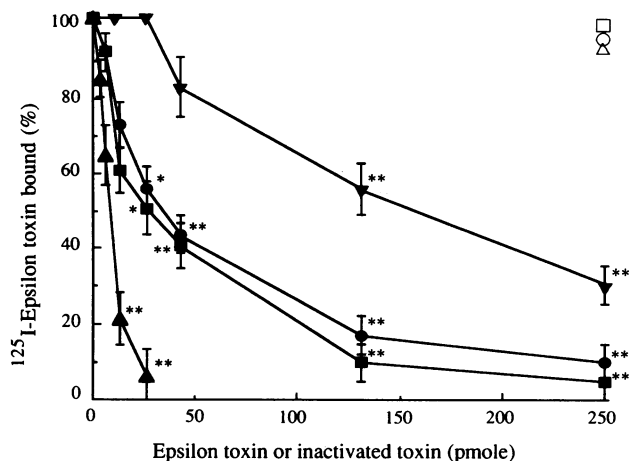
FIG. 2. Effect of epsilon-toxin inactivated by various agents on binding of epsilon-toxin to synaptosomal membrane fractions from rats. The membrane fractions (12.5 μg) were incubated with inactivated epsilon-toxins in 500 μl of 0.01 M phosphate buffer (pH 7.0) containing 0.5% BSA at 37°C for 10 min and then were washed twice with the fresh buffer by centrifugation (10,000 × g, 5 min). The resulting pellets were resuspended in the fresh buffer, and then the labeled toxin (1.8 pmol) was added. The mixture was incubated at 37°C for 10 min. After pellets were washed twice by centrifugation (10,000 × g, 5 min), the radioactivity of the pellets was counted. Each point is the mean value of 9 or 10 determinations. The standard errors are shown by vertical lines. Binding of ^{125}I-epsilon-toxin to the membrane fractions was expressed as a percentage of the binding of the labeled toxin under control conditions. Symbols: *, $P < 0.01$ compared with control; **, $P < 0.001$ compared with control; ■, TNBS-inactivated toxin; ▼, SA-inactivated toxin; ●, EDC-inactivated toxin; ○, NAI-inactivated toxin; □, TNM-inactivated toxin; △, NBS-inactivated toxin; ▲, prototoxin.

FIG. 3. Effect of botulinum toxin or β-bungarotoxin on epsilon-toxin binding to synaptosomal membranes. ^{125}I-epsilon-toxin (1.8 pmol) was incubated with synaptosomal membrane fractions (12.5 μg) in 200 μl of 0.01 M phosphate buffer (pH 7.0) containing 0.5% BSA in the presence of various concentrations of botulinum toxin (Type A; Wako Pure Chemical Industries, Ltd., Osaka, Japan) or β-bungarotoxin (Wako Pure Chemical Industries) for 20 min at 37°C. The mixture was washed twice with the fresh buffer by centrifugation (10,000 × g, 5 min). The radioactivity of the pellets was counted. Symbols: □, botulinum toxin; ○, β-bungarotoxin; ■, prototoxin.

epsilon-prototoxin specifically inhibited biological activities of epsilon-toxin and accumulation of the labeled toxin in mouse brain (14). It therefore appears that epsilon-toxin specifically binds to and then acts on the brain.

The labeling of epsilon-toxin was performed through the conjugation of amino groups in the toxin with iodinated ester by the Bolton-Hunter method (1). We have reported that modification of free amino groups in epsilon-toxin by TNBS and SA caused significant loss of the biological activities of the toxin (19) but did not have a significant effect on binding of the toxin to the receptors (15). It therefore seemed reasonable to examine the binding of epsilon-toxin to the membrane fractions prepared from rat brain by using ^{125}I-epsilon-toxin.

We examined the binding properties of epsilon-toxin to rat brain homogenates and membrane fractions. The data showed that there was a single class of binding site with high affinity in the brain (K_d, 2.5 nM) and in the membranes (K_d, 3.3 nM) and that there were a small number of sites in the brain (B_{max}, 9.0 pmol of toxin bound per mg of protein) and in the synaptosomal membranes (B_{max}, 25.5 pmol of toxin bound per mg of protein), suggesting that the specific binding site may be located in the synaptosomes. Treatment of the membrane fractions with trypsin and phospholipase C resulted in no effect on binding. On the other hand, binding of the ^{125}I-labeled toxin was significantly reduced by pretreating the fractions with pronase or heat, suggesting that the binding site for the toxin may be a protein. Furthermore, decrease of the binding of the toxin brought about by neuraminidase seems to indicate the importance of sialic
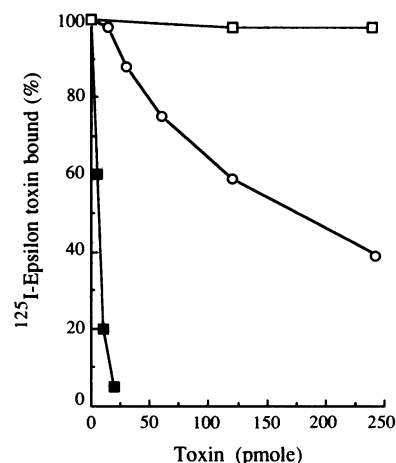
acid residues. On the basis of these data, the binding site of the toxin may be a sialoglycoprotein. However, treatment with these enzymes did not cause a complete loss of binding, even when treatment conditions, increases in enzyme concentrations, and incubation times were severe. Therefore, it is unlikely that the site is composed of protein components only. In addition, binding was sensitive to pretreatment of the fractions with lipase, suggesting that interaction of epsilon-toxin with synaptosomal membranes may be facilitated by a lipid component and/or may require an appropriate lipid environment. There also is no denying the possibility that the reduction in binding obtained with lipase may be due to the disruption of membrane properties following the extensive solubilization of membrane proteins by this enzyme.

β-Bungarotoxin was reported to inhibit the evoked release of acetylcholine from the terminals of motor neurons (10). Botulinum toxin inhibits the evoked release of the transmitter from cholinergic nerve terminals (24). Furthermore, it has been reported that these toxins exhibit binding to synaptosomes from rat brain (16, 25). In the present work, epsilon-toxin was found to bind specifically to the membrane fractions from rat brain. β-Bungarotoxin inhibited the binding of the labeled epsilon-toxin. Botulinum toxin had no effect on epsilon-toxin binding. However, the binding site of β-bungarotoxin is reported to be different from that of botulinum toxin (16). The binding site of epsilon-toxin seems specific for epsilon-toxin in the sense that competition binding was observed with the presynaptically acting protein β-bungarotoxin but not with botulinum toxin.

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