BINDING PROTEINS ON SYNAPTIC MEMBRANES FOR CERTAIN PHOSPHOLIPASES A_2 WITH PRESYNAPTIC TOXICITY

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Many steps in the process of neurotransmitter release are vulnerable to various neurotoxins. Some of these presynaptic toxins exhibit phospholipase A_2 (PLA_2) activity. These neurotoxic PLA_2s (or PLA_2 neurotoxins) are members of a group of extracellular (or secreted) PLA_2 proteins found in most if not all animals. Besides phospholipid metabolism, these PLA_2s exhibit a variety of biological effects, including host defense, neurotoxicity (presynaptic and/or postsynaptic), myotoxicity, and alteration of coagulation, which may or may not be related to hydrolysis of phospholipids. Despite large differences in biological actions, the PLA_2 chains of these proteins show high degrees of homology in the primary, secondary and possibly tertiary structures. A small number of these proteins, mostly isolated from the venoms of a number of snakes, act primarily at the presynaptic level to cause synaptic blockade by inhibiting the release of neurotransmitters, though most of them also produce postsynaptic toxicity and other effects. These presynaptic PLA_2 toxins may be classified into three classes. The toxins differ in their subunit structures, but in every case, at least one subunit is an active PLA_2 with M.W. of 12,000 to 16,000. Each toxin in the first class is a single-chained protein. In the second class, a toxin may comprise 2 to 4 homologous subunits associated noncovalently. The toxins in the third class are composed of a phospholi-

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pase chain in disulfide linkage to a non-PLA_2 chain. Different facets of these toxins have been studied by many investigators (see Ref. 1-9 for recent reviews).

Most of the earlier studies on these toxins were concerned about the structural, pharmacological and electrophysiological aspects. At the neuromuscular junction, these presynaptic neurotoxins have been shown to affect neuromuscular transmission. The time courses vary with the toxins and the experimental conditions. An initial decrease in neurotransmission followed by a transient increase is most common. And in the final stage, when sufficient amounts were used, all of these toxins cause neuromuscular blockade.

A number of studies indicate that the PLA_2 activity is required for the blocking effect of these toxins under normal condition. On the other hand, many PLA_2s bear little if any neurotoxicity. There are wide differences in toxic potencies between the PLA_2s, but the differences in enzymatic activities are small. Furthermore, the relative toxic potencies of the PLA_2 toxins are not proportional to their PLA_2 activities. In order to explain this phenomenon, it has been postulated that the distinction lies in disparities in the strength of binding to the presynaptic membranes, strong for the presynaptic toxins against poor for the non-neurotoxic PLA_2s. Indirect evidence for the existence of high-affinity binding sites in the target membranes has been provided by the observations that neuromuscular blockade still occurs when the nerve-muscle preparation has been exposed to the toxins for only a brief period followed by thorough washing. Since the PLA_2 activity is greatly attenuated by lowering the temperature to near 0°C or when Ca^{2+} is replaced by Sr^{2+}, whereas toxin binding persists, it is even possible to temporally separate the binding step from the subsequent event of synaptic blockade. As all known toxic PLA_2s are from snake venoms, most of the many pharmacological studies of these toxins were done on the peripheral tissues with the consideration that in snakebites the toxins do not reach the brain because of the blood-brain barrier. However, when the toxins were injected directly into the brain or tested on its isolated preparations, the neurotoxic PLA_2s showed high degrees of toxicity.

Direct evidence for binding to presynaptic membranes has been obtained for several toxins in recent years (10-22). Membrane preparations from the brain have been used in most of the endeavors. One study has been conducted with nerve ending preparation from the electric organ. The scarcity of the mammalian peripheral nerve endings has impeded their use for studies in this respect.

Specific binding of high affinity to synaptosomes or to synaptic membrane fragments prepared from the brain has been reported (in chronological order) for β-bungarotoxin, crotoxin, Mojave toxin, taipoxin, PLA_2 from *Naja nigricollis*, PLA_2 from *Naja naja atra*, *Pseudocerastes* neurotoxin, OS_2 (a PLA_2 from *Oxyuranus scutellatus*), Pa-11 (a PLA_2 from *Pseudechis australis*), ammodytoxin C, and Pseudexin B (10-21). Among the above PLA_2s, it is not known whether OS_2 is a presynaptic toxin, and the PLA_2s from *Naja nigricollis* and from *Naja naja atra* showed little effect on the peripheral nervous system. Our own studies in this respect (10-13) will be described below. In our experiments, crotoxin, Mojave toxin, taipoxin, β-bungarotoxin, Pa-11 and pseudexin B were purified from the crude venoms and then radioactively labeled with Na[^{125}I]. The neurotoxicity of each of the iodinated toxins was found to be fully retained when assayed on nerve-muscle preparations from the chick. Each ^{125}I-toxin was incubated with synaptosome or synaptic membrane (23,24) fractions from the cerebral cortex of guinea pig in the absence or in the presence of large excess (≥100-fold) of the unlabeled toxin for a suitable time. The membrane bound radioactivity was separated from the unbound by centrifugation. For each radioactive toxin, specific binding, i.e., that displaceable by the unlabelled toxin, to synaptosomes or synaptic membranes was observed. The specific binding is saturable for each toxin, being consistent with the presence of a limited number of binding sites. The degree of nonspecific binding is low (generally <10%) in each case. In sharp contrast, when pancreatic PLA_2, which is non-toxic, was tested, specific binding was undetectable. The affinities of the toxins to the membrane

Table I. A list of the parameters reported for the binding of PLA_2 neurotoxins

Toxin	Membrane preparation	K_D (nM)	B_{max} (pmol/mg protein)	References
β-bungarotoxin	rat synaptic membrane	high affinity, 0.26	0.16	14, 18
		low affinity, 6.1	2.6	
	chick synaptic membrane	0.47	~0.05	15
	guinea pig synaptosome	0.7	0.1	
crotoxin	guinea pig synaptosome	< 10	~10	10, 11
	guinea pig synaptosome	high affinity, 4	2	20
		low affinity, 87	4	
	Torpedo presynaptic membrane	700	240	22
Mojave toxin	guinea pig synaptosome	< 8	~10	10
OS_2	rat synaptic membrane	high affinity, 0.0015	1	19
		low affinity, 0.045	3	
Pa-11	guinea pig synaptic membrane	high affinity, 0.25	6.9	12
		low affinity, 4.6	23	
ammodytoxin C	bovine synaptic membrane	6.0	5.7	21
pseudexin B	guinea pig synaptosome	high affinity, 1.2	2	13
		low affinity, 10	77	
taipoxin	guinea pig synaptosome	< 12	~60	

preparations and the density of the binding sites are shown in Table I, which also includes data obtained for other toxins by other investigators. We want to stress that we have chosen the condition that minimizes the PLA_2 activity by using a solution containing 10 mM Sr^{2+}, 0.5 mM EGTA and no Ca^{2+} for the binding assay in our studies to avoid unwanted complications which may arise from hydrolysis of membrane phospholipids (see Ref. 1 for more information). Ca^{2+} is known to be required for the enzymatic activity of the secreted PLA_2s, while Sr^{2+} is inhibitory (see Ref. 1-4 for reviews).

Crotoxin is produced by the South American rattlesnake *Crotalus durissus terrificus* and is the first neurotoxin isolated and crystalized. This toxin is composed of two differnt subunits, an acidic subunit A of 9,000 Da and a basic subunit B of 14,400 Da. Subunit B is an active PLA_2 and is weakly toxic on its own. Subunit A can potentiate the toxicity of subunit B, but otherwise no biological activity by itself is known (Ref. 25 and 26 for recent reviews). Since there are two subunits in the crotoxin molecule, it is of interest to know whether the whole complex or only one subunit is involved in the binding. For this purpose, we subjected both the pellets and the supernatants after centrifugation to sodium dodecyl sulfate-polyacrylamide gel electropheresis (SDS-PAGE). From the autoradiogram of the gel, it was found that only the subunit B bound to the synaptosomal membrane, while the subunit A remained in the supernatant (10, 11). Previously this phenomenon was also demonstrated with the postsynaptic membrane from electric fish using a different technique by Bon *et al.* (27). Since it is difficult to accurately determine the concentration of the unassociated subunit B in the reaction mixture, only IC_{50} value of ~10 nM for unlabeled crotoxin to compete for the binding of labeled toxin was given by us. The dissociation constant (K_D) would be considerably lower. The density of binding sites (B_{max}) was ~10 pmol/mg of total protein (10, 11).

Since subunit A did not bind to the membranes in the end of the equilibrium binding assay, obviously the dissociation of the crotoxin complex at some point is an important step in the action of the toxin. Hendon and Tu (28) converted crotoxin complex into a non-dissociable conjugate by cross-linking the two subunits with a bifunctional cross-linker, dimethyl suberimidate. This conversion abolished the lethality of crotoxin but did not significantly reduce its PLA_2 activity. When we used the non-dissociable crotoxin in binding

assay, we found that it did not bind to the membranes. On the other hand, subunit A was capable of inhibiting the binding of subunit B (20). This may not be surprising, for excess subunit A will reduce the effective concentration of free subunit B by mass action. We may deduce from these observations that subunit A competes with membrane binding sites for combining with subunit B, and thus prevents subunit B from binding to low-affinity sites at various places. Accordingly, the presence of subunit A allows subunit B to bind only to the high-affinity site. This scheme is compatible with the proposal that subunit A acts as a chaperone, as put forward by Folman, Habermann and others (29,30), although more than one mechanism is possible with that proposition. A different view has been expressed by other investigators (22).

We then examined the effects of other PLA_2s on the binding of crotoxin. β–Bungarotoxin had no effect at all (11). Pancreatic PLA_2 was also without effect. On the other hand, in contrary to our preliminary report (11), taipoxin inhibited up to 40% of the binding of ^{125}I-crotoxin (1). Caudoxin, notexin, and pseudexin A behaved like taipoxin in this respect. Unlabeled crotoxin inhibited to completion, as would be expected.

Taipoxin, found in the venom of the Australian taipan *Oxyuranus scutellatus*, is made of three subunits, α, β, and γ of 13,750, 13,500 and 18,350 Da, respectively, each showing sequence homologous to other PLA_2s. The α–subunit is weakly toxic itself. The β and γ–subunits are non-toxic, but can greatly enhance the toxicity of the α–subunit (29, 31). And similar to the case of crotoxin, only the α–subunit was found to bind to the synaptosome (1). By analogy with crotoxin, it may be proposed that dissociation of the taipoxin complex occurs before binding of the α–subunit. The β– and γ–subunits may well act like the subunit A of crotoxin. The IC_{50} for the unlabeled taipoxin to inhibit the binding of the radioactive toxin was ~12 nM (11), and the binding sites of taipoxin appeared to be about 60 pmol/mg protein of synaptosomes. The binding of taipoxin was not antagonized by β–bungarotoxin. Unexpectedly, high concerntration of crotoxin appeared to increase the binding to some extent. The nonreciprocal antagonism between crotoxin and taipoxin binding would indicate that some binding site (or sites) is common for the two toxins, while others are not shared.

As the binding of crotoxin is strongly protease sensitive, the involvement of proteins in the plasma membrane of the synaptosome is indicated. We went on to identify the binding protein(s) for crotoxin (11, 32) first by using the photoaffinity labeling technique in collaboration with Dr. R. J. Guillory of the University of Hawaii. We were able to attach a photoactivatable group to crotoxin by coupling iodinated crotoxin with N-hydroxysuccinimidyl-4-azidobenzoate (HSAB) without impairing the neurotoxicity. This crotoxin derivative was allowed to bind to the synaptosomal membrane and then irradiated with UV light. When the membrane pellet was analyzed by SDS-PAGE, a major radioactive conjugate of 100 kDa was found in the autoradiogram. The formation of this conjugate was prevented by unlabeled crotoxin, indicating the binding is specific. Prior treatment of the membrane by trypsin, papain, or protease V8 from *Staphylococcus aureus* also abolished the conjugation. Therefore this putative receptor is a protein. The M.W. of this protein was estimated to be about 85 k by subtracting the mass of subunit B, which is 15 k, from that of the conjugate. The formation of this conjugate was also inhibited by serveral other neurotoxic PLA_2s, such as Mojave toxin, taipoxin, caudoxin, but not by β–bungarotoxin and pancreatic PLA_2. The IC_{50} of unlabeled crotoxin to abolish this conjugation was ~10 nM (32). When the synaptosome was subfractionated, this polypeptide was chiefly found in the synaptic membrane fraction, and not present in the intra-synaptosomal mitochondrial fraction. We have also tested membranes from non-neural tissues, such as liver, kidney, erythrocytes, and did not find the 85-k polypeptide in these membranes.

When purely chemical cross-linking reagent disuccinimidyl suberate was used, a different conjugate of ~60 kDa was found. As prior treatment of the synaptosome with proteinase K, trypsin, subtilisin, α-chymotrypsin, elastase or heat destroyed this conjugation

capability of the membrane, the membrane component in the conjugate was a single-chained protein of ~45 kDa. Similar results were obtained when dithio*bis*(succinimidylpropionate), or ethylene glycol*bis*(succinimidylsuccinate) was used as the cross-linker. Of the subfractions of synaptosomes, this binding protein was mostly found in the synaptic membrane fraction, and not present in the mitochondrial fraction. Plasma membranes from several non-neural tissues also did not contain this binding protein. The IC_{50} of unlabeled crotoxin to inhibit the formation of this adduct was ~10 nM. Mojave toxin, taipoxin, Pa-11, and pseudexin A were also highly inhibitory to in this conjugation, notexin and caudoxin were less effective, while β-bungarotoxin and pancreatic PLA_2 were totally ineffective. The results may signify that the 45-k polypeptide preferentially present in neuronal membranes is another putative receptor responsible for the binding of crotoxin, but it is also possible that this polypeptide is another subunit of the receptor (33).

Besides membrane proteins, crotoxin appears to bind also to lipid components of the membrane. Radvanyi *et al.* (34) have shown that incubating crotoxin with negatively charged vesicles of phospholipids resulted in the binding of subunit B with K_D ranging from <1 to 100 μM, depending on the lipid composition, whereas binding was not observed with uncharged vesicles. Therefore, it is possible that some negatively charged lipids may be components of the binding sites for crotoxin in its target membranes, but the relative contribution remains to be determined.

When similar cross-linking experiments were performed with ^{125}I-taipoxin, a 60-k radioactive conjugate was also formed using disuccinimidyl suberate as the cross-linker. The IC_{50} for the unlabeled taipoxin to compete for the formation of this conjugate was somewhere between 1 and 5 nM. This adduct was not observed when the synaptosomes were treated priorly with trypsin, proteinase K or chymotrypsin. From these experiments, we deduced that a 45-k polypeptide in the synaptic membrane is a taipoxin-binding protein or a subunit of it. Again, this polypeptide was not present in the nonneural membranes from erythrocytes, kidney and liver. Although the M.W. of this polypeptide is indistinguishable from that of one of the crotoxin-binding proteins, crotoxin was not very efficacious in blocking the formation of the taipoxin-conjugate. Hence the two 60-k polypeptides are not identical, and crotoxin is a poor ligand for this taipoxin-binding protein. In contrast, taipoxin appears to be a good ligand for the two crotoxin-binding proteins, as taipoxin can inhibit the conjugation of crotoxin to its two binding proteins with high potency. Caudoxin, notexin, Pa-11 behaved like crotoxin, whereas β–bungarotoxin and pancreatic PLA_2 showed no effects at all (35).

We have also undertaken purification of the binding proteins for crotoxin. The synaptic membrane fraction from guinea pig brain was extracted with 4% Triton X-100. Crotoxin-binding activities were solubilized in active form as assayed by a gel filtration procedure and by affinity labeling experiment. The detergent extract was then subjected to gel filtration with Sepharose 6B followed by affinity chromatography with crotoxin linked to Sepharose 4B. After elution from the affinity column, only three bands with molecular weights of ~85 k, 65 k and 50 k were present when analyzed by SDS-PAGE under reducing condition. For the purpose of purifying the binding proteins in large scale, porcine brain was used instead. Only two bands of ~65 k and 50 k were observed when the material purified by affinity chromatography was analyzed by SDS-PAGE. A partial amino acid sequence of KPTEKKDRVHHEPQLL has been obtained for the 50-k band.

We are also interested in learning the determinants in the crotoxin molecule involved in binding to synaptic membranes. An illuminating result was obtained by modifying the toxin with *p*-nitrobenzenesulfonyl fluoride, which is known to react quite selectively with Tyr residues. When crotoxin complex was allowed to react with the reagent, one *p*-nitrobenzenesulfonyl group was readily incorporated into subunit B, while subunit A was modified insignificantly. After separating the modified subunit from the unmodified, we cleaved each protein with trypsin, then separated the peptide fragments with HPLC. When the elution

patterns of the modified subunit B and the unmodified were compared, a new peak in the elution profile of the modified subunit B was identified. The amino acid sequence of this peptide was found to correspond to residues 70-77 (SGYITCGK) of subunit B, thus the residue modified was Tyr72. This modification had little effect on the PLA_2 activity, the affinity for binding to synaptosomes, or the neurotoxicity.

We then subjected the subunit B to further modification and separated the modified proteins into fractions modified to different extents by reversed-phase HPLC. When two *p*-nitrobenzenesulfonyl groups were incorporated, there were drastic reductions in the binding affinity and in neurotoxicity. When three residues were modified, these two activities were virtually completely abolished. In sharp contrast, the PLA_2 activity was largely retained. These results reinforced the importance of binding and confirmed that the PLA_2 activity alone is not sufficient for neurotoxicity of crotoxin. The second and the third modified residues were found to reside in the same peptide fragment (NAIP-FYAFYGCYCGWGGR) corresponding to residues 16-33, which contains three Tyr residues at positions 21, 24, and 27. The modifications occurred mostly at residues 21 and 24. Since Tyr24 and Tyr27 (25 and 28, respectively, in alignment number according to Ref. 36, 37) are conserved for all of the secreted PLA_2s sequenced to date, toxic or not, and Tyr21 (22 in alignment number) is found in the neurotoxic PLA_2s but not in the great majority of the nontoxic ones, Tyr21 would be more important for binding.

In the bovine pancreatic PLA_2, as well as many other nontoxic PLA_2s, the residue at the position corresponding to Tyr21 of subunit B is Phe (and the number is 22 for the bovine enzyme). We found that when the bovine pancreatic PLA_2 mutant F22Y, in which the Phe22 of the wild type was replaced by Tyr (38) by site-directed mutagenesis utilizing the phosphorothioate method (39), was present during the binding period, the subsequent formation of the 60-k radioactive conjugate was suppressed with an IC_{50} of 1 μM. In sharp contrast, the wild-type PLA_2 purified from the bovine pancreas gave no effect even at concentration as high as 50 μM. The wild-type pancreatic enzyme produced by cloning techniques and another mutant F22A, which has Ala at residue 22, were also without effect at the highest concentration used. The one- and two-dimensional NMR spectra of F22Y and the wild type are almost identical except for the obvious changes arising from the new phenolic OH group and a 0.19 ppm change in one of the three chemical shifts of Phe106, which is in close proximity to residue 22, forming the second half of the Phe22-Phe106 aromatic sandwich (40). The enzymatic activities of the two mutants are also comparable with that of the wild-type enzyme (38). Besides, we have performed the competitive binding assay under Ca^{2+}-free condition. Hence it is unlikely that the inhibitory effect of the F22Y mutant is due to the hydrolysis of membrane phospholipids. We thus conclude that the F22Y mutant blocked the formation of the radioactive conjugate by competing the binding of ^{125}I-crotoxin to the binding protein (40). However, because the binding affinity of the F22Y mutant was not high relative to that of crotoxin ($K_D < 10$ nM), there must be other residues also involved in binding and thereby in neurotoxicity. We are currently trying to find them out.

Judging from the affinity of F22Y, we would not expect it to be neurotoxic. All mice injected with the F22Y mutant, either intraperitoneally or intracisternally, lived and behaved normally even at a dose of 18 mg of protein/kg of body weight. We have iodinated the F22Y mutant and attempted to demonstrate its binding to the synaptic membrane directly. Specific binding was not evident, apparently because the affinity is too low.

Obviously, nerve cells produce these toxin-binding proteins not for the purpose of self-destruction. In order to learn the normal function of these binding proteins, we have looked into the effects on the synaptosomes of the two toxins not related to their PLA_2 actions. We found that in a Ca^{2+}-free, Sr^{2+}-containing Tyrode solution, taipoxin caused a marked inhibition of the synaptosomal Na^+/Cl^--dependent uptake of tritiated norepinephrine

and glycine, with IC_{50}s of ~20 and 30 nM, respectively, to be compared with low potencies (IC_{50} > 0.2 μM) in blocking the uptake of dopamine, serotonin, lysine, and phenylalanine, and no effect on choline uptake into synaptosomes under the same condition. Suppression of the uptake of tritiated norepinephrine was also observed with crotoxin, although with low potency (IC_{50} = 1 μM). Again, choline uptake was not affected.

Taipoxin was also capable of antagonizing the binding of desipramine, which is a specific blocker of norepinephrine transporter, to its high-affinity site(s) with IC_{50} of ~15 nM. Taken together, these results strongly indicate that the Na^+/Cl^--dependent norepinephrine and glycine transporters act as two high-affinity binding proteins for taipoxin. The genes for norepinephrine, glycine, dopamine, and serotonin transporters have been cloned just recently (Ref. 41-45 for reviews). They are homologous to each other. But purification of them from the nervous tissue has been difficult. We hope that the PLA_2 neurotoxins can also contribute to the study of these transporters. It is probable that the structural similarity of these transporters is one of the reasons for the multiplicity in the binding proteins for these PLA_2 toxins. As to the receptors for the two toxins at the neuromuscular junction, we consider it possible that they are members (as yet unidentified ?) of the Na^+/Cl^--dependent transporters or molecules related to these transporters in structure, for it is becoming canonical that many different proteins may be grouped into family or superfamily evolutionarily related according to their structures.

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