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BINDING PROTEINS ON SYNAPTIC MEMBRANES FOR CROTOXIN AND TAIPOXIN, TWO PHOSPHOLIPASES A₂ WITH NEUROTOXICITY

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M.-C. Tzeng, C.-H. Yen, M.-J. Hseu, C.-C. Tseng, M.-D. Tsai and C. M. Dupureur. Binding proteins on synaptic membranes for crotoxin and taipoxin, two phospholipases A₂ with neurotoxicity. *Toxicol* **33**, 451-457, 1995.— Crotoxin and taipoxin are both neurotoxic phospholipases A₂ capable of affecting the presynaptic activity to bring about ultimate blockade of synaptic transmission. The enzymatic activity has generally been considered to be necessary but not sufficient for the blockade. Since many phospholipases A₂ with comparable or even higher enzymatic activity are not toxic, it has been postulated that the difference lies in the affinity of binding to the presynaptic membrane. In confirmation of this proposition, we and others have previously shown that iodinated crotoxin and taipoxin bind specifically with high affinity to the isolated synaptic membrane fraction from guinea-pig brain, whereas specific binding is not detected with the nontoxic pancreatic phospholipase A₂. Experiments based on photoaffinity labeling and simple chemical cross-linking techniques have led to the identification of three polypeptides preferentially present in neuronal membranes as (subunits of) the binding protein(s) for crotoxin and/or taipoxin. Some, but not all, other toxic phospholipases A₂ also appear to be ligands for the three polypeptides. We now report studies on partial purification of these polypeptides using affinity chromatography and other techniques. In order to learn the normal physiological roles played by the toxin-binding proteins, the phospholipase-independent effects of the toxins on the synaptosomes have been sought. We have found that under Ca²⁺-free condition, taipoxin or crotoxin inhibits with IC₅₀ of 20-1000 nM the Na⁺-dependent uptake of norepinephrine, dopamine and serotonin by the synaptosomes. In contrast, choline uptake is not affected. Furthermore, the high-affinity site for [³H]desipramine binding, known to be the norepinephrine transporter, is inhibited with an IC₅₀ of 14 nM by taipoxin independent of phospholipase activity. These results are strong indications that certain synaptosomal biogenic amine transporters are part of the binding proteins for taipoxin and crotoxin. Chemical modification at Tyr-21 of the phospholipase subunit of crotoxin greatly reduces the neurotoxicity and the binding affinity with little effect on the enzymatic activity of the toxin. Hence, Tyr-21 may be an important residue for the binding of crotoxin and perhaps other

phospholipase A₂ neurotoxins. Even stronger evidence comes from the finding that replacement of the corresponding Phe residue of bovine pancreatic phospholipase A₂ with Tyr by site-directed mutagenesis enables it to compete with an IC₅₀ of 1 μM for the binding of [¹²⁵I]crotoxin, contrasting sharply with the complete lack of such ability with the wild type even at 50 μM. In addition, rat phospholipase A₂, which has a corresponding Tyr residue, can inhibit the binding of [¹²⁵I]crotoxin.

INTRODUCTION

Crotoxin and taipoxin, among others, are phospholipases A₂ (PLA₂s) with presynaptic neurotoxicity, although most of them also show postsynaptic toxicity and other effects. These presynaptic toxins differ in their subunit organizations, but in every case, a PLA₂ of 12,000–16,000 mol. wt is present. Crotoxin from the venom of the South American rattlesnake *Crotalus durissus terrificus* is composed of two subunits, subunit A (acidic) of 9000 mol. wt, and subunit B (basic) of 14,400 mol. wt, with PLA₂ activity (Slotta and Fraenkel-Conrat, 1938; Rubsamen *et al.*, 1971; Hendon and Fraenkel-Conrat, 1971). Taipoxin from the taipan *Oxyuranus scutellatus* is made up of three subunits with mol. wts of 13,500, 13,750 and 18,350, each having a sequence homologous to that of pancreatic PLA₂ (Fohlman *et al.*, 1976, 1977). At the neuromuscular junction, the effects in the early stages vary with the toxins and experimental conditions, an initial decrease in transmission followed by a transient increase being observed with crotoxin and with taipoxin. But the blockade of transmission in the final stage is common to these presynaptic toxins and is the underlying cause of their neurotoxicity (Tzeng, 1993; Harris, 1991; Hawgood and Bon, 1991; Harvey, 1990; Rosenberg, 1990). As all known toxic PLA₂s 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 injected directly into the brain or tested on isolated preparations, the neurotoxic PLA₂s showed high degrees of toxicity (Hanley and Emson, 1979; Halliwell and Dolly, 1982*a, b*; Othman *et al.*, 1982; Rehm *et al.*, 1982; Rehm and Betz, 1982; Ling *et al.*, 1988; Lambeau *et al.*, 1989).

Based on many studies, the PLA₂ activity has generally been considered to be a necessary condition for the blocking effect of these toxins under normal situations. In addition, several of the PLA₂ presynaptic toxins, including crotoxin and taipoxin, have been shown to bind specifically with high affinity to the membrane fractions rich in synaptic regions, while specific binding was not detectable with the nontoxic pancreatic PLA₂ (Othman *et al.*, 1982; Rehm and Betz, 1982; Lambeau *et al.*, 1989; Yang and Tzeng, 1983; Tzeng *et al.*, 1986; Degn *et al.*, 1991). Three polypeptides preferentially present in neuronal membranes have been identified as (subunits of) the binding protein(s) for crotoxin and/or taipoxin by photoaffinity labeling and chemical cross-linking techniques (Tzeng *et al.*, 1986, 1989; Hseu *et al.*, 1990; Yen and Tzeng, 1991).

Previous chemical modification studies with *p*-bromophenacyl bromide and other reagents have established the importance of His-48 and other residues in the catalytic activity of PLA₂s. We consider it also of interest to determine the residues required for the binding of the PLA₂ neurotoxins to their targets.

Apparently the cell produces the toxin-binding proteins for some other purpose. It may be reasoned that strong binding of the toxins to the synaptic membrane alone will affect

the physiological functions of the toxin-binding proteins without the participation of the enzymatic reaction. We have therefore investigated the phospholipase-independent action of crotoxin and taipoxin on the functioning of the synaptosomes.

MATERIALS AND METHODS

Neurotoxicity assay

The chick biventer cervicis nerve-muscle preparation (Ginsborg and Warriner, 1960) was used to test the neurotoxicity of purified toxins. The muscle was incubated in Tyrode's solution gassed with 95% O₂/5% CO₂ continuously. The nerve was stimulated with supramaximal rectangular pulses of 0.05 msec at 0.2 Hz and isometric contraction recorded with a polygraph.

Protein modification

Modification with *p*-nitrobenzenesulfonyl fluoride was carried out in 0.1 M Tris-HCl, pH 8.0, at 25°C for 60 min (Liao *et al.*, 1982). The modified proteins were separated from the unmodified by a Synchronpak RP column with a gradient of 24–48% acetonitrile. The proteins were reduced by dithiothreitol, carboxymethylated by iodoacetic acid, and then fragmented by TPCK-trypsin. The peptides generated by proteolytic cleavage were separated by HPLC with a Synchronpak column with a gradient of 0–40% acetonitrile. Pertinent fragments were selected for amino acid sequence determination by a sequencer.

Cross-linking of [¹²⁵I]crotoxin with membrane components

Crotoxin labeled with ¹²⁵I by the chloramine-T method was mixed with the synaptic membrane preparation from guinea-pig brain in the presence or absence of other unlabeled PLA₂s at 25°C for 2.5 hr. The membrane was pelleted down, washed and resuspended. Disuccinimidyl suberate (DSS) was added and the mixture incubated for 4 min at 25°C. The reaction was stopped by adding 1 N glycine. The membrane pellet was recovered and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography.

Solubilization and purification of the crotoxin-binding proteins

The synaptic membrane fraction from guinea-pig brain was suspended in solution A (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 10 mM SrCl₂, 0.5 mM EGTA) containing 4% Triton X-100. After agitation at 4°C for 2 hr, the mixture was centrifuged at 105,000 × *g* for 1 hr at 4°C. The resulting supernatant was fractionated by gel filtration with a Sepharose 6B column. The eluate was assayed for [¹²⁵I]crotoxin-binding activity. The active fractions were pooled and further purified by affinity chromatography with crotoxin coupled to CNBr-activated Sepharose 4B.

Binding assay

To measure the binding activity of the solubilized preparations, each sample was mixed with [¹²⁵I]crotoxin in solution A. Parallel incubation containing excess unlabeled crotoxin in addition was carried out in order to determine nonspecific binding. The mixtures were incubated for 2.5 hr at 30°C. The bound [¹²⁵I]crotoxin was separated from the unbound by a rapid gel filtration method with Sephadex G-75.

Neurotransmitter uptake by the synaptosome

Synaptosomes (Whittaker, 1959) were suspended in Sr²⁺-Tyrode solution of the following composition (mM): NaCl 137; NaHCO₃ 12; MgCl₂ 1.0; KCl 2.7; NaH₂PO₄ 0.6; glucose 11; Sr(NO₃)₂ 3.8; EGTA 0.5, equilibrated with 95% O₂/5% CO₂. Incubations were performed at 37°C in the presence or absence of specified concentrations of toxins. Tritiated norepinephrine, dopamine, serotonin, or choline was then added. After an additional 6 min incubation, the mixture was diluted with ice-chilled Sr²⁺-Tyrode solution and the synaptosomes were collected on Whatman GF/F filters. After washing, the radioactivity trapped by the filters was measured by liquid scintillation counting.

Desipramine binding

Guinea-pigs were decapitated and each cerebral cortex was homogenized in 20 ml of ice-chilled assay buffer (composition, in mM: Tris-HCl 50; NaCl 120; KCl 5; Sr(NO₃)₂ 10; EGTA 0.5; pH 7.4) using a Kinematica polytron homogenizer. The brain homogenates were incubated with 1.5 nM [³H]desipramine and specified concentrations of taipoxin in the assay buffer for 2 hr at 0°C. Then the mixture was diluted with ice-chilled assay buffer and filtered through Whatman GF/F filters. After washing, the radioactivity on each filter was measured.

RESULTS AND DISCUSSION

The subunit B of crotoxin (CXB) was modified with iodoacetate, phenylglyoxal, *p*-nitrobenzenesulfonyl fluoride, or trinitrobenzenesulfonic acid. Among these modifications, only the one with *p*-nitrobenzenesulfonyl fluoride, which modifies Tyr residues, impaired the ability of binding to the synaptic membrane as judged from the effectiveness (or lack of it) of the modified proteins to inhibit the binding of [¹²⁵I]crotoxin. Hence, this modification was chosen for more detailed studies.

After reacting with *p*-nitrobenzenesulfonyl fluoride, the modified proteins were separated by a reversed-phase HPLC column into fractions modified to different extents. When the whole crotoxin complex was subjected to the modification reaction, Tyr-72 of CXB was the residue most readily modified, as determined by peptide sequence analysis after separating the modified toxin subunits from the unmodified. When the above modified CXB was compared with the unmodified for PLA₂ activity, neurotoxicity and the affinity for binding to synaptosomal membranes, only small changes (2–24%) were observed. Then the CXB was modified further, and analyses after separation of the reaction products showed that there were parallel and drastic reduction in neurotoxicity and in binding activity when two residues were modified, and virtually complete loss of both when three residues were modified. In sharp contrast, the phospholipolytic activity was largely retained. These results demonstrate again that PLA₂ activity alone is not sufficient for the toxicity of crotoxin and provide additional evidence for the necessity of strong binding to membranes. Amino acid sequence determination following peptide mapping of tryptic digest revealed that Tyr 21, 24 and/or 27 (all in the same peptide fragment of residues 16–33 with the sequence of NAIPFYAFYGCYCGWGGR) were the one(s) modified. From these results, we may deduce that Tyr-21, Tyr-24 or Tyr-27 is an important determinant for the binding of CXB to its target protein(s). Since Tyr-24 and 27 (25 and 28 in alignment number) are conserved for all of the PLA₂s sequenced to date, whether toxic or not, and Tyr-21 (22 in alignment number) is found in the neurotoxic PLA₂s but not in the great majority of the nontoxic PLA₂s, we consider that Tyr-21 is the more important one. Additional evidence below lends even stronger support for the importance of Tyr-21 in binding. However, there are certainly other determinants important for binding. This may well be the reason why some of the PLA₂s with Tyr-21/22 are not neurotoxic.

Covalent attachment of [¹²⁵I]CXB to the synaptic membrane preparation by the chemical cross-linker disuccinimidyl suberate yields a 60,000 mol. wt conjugate, as reported previously (Yen and Tzeng, 1991). The formation of this radioactive conjugate was completely suppressed by the presence of sufficient F22Y, a mutant bovine pancreatic PLA₂ generated by site-directed mutagenesis of Phe-22 of the wild-type to Tyr, in the binding stage of the experiment. The IC₅₀ was estimated to be ~1 μM from the dose-response curve. In sharp contrast, no effect was observed with the wild-type even at 50 μM, the highest dose used. The enzymatic activity was not changed by this mutation and NMR analysis ascertained that the conformations of the mutant and the wild-type were virtually identical. Somewhat later we became aware that residue 22 of the rat pancreatic PLA₂ is Tyr. There is good reason to expect that the rat enzyme will behave like the F22Y mutant above. After purification of the rat PLA₂, examination of its ability to nullify the conjugation of [¹²⁵I]CXB to the membrane component fully supports this prediction. These results demonstrate again the importance of Tyr-21/22 in the binding to membrane protein. Site-directed mutagenesis of the crotoxin subunit B is being carried out to gain more information about the residues involved in binding.

In order to solubilize the binding proteins for crotoxin and taipoxin, the synaptic membrane fraction from guinea-pig brain was extracted with detergents. The extracts were then incubated with [125 I]crotoxin in the absence or the presence of unlabeled crotoxin and assayed for binding of [125 I]crotoxin using a gel filtration procedure. When a buffer containing 4% Triton X-100 was used, the crotoxin-binding proteins were found to be solubilized and in biologically active form. The affinity of the solubilized proteins for [125 I]crotoxin was unimpaired by solubilization, judging by the IC_{50} of 7 nM for the inhibition of the binding of [125 I]crotoxin to the detergent extract by the unlabeled crotoxin. After binding complexes of [125 I]crotoxin and its binding proteins had formed, chemical cross-linking with disuccinimidyl suberate resulted in a predominant band of conjugation with a mol. wt of approx. 60,000, similar to the result of cross-linking with synaptic membrane fragments. Following gel filtration with Sepharose 6B, affinity chromatography with crotoxin coupled to Sepharose 4B was carried out to purify the crotoxin-binding proteins. Binding activity for [125 I]crotoxin was desorbed by eluting with 50 mM Tris-HCl, pH 7.4, 10 mM EDTA, 50 mM NaCl, and 0.2% Triton X-100. When this eluate was analyzed by SDS-PAGE, only three major bands, presumably proteins, were found under reducing condition, the mol. wts being 87,000, 65,000 and 50,000. Additional steps are necessary to achieve complete purification of the crotoxin-binding proteins.

In Ca^{2+} -free, Sr^{2+} -containing Tyrode solution, taipoxin caused a marked inhibition of the synaptosomal Na^{+} -dependent uptake of tritiated norepinephrine (NE), dopamine (DA) and serotonin (5-HT) with IC_{50} of 20, 100 and 500 nM, respectively. In sharp contrast, taipoxin showed very little if any effect on choline uptake into brain synaptosomes under Ca^{2+} -free conditions. Suppression of the uptake of tritiated NE, DA, and 5-HT was also observed with crotoxin, IC_{50} being 1 μ M, 0.3 μ M, and 0.5 μ M, respectively, under the same condition. Again, choline uptake was not affected. For comparison, β -bungarotoxin (also a neurotoxic PLA_2) has been reported to cause Ca^{2+} -dependent inhibition of synaptosomal uptake of choline, but not NE (Werniche *et al.*, 1974; Sen *et al.*, 1976), and our own data have confirmed these observations. Hence, the Ca^{2+} -independent inhibitions of NE, DA and 5-HT uptake are not shared by all toxic PLA_2 s.

Desipramine has been known to be a blocker of the NE transporter (Lee and Snyder, 1981; Backstrom *et al.*, 1989). According to the literature, two specific binding sites for [3 H]desipramine exist, the IC_{50} being 1 nM and 600 nM for the high-affinity and the low-affinity sites, respectively. The high-affinity site resides on the NE transporter. Our experiments showed that the high-affinity, but not the low-affinity, site for desipramine binding was inhibited by taipoxin with an IC_{50} of 14 nM.

In order to avoid unwanted perturbations of the synaptosomes by the PLA_2 action of the toxins, we used a medium containing Sr^{2+} , EGTA and no Ca^{2+} in the experiments concerning the inhibition of [3 H]desipramine binding and performed the experiments near 0°C. In the synaptosomal uptake experiments, it is impractical to operate near 0°C, but it is still workable to include EGTA and replace Ca^{2+} with Sr^{2+} in the incubation solution. We have tested taipoxin and crotoxin under the Sr^{2+} -EGTA (Ca^{2+} -free) condition for PLA_2 activity, and none was detected. Furthermore, the synaptosomes appeared to be intact at the end of the experiments as judged from the retention of lactate dehydrogenase identical to control. The integrity of the synaptosomes was further assured by the lack of effect by taipoxin and crotoxin on choline uptake. Therefore, it is reasonable to conclude that the observed effects of taipoxin and crotoxin in the present study do not require the PLA_2 activity of the toxins, since the enzymatic reaction did not occur to any significant degree.

In order to find clues about the physiological roles played by the unliganded binding proteins for the neurotoxic PLA₂ in the normal functioning of the neuron, we believe that the search for the phospholipase-independent action of the toxins should be promoted. Working along this line, we have obtained reliable results to indicate strongly that some biogenic amine transporters in the synaptic membranes are the major binding proteins for taipoxin and crotoxin. This is the first report of the phospholipase-independent actions of taipoxin and crotoxin and the first of the phospholipase-independent effects on biogenic amine transport systems for any neurotoxic PLA₂.

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