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Chemical Modification of Taipoxin and the Consequences for Phospholipase Activity, Pathophysiology, and Inhibition of High-Affinity Choline Uptake

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Treatment of taipoxin with *p*-bromophenacyl bromide resulted in modification of single histidine residues in the α and β subunits. The modification decreased the neurotoxicity (lethality) 350-fold, but the inhibitory action on high-affinity choline transport was reduced only threefold. The phospholipase activity and Ca^{2+}-association constants for taipoxin and its subunits were determined. A model for the neurotoxicity of taipoxin indicates the α subunit as the ultimate cause of the disruption of synaptic transmission.

Upon nervous stimulation, cholinergic nerve endings release quanta of acetylcholine packaged in small vesicles, apparently by an exocytotic mechanism [1]. By retrieval of the membrane, vesicles are reformed and reloaded with transmitter [2,3]. A membrane pump provides choline for the synthesizing enzyme choline acetyltransferase, and acetyl coenzyme A is probably furnished by the mitochondrion [4]. Snake venom neurotoxins, black widow spider venom and the bacterial botulinus toxin can interfere with different parts of this cycle.

Black widow spider venom acts by releasing the transmitter as evidenced by an avalanche of miniature endplate potentials and the disappearance of vesicle structure [5,6]. Botulinus toxin inhibits the release process, probably directly, without visible ultrastructural alterations [7–9]. The snake venom neurotoxins have a slight enhancing action on the release process, but the final effect seems to be inhibited reformation of functional vesicles [10,11]. The biochemical correlate may be inhibition of high-affinity choline uptake, as we have demonstrated on isolated nerve endings from *Torpedo marmorata* [12] or as others have shown with brain synaptosomes [13].

Taipoxin has been shown to be composed of three subunits, which are linked together by non-covalent forces [14]. Data on the nature of the interaction of the subunit polypeptide chains will be presented elsewhere (Fohlman et al., unpublished). The complete covalent structure of the polypeptide backbone of the γ subunit has been determined and the attachment point of the carbohydrate moiety has been assigned [15]. It is homologous to pancreatic prophospholipase [16]. The N-terminal residues of the α and β subunits have been shown to be highly homologous to each other and to vertebrate digestive-tract phospholipases including presynaptic toxins. Microheterogeneity, at least of the β subunits, is present and three very similar but clearly different amino acid compositions have been obtained [14] (and unpublished observation). Further sequence analysis of these polypeptides is under way. Fig. 1 gives a schematic picture of the taipoxin complex and the derivatives used in this study.

Four known presynaptic snake venom neurotoxins (crotoxin, β-bungarotoxin, notexin and taipoxin) have been used in neurochemical studies. Notexin, one of the chains of crotoxin and β-bungarotoxin and all three subunits of taipoxin are known to be structural homologues of the pancreatic phospholipase A_2 or its proenzyme. Moreover, all of these toxins have at least some catalytic activity in the presence of deoxycholate. A central issue is whether the phospholipase activity is involved in the neurotoxic action and if so, how.

MATERIALS AND METHODS

Isolation of Taipoxin

Taipoxin was purified from the venom of the taipan, *Oxyuranus s. scutellatus* by gel filtration on Sephadex

Abbreviations. BrPhAc, *p*-bromophenacyl; CD, circular dichroic.

Enzyme. Phospholipase A_2 (EC 3.1.1.4).

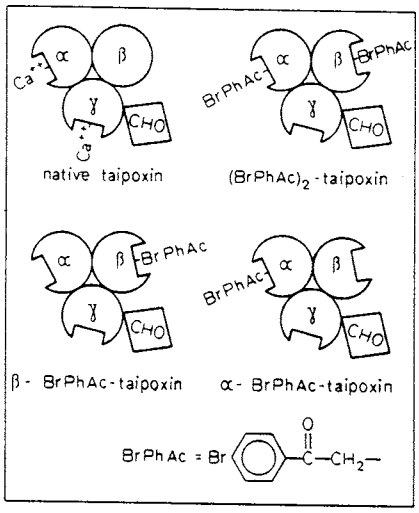


Fig. 1. *Schematic representation of the taipoxin complex and the modified derivatives.* It is assumed from unpublished data that all three subunits interact with each other; BrPhAc is bound to a histidine residue, but the Ca^{2+} ion is associated with as yet unidentified residues, most likely acidic and close to the histidyl residue in the three-dimensional structure. CHO = carbohydrate

G-75 followed by column zone electrophoresis as described earlier [14].

Alkylation with p-Bromophenacyl Bromide

Taipoxin was modified essentially as previously published for pork pancreas phospholipase A_2, notexin, and a sea snake myotoxin [17–19] with the following changes. Taipoxin (380 nmol) was dissolved in 10 ml 0.1 M sodium cacodylate/HCl buffer, pH 6.0, containing 0.1 M NaCl. 50 µl 0.06 M *p*-bromophenacyl bromide was added (= eightfold molar excess) and was allowed to react for 22 h at 30 °C. The reaction mixture was lyophilized to 2 ml, gel filtered on a Sephadex G-25 column (1.5 × 14 cm) equilibrated with 0.1 M ammonium acetate and the protein fraction lyophilized to dryness. The modified protein was then reacted as above for a second cycle to ensure complete reaction, since the separation of native from reacted taipoxin is technically and theoretically difficult, perhaps impossible with present techniques.

Subunit Separation

The polypeptide chains constituting taipoxin were prepared by gel filtration in 6 M guanidine hydrochloride on a Sepharose 6B column, by column zone electrophoresis at pH 1.9 and ion-exchange chromatography on SP-Sephadex G-25 as previously reported [14].

Amino Acid Analysis

The content of unmodified histidine was determined from the amino acid analysis. Prior to hydrolysis with 6 M HCl, samples were oxidized with performic acid, diluted tenfold with water and freeze-dried. This is to avoid cysteine derivatives which would interfere with the integration of the histidine peak. Spectra were run in conjunction with the analysis to give molar absorption values.

Toxicity Assay

Toxicity was assayed in mice as previously [14].

Phospholipase Assay

Using 2 ml 50% egg yolk in water suspension as substrate, the free fatty acids liberated were titrated with 0.01 M NaOH using a Radiometer TTT-1 autotitrator at pH 8.0, 18 °C, in the presence of 2.5 mM sodium deoxycholate and 2 mM $CaCl_2$ [20].

Calcium Binding

Association of calcium with protein was assayed by ultraviolet difference spectroscopy, since binding of calcium causes a change in the absorption with a maximum at 242 nm [21]. This was used to calculate the dissociation constant and number of bound calcium atoms.

Circular Dichroism Measurements

Circular dichroism (CD) spectra were obtained at room temperature with a Jasco J-41A spectropolarimeter. The instrument was calibrated with D-10-camphorsulphonic acid. The results are expressed as molar ellipticity $[\theta]$ beyond 250 nm and as mean residue ellipticity $[\theta]_{\bar{w}}$ below 250 nm, assuming a mean residue weight $\bar{w}$ of 117. The latter value was obtained by dividing the molecular weight by the sum of the number of amino acid and carbohydrate residues. Calculations on secondary structure were made according to Chen et al. [22] and Greenfield and Fasman [23].

Inhibition of Choline Uptake

T sacs can be purified from *Torpedo marmorata* electric organs [24]. They represent resealed fragments of cholinergic nerve teminals with the high-affinity choline uptake mechanisms intact. We have shown that a variety of presynaptic neurotoxins inhibit uptake [12]. This 'toxicity assay' is independent of pharmacokinetic parameters and might thus be taken to reflect molecular events at the target site. Choline uptake was determined using a filtration assay exactly as described recently [25]. Inhibition by toxins was determined by the simultaneous exposure of T sacs

to 1 μM [^{3}H]choline and varying concentrations of toxin at room temperature for 15 min.

Inhibition of Neuromuscular Transmission

Experiments were made on isolated phrenic-hemidiaphragm preparation of adult male mice. The preparation was mounted in a constant-temperature bath at 30 °C and perfused with an oxygenated medium of the following composition: 135 mM NaCl, 15.0 mM $NaHCO_3$, 1.0 mM Na_2HPO_4, 5.0 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 11.0 mM glucose. The pH of the solution was 7.2–7.4. To record isometric twitch tension the tendon part of the muscle was attached to a Grass FT-03 transducer connected to an ink-writing oscillograph. The phrenic nerve was stimulated by a glass capillary suction electrode with supramaximal voltage and pulses of 0.05-ms duration at 0.1 Hz. The resting tension of the muscle was adjusted to give maximal twitch responses. Native or modified taipoxin was present in the bath for the first 30 min of each experiment. The bath was then perfused with normal solution (toxin-free) throughout the experiment.

Changes in the Electron-Microscopic Appearance

For ultrastructural studies, diaphragms were taken from two mice at each dose level following an intravenous injection of the modified toxin at doses of 1 μg, 10 μg, 100 μg and from untreated control animals. For comparison with native taipoxin, motor endplates from mice of a previous series [11] were inspected. In these latter experiments diaphragms were obtained from animals which had died of respiratory paralysis 90–120 min following an intravenous injection of native taipoxin (1 μg). After removal the muscles were processes for microscopical and electrophysiological identification of the endplate regions as described earlier [11]. The tissue was fixed in 3% glutaraldehyde and the endplate regions were dissected out. The samples were prepared for routine electron microscopy as described previously [11].

RESULTS

Alkylating Reaction

After two modification cycles (see Methods) taipoxin was gel-filtered on a Sephadex G-75 column (Fig. 2). The heterogeneity is due to dimer formation, as substantiated by amino acid analysis. The 'dimers' are non-covalently linked and separable into the taipoxin subunits in 6 M guanidine hydrochloride as reported [14]. The 'dimer' was used for preparing subunits. The monomer used for the physiological assays eluted at the position of native taipoxin on both Sephadex G-150 and G-75 [14].

Chemical Characterization

The amino acid data (Table 1) clearly indicate the loss of one histidine residue from the α and β subunits, and the spectral data indicate the incorporation of one bromophenacyl moiety into each. The γ subunit does not react at histidine although the spectrum gives a high value and indicates some reaction elsewhere, perhaps in the carbohydrate moiety. Even when the alkylation reaction was done with the purified γ subunit no loss of histidine was observed by amino acid analysis.

Ca^{2+} Binding

Two taipoxin subunits (α and γ) can bind Ca^{2+} (Table 2 and Fig. 3) in apparently analogous fashion,

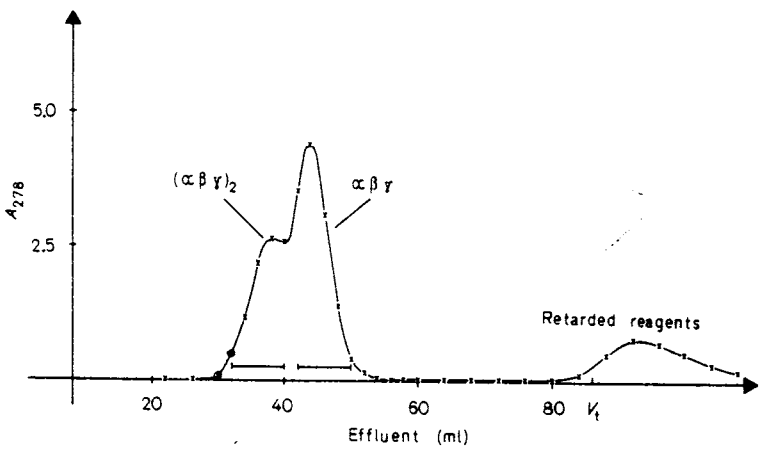


Fig. 2. *Gel filtration of modified taipoxin in a column (100 × 1.02 cm) of Sephadex G-75 in 0.1 M ammonium acetate.* Flow rate 10 ml × h^{-1}, 2-ml fractions

Table 1. *Reactivity of taipoxin histidine residues toward* p-*bromophenacyl bromide*

Toxin	Residues histidine per molecule			Molar absorption, ε_{271}
	amino acid analysis	difference from amino acid analysis	difference from spectrum[a]	
				$M^{-1}\,cm^{-1}$
Taipoxin	7.0	–	–	60500
α Subunit	1.9	–	–	30500
β Subunit	3.4	–	–	13200
γ Subunit	1.8			10000
Modified taipoxin	5.0	− 2.0	− 2.0	94000
Modified α	1.1	− 0.8	− 1.2	53800
Modified β	2.4	− 1.0	− 1.0	30600
Modified γ	1.6	−(0.2)	−(0.3)	14900
Modified γ (treated alone)	1.8	0	−(1.0)	27200

[a] $\varepsilon_{271} = 17000\ M^{-1}\,cm^{-1}$ [16].

Table 2. *Calcium dissociation constants for taipoxin and its subunits at pH 7.4 as obtained by difference spectroscopy*

Toxin	ε	$K_{Ca^{2+}}$	Ca^{2+} content	λ_{max}
	$M^{-1}\,cm^{-1}$	M	mol^{-1}	nm
Taipoxin	3900	0.7×10^{-4}	2	241
α	1800	0.4×10^{-4}	1	242
β[a]	0	–	0	–
γ	2000	0.5×10^{-4}	1	239

[a] Solubility is 5 μM.

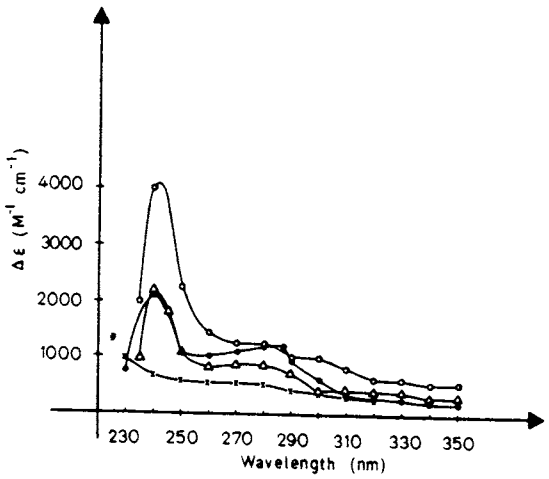


Fig. 3. *Ca^{2+}-dependent adsorption difference spectra of taipoxin and subunits.* (△) α subunit; (×) β subunit; (●) γ subunit; (○) taipoxin

as evidenced by ultraviolet difference spectroscopy. The dissociation constants are close to 0.1 mM, comparable to results for similar phospholipases [18, 26]. The β subunit shows no difference spectrum.

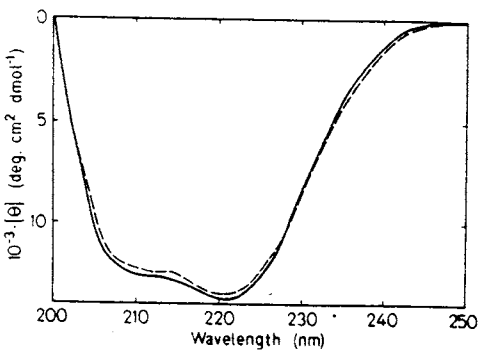


Fig. 4. *Circular dichroism spectra of taipoxin (○——○) and BrPhAc-taipoxin (○----○) in 0.1 M NH_4HCO_3.* The concentrations were 5.7 μM and 8.6 μM, respectively

Circular Dichroism Measurements

CD spectra in the aromatic and in the peptide bond absorption range were run with native and modified taipoxin. In the former case (near ultraviolet) the spectra are shown as difference spectra (Fig. 5). In the figure is also included the absorption spectrum of protein-bound BrPhAc as a difference spectrum between $(BrPhAc)_2$-taipoxin and the virgin protein. In the far ultraviolet the CD spectra for taipoxin and (BrPhAc)-taipoxin are identical (Fig. 4) and within the sensitivity limits of the methods used for calculation of secondary structure no difference could be detected (Table 3). However, the two methods gave different values, especially concerning the content of β structures.

Catalytic Activity

The basic α subunit shows the highest phospholipase activity against egg yolk in the presence of sodium deoxycholate. The acidic γ subunit has a hydrolytic potency comparable to the pork pancreatic prephospholipase and is furthermore not activated by sodium deoxycholate [26, 27]. The neutral β subunit has no activity. The catalytic activity of whole taipoxin is less than the sum of the activities of its constituents, indicating weak inhibition in the complex. The hydrolytic capability of the modified protein was greatly reduced and below the detection limit of the method.

Lethality

As shown previously taipoxin is the most potent presynaptic animal neurotoxin as assayed in mice [14] (Table 4). Other animals might be more or less sensitive [10]. The only lethal subunit (α) is about 500-fold less potent on a molar basis than the complex. $(BrPhAc)_2$-taipoxin is still toxic. This is probably not due to 0.3% remaining native protein (or spontaneous reversion) [18].

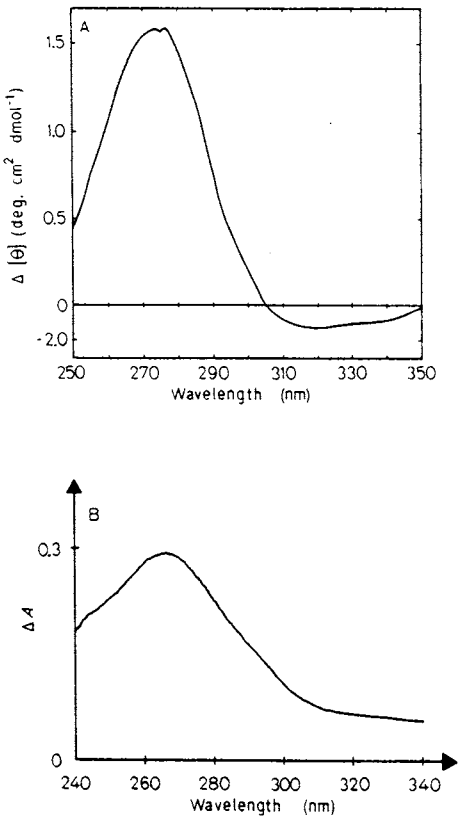


Fig. 5. *Circular-dichroism and ultraviolet-absorption difference spectra of taipoxin and BrPhAc-taipoxin.* (A) Calculated difference circular dichroism spectrum of BrPhAc-taipoxin obtained after subtraction of the CD spectrum of taipoxin alone. The concentrations were 32 μM taipoxin and 21.4 μM BrPhAc-taipoxin in 0.1 M NH_4CO_3. (B) Ultraviolet absorption difference spectrum of BrPhAc-taipoxin and taipoxin is shown to indicate that the change in CD spectrum is most likely due to the modifying groups

ıble 3. *Secondary structure of taipoxin calculated as the percentage of α helix (α), β-pleated sheet (β) and random structure (R)*

Toxin	Structure calculated according to					
	Chen et al.[22]			Greenfield & Fasman [23]		
	α	β	R	α[a]	β[b]	R
	%					
Taipoxin	37	10	53	28	37	35
BrPhAc-taipoxin	36	7	57	27	37	36

[a] Calculated at 208 nm.
[b] Calculated at 222 nm.

Inhibition of Choline Uptake

The effects of the different chains on the high-affinity choline uptake of T sacs are compatible with the lethality data (Table 4). The modified taipoxin has

Table 4. *Biochemical and physiological activities of native and modified taipoxin and its subunits*
ID_{50} = toxin concentration required to block choline uptake by 50% at 1 μM external choline concentration. Phospholipase activity was measured at 18 °C in the presence of 2 mM Ca^{2+} and 2.5 mM sodium deoxycholate. n.d. = not determined

Toxin	ID_{50}	Phospholipase activity	LD_{50}
	μg/ml	μmol min^{-1} mg^{-1}	μg/kg
Taipoxin	0.3	0.4	2
α Subunit	0.3	3.8	300
β Subunit	>10	0	>2000
γ Subunit	>10	0.7[a]	>2000
$(BrPhAc)_2$-taipoxin	0.8	<0.01	700
(β-BrPhAc)-taipoxin	0.2	n.d.	10
(α-BrPhAc)-taipoxin	0.7	n.d.	700

[a] Activity obtained even without sodium deoxycholate; the Q_{10} is close to 2 over the interval 20–60 °C; raising the Ca^{2+} concentration to 6 mM increases the activity 2–3-fold.

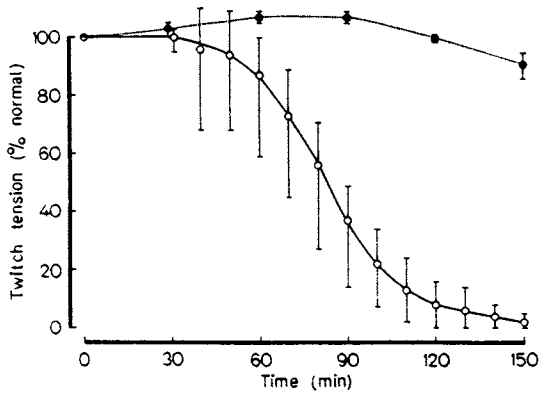
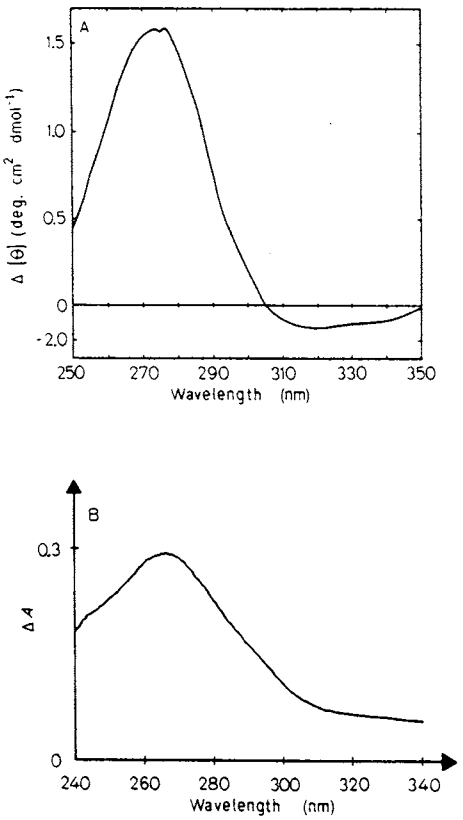


Fig. 6. *Isometric twitch tensions, in response to indirect stimulation at 0.1 Hz, of isolated mouse diaphragm muscles in the presence of native taipoxin, 5 μg/ml (○——○), and modified taipoxin, 20 μg/ml (●·····●).* Each value expresses the mean of 2–5 muscles and the range of tensions. Time after addition of toxin is plotted against twitch tension as proportion of that in untreated muscle

a threefold higher ID_{50} than native taipoxin. If Ca^{2+} is replaced by Sr^{2+} the toxins are totally ineffective [12]. Similar observations have been made with pork pancreatic phospholipase A_2 [28] and β-bungarotoxin [29, 30].

Inhibition of Neuromuscular Transmission

The development of the neuromuscular block produced in nerve-muscle preparations stimulated at 0.1 Hz in the presence of native taipoxin at a concentration of 5 μg/ml is shown in Fig. 6. Modified taipoxin even at 20 μg/ml had practically no effect.



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β Subunit	>10	0	>2000
γ Subunit	>10	0.7[a]	>2000
$(BrPhAc)_2$-taipoxin	0.8	<0.01	700
(β-BrPhAc)-taipoxin	0.2	n.d.	10
(α-BrPhAc)-taipoxin	0.7	n.d.	700

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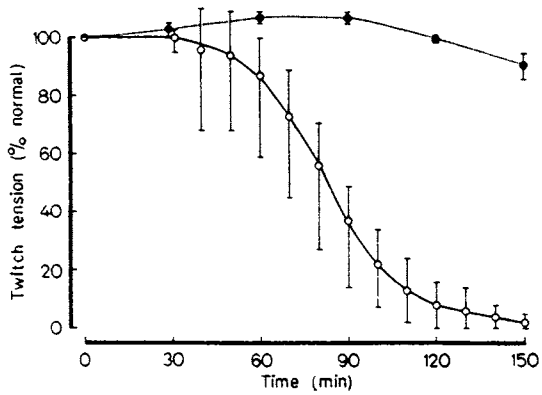

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Alteration in the Electron-Microscopic Appearance of the Neuromuscular Junction

The chemically modified taipoxin had lost much of its potency to interfere with neuromuscular transmission. In previous experiments [11] with the native toxin a dose of 1 μg was sufficient to induce respiratory paralysis within 90 – 120 min. With the modified toxin, a dose of 100 μg was required to obtain respiratory paralysis within 3 h. Electrophysiologically, the number of miniature endplate potentials was reduced. 3 h after injection of 10 μg of the modified toxin the animals showed some muscular weakness, but no respiratory paralysis occurred. A dose of 1 μg of the modified toxin had no effect.

The functional disturbances correlated well with the ultrastructural findings. The morphological effects upon motor axon terminals of native taipoxin have been described [11]. Briefly, the late stage of intoxication, i.e. when the animals had died of respiratory paralysis, was characterized by the following features (Fig. 7a). In most of the motor axon terminals the synaptic vesicles were greatly reduced in number, varied greatly in size, the axoplasm displayed increased electron density and the mitochondria were swollen. In contrast, 3 h after an equal dose (1 μg) of the modified toxin, all inspected endplates ($n = 15$) appeared entirely normal (Fig. 7b) as compared with untreated controls; 3 h after a 10-μg dose of $(BrPhAc)_2$-taipoxin all endplates examined ($n = 22$)/still appeared normal. In a few terminals the number of vesicles appeared slightly reduced. When the animals died 3 h following injection of 100 μg of the modified toxin, the majority of inspected endplates ($n = 40$) closely resembled the picture (Fig. 7c) usually observed with the low dose (1 μg) of the native toxin.

Competition Experiments

If a specific binding is involved in the neurotoxicity one might expect protection by the modified protein, since the latter might bind to the same target without being able to destroy it.

Modified taipoxin corresponding to 350 μg/kg mouse (0.5 LD_{50}) injected intravenously did not protect mice against taipoxin, at doses of 10 – 20 μg/kg (5 – 10 LD_{50}) injected 5 min or 2 h later. This is also corroborated by experiments with nerve-muscle preparations. A preincubation for 30 min with modified taipoxin before using native taipoxin in no way affected the neuromuscular block caused by the native toxin.

Recombination Experiments

Two different recombined taipoxins modified on α and β subunits, respectively, were made by mixing in 0.1 M ammonium acetate and gel filtration as described earlier [14]. Their toxicities in mice and their inhibitory actions on choline transport (Table 4) strongly indicate that the α subunit is responsible for the ultimate neuromuscular blockade.

Antibody Production and Experiments

The modified taipoxin lends itself superbly to antibody production, as presented elsewhere [31]. When the injection is intravenous, the antibodies are only effective in blocking the toxic sequelae if injected before or together with taipoxin. If taipoxin is injected first followed within five minutes by antibodies, even in tenfold excess, no protection is observed.

DISCUSSION

The reasons for dimerization are not quite understood. If the first lyophilization and Sephadex G-25 step are avoided and replaced by dialysis before the second modification, aggregation occurs to a much lesser extent. A dimer could be of value for determining the optimal molecular weight of a presynaptic neurotoxin. The size is limited at the extrems by renal elimination if too small and non-penetration into the synaptic cleft if too big. Possibly other factors contribute as well and one would probably need to have a covalent dimer to perform the experiment. Taipoxin is very probably optimal in this respect since a quaternary structure with three subunits is most unusual.

In the light of the recent elucidation of the primary structure of the γ subunit [15] it is surprising that no histidine modification occurs. It has exactly the same linear sequence around the 'active site' as other

Fig. 7. *Effects of native and of modified taipoxin on the ultrastructure of motor axon terminals.* (a) Neuromuscular junction from the diaphragm of a mouse which had died of respiratory paralysis approximately 100 min following intravenous injection of native taipoxin (1 μg). The axoplasm of the terminal (AX) displays high electron density; the synaptic vesicles are reduced in number and are large as compared with those in controls or in (b). MF, muscle fibre; × 36900. (b) Neuromuscular junction from the diaphragm of a mouse sacrified 3 h following intravenous injection of modified taipoxin (1 μg). The axon terminal (AX) appears entirely unaltered as compared with controls (not shown). MF, muscle fibre; × 39000. (c) Neuromuscular junction from the diaphragm of a mouse which had died of respiratory paralysis 3 h following intravenous injection of modified taipoxin (100 μg). There are close similarities with the terminal shown in (a): increased density of the axoplasm (AX), reduced number and variable sizes of the synaptic vesicles, mitochondrial swelling. MF, muscle fibre: × 39000

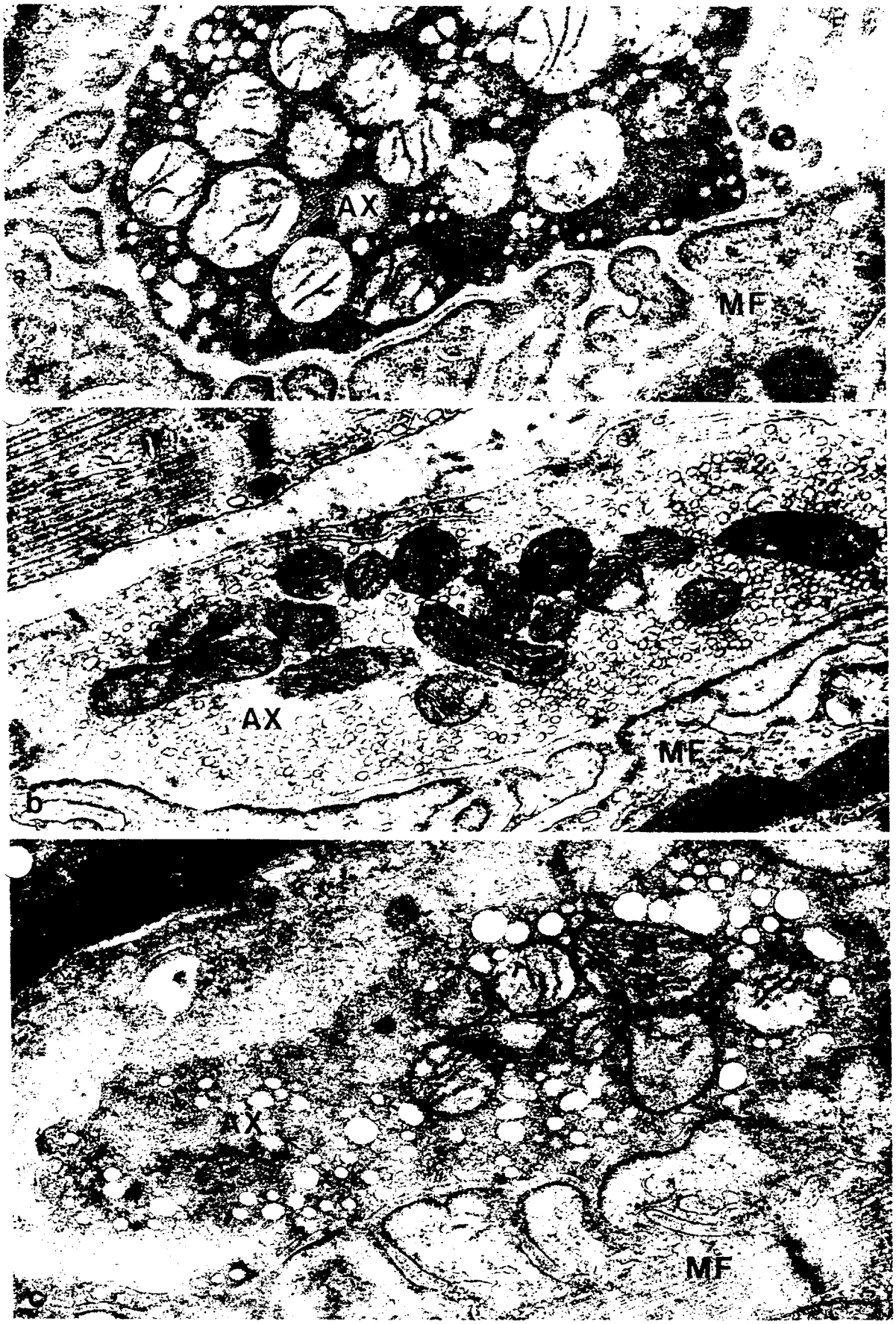

Fig. 7

vertebrate phospholipases. Even though it has the structure and properties of a prophospholipase, it still ought to be modified. The acidic nature, the extra disulfide loop or the carbohydrate moiety might be the reason. By comparison with the three-dimensional structure as reported by Drenth et al. [16] the carbohydrate moiety would in fact be situated at or very close to the catalytic cavity. This very bulky and hydrophilic group could then possibly inhibit membrane or micelle interaction.

The sequence around the active site of the β chain is probably intact since it does react specifically with the alkylating reagent. The apparent inability to bind Ca^{2+} (e.g. the absence of a spectral shift) might explain the lack of catalytic activity, since many other elapid and vertebrate phospholipases have an absolute requirement for Ca^{2+}. Work on the primary structure (in progress) could perhaps reveal some feature of the Ca^{2+}-binding function.

The new Cotton effects obtained in $(BrPhAc)_2$-taipoxin are most probably extrinsic, as the CD difference spectrum agrees well with the absorption spectrum of protein-bound $BrPhAc_2$ (Fig. 5). The changes in the intrinsic Cotton effects of taipoxin itself must be small, which means that the modification has little or no effect on the fine structure of this protein. The polypeptide backbone conformation is not changed as taipoxin and $(BrPhAc)_2$-taipoxin give identical CD spectra in the far-ultraviolet region. However, calculations regarding the amounts of α helix and β structures were not reliable. The content of α helix seems to be approximately 30% according to both methods but the values for the content of β structure were very different. As pointed out earlier [32], this is not surprising considering the variety of standard curves obtained by different methods and because of the many types of conformation designated as β forms.

We have also looked at CD spectra of notexin [18], a similar type of neurotoxic phospholipase consisting of one polypeptide chain. The spectra in the presence and absence of Ca^{2+} are identical, so the effect of Ca^{2+} can not be to induce gross conformational changes of the protein.

Only the α subunit is active at the target as demonstrated with the mono-substituted derivatives. The increased potency of the α chain in the taipoxin complex could be ascribed to pharmacokinetic factors. The increase is not observed in the choline inhibition assay. The larger size of whole taipoxin (as compared to one-chain presynaptic toxins), together with the sialoglycoprotein nature of the γ subunit, might place taipoxin in the class of proteins for which the rate of renal elimination is practically zero. The carbohydrate might thus have at least a twofold function: to increase the hydrodynamic radius and protect against proteolysis. It may also be important in keeping the α chain in an optimized environment perhaps orienting the γ chain away from the membrane in order to allow good cell contact for the α chain.

The antibodies are not effective in preventing neuromuscular blockade unless injected before the toxin. In an actual snake bite this is not so serious since most of the venom is deposited subcutaneously. An intravenous injection of antiserum will be effective in preventing development of paralysis if injected very soon after the bite since anti-taipoxin will then be circulating ('waiting') in the blood vessels to take care of released neurotoxin. The reason(s) for the ineffectiveness of the antibodies in the experimental situation may be one or more of the following: (a) the neurotoxin acts intracellularly; (b) catalysis can not be reversed by antibodies; (c) high affinity-binding; (d) dissociation of the complex after binding leaving no exposed antigenic sites.

The reasons for implicating phospholipase activity in neurotoxicity are as follows. All presynaptic snake venom toxins known so far either are, or contain as an indispensable part, a basic phospholipase. No one has yet found a means to abolish the phospholipase A activity without essentially eliminating the lethality. Substitution of Sr^{2+} for Ca^{2+} inhibits both activities, as does the chemical modification with *p*-bromophenacyl bromide.

Based mainly on studies with β-bungarotoxin, several hypotheses have been advanced regarding the subcellular point of attack of these phospholipase-type toxins. Mitochondria have been proposed as the primary target [33–36] and some authors have reported saturable binding to brain synaptosomal membranes with a dissociation constant of 10^{-9} M [37]. Others have reported effects on sarcoplasmatic reticulum [38], on the storage of non-specific transmittors in brain [39] and on choline uptake by brain synaptosomes but independant of phospholipase activity [13]. However, since these toxins exert their fatal action at peripheral vertebrate motor nerve terminals, the relevance of the above findings to the neurotoxic mechanism are questionable. By contrast the nerve terminals innervating the electric organ of *Torpedo* are analogous to those innervating skeletal muscles and can therefore be regarded as a more physiological target. The only known biochemical effect observed is associated with inhibition of high-affinity choline uptake [12].

Catalysis at the axolemma itself would thus be very straightforward with no penetration problems and fully in accordance with the preference of phospholipases A for organized lipid [27]. The following model for the neurotoxicity of taipoxin is suggested (Fig. 8).

a) A high proportion of the taipoxin molecules reach their target site due to the 'chaperone' effect of γ and β and with the α in an optimal orientation.

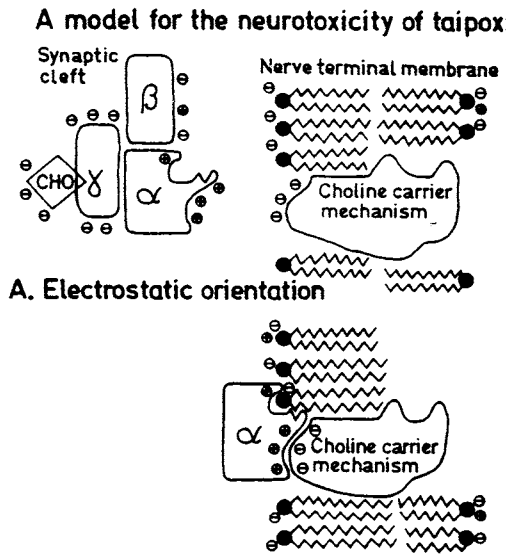

Fig. 8. *Tentative model for the toxicity of taipoxin.* The involvement of positive charges is suggested by the fact that all presynaptic snake toxins are basic

b) Subunit α binds to the target site (parts of the choline carrier?) and this changes the activities of membrane-dependant processes (e.g. instantaneous blockade of choline transport [12] and enhancement of neurotransmitter release [10]). The binding may be enhanced since there are two affinities, one for the lipid (enzyme-substrate interaction) and one hypothetically specific.

c) Limited phospholipid hydrolysis occurs leading to permanent disruption (neurotoxicity?). At this stage the toxin may be released, since substrate affinity is diminished.

This model is supported by the fact that all homologous phospholipases are not neurotoxins. Furthermore, there is no simple correlation between neurotoxicity and phospholipase activity [12] (Table 3). The specificity for presynaptic elements accounts for the fact that the inhibitory activity of modified taipoxin in the assay *in vitro* is only slightly less than that of native taipoxin. An extracellular site of attack was also suggested by Howard and Wu [40], who used fully active immobilized β-bungarotoxin.

Finally, we may suggest here that the modification reaction used in this study could be a diagnostic instrument to ascertain whether a toxin is of the phospholipase type.

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