Met-8 of the β_1 -bungarotoxin phospholipase A_2 subunit is essential for the phospholipase A_2 -independent neurotoxic effect

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β_1 -Bungarotoxin consists of a phospholipase A_2 subunit and a non-phospholipase A_2 subunit. The toxin was oxidized with a 100-fold molar excess of chloramine T with respect to the methionine content of the protein in 0.1 M Tris/HCl at pH 8.5 and at room temperature. Reactivities of the two methionine (Met-6 and Met-8 of the phospholipase A_2 subunit), five histidine, 14 tyrosine and one tryptophan residues of one toxin molecule with chloramine T were assessed from the change in intrinsic fluorescence and amino acid composition of the protein. Met-8 and one tyrosine on the phospholipase A_2 subunit and less than one histidine were oxidized, while Met-6 remained intact after 30 min of reaction. One histidine and approx. two tyrosine residues were oxidized when both methionine residues were

oxidized after 90 min of reaction. The sole tryptophan was oxidized slightly throughout the reaction. The chloramine T oxidation did not destroy the two Ca^{2+} -binding domains, though it modified the toxin to become less effective at binding Ca^{2+} . The modified toxin obtained after 30 or 90 min reaction time retained 65% or 40% of the phospholipase A_2 activity of the parent toxin, but both were not lethal to mice and showed a very weak ability to induce the indirectly evoked contraction of chick biventer cervicis muscle. It is suggested that Met-8 may play an important role in the phospholipase A_2 -independent interaction with the nerve terminal membrane during the neurotoxic effect of β_1 -bungarotoxin.

INTRODUCTION

The presynaptically active toxins in snake venoms exhibit weak phospholipase A_2 (PLA $_2$) activity but are very potent in blocking neuromuscular transmission (Chang, 1985). β_1 -Bungarotoxin (β_1 -BuTX), the main component of β -bungarotoxins in the venom of Taiwan banded krait (*Bungarus multicinctus*) (Chen et al., 1982), is a representative of this kind of toxin. The neurotoxic effect of β_1 -BuTX comprised a triphasic effect on cholinergic synapses (Caratsch et al., 1981). The early biphasic effects are associated with a change in the nerve-terminal membrane as a result of a PLA $_2$ -independent interaction between the toxin and its receptor. Next, there is an effect involving the hydrolysis of phospholipid in the membrane surrounding the bound toxins. Apparently, the ability of toxins to bind to their receptors plays a crucial role in evoking the serial actions which first induce facilitation and irreversible disruption of acetylcholine release from cholinergic synapses before disruption of neuromuscular transmission. Since the action is very specific and unique, β_1 -BuTX has been exploited as an important tool in physiological, biochemical and pharmacological studies.

β_1 -BuTX is composed of two subunits linked by a disulphide bond (Kondo et al., 1978). One is a PLA $_2$ subunit having 120 amino acid residues, which shows remarkable structural similarity to other vertebrate PLA $_2$ enzymes (Kini and Evans, 1987). The other is a rather small subunit with 60 amino acid residues. This subunit shows sequence similarity to dendrotoxin and toxins I and K from mamba venoms, which are representative of Kunitz protease inhibitors (Dufton, 1985) and themselves show facilitation of transmitter release, interference with the binding of β_1 -BuTX in chick muscle, and enhancement of the presynaptic effect of crotoxin and notexin (Harvey and Karlsson, 1982).

The β_1 -BuTX molecule should possess active sites for both

receptor binding and PLA $_2$ activity in order to fulfil its neurotoxic effect. Finding the amino acid residues responsible for the activities mentioned above on the β_1 -BuTX molecule is important in order to elucidate its structure and function. The present work was carried out to establish the role of the chloramine T-reactive amino acid residue on β_1 -BuTX for this purpose.

EXPERIMENTAL

Materials

Crude venom from the Taiwan banded krait was supplied by Chen Hsin Tong Chemical Co., Taipei, Taiwan. CM-Sephadex C-25, Sephadex G-50 and column PD-10 Sephadex G-25 were obtained from Pharmacia, Uppsala, Sweden. All other chemicals were of reagent grade.

Chemical modification of β_1 -BuTX

β_1 -BuTX was isolated from venom on a CM-Sephadex C-25 column (Chen et al., 1982) and purified further on a Sephadex G-50 column (Lin et al., 1984). It was cleaved with CNBr according to our previously described method (Chu and Chen, 1991). Finally, the reaction mixture was dialysed against water to remove reagent and small peptide fragments.

β_1 -BuTX was oxidized with chloramine T by a modified method of Shechter et al. (1975). In 0.5 ml of 0.1 mM Tris/HCl at pH 8.5, 1 mg of toxin was reacted with a 100-fold molar excess of chloramine T with respect to the methionine content of the protein. The chloramine T-modified toxins obtained from desalting the reaction mixture on a PD-10 column (1.5 cm $\times$ 5.0 cm) in 0.07 M ammonium bicarbonate buffer at pH 8.0 are named CMT. The gel-filtration chromatography was completed within 5–10 min. The reaction times, given in minutes, are denoted by

Abbreviations used: β_1 -BuTX, β_1 -bungarotoxin; CMT, chloramine T-modified β_1 -bungarotoxins; PLA $_2$, phospholipase A_2 ; PVDF, poly(vinylidene difluoride).

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subscripts: CMT₀ are the control samples and CMT₃₀ and CMT₉₀ represent the modified toxins from 30 and 90 min of reaction respectively. The histidine, methionine or tyrosine content of the proteins was determined from direct analysis of amino acid composition (Spackman et al., 1958). Subtraction of the amino acid content of the modified toxin from that of the parent toxin gave the amount of amino acid that was oxidized by chloramine T.

The two subunits of both β_1 -BuTX and CMT₃₀ were resolved by SDS/PAGE on a 15% (w/v) polyacrylamide slab gel (7 cm × 9 cm) in Tricine buffer at pH 8.9 according to the method of Schägger and von Jagow (1987). The electrophoresis was conducted at 100 V for 2 h. The polypeptides on the gel were transferred to poly(vinylidene difluoride) (PVDF) membrane (Millipore) by a diffusion method (Bowen et al., 1980). Each polypeptide on the filter was hydrolysed in the vapour phase of 7.0 M HCl containing 10% (v/v) trifluoroacetic acid and 0.1% phenol at 158°C for 45 min, according to the method of Tsugita and Scheffler (1982) (C. S. Liu, personal communication) and its amino acid composition determined.

Transformation of methionine to methionine sulphoxide was assayed according to the method of Shechter et al. (1975). Briefly, protein was cleaved with CNBr according to the method of Gross and Withop (1962). The reaction mixture was subjected to gel filtration on a PD-10 column to remove the reagent and the small peptide fragment. The CNBr-cleaved protein was reduced with dithiothreitol before the determination of its methionine content, which represented the CNBr-resistant methionine that had been oxidized to methionine sulphoxide.

Assay of PLA₂ activity and toxicity

β_1 -BuTX released the fatty acid from 10 μ mol of egg phosphatidylcholine suspended in 4 ml of 5 mM deoxycholate at 37 °C. The amount of fatty acid was measured by H⁺ titration, using a Radiometer pHM8 standard pH meter attached to a T80 titrator and an ABU80 autoburette. One unit of PLA₂ activity was defined as the amount of enzyme needed for the release of 1 μ equiv. of fatty acid from phospholipid/min.

Indirectly evoked contraction of the neuromuscular preparation

The cervicis-nerve-muscle preparation was isolated from baby chicken biventer. The organ bath contained 10 ml of Tyrode's solution: NaCl, 137 mM; KCl, 2.7 mM; CaCl₂, 2.8 mM; MgCl₂, 1.0 mM; NaH₂PO₄, 0.36 mM; NaHCO₃, 12.0 mM; glucose, 11.0 mM. The twitch response of biventer cervicis muscle at 37 °C was evoked by a supramaximal indirect stimulation of 0.2 Hz and the force displacement was measured isometrically with a driver amplifier (Grass polygraph D.C. model RPS 7C8).

Measurement of fluorescence

The concentration of β_1 -BuTX was determined from absorbance at 280 nm, by using a value for $A_{1\text{ cm}}^{1\%}$ of 12.5 (Lin et al., 1984). The fluorescence intensity, expressed in arbitrary units, was measured at room temperature with a Hitachi F-4000 fluorescence spectrophotometer. Both the excitation and the emission slit-widths were 10 nm. Raman emission due to the scattering of solvent was minimized by adjusting the intensity scale. It took no more than 5 min to scan a spectrum, avoiding protein denaturation. $F_{\lambda_1}^{\lambda_2}$ represents the fluorescence intensity at wavelength λ_2 (nm) when the fluorophore was excited at wavelength λ_1 (nm).

Analysis of fluorescence data

The modified Scatchard plot (Epstein et al., 1974) was constructed to analyse the fluorescence data of a complex formed between Ca²⁺ and β_1 -BuTX or the modified derivative:

$$|\Delta F|/[Ca^{2+}]_{\text{free}} = K_a \cdot F_{\infty} - K_a \cdot \Delta F \quad (1)$$

where ΔF is the change in protein fluorescence on adding Ca²⁺ and F_{∞} the protein fluorescence in the absence of Ca²⁺. K_a is the association constant of the complex. $|\Delta F|/[Ca^{2+}]_{\text{total}}$ was plotted against $|\Delta F|$, since $[Ca^{2+}]_{\text{free}}$ was close to $[Ca^{2+}]_{\text{total}}$.

RESULTS

Chloramine T oxidation of β_1 -BuTX

Chloramine T is able to oxidize methionine, cysteine, histidine, tyrosine and tryptophan residues of proteins; however, the oxidation of the aromatic amino acids can be suppressed at alkaline pH values (Shechter et al., 1975). β_1 -BuTX contains five histidines, two methionines (Met-6 and Met-8 of the PLA₂ subunit), 14 tyrosines and one tryptophan residue (Trp-19 of PLA₂ subunit) (Kondo et al., 1978). We searched for suitable experimental conditions for the chloramine T oxidation of β_1 -BuTX which would oxidize the methionine residues but suppress the reactivity of the aromatic residues. Figure 1 displays the emission spectra of β_1 -BuTX and the chloramine T-modified toxins (see the method for their preparation in the Experimental section) in 0.02 M Pipes buffer at pH 7.6. When excited at 275 nm, CMT₉₀ shared with β_1 -BuTX a nearly identical shoulder in the range of 300–310 nm and a 339 nm peak of similar shape but lower fluorescence intensity (Figure 1a). The shoulder results from tyrosine residues that do not transfer their resonance energy to Trp-19 and the main peak arises from Trp-19, which is excited directly with absorption energy or indirectly through resonance-energy transfer from the proximal tyrosine residues (Chu and Chen, 1991). Both proteins gave a single peak at 339 nm when they were excited at 295 nm, although β_1 -BuTX showed a stronger intensity than CMT₉₀ (Figure 1b). We recovered the modified toxins from the reaction mixture of chloramine T and β_1 -BuTX by gel chromatography on a PD-10 column with an alkaline solution instead of the acidic solution which was used previously (Chang and Yang, 1988). This was critical, as we found that the main fluorescence peak disappeared when CMT₃₀ was recovered from an acidic solution (Figure 1).

Figure 2 depicts the retention of β_1 -BuTX fluorescence during the chloramine T oxidation. The ratio of F_{275}^{305} , F_{275}^{339} or F_{295}^{339} of the modified toxin to that of the parent toxin would reflect the extent of oxidation of the fluorescence-associated amino acid. The smaller the ratio, the greater the extent of oxidation. F_{275}^{305} remained constant even after the oxidation had proceeded for 120 min. Both F_{275}^{339} and F_{295}^{339} decreased only very slightly before 30 min of reaction but decreased thereafter. The reduction of F_{295}^{339} was more prominent than that of F_{275}^{339} . For instance, CMT₉₀ remained around 85% of F_{275}^{339} and 76% of F_{295}^{339} of the parent toxin. These fluorescence characteristics suggested that: (a) tyrosine residues distant from Trp-19 remained intact during the chloramine T oxidation; (b) Trp-19 and its proximal tyrosine residues reacted only very slightly with chloramine T in the reaction before 30 min; however, they might be partially oxidized with longer reaction.

Figure 3 displays the reactivity of the histidine, methionine and tyrosine residues on β_1 -BuTX with chloramine T. These three kinds of amino acids remained almost intact in CMT₀. Oxidation of both tyrosine and histidine was not extensive under the experimental conditions suitable for methionine oxidation.

Figure 1 Fluorescence emission spectra of β_1 -BuTX and the modified toxin in 0.02 M Pipes buffer at pH 7.6

The emission spectra were scanned with excitation wavelengths at 275 nm (a) and 295 nm (b). All proteins were present at 5.0 μ M: β_1 -BuTX (—); CMT₉₀ (---); the modified toxin prepared from the chloramine T oxidation of β_1 -BuTX after reaction for 30 min and recovered by PD-10 chromatography in 0.1 M acetic acid at pH 3.0 (-.-).

Figure 2 Retention of β_1 -BuTX fluorescence during the chloramine T oxidation

The fluorescence retention is represented by the ratio of the fluorescence of the modified toxin to that of β_1 -BuTX. The estimation was based on the change in F_{275}^{305} ($\blacktriangle$), F_{275}^{339} ($\blacksquare$) or F_{295}^{339} ($\bullet$).

Approx. one methionine, one tyrosine and 0.7 histidine residues were oxidized on one CMT₃₀ molecule, while, on one CMT₉₀ molecule, approx. two methionine, two tyrosine and one histidine were oxidized.

The two subunits of both β_1 -BuTX and CMT₃₀ were resolved

Figure 3 The chloramine T-reactive amino acid residues on β_1 -BuTX

The chloramine T oxidation of histidine ($\blacksquare$), methionine ($\blacktriangle$) or tyrosine ($\bullet$) residue(s) on one β_1 -BuTX molecule was determined as described in the text.

further by SDS/PAGE (Figure 4). After the polypeptides were transferred to PVDF membrane and hydrolysed, the tyrosine or histidine content of each polypeptide was determined (see the Experimental section). We determined contents of approx. 8.7 tyrosine and 2.6 histidine residues for the PLA₂ subunit of CMT₃₀, and approx. 3.9 tyrosine and 1.7 histidine for the non-PLA₂ subunit of CMT₃₀. In comparison with β_1 -BuTX, which contains ten tyrosine and three histidine residues in its PLA₂

Figure 4 Separation of the two subunits of β_1 -BuTX and CMT₃₀

A sample (8 μ g) of protein was reduced by mercaptoethanol and the reaction mixture was subjected to SDS/PAGE on a polyacrylamide gel slab (7 cm $\times$ 9 cm). Lane A, protein standards: phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soy-bean trypsin inhibitor (21 kDa) and α -lactalbumin (14 kDa); lane B, β_1 -BuTX; lane C, CMT₃₀. The PLA₂ and the non-PLA₂ subunits are denoted.

Figure 5 Modified Scatchard plot for the binding of Ca^{2+} to either β_1 -BuTX or the modified toxins

At pH 7.6 and room temperature, β_1 -BuTX (a), CMT₀ (b), CMT₃₀ (c), and CMT₉₀ (d), each present at 5 μ M, were titrated with the same protein solution in the presence of 0.1 M CaCl_2 . The fluorescence data were analysed using eqn. (1) with linear regression fitting. The correlation coefficient was calculated to be more than 0.95 for each curve; ●, estimation from F_{275}^{305} ; ○, estimation from F_{295}^{339} .

subunit and four tyrosine and two histidine residues in its non-PLA₂ subunit (Kondo et al., 1978), one tyrosine on the PLA₂ subunit was apparently oxidized while all of the tyrosine residues of the non-PLA₂ subunit remained intact in the CMT₃₀ molecule.

Table 1 Functional characteristics of β_1 -BuTX and the modified toxins

The enzymic PLA₂ activity of the native toxin was set at 100%. K_{a1} and K_{a2} are the association constants for the low-affinity and the high-affinity site respectively.

Toxin	LD ₅₀ (μ g/g body wt.)	PLA ₂ activity (%)	$10^{-3} \times K_i$ (M^{-1})*	
			K_{a1}	K_{a2}
Native	0.017	100	0.58 ± 0.03	2.40 ± 0.21
CMT ₀	0.023	95	0.37 ± 0.05	1.96 ± 0.70
CMT ₃₀	> 2.0	65	0.19 ± 0.04	1.68 ± 0.37
CMT ₉₀	> 2.0	40	0.11 ± 0.06	1.32 ± 0.32
CNBr-modified β_1 -BuTX†	> 2.0	0	—	0.56 ± 0.06

* The correlation coefficients were calculated to be larger than 0.95 by linear regression fitting.

† Data from our previous study (Chu and Chen, 1991).

However, we were unable to locate unequivocally the chloramine T-oxidized histidine on CMT₃₀ from the histidine content data.

With regard to which one of the two methionine residues was oxidized at 30 min, one possibility was that Met-6 remained intact and Met-8 was transformed to a sulfoxide derivative, or vice versa. Modification of CMT₃₀ with CNBr would delete from the protein molecule a hexapeptide from the N-terminus, up to Met-6 in the former situation, or an octapeptide from the N-terminus, up to Met-8 in the latter situation, considering that methionine reacts with CNBr while methionine sulfoxide is resistant. Since we determined a content of one methionine sulfoxide per molecule of the CNBr-cleaved CMT₃₀ (see the Experimental section), the former situation was most likely to occur.

Functional characterization of CMT

Using our previous method, which was able to identify the two distinct Ca^{2+} -binding sites on β_1 -BuTX molecule (Chu and Chen, 1991), we analysed the fluorescence data obtained when Ca^{2+} was added to β_1 -BuTX, CMT₀, CMT₃₀, or CMT₉₀ in 0.02 M Pipes at pH 7.6 according to eqn. (1). The modified Scatchard plot for the effect of Ca^{2+} on F_{295}^{339} showed the characteristics of the high-affinity site and that for the effect of Ca^{2+} on F_{275}^{305} the low-affinity site. Each protein gave a linear curve for both the high-affinity site and the low-affinity site (Figure 5). The association constant determined for either site on each protein had the same order of magnitude, despite the fact that the order of the binding strengths was $\text{CMT}_{90} < \text{CMT}_{30} < \text{CMT}_0 < \beta_1$ -BuTX (Table 1). Apparently, the chloramine T oxidation modified β_1 -BuTX so that it became less effective at Ca^{2+} binding, but oxidation did not disintegrate the two Ca^{2+} -binding sites, even when the reaction had proceeded for 90 min.

PLA₂ activity and LD₅₀ values of five proteins were compared (Table 1). β_1 -BuTX showed weak PLA₂ activity but very potent toxicity. Its enzymic activity in 10 mM CaCl_2 at 37 °C was 115 units/mg of protein and its LD₅₀ was assayed to be 0.017 μ g/g body weight of mice. CMT₀ was as potent for both activities as β_1 -BuTX. Both CMT₃₀ and CMT₉₀ were not lethal, even when a dose as high as 2.0 μ g/g body weight of mice was tested. However, they showed PLA₂ activity, although they were less active than β_1 -BuTX. CMT₃₀ and CMT₉₀ maintained approx. 65% and 40% of the enzymic activity of β_1 -BuTX respectively.

Figure 6 displays the twitch response of baby chicken biventer cervicis nerve-muscle preparations in Tyrode's solution to each protein at a concentration of 0.045 μ M. β_1 -BuTX and CMT₀

Figure 6 The indirectly evoked contraction of chick biventer cervicis muscle by β_1 -BuTX and the modified toxins

Twitches were elicited by indirect stimulation of chick cervicis nerve-muscle preparation in Tyrode's solution at 0.2 Hz. The concentration of each protein solution was $0.045 \mu\text{M}$: β_1 -BuTX (a); CMT₀ (b); CMT₃₀ (c); CMT₉₀ (d); and CNBr-modified β_1 -BuTX (e). Addition of proteins to the organ bath is denoted by the arrow.

were potent, although CMT₀ showed slightly less ability than β_1 -BuTX to block the twitch response. In a similar manner to that observed in previous work (Chang, 1985), both proteins exhibited early biphasic presynaptic effects including an immediate depression and a restoration period of neuromuscular transmission over the first 5.0 min of action before a later phase of neuromuscular blockage (Figures 6a and 6b). β_1 -BuTX took 24 min to inhibit completely the indirectly evoked contraction of biventer cervicis muscle, while CMT₀ took 30 min. The early biphasic effects were not evident in the action of either CMT₃₀ or CMT₉₀ and they showed very little ability to evoke late-phase neuromuscular blockage. They both took more than 60 min to block the neuromuscular transmission completely (Figures 6c and 6d). The time only shortened slightly to 56 min even when CMT₃₀ or CMT₉₀ concentrations were increased to $0.45 \mu\text{M}$ in the organ bath (results not shown). Moreover, CNBr-modified β_1 -BuTX showed no neuromuscular blocking activity over the time period tested (Figure 6e).

DISCUSSION

Vertebrate PLA₂ enzymes share a very similar tertiary structure (Keith et al., 1981; Dijkstra et al., 1983; White et al., 1990). In the β_1 -BuTX molecule the rather hydrophobic NLINFMEMIRYT segment of the N-terminal region of the PLA₂ subunit forms a helix whose steric arrangement in the protein molecule is dominated by the confinement of Asn-1 that is stabilized by Asn-4, Gln-67 and a hydrogen-bonded network involving His-48, Try-52, Pro-63, Try-68 and Asp-94 (Chu and Chen, 1991). Met-6 and Met-8 are in the same helical turn, although the environment around Met-6 differs from that of Met-8; note that the side chains of Leu-2, Met-6 and Arg-10 are

on one side and those of Asn-4, Met-8 and Thr-12 are on the opposite side of the helical core. We found that selective modification of the two methionine residues could be achieved by controlling the reaction time for the chloramine T oxidation of β_1 -BuTX in alkaline solution under which conditions oxidation of the aromatic residues was limited but could not be suppressed completely (Figure 3).

The c.d. characteristics of β_1 -BuTX suggest a stable tertiary structure of the protein molecule in a segmental sense, and 80 % of the helical structure of β_1 -BuTX remains in CNBr-modified β_1 -BuTX, in which a helical segment of octapeptide is deleted from the N-terminal region of the PLA₂ subunit (Chu and Chen, 1991). In line with the evidence that the chloramine T oxidation of β_1 -BuTX did not disintegrate its two Ca²⁺-binding domains (Table 1), CMT₃₀ may remain in the gross conformation of β_1 -BuTX. We confirmed that the chloramine T-reactive amino acid residues, including mainly Met-8 and one tyrosine, are on the PLA₂ subunit of CMT₃₀, suggesting that its non-PLA₂ subunit may remain intact. Together with the functional characteristics of CMT₃₀, this suggests that these two residues on the PLA₂ subunit have an essential role in the PLA₂-independent neurotoxic effect of β_1 -BuTX. However, one should not neglect the importance of the non-PLA₂ subunit, which may interact with the PLA₂ subunit in the maintenance of the active conformation needed for the neurotoxic effect (Lin et al., 1984; Chu and Chen, 1991) or/and may itself cause a presynaptic effect as suggested previously (Harvey and Karlsson, 1982).

Met-6 was not oxidized, but Met-8 was transformed to a sulphoxide derivative in the CMT₃₀ molecule. According to the results of a spectrophotometric titration for the tyrosine residues of β_1 -BuTX (Yang and Lee, 1986), there are only three 'exposed' residues which can be titrated at pH values lower than 10.5. Most of the tyrosine residues are 'buried' in or confined on the protein molecule since they are titratable only at pH values higher than 11.0. Chemical modification of β_1 -BuTX with tyrosine-specific reagents, which should most likely occur at the exposed residues, may not lead to the inactivation of β_1 -BuTX as suggested in previous studies: (a) iodination of β_1 -BuTX with Na¹²⁵I, by either the lactoperoxidase or the chloramine T method, shows no significant effect on the toxicity and the binding affinity of β_1 -BuTX (Rehm and Betz, 1982; Tzeng et al., 1989); (b) selective sulphonation of Tyr-68 ($pK_a = 10.1$) of the PLA₂ subunit by *p*-nitrobenzenesulphonyl fluoride makes the modified toxin more potent in both its enzymic activity and the induction of indirectly evoked contraction of the phrenic nerve diaphragm preparation, though it results in a partial decrease in lethal potency when compared with the activities of the parent toxin (Rosenberg et al., 1989). These findings, together with the present results, imply that oxidation of Met-8 may be the primary cause of the loss of neurotoxic effect by CMT₃₀.

There are two binding sites for calcium ions on different domains of the PLA₂ subunit of β_1 -BuTX (Chu and Chen, 1989, 1991). The high-affinity-site domain is associated with the catalytic site for phospholipid hydrolysis. The low-affinity-site domain is involved, in the presence of Ca²⁺, in the interaction process with micellar lipid/water. This is the first step in the catalysis of PLA₂ and the N-terminal region is believed to be part of the so-called interface recognition site for this process. Removal of the N-terminal octapeptide of the PLA₂ subunit disintegrates the Ca²⁺-binding domain of the low-affinity site but does not disrupt that of the high-affinity site (Table 1). CNBr-modified β_1 -BuTX is not lethal and shows neither the enzymic activity nor the ability to elicit the indirectly evoked contraction of a neuromuscular preparation (Table 1 and Figure 6e). It raises the question of what role the N-terminal region plays in the

neurotoxic effect of β_1 -BuTX. The functional characteristics of CMP_{30} indicate that Met-8 may play an important role in an active site other than the two Ca^{2+} -binding domains and is required for a PLA_2 -independent interaction with the nerve terminal membrane, which occurs before the hydrolysis of the membrane phospholipid during the neurotoxic effect of β_1 -BuTX (Caratsch et al., 1981). The chloramine T oxidation of Met-8 may effect a reduction of hydrophobicity or dipole moment of the N-terminal region, in addition to a conformational change that results in a partial decrease of the PLA_2 activity of β_1 -BuTX. Both of these physical properties are thought to make an important contribution to the neurotoxicity of the PLA_2 neurotoxin family (Tsai et al., 1987). It is unclear whether Met-8 binds directly to the β_1 -BuTX receptor, which is suspected to be a 65 kDa K^+ channel (Scott et al., 1990), or to the phospholipid surrounding the protein receptor in order to evoke the acetylcholine release from cholinergic synapses.

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