The non-phospholipase A₂ subunit of β -bungarotoxin plays an important role in the phospholipase A₂-independent neurotoxic effect: characterization of three isotoxins with a common phospholipase A₂ subunit

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Three isotoxins (SP I–III) of the β -bungarotoxin family were purified to homogeneity via a series of isolation procedures including a final step of h.p.l.c. on an SP column washed with a linear gradient of 0.2–0.6 M sodium acetate at pH 7.4. Their proportions varied greatly with the batch of venom. Each isotoxin was demonstrated by SDS/PAGE to contain a phospholipase A₂ subunit and a non-phospholipase A₂ subunit. The three proteins were reductively alkylated with 4-vinylpyridine and the alkylated derivatives of the two subunits of each isotoxin were separated. N-Terminal sequence analysis of the alkylated derivatives revealed that the three isotoxins probably share a common phospholipase A₂ subunit but differ in their non-phospholipase A₂ subunits. The non-phospholipase A₂ subunits of SP II and SP III were identical with those of β_2 - and β_1 -toxin respectively, except that there was an additional valine inserted between

Thr-18 and Val-19 in β_2 -toxin and Pro-18 and Val-19 in β_1 -toxin. The non-phospholipase A₂ subunit of SP I differed greatly from that of SP III but was almost identical with that of SP II, except that Lys-14 and Ala-29 in SP II were replaced by Arg-14 and Glu-29 in SP I. Analysis of the effect of CaCl₂ on protein fluorescence showed the existence of a low- and a high-affinity site on the different domains of each isotoxin for Ca²⁺ binding. The three isotoxins showed no great difference in their ability to bind Ca²⁺ on both the high- and low-affinity site. They had slightly different phospholipase A₂ activities but differed to a great extent with respect to their neurotoxic effects. LD₅₀ values increased in the order SP I > SP II > SP III. In contrast, the ability to inhibit the indirectly evoked contraction of chick biventer cervicis muscle was in the order SP III > SP II > SP I.

INTRODUCTION

The β -bungarotoxin (β -Butx) family consists of a group of isotoxins which constitute the main portion of Taiwan banded krait (*Bungarus multicinctus*) venom, and β_1 -Butx is defined as the most toxic. The toxin is a phospholipase A₂ (PLA₂) neurotoxin that acts presynaptically blocking neuromuscular transmission (Chang, 1985). The presynaptic action is associated with an early effect which causes a change in the nerve-terminal membrane of cholinergic synapses as a result of a PLA₂-independent interaction between the toxin and its receptor (Caratsch et al., 1981). Next, there is an effect involving the hydrolysis of phospholipid in the membrane surrounding the bound toxins. As the toxin is specific and unique in evoking the serial actions that initiate facilitation and irreversible disruption of acetylcholine release from cholinergic synapses before blockade of neuromuscular transmission, it has been exploited as an important tool in physiological, biochemical and pharmacological studies.

The isotoxins of the β -Butx family have similar primary structures. Each is composed of two subunits linked by a disulphide bond (Kondo et al., 1978, 1982a,b). One is a PLA₂ subunit having 120 amino acid residues, which shows remarkable structural similarity to other vertebrate PLA₂ enzymes (Kini and Evans, 1987). The other is a rather small subunit of 60 amino acid residues, showing sequence similarity to dendrotoxin and toxins I and K from mamba venom, which are representative of Kunitz protease inhibitors (Dufton, 1985) and themselves are able to facilitate the transmitter release. Each isotoxin molecule

possesses active sites for both receptor binding and PLA₂ activity in order to fulfil its neurotoxic effect. The present work reports the purification of three well-defined β -Butxs with identical PLA₂ subunits but different non-PLA₂ subunits. On the basis of their functional characteristics, we have elucidated the protein structure of the non-PLA₂ subunit responsible for the PLA₂-independent neurotoxic effect.

EXPERIMENTAL

Materials

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

Protein purification and analysis

Crude venom was subjected to chromatography on a CM-Sephadex C-25 column (Chen et al., 1982). The β_1 -Butx fraction was passed through a Sephadex G-50 column (Lin et al., 1984), and was further resolved by h.p.l.c. on a Protein PAK SP 5PW column (Waters Associates, Milford, MA, U.S.A.) pre-equilibrated with 20 mM phosphate buffer at pH 6.5 or 7.4. The column was washed with a linear gradient of 0–0.8 M NaCl, 0–0.8 M sodium acetate or 0.2–0.6 M sodium acetate in the buffer.

The proteins were incubated in 6 M guanidinium chloride/50 mM Tris/HCl buffer at pH 8.6 in the presence of 20 mM

Abbreviations used: β -Butx, β -bungarotoxin; PLA₂, phospholipase A₂.

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2-mercaptoethanol at 60 °C for 1 h. The reduced samples were alkylated in 20 mM 4-vinylpyridine in the dark at 25 °C for 3 h (Flannery et al., 1989). The alkylated derivatives were resolved by h.p.l.c. on a Chemcosorb-ODSH C18 column washed with a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid.

Amino acid sequences were determined by automated Edman degradation with a gas-phase microsequencer [477A protein sequencer with on-line 120A analyser (Applied Biosystems, Foster City, CA, U.S.A.)].

Protein size was determined by SDS/PAGE on a 15% (w/v) gel slab (7 cm × 5 cm) in Tris/glycine buffer (Laemmli, 1970). The electrophoresis was conducted at 60 V for 30 min and then at 120 V for 1 h. Protein bands were visualized by staining with Coomassie Blue.

Assay of PLA₂ activity and toxicity

A mixture of 0.5 µg of β -Butx and 8 µmol of 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine (Sigma) suspended in 4 ml of 1.85 mM sodium deoxycholate was incubated at 37 °C. Fatty acid was measured by H⁺ titration at pH 8.0, using a Radiometer pHM8 standard pH-meter attached to a TTT80 titrator and an ABU80 autoburette. One unit of PLA₂ activity was defined as the amount of enzyme needed for the release of 1 µequiv. of fatty acid from phospholipid/min.

Albino mice weighing 20–25 g were injected intraperitoneally with toxin. LD₅₀ was determined by the method of Litchfield and Wilcoxon (1949).

Indirectly evoked contraction of the neuromuscular preparation

The biventer cervicis nerve muscle preparation was isolated from baby chicken (3–6 days). The organ bath contained 10 ml of Krebs's solution (126 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 23.8 mM NaHCO₃ and 11.2 mM glucose). The isolated preparation was maintained in the organ bath in the presence of 95% O₂/5% CO₂ at 37 °C. The twitch response of biventer cervicis muscle was indirectly evoked by a supramaximal square pulse of 0.5 ms duration at a frequency of 0.2 Hz and the isometric contraction was recorded with a physiograph (Grass FT 03 force-displacement transducer attached to a Grass low-level d.c. preamplifier, model 7p1F).

Measurement of fluorescence

β -Butx concentration 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. The excitation slit width was 5 nm and the emission slit width was 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 to avoid protein denaturation. $F_{\lambda_2}^{\lambda_1}$ represented the fluorescence intensity at wavelength λ_2 (nm) when the fluorophore was excited at wavelength λ_1 (nm).

Analysis of fluorescence data

A modified Scatchard plot (Epstein et al., 1974) was constructed to analyse the fluorescence data of a complex formed by a metal ion and β -Butx:

$$|\Delta F|/[L]_{\text{free}} = (K_L F_{\infty}) - (K_L |\Delta F|) \quad (1)$$

where ΔF is the change in protein fluorescence on adding metal ion, L , and F_{∞} the protein fluorescence in the absence of metal ion. K_L is the association constant of the complex concerned. Throughout the titration, $|\Delta F|/[L]_{\text{total}}$ was plotted against $|\Delta F|$, because $[L]_{\text{free}}$ was close to $[L]_{\text{total}}$.

RESULTS

Purification of three isotoxins of β -Butx family

Resolution of the protein components in Taiwan banded krait venom has been attempted many times by CM-Sephadex C-25 column chromatography (Clark et al., 1972; Lee et al., 1972; Eterovic et al., 1975; Abe et al., 1977; Chen et al., 1982). However, the reported chromatograms varied greatly with the batch of venom. This made it difficult to clearly assign the isotoxins of the β -Butx family. In fact, the β_1 -Butx fraction obtained by chromatography could be resolved into several components by h.p.l.c. on an SP column when the column was eluted with a linear gradient of 0.2–0.6 M NaCl at pH 6.5, but the resolution was unsatisfactory (Breeze and Dolly, 1989). We

Figure 1 Resolution of the β_1 -Butx fractions obtained by CM-Sephadex C-25 chromatography of crude venom

Protein sample (40 µg) was subjected to h.p.l.c. on a Waters Protein-PAK SP-5PW column pre-equilibrated with 20 mM NaH₂PO₄/Na₂HPO₄ at pH 6.5 or 7.4. A linear gradient of 0–0.8 M NaCl at pH 6.5 (a) and pH 7.4 (c), 0–0.8 M sodium acetate at pH 6.5 (b) and pH 7.4 (d), or 0.2–0.6 M sodium acetate at pH 7.4 (e) was applied to the column at a flow rate of 1 ml/min for 30 min. SP I–III are indicated in (d) and (e).

Figure 2 Characterization of SP I–III by SDS/PAGE

SP I (lane 1), SP II (lane 2) and SP III (lane 3), which were reduced by 2-mercaptoethanol (a) or with the disulphide bonds left intact (b), were subjected to SDS/PAGE on a gel slab (7 cm $\times$ 9 cm). Protein was visualized by Coomassie Blue staining.

therefore attempted to purify β_1 -Butx without contamination by other isotoxins.

We resolved the β_1 -Butx fraction prepared from CM-Sephadex C-25 according to previous procedures (see the Experimental section) by an SP column pre-equilibrated with 20 mM phosphate buffer at pH 6.5 or 7.4 to test the protein homogeneity. Figure 1 shows the chromatograms of several elutions. Only one major peak with a rather broad shape appeared when the column was washed at pH 6.5 with a linear gradient of either 0–0.8 M NaCl or 0–0.8 M sodium acetate (Figures 1a and 1b). However, three main components (SP I–III in Figure 1d) could be clearly seen on elution with a linear gradient of 0–0.8 M sodium acetate at pH 7.4. A better resolution of the three components could be achieved using 0.2–0.6 M sodium acetate (Figure 1e). Resolution of protein components was unsatisfactory when the column was washed with a linear gradient of 0–0.8 M NaCl at pH 7.4 (Figure 1c). Thus we applied the h.p.l.c. method described in Figure 1(d) to resolve the protein components directly from crude venom. Peaks SP I–III could be identified in each chromatogram but their proportion varied greatly with the batch of venom used (not shown).

Identification of SP I–III

SP I–III samples were characterized by SDS/PAGE (Figure 2). In each there appeared a 20 kDa band when the protein disulphide bonds remained intact (Figure 2b). Cleavage of the disulphide bonds resulted in two bands, a 13 kDa band corresponding to the PLA₂ subunit and a 7 kDa band corresponding to the non-PLA₂ subunit (Figure 2a). Both their PLA₂ subunits and non-PLA₂ subunits showed the same molecular mass, thereby making it impossible to distinguish between the three isotoxins by SDS/PAGE.

The SP I–III samples were reductively alkylated (see the Experimental section) and the alkylated derivatives were resolved by h.p.l.c. on a reversed-phase C18 column. Two alkylated products, denoted SP I-A and SP I-B on Figure 3(a), SP II-A and SP II-B on Figure 3(b) and SP III-A and SP III-B on Figure 3(c), appeared. Analysis by SDS/PAGE confirmed that SP I-B, SP II-B and SP III-B were derived from the non-PLA₂ subunits, and SP I-A, SP II-A and SP III-A from the PLA₂ subunit. The

non-PLA₂ derivatives were washed from the column at the same solution polarity as were the PLA₂ derivatives, demonstrating that there was no difference in the hydrophilicity among either the three non-PLA₂ derivatives or the three PLA₂ derivatives. Under the same experimental conditions, SP I–III were washed from the column at the same retention time which was longer than that of the non-PLA₂ derivatives but slightly shorter than that of the PLA₂ derivatives (Figure 3d). These chromatographic characteristics suggest that (a) the non-PLA₂ subunit was more hydrophilic than the PLA₂ subunit and (b) there might be some kind of interaction between the two subunits to make the hydrophobicity of the parent protein close to that of its PLA₂ subunit.

At least five isotoxins, β_1 – β_5 Butx, have been identified thus far in the β -Butx family. According to their primary structures (Kondo et al., 1978, 1982a,b), three types of PLA₂ subunits designated A1, A2 and A3 and two types of non-PLA₂ subunits designated B1 and B2 have been defined (Figure 4). Each isotoxin is formed by one type of PLA₂ subunit and one type of non-PLA₂ subunit. For instance, β_1 -Butx contains A1 and B1 and β_2 -Butx A1 and B2. We correlated SP I–III with these isotoxins. Automated Edman degradation for at least 30 cycles of each of the alkylated derivatives gave reliable data (Figure 4). The same amino acid was identified for three PLA₂ derivatives at each corresponding cycle, suggesting that SP I–III may share a common PLA₂ subunit which is most likely to be A1 or A2 but not A3. From alignment of the N-terminal sequences of the three non-PLA₂ derivatives with those of B1 and B2 as references emerged two features. First, SP II-B and SP III-B were identical with B2 and B1 respectively, except that SP II-B had an additional valine inserted between Thr-18 and Val-19 of B2 and SP III-B had an additional valine inserted between Pro-18 and Val-19 of B1. This might be due to the limitation of Edman degradation by manual procedures to sequence B1 and B2 which often causes confusion in protein sequences when the same amino acid is detected in consecutive cycles. For the time being, no amino acid sequences deduced from the corresponding cDNA are available to clarify this point. It is of interest to note that insertion of this valine in B1 or B2 allows better alignment of the non-PLA₂ subunit with kunitz trypsin inhibitor with respect to cysteine residues along the polypeptide chain (Dufton, 1985). Second, SP I-B differed greatly from SP III-B but was almost identical with SP II-B, except that Lys-14 and Ala-29 in SP II-B were replaced by Arg-14 and Glu-29 in SP I-B.

Characterization of the Ca²⁺-binding sites on SP I–III

The aromatic residue contents of the isotoxins of the β -Butx family are all the same according to the primary structures reported thus far (Kondo et al., 1978, 1982a,b). Each isotoxin molecule contains 14 tyrosines and one tryptophan residue (Trp-19 of the PLA₂ subunit). We compared the effect of CaCl₂ on the characteristics of intrinsic fluorescence of these aromatic residues in SP I–III in 0.02 M Pipes buffer at pH 7.6. SP I–III shared a similar emission spectrum when they were excited at 275 nm (not shown), exhibiting a shoulder in the range of 300–310 nm and a peak at 339 nm. 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. Addition of CaCl₂ to each protein solution caused a considerable decrease in both F_{339}^{339} and F_{275}^{305} . We analysed the fluorescence data obtained by adding Ca²⁺ to the protein solution according to eqn. (1). On the basis of our previous study (Chu and Chen, 1991), a linear curve obtained in

Figure 3 Resolution of the two subunits of SP I–III

SP I (a), SP II (b) and SP III (c) were reductively alkylated and the reaction mixture subjected to h.p.l.c. on a reversed-phase C18 column washed with a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The chromatogram of SP III (d) under the same experimental conditions of h.p.l.c. is included for comparison.

the modified Scatchard plot for the effect of Ca^{2+} on F_{275}^{339} and F_{275}^{305} characterizes the high- and low-affinity site respectively of the toxin– Ca^{2+} complex. Two distinct Ca^{2+} -binding sites appeared on each SP I–III (Figure 5). The association constants determined for each site were found to be of the same order of magnitude for each protein (Table 1), demonstrating that the three isotoxins may not differ greatly in their ability to bind Ca^{2+} at either the high- or the low-affinity site.

Functional characterization of SP I–III

PLA₂ activity, LD₅₀ value and the neuromuscle-blocking activity of SP I–III were compared (Table 1). SP III showed weak PLA₂ activity but potent toxicity. Its enzyme activity in 10 mM CaCl_2 at 37 °C was 373 units/mg of protein and its LD₅₀ was 0.012 µg/g body weight of mice. Both SP I and SP II had slightly higher PLA₂ activity but were less toxic than SP III.

Figure 6 shows the twitch response of baby chicken nerve-muscle preparation in Krebs's solution to each isotoxin at a

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SP I-A	N	L	I	N	F	M	E	M	I	R	Y	T	I	P	C	E	K	T	W	G	E	Y	A	D	Y	G	C	Y	C	G	A	G	G	S					
SP II-A	N	L	I	N	F	M	E	M	I	R	Y	T	I	P	C	E	K	T	W	G	E	Y	A	D	Y	G	C	Y	C	G	A	G	G	S					
SP III-A	N	L	I	N	F	M	E	M	I	R	Y	T	I	P	C	E	K	T	W	G	E	Y	A	D	Y	G	C	Y	C	G	A	G	G	S					
A1	N	L	I	N	F	M	E	M	I	R	Y	T	I	P	C	E	K	T	W	G	E	Y	A	D	Y	G	C	Y	C	G	A	G	G	S					
A2	N	L	I	N	F	M	E	M	I	R	Y	T	I	P	C	E	K	T	W	G	E	Y	A	D	Y	G	C	Y	C	G	A	G	G	S					
A3	N	L	I	N	F	M	E	M	I	R	Y	T	I	P	C	E	K	T	W	G	E	Y	T	D	Y	G	C	Y	C	G	A	G	G	S					
		:		:		:		:		:		:		:		:		:		:		:		:		:		:		:		:		:		:			
SP III-B	R	Q	R	H	R	D	C	D	K	P	P	D	K	G	N	C	G	P	V	V	R	A	F	Y	Y	D	T	R	L	K	T	C	K	A	F	Q	Y	R	G
B1	R	Q	R	H	R	D	C	D	K	P	P	D	K	G	N	C	G	P	-	V	R	A	F	Y	Y	D	T	R	L	K	T	C	K	A	F	Q	Y	R	G
B2	R	K	R	H	P	D	C	D	K	P	P	D	T	K	I	C	Q	T	-	V	R	A	F	Y	Y	K	P	S	A	K	R	C	V	Q	F	R	Y	G	G
SP II-B	R	K	R	H	P	D	C	D	K	P	P	D	T	K	I	C	Q	T	V	V	R	A	F	Y	Y	K	P	S	A	K	R	C	V	Q	F	R	Y	G	G
SP I-B	R	K	R	H	P	D	C	D	K	P	P	D	T	R	I	C	Q	T	V	V	R	A	F	Y	Y	K	P	S	A	K	R	C	V	Q	F	R	Y	G	G

Figure 4 N-Terminal sequences of the two subunits of SP I–III

The amino acid sequences of PLA₂ subunits designated A1, A2 and A3 and non-PLA₂ subunits designated B1 and B2 (see the text) are also listed for comparison. The amino acid sequences along polypeptide chains were aligned and the homologous residues are indicated by vertical bars.

Figure 5 Modified Scatchard plot for the binding of Ca^{2+} to SP I–III

SP I (a), SP II (b) or SP III (c) each at 7.5 µM was titrated with the same protein solution in the presence of 1.0 M CaCl_2 at pH 7.6 and room temperature. The fluorescence data were analysed using eqn. (1) with linear regression fitting. The correlation coefficient was calculated to be larger than 0.90 for the high-affinity site and 0.71 for the low-affinity site in each curve: ●, estimation from F_{275}^{305} for the low-affinity site; ○, estimation from F_{275}^{339} for the high-affinity site.

concentration of 0.01 µg/ml. In a similar manner to that observed in previous work (Chang, 1985), SP I–III each exhibited two stages of neurotoxic action. SP III took a shorter time than either

Table 1 Functional characteristics of SP I–III

Values are means $\pm$ S.E.M. except for LD₅₀ where the 95% confidence limit is given in parentheses. For transmission-block activity, protein at 0.01 μ g/ml inhibited the indirectly evoked contraction of chick biventer cervicis muscle. The values in parentheses are the number of experiments. K_a and K_b are the association constants for the low- and high-affinity site respectively of the toxin–Ca²⁺ complex.

Toxin	PLA ₂ activity (units/mg)	LD ₅₀ (μ g/g body weight)	Transmission-block activity (time for 90% inhibition; min)	10 ⁻³ $\times$ K (M ⁻¹)	
				K_a	K_b
SP I	393.3 $\pm$ 23.1	0.123 (0.090–0.168)	101.3 $\pm$ 14.6 (6)	0.22 $\pm$ 0.02	2.39 $\pm$ 0.98
SP II	430.0 $\pm$ 10.0	0.043 (0.039–0.049)	79.0 $\pm$ 13.6 (8)	0.23 $\pm$ 0.03	2.50 $\pm$ 0.64
SP III	373.3 $\pm$ 23.1	0.012 (0.010–0.014)	65.0 $\pm$ 4.7 (6)	0.15 $\pm$ 0.02	5.01 $\pm$ 1.27

Figure 6 Inhibitory effect of SP I–III on the indirectly evoked contraction of chick biventer cervicis muscle

Twitches were elicited by indirect stimulation of chick cervicis nerve–muscle preparation in Krebs' solution at 0.2 Hz. The concentration of each protein solution was 0.01 μ g/ml: (a) SP I; (b) SP II; (c) SP III. Addition of protein to the organ bath is denoted by the arrow.

SP I or SP II to evoke the early effect. This was parallel to the duration of the later action. SP I, SP II and SP III took around 101, 79 and 65 min respectively to inhibit 90% of the indirectly evoked contraction of biventer cervicis muscle (Table 1).

DISCUSSION

The isotoxins of the β -Butx family show a wide range of lethal potency. Therefore their separation to avoid contamination with one another is critical in order to correlate their structures with neurotoxicity. We achieved good resolution of SP I–III. We showed that, in addition to the same N-terminal sequences of their PLA₂ subunits, they have a slightly different PLA₂ activity and a similar Ca²⁺-binding characteristic. The N-terminal sequences of their non-PLA₂ subunits confirmed SP II and SP III to be β_2 - and β_1 -Butx respectively. According to the primary structures reported by Kondo et al. (1978, 1982a,b), 63% of the 60 amino acid residues of the non-PLA₂ subunit in β_1 -Butx can be aligned identically with the amino acid sequence of β_2 -Butx. We showed that SP I, which has not been reported in the literature so far, has high structural similarity to SP II. These data together reveal that SP I–III may share a common PLA₂ subunit but have different non-PLA₂ subunits, demonstrating that the structure of the non-PLA₂ subunit has only a slight effect on the two Ca²⁺-binding domains on the PLA₂ subunit.

Investigating the amino acid residues responsible for the neurotoxic effect of β -Butx has been approached with a certain

degree of success by chemical modification of a specific residue on the protein molecule. The study of Rosenberg et al. (1989) reveals that selective sulphonation of Tyr-68 on the PLA₂ subunit increases the enzyme activity and the inhibitory effect on the indirectly evoked contraction of the phrenic nerve diaphragm preparation, but this results in a partial decrease in lethal potency when compared with the activities of the parent toxin. On transformation of Met-8 of PLA₂ subunit to a sulfoxide derivative, most of the PLA₂ activity of the parent toxin is retained but there is loss of neurotoxicity (Chu et al., 1993). These findings suggest that the active site for the PLA₂-independent neurotoxic effect is separate from the two Ca²⁺-binding sites required absolutely for both catalytic activity of the PLA₂ subunit and the receptor-binding activity (Rehm and Betz, 1982). However, one should not neglect the involvement of the non-PLA₂ subunit in the structure and function of β -Butx, considering that the binding of this toxin in chick muscle can be interfered with dendrotoxin and toxins I and K from mamba venom (Harvey and Karlsson, 1982). Its role cannot be studied directly unless the non-PLA₂ subunit in its native form can be separated from the PLA₂ subunit, which is difficult if not impossible. Note that both the PLA₂ subunit and the non-PLA₂ subunit, which could be separated from each other after the disulphide bond linking them together was selectively cleaved, showed neither enzyme activity nor lethality (Chang and Yang, 1993). Apparently, neither the PLA₂ subunit nor the non-PLA₂ subunit alone is sufficient to maintain the active conformation for the neurotoxic effect. The characteristics on h.p.l.c. (Figure 3) together with our previous results (Lin et al., 1984; Chu et al., 1993) suggest that the two subunits may interact with each other to maintain the protein structure of the native toxin. Under the circumstances, comparing the functional characteristics of isotoxins with the same PLA₂ subunit but different non-PLA₂ subunits may shed some light on the role of the non-PLA₂ subunit in the neurotoxic effect. SP I–III can be used for this purpose.

SP I–III differ in their neurotoxic potency. This can probably be attributed to the structural differences in their non-PLA₂ subunits which may themselves cause a presynaptic effect as suggested previously (Harvey and Karlsson, 1982) and/or modulate the structure of the PLA₂ subunit so that it becomes active in the PLA₂-independent neurotoxic effect. Correlation of the N-terminal sequence of the non-PLA₂ subunit in SP I with that in SP II suggests that the difference between Arg-14 of SP I and Lys-14 of SP II and/or the difference between Glu-29 of SP I and Ala-29 of SP II in either their receptor-binding reactivity or their effect on protein conformation may be the reason why SP II is more potent than SP I. The former may result in different basicity and the latter different hydrophobicity. Considering

with Gly-14 and Leu-29 in the non-PLA₂ subunit of SP III, it may rule out the former but supports the latter to involve the PLA₂-independent neurotoxic effect.

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