Dissociation of lethal toxicity and enzymic activity of notexin from *Notechis scutatus scutatus* (Australian-tiger-snake) venom by modification of tyrosine residues

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Notexin from *Notechis scutatus scutatus* snake venom was subjected to tyrosine modification with *p*-nitrobenzenesulphonyl fluoride (NBSF), and four modified derivatives were separated by h.p.l.c. The results of amino acid analysis and sequence determination revealed that only Tyr-7, Tyr-70 and Tyr-77 were modified in notexin. Modification of Tyr-7 resulted in decreases in lethal toxicity and enzymic activity by 70.2% and 22.7% respectively. Conversely, modification of Tyr-77 caused a 1.8-fold increase in enzymic activity, in contrast with the loss of 52.5% of lethality. A drastic decrease in lethal toxicity was observed when both Tyr-7 and Tyr-70 were modified, whereas the enzymic activity decreased by only 35.8%. Likewise, the derivative in which Tyr-7 and Tyr-77 were modified retained 44.4% of enzymic activity, but showed a marked decrease in lethal toxicity. It is obvious that modification of tyrosine residues causes a decrease in lethal toxicity of notexin, which does not directly correlate with the change in enzymic activity. On the other hand, the antigenicity of NBS derivatives remained unchanged. The modified derivatives retained their affinity for Ca²⁺, indicating that the modified tyrosine residues did not participate in Ca²⁺ binding. These results indicate that modification of tyrosine residues can differentially influence the enzymic activity and lethal toxicity of notexin, and suggest that notexin might possess two functional sites, one being responsible for the catalytic activity and the other associated with its lethal effect.

INTRODUCTION

Notexin, a presynaptic phospholipase A₂ (PLA₂) neurotoxin purified from the venom of *Notechis scutatus scutatus*, is a basic protein with 119 amino acid residues and seven disulphide bridges (Halpert & Eaker, 1975; Chwetzoff *et al.*, 1990). Besides its presynaptic neurotoxicity, which blocks neuromuscular transmission by decreasing the release of acetylcholine (Harris *et al.*, 1973; Cull-Candy *et al.*, 1976), notexin exhibits PLA₂ activity and structurally shows sequence similarity to PLA₂ enzymes from mammalian pancreas and other snake venoms (Dufton *et al.*, 1983; Waite, 1987).

The molecular structures of presynaptic PLA₂ neurotoxins are quite varied. Some of them, like notexin and caudotoxin, are single polypeptide chains, whereas others are binary (crotoxin and β -bungarotoxins), ternary (taipoxin) or quaternary (textilotoxin) complexes (Bon *et al.*, 1986). However, either the single polypeptide chain itself or at least one of the subunits in complex toxins shares sequence similarity to PLA₂ enzymes (Dufton *et al.*, 1983). The variations in molecular structure were taken to mean that these toxins might interact at different locations on the presynaptic site of the neuromuscular junction. This inference was supported by the observed synergism (Chang & Su, 1980) and lack of competition for binding between these neurotoxins (Tzeng *et al.*, 1986, 1989). However, presynaptic PLA₂ neurotoxins show both PLA₂ activity and presynaptic neurotoxicity (Harris, 1985); thus it is important to clarify the role of enzymic activity in inducing the toxic effects. There is considerable evidence in favour of an essential role of PLA₂ activity in the involvement of presynaptic neurotoxicity (Strong *et al.*, 1976; Halpert *et al.*, 1976; Halpert & Eaker, 1976; Howard & Truong, 1977; Howard & Gunderson, 1980; Harris, 1985). Nevertheless, Chang & Su (Su & Chang, 1981, 1984; Chang & Su, 1982; Chang, 1985) found that the immediate presynaptic depression

caused by presynaptic PLA₂ neurotoxin appears to be due to the binding of the toxin molecule as such, and is not related to its PLA₂ activity. Moreover, there is no quantitative relationship between PLA₂ activity and presynaptic potency (Chang, 1985), and many groups have reported that chemical modification of presynaptic PLA₂ neurotoxins can differentially influence their enzymic activity and lethal toxicity (Halpert, 1979; Ng & Howard, 1978, 1980; Yang & Lee, 1986; Mollier *et al.*, 1989). On the basis of these observations it is plausible that, besides the catalytic site, additional molecular features must be present in molecules of presynaptic PLA₂ neurotoxins in order for them to exert their lethal toxicity and pharmacological effects (Rosenberg, 1986; Kini & Evans, 1989). However, the structural characteristics that make presynaptic PLA₂ neurotoxin such a potent toxin are still not unequivocally identified. Therefore chemical modification of notexin was undertaken in the hope of shedding more light on the molecular features contributing to the biological activities of presynaptic PLA₂ neurotoxins.

In the present study the tyrosine residues of notexin were selectively modified with *p*-nitrobenzenesulphonyl fluoride (NBSF), and four modified derivatives were obtained. We found that the derivatives showed a decrease in lethal toxicity that was not in parallel with the change in enzymic activity, suggesting that the lethal effect of notexin does not depend heavily on its enzymic activity.

MATERIALS AND METHODS

Materials

Notechis scutatus scutatus venom was obtained from Venom Supplies, Tanunda, South Australia, Australia. Notexin was isolated from the venom on an SP-Sephadex C-25 column according to the method described by Yang & Chang (1990). NBSF and 8-anilino-naphthalene-1-sulphonate were obtained

Abbreviations used: PLA₂, phospholipase A₂; NBSF, *p*-nitrobenzenesulphonyl fluoride.

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Fig. 1. Separation of NBS-notexin on a SynChropak RP-P column

The column (4.6 mm $\times$ 25 cm), equilibrated with 0.1 % trifluoroacetic acid, was eluted with a linear gradient of 30–43 % acetonitrile in 0.1 % trifluoroacetic acid for 46 min. The flow rate was 0.8 ml/min, and the eluent was monitored at 280 nm.

from Pierce Chemical Co.; acetonitrile, trifluoroacetic acid and the LiChrospher 100 RP-18 column (4 mm $\times$ 25 cm) were from E. Merck. The SynChropak RP-P column (4.6 mm $\times$ 25 cm) was purchased from SynChrom (Lafayette, IN, U.S.A.). SP-Sephadex C-25 and Sephadex G-25 were the products of Pharmacia Fine Chemicals. All other reagents were of analytical grade.

Modification of tyrosine residues with NBSF

Notexin was modified with NBSF according to the procedure described by Yang *et al.* (1985), with some modification. Notexin (1 μ mol) in 14 ml of 0.05 M-NaCl/0.1 M-Tris/HCl buffer, pH 8.0, was incubated with a 25-fold molar excess of NBSF. The reaction was allowed to proceed at 30 $^{\circ}$ C for 2 h, and the mixture was desalted by passage through a Sephadex G-25 column (2 cm $\times$ 100 cm) equilibrated with 0.2 M-acetic acid.

The *p*-nitrobenzenesulphonylated (NBS) derivatives were separated by h.p.l.c. on a SynChropak RP-P column (4.6 mm $\times$ 25 cm) equilibrated with 0.1 % (v/v) trifluoroacetic acid and eluted with a linear gradient of 30–43 % (v/v) acetonitrile in 0.1 % trifluoroacetic acid for 46 min. The effluent was monitored at 280 nm. Notexin and tyrosine-modified derivatives were rechromatographed on a SynChropak RP-P column under the same conditions as described above. It was found that their biological activities remained unchanged. This indicates that separation of the modified derivatives with an acetonitrile gradient did not cause irreversible changes in their biological activities.

Identification of the tyrosine residues modified

To determine the position(s) of the incorporated NBS group(s) in the sequence of notexin, the NBS derivatives were oxidized by performic acid according to the method described by Yang *et al.* (1970) followed by tryptic digestion. The oxidized proteins were dissolved in 0.2 M-ammonium bicarbonate buffer, pH 7.8, and trypsin was added (30:1, w/w). Digestion was carried out at 37 $^{\circ}$ C for 3 h and the hydrolysates were freeze-dried.

The tryptic hydrolysates were separated by h.p.l.c. on a LiChrospher 100 RP-18 column (4 mm $\times$ 25 cm) equilibrated with 0.1 % trifluoroacetic acid and eluted with a linear gradient of 10–35 % acetonitrile in 0.1 % trifluoroacetic acid for 80 min.

Fig. 2. Separation of the tryptic-digest peptides from performic acid-oxidized notexin and its NBS derivatives

Tryptic hydrolysates of the oxidized protein were applied on a LiChrospher RP-18 column (4 mm $\times$ 25 cm) equilibrated with 0.1 % trifluoroacetic acid and eluted with a linear gradient of 10–35 % acetonitrile in 0.1 % trifluoroacetic acid for 100 min. The flow rate was 0.8 ml/min and the effluent was monitored at 230 nm.

The flow rate was 0.8 ml/min and the effluent was monitored at 230 nm.

Other tests

Determination of PLA₂ activity, lethal toxicity, antigenicity, PAGE, amino acid analysis and sequence determination, Ca²⁺-induced difference spectroscopy and fluorescence measurement were performed in essentially the same manner as previously described (Yang & Chang, 1989, 1990).

RESULTS

p-Nitrobenzenesulphonylation of notexin with NBSF

Notexin from *Notechis scutatus scutatus* venom was treated with NBSF for 2 h at pH 8.0. The modified derivatives were separated by h.p.l.c. on a SynChropak RP-P column, and four

Table 1. Amino acid sequences of tryptic-digest peptides of notexin and tyrosine-modified notexin

Peptide(s)	Position	Sequence
1	64-79	Met-Ser-Ala-Tyr-Asp-Tyr-Tyr-Cys-Gly-Glu-Asn-Gly-Pro-Tyr-Cys-Arg
2	6-17	Ser-Tyr-Leu-Ile-Gln-Cys-Ala-Asn-His-Gly-Lys-Arg
3	3-12	Val-Gln-Phe-Ser-Tyr-Leu-Ile-Gln-Cys-Ala
2a, 4a	64-79	Met-Ser-Ala-Tyr-Asp-Tyr-Tyr-Cys-Gly-Glu-Asn-Gly-Pro-Tyr-Cys-Arg NBS
3a	64-79	Met-Ser-Ala-Tyr-Asp-Tyr-Tyr-Cys-Gly-Glu-Asn-Gly-Pro-Tyr-Cys-Arg NBS
1b, 2b, 4b	6-17	Ser-Tyr-Leu-Ile-Gln-Cys-Ala-Asn-His-Gly-Lys-Arg

Table 2. Properties of tyrosine-modified notexin

	Incorporated NBS group(s)		Enzymic activity (%)	LD ₅₀ (% of native) (reciprocal)	Antigenic activity* (%)
	Number	Position			
Native notexin	0		100	100	100
NBS-1 derivative	1	Tyr-7	77.3	29.8	99
NBS-2 derivative	1	Tyr-77	180.6	47.5	99
NBS-3 derivative	2	Tyr-7 and Tyr-70	64.2	3.0	99
NBS-4 derivative	2	Tyr-7 and Tyr-77	44.4	2.7	100

* Antigenic activity was determined by a quantitative precipitin reaction as described by Yang & Chang (1984).

NBS derivatives were isolated (Fig. 1). PAGE revealed that all the NBS derivatives are homogeneous (results not shown). Amino acid analysis showed that only one tyrosine residue is modified in each of derivatives NBS-1 and NBS-2, and two tyrosine residues in each of derivatives NBS-3 and NBS-4.

Identification of the modified tyrosine residues

In order to determine the positions of the tyrosine residues modified in the sequence of notexin, notexin and the tyrosine-modified derivatives were digested with trypsin after performic acid oxidation. The tryptic hydrolysates were separated by h.p.l.c. on a LiChrospher 100 RP-18 column and the elution profiles are shown in Fig. 2. The results of amino acid analysis show that peak 1 in the native notexin, which represents the segment at positions 64-79, disappeared from the maps of derivatives NBS-2, NBS-3 and NBS-4. Instead, new peaks 2a, 3a and 4a appeared, which correspond to the peak 1 in native notexin. Sequence determination revealed that the peptide fragments 2a and 4a contained a modified tyrosine residue at position 77, whereas Tyr-70 was modified in peptide fragment 3a (Table 1). Peaks 2 and 3 in the native notexin represent the peptide segments at positions 6-17 and 3-12 respectively. Amino acid analysis and sequence determination of new peaks 1b, 3b and 4b that appeared in the tryptic digests of derivatives NBS-1, NBS-3 and NBS-4 revealed that they were derived from the peaks 2 and 3 in native toxin, but were missing a tyrosine residue at position 7 (Table 1). These results unambiguously indicate that Tyr-7 and Tyr-77 are modified in derivatives NBS-1 and NBS-2 respectively, as are Tyr-7 and Tyr-70 in derivative NBS-3 and Tyr-7 and Tyr-77 in derivative NBS-4.

Properties of tyrosine-modified notexin

As shown in Table 2, modification of Tyr-7 resulted in decreases in enzymic activity and lethal toxicity of notexin by 22.7% and 70.2% respectively. In sharp contrast with the loss of 52.5% of lethal toxicity, modification of Tyr-77 caused a 1.8-fold

increase in enzymic activity. A drastic decrease in lethal toxicity was observed when both Tyr-7 and Tyr-70 were modified, whereas the enzymic activity decreased by only 35.8%. Likewise, the derivative in which Tyr-7 and Tyr-77 were modified retained 44.4% of enzymic activity but showed a marked decrease in lethal toxicity. However, the antigenicity of NBS derivatives remained unchanged (Table 2). These results imply that Tyr-77 is closely related to the lethal toxicity, and that Tyr-7 and Tyr-77 are important for the biological activity of notexin.

The fluorescence spectra of notexin and tyrosine-modified derivatives are shown in Fig. 3. Native toxin and modified derivatives enhanced the emission intensity of 8-anilino-naphthalene-1-sulphonate, and the emission intensity of 8-anilino-naphthalene-1-sulphonate-enzyme complexes increased in parallel with increasing concentration of Ca²⁺ until the saturation level was reached. Since the binding of Ca²⁺ is associated with a marked increase in fluorescence of the 8-anilino-naphthalene-1-sulphonate-toxin complex, the Ca²⁺-toxin interaction can be monitored by the change in fluorescence intensity of the 8-anilino-naphthalene-1-sulphonate-toxin complex caused by the addition of Ca²⁺. As shown in the insets to Fig. 3, notexin and its NBS derivatives exhibit a linear relationship between $(\Delta F/\Delta F_{\max})/[\text{Ca}^{2+}]$ (mM) and $\Delta F/\Delta F_{\max}$. The linearity indicates that native notexin and tyrosine-modified derivatives possess one type of Ca²⁺-binding site. However, the intercepts on the $\Delta F/\Delta F_{\max}$ scale do not extrapolate to a value of 1. Since measurements of ligand-protein interaction by spectroscopic techniques are based on the assumption that the fractional change in the optical response, $\Delta F/\Delta F_{\max}$, is linearly proportional to the fractional saturation of binding sites (Ward, 1985), this deviation might be due to non-proportionality between ligand binding and optical response. The dissociation constants for Ca²⁺ calculated from the slopes of the lines (Ward, 1985; Oberfelder & Lee, 1985; Bagshaw & Harris, 1987) were 0.25 mM, 0.21 mM, 0.22 mM, 0.48 mM and 0.33 mM for notexin and derivatives NBS-1, NBS-2, NBS-3 and NBS-4 respectively. It is evident that the NBS

Fig. 3. Effect of Ca^{2+} on the interaction of native and tyrosine-modified notexin with 8-anilino-1-naphthalene-1-sulphonate at 25 °C

The sample cuvette contained 0.105 mg of native or tyrosine-modified protein/ml of 0.1 M-NaCl/0.025 M-Tris/HCl buffer, pH 8.0, and $7.5 \mu\text{M}$ -8-anilino-1-naphthalene-1-sulphonate in the presence of various concentrations of Ca^{2+} (mM) as indicated. The plots of data were obtained by fluorescence emission enhancement at 485 nm (insets). The different symbols, $\times$ and $\cdot$, refer to two separate experiments and the straight lines were determined by a linear-regression method.

derivatives still retained their binding affinity for Ca^{2+} . Ca^{2+} -induced difference spectra also revealed that the binding affinity of notexin for Ca^{2+} is not impaired after *p*-nitrobenzenesulphonylation (results not shown).

DISCUSSION

In this study we found that modification of tyrosine residues resulted in a decrease in the lethal toxicity of notexin, but that the modified derivatives retained a greater proportion of enzymic

activity and in one case even showed an increase in enzymic activity. This observation indicates that modification of tyrosine residues in notexin causes a differential effect on its enzymic activity and lethal toxicity, and suggests that notexin might possess two functional sites, one being responsible for the catalytic activity and the other associated with its lethal effect. Modification of tryptophan residues in notexin also favours the existence of a specific toxic site that is independent of catalytic site (Mollier *et al.*, 1989). The fact that the NBS derivatives were similar to native toxin in their affinity for Ca^{2+} indicates that the

Fig. 4. Tertiary structure of *Naja naja atra* PLA₂, showing the relative locations of the tyrosine residues, the Ca²⁺-binding site and the residues of which the 'catalytic network' is composed

The model is based on the results reported by White *et al.* (1990). The carbonyl oxygen atoms of Tyr-27, Gly-29 and Gly-31, the carboxylate oxygen atom of Asp-48 and two water molecules form an oxygen cage that sequesters a Ca²⁺ ion. The 'catalytic network' is formed by the side chains of His-47, Tyr-51, Tyr-67 and Asp-93. Numbers in parentheses indicate the positions of Tyr-7, Tyr-70 and Tyr-77 in notexin.

loss of the biological activity is not related to cofactor binding. Moreover, the antigenicity of NBS derivatives remained unchanged, suggesting that the gross structure of the toxin molecule was not significantly affected by tyrosine modification. Therefore alteration of the biological activities of notexin could be exclusively attributed to modification of the tyrosine residues in these derivatives.

Since the amino acid sequences of notexin and PLA₂ enzymes resemble each other, one might assume that the overall structure of notexin is similar to that of the PLA₂ enzymes (Dufton *et al.*, 1983). Inspection of the tertiary structure of *Naja naja atra* PLA₂ (White *et al.*, 1990; Scott *et al.*, 1990) showed that a hydrogen-bonding network connects the 'catalytic network' formed by the side chains of His-47, Tyr-51, Tyr-67 and Asp-93 to the *N*-terminal region (Fig. 4). Brunie *et al.* (1985) and White *et al.* (1990) suggested that the hydrogen-bonding network is an 'activation network' that could stabilize the productive conformation of PLA₂ enzymes for aggregated substrate. This inference was consistent with our findings (Yang & Chang, 1988, 1989, 1990; Chang & Yang, 1988) that the *N*-terminal region is of utmost importance for the maintenance of a functional catalytic site in snake-venom PLA₂ enzymes and presynaptic PLA₂ neurotoxins. Incorporation of the NBS group on the *N*-terminal Tyr-7 or on the tyrosine residues around the catalytic network is likely to perturb this hydrogen-bonding network, thereby causing notexin to change its enzymic activity. The three-dimensional structure of PLA₂ (Fig. 4) showed that Tyr-7 and Tyr-70 are located at the same face as the residues (at positions

2, 3, 5, 6, 9, 19, 30 and 63) involved in the interaction of enzyme with lipid/water interfaces, whereas Tyr-77 lies in the other plane. This difference in their architectural positions might result in the differential effect on the enzymic activity of notexin that is observed with tyrosine modification.

On the basis of comparative analysis of the amino acid sequence of presynaptic PLA₂ neurotoxins, Tsai *et al.* (1987) proposed that the toxicity of the presynaptic PLA₂ neurotoxins is associated with a cluster of basic residues at positions 59–73 and 87 or 88, and the nearby hydrophobic regions 1–10, 64–78 and 87–106 (Fig. 4). Chemical-modification studies (Mollier *et al.*, 1989; Yang & Chang, 1990) confirmed that the *N*-terminus and *C*-terminal region of notexin are important for its lethal toxicity. Modification of Tyr-68 (which corresponds to Tyr-67 in notexin and *Naja naja atra* PLA₂) in the A chain of β_1 -bungarotoxin (which is composed of the A-chain and the B-chain, the former having structural similarities to notexin and PLA₂) causes a selective loss in lethal toxicity (Yang & Lee, 1986); this suggests that the neighbouring region surrounding Tyr-68 is related to the lethal toxicity. Studies on the modified β -bungarotoxin and notexin (Rosenberg *et al.*, 1989) are also in agreement with the proposal by Tsai *et al.* (1987). Therefore it is likely that modification of tyrosine residues at positions 7, 70 and 77 affects the molecular feature responsible for the lethal toxicity of notexin, thereby causing notexin to lose its lethal effect.

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