THE ROLE OF PHOSPHOLIPASE ACTIVITY IN THE ACTION OF A PRESYNAPTIC NEUROTOXIN FROM THE VENOM OF *NOTECHIS SCUTATUS SCUTATUS* (AUSTRALIAN TIGER SNAKE)

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1. Introduction

Notexin from the venom of *Notechis scutatus scutatus* is a basic protein with 119 amino acid residues and seven disulfide bridges which blocks neuromuscular transmission by interfering with the release of acetylcholine from the motor nerve terminals [1,2]. Notexin exhibits phospholipase activity and is highly homologous to phospholipase A from porcine pancreas and various snake venoms [3–5], raising the possibility that the neurotoxicity is catalytic in nature, involving the hydrolysis of a special phospholipid structure in the nerve terminal. Furthermore, it has recently been suggested that another presynaptic snake venom neurotoxin, β-bungarotoxin, acts by inhibiting oxidative phosphorylation in the mitochondria of nerve terminals through a phospholipase A activity [6].

The discovery that porcine pancreatic phospholipase A_2 can be completely inactivated by the modification of a single histidine residue with *p*-bromophenacyl bromide [7] appeared to offer a means of assessing the role of phospholipase activity in the presynaptic blocking action of notexin. Notexin exhibited a 99.8% loss of both phospholipase A activity and lethal neurotoxicity upon treatment with *p*-bromophenacyl bromide. Ultraviolet difference spectroscopy and amino acid analysis showed that the inactivation was accompanied by the incorporation of 1 *p*-bromophenacyl residue and the disappearance of 1 histidine residue per mole of protein. The modified residue was identified as histidine-48, which corresponds to the histidine residue shown to be part of the catalytic site of the pancreatic enzyme. Calcium protects notexin against the modification and changes the ultraviolet absorption spectrum of both the native and modified protein. Native and modified notexin bind calcium in a 1:1 molar ratio at calcium concentrations ranging from 2×10^{-5} to 2×10^{-1} M. At pH 7.4 the dissociation constant of the native toxin–calcium complex is 1.4×10^{-4} M and that of the modified toxin–calcium complex 2.5×10^{-2} M.

2. Materials and methods

Notexin was isolated as described previously [3]. Staphylococcal protease was a gift from Dr A.-C. Rydén. *p*-Bromophenacyl bromide was purchased from Fluka AG. All other reagents and materials were of the highest grade available and were used without further purification.

Toxicity assays were performed by intravenous injection into the tail vein of white mice weighing between 25 and 30 g. Three to four mice were used at each dose level. The toxin concentration was determined spectrophotometrically using a predetermined extinction coefficient.

Phospholipase A assays were performed by a slight modification of the titrimetric method of de Haas et al. [8]. Titrations were done with 0.01 M NaOH on a TTT-1 Radiometer autotitrator at 25°C and pH 8.0. As substrate 2 ml of an aqueous emulsion of egg yolk was used in the presence of 2.7×10^{-3} M solium deoxycholate and 2×10^{-2} M calcium.

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Modification with *p*-bromophenacyl bromide was done at 30°C at a molar reagent:protein ratio of 5:1 in 0.1 M sodium cacodylate–HCl buffer (pH 6.0) containing 0.1 M NaCl [7]. The protein was dissolved in buffer at a concentration of 7×10^{-5} M (1 mg/ml), and 25 μl 1.4×10^{-2} M *p*-bromophenacyl bromide in acetone was added per mg of protein at 0 time. The kinetics of the modification in the presence and absence of calcium were studied by removing 1 ml aliquots at suitable times and acidifying them with 25 μl glacial acetic acid to stop the reaction. After dialysis for 6 h against 1 litre 0.1 M acetic and 2 × 12 h against 1 litre portions of 0.43 M ammonium acetate the sample was applied to a column of Bio-Rex 70 for separation and quantification of modified and native protein.

Ion-exchange chromatography was performed on a 1 × 33 cm column of Bio-Rex 70 (–400 mesh) equilibrated with 0.43 M ammonium acetate. The sample was applied in 1 ml 0.43 M ammonium acetate and eluted at 9.3 ml per h with the same buffer. The runs were monitored with a Multiref 901 differential refractometer (Optilab, Vällingby, Sweden), and 2-ml fractions were collected. The areas of the peaks were estimated by triangulation.

Amino acid analysis was done with a Durrum D-500 analyzer following hydrolysis for 24 h with 6 N HCl at 110°C in thoroughly sealed evacuated tubes.

Direct and difference spectra were recorded on a Beckman double-beam spectrophotometer using cuvettes of 1-cm path length. Molar extinction coefficients were determined by amino acid analysis of samples of known absorbance.

The number of *p*-bromophenacyl residues incorporated per mole of protein was determined by comparison of the molar difference extinction coefficient of modified vs. native protein with the molar extinction coefficient of the reagent.

Determination of the dissociation constant of the native toxin–calcium and modified toxin–calcium complexes was performed by ultraviolet difference spectroscopy as described for porcine pancreatic phospholipase A_2 [9]. The protein was dissolved at a concentration of 5×10^{-5} M in 0.1 M sodium cacodylate-HCl (pH 7.4) containing 0.1 M NaCl. Calcium ion stock solutions with a concentration of about 25 times the estimated dissociation constant were added to the sample cell and an equal volume of buffer to the reference cell. A double reciprocal plot of ΔA vs. $[Ca^{2+}]$ gave a straight line from which the maximal absorbance (ΔA_{max}) and the molar difference extinction coefficient were calculated. The dissociation constant and the number of binding sites were determined by a Scatchard plot of $V_{Ca^{2+}}/[Ca^{2+}]$ vs. $V_{Ca^{2+}}$ where $V_{Ca^{2+}} = \Delta A/\Delta A_{max}$ [10].

Reduction and *S*-carboxymethylation of modified notexin was performed in 6 M guanidine hydrochloride buffered at pH 8.3 with 0.86 M Tris–HCl and containing 0.3% EDTA. To 0.6 μmol of protein in 3 ml buffer 6.5 mg dithioerythritol was added, corresponding to a 10-fold excess of reagent over disulfide. After 4 h 84 μmol (17.5 mg) sodium iodoacetate was added in 1 ml distilled water. After 15 min the sample was dialyzed for 3 × 12 h against 1 litre portions of distilled water.

Identification of the modified residue was accomplished by digesting the reduced and *S*-carboxymethylated modified protein with staphylococcal protease and isolating the peptide containing a *p*-bromophenacyl residue by gel filtration and column electrophoresis [3]. Spectra were run on the three fractions obtained from the digest by gel filtration on a 1 × 142 cm column of Sephadex G-25, and the fraction showing an adsorption maximum at 262 nm (λmax for the reagent) was submitted to column electrophoresis for 22 h at 1000 V at pH 5.0.

The susceptibility of the native and modified protein to proteolytic digestion was studied by incubation of protein (10 mg/ml) with trypsin (0.37 mg/ml) at 37°C in 0.1 M *N*-ethylmorpholine acetate buffer (pH 7.5) containing 5×10^{-3} M calcium. After 1 h the digestion was terminated by addition of acetic acid, and the digests were lyophilized. Half of each digest was examined by paper electrophoresis at pH 3.5 for 1 h at 3000 V, and the remaining half was subjected to performic acid oxidation [11] and then lyophilized and examined by paper electrophoresis at pH 6.5 for 1 h at 3000 V.

3. Results

As shown in fig.1 modified notexin can be completely separated from native toxin by elution chromatography on Bio-Rex 70. The properties of the native and modified toxin are summarized in table 1.

Fig.2 is a semilogarithmic plot illustrating the first-order dissappearance of native notexin upon incubation with *p*-bromophenacyl bromide. The presence of 1 mM calcium increases the half-time from 187 min to 442 min, suggesting that calcium binds to the same site as the reagent.

The binding of calcium to both native and modified notexin changes the protein absorption spectrum. The ultraviolet difference spectrum of native notexin is very similar to that of porcine pancreatic phospholipase A_2, i.e., there is a strong band at 242 nm and two smaller bands at 282 and 288 nm [9]. However, in contrast to modified pancreatic phospholipase [12], modified notexin is still able to bind calcium. The ultraviolet difference spectrum of modified notexin is characterized by a well extending from 242 to 280 nm, the minimum of which shifts from 250 to 260 nm as the calcium concentration is increased from 6×10^{-4} to 1×10^{-1} M. The small bands at 282 and 288 nm are still present.

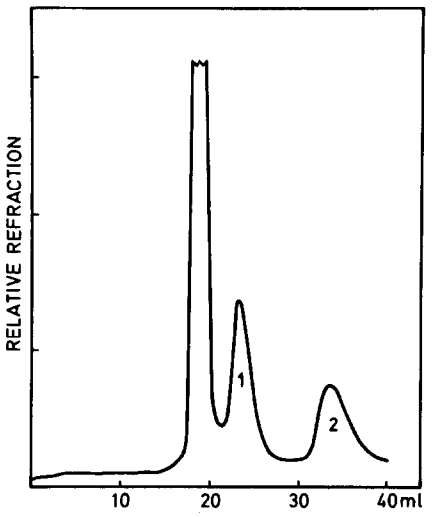

Fig.1. Separation of native (Peak 1) and modified (Peak 2) notexin by elution chromatography on a 1 X 33 cm column of Bio-Rex 70 in 0.43 M ammonium acetate as described in Methods. The breakthrough peak at 19 ml is due to residual buffer salts and contains no protein.

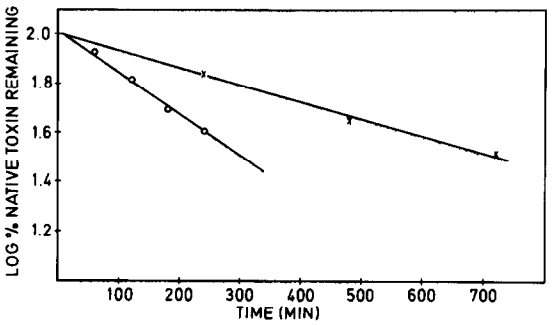

Fig.2. Disappearance of native notexin as a function of time of incubation with a 5:1 molar excess of *p*- bromophenacyl bromide as described in Methods.
(o) No calcium in incubation mixture.
(x) 1 mM calcium.

In order to determine whether the loss of lethal neurotoxicity accompanying the modification was the result of an enhanced susceptibility to proteolytic degradation, native and modified notexin were incubated with trypsin. No difference could be detected between the digests upon examination by high voltage paper electrophoresis either before or after performic acid oxidation. The digest of the native protein showed 20% residual phospholipase activity after the treatment with trypsin.

4. Discussion

Although the loss of lethal neurotoxicity seems to parallel the loss of phospholipase A activity, we feel that it would be premature to conclude definitely that the phospholipase activity is essential to the presynaptic blocking action of notexin. Preliminary experiments in isolated nerve-muscle preparations seem to indicate that the modified toxin has a residual in vitro activity exceeding that expected on the basis of the 0.2% residual phospholipase activity [13]. Unfortunately no quantitative data on the in vitro activity are available at present. Furthermore, notexin exhibits a direct dystrophic action on striated muscle

Table 1
Properties of native and *p*-bromophenacyl bromide-modified notexin

	Native	Modified
Number of histidine residues per mole of protein	3	2
Molar extinction coefficient	27 600 (278 nm)	45 500 (276 nm)
Number of *p*-bromophenacyl residues per mole of protein	0	1.0[a]
Modified residue		His-48[b]
Specific phospholipase activity (μequiv OH per min per mg protein)	850	1.8
LD_{100} (mg per kg mouse)	0.025	10
Molar difference extinction coefficient upon calcium binding[c]	1200 (242 nm)	1200 (260 nm)
Number of calcium binding sites[c]	1.1	1.0
Protein–calcium dissociation constant (M)[c]	1.4×10^{-4}	2.5×10^{-2}

[a] The molar difference extinction coefficient of modified vs. native notexin was 18 500 with a maximum at 271 nm. The extinction coefficient of the reagent was 17 600 with a maximum at 262 nm (determined in methanol).

[b] Based on the isolation of a peptide corresponding in amino acid composition to residues 41–54 in the notexin sequence except for the lack of histidine. The molar extinction coefficient was 19 800 at 262 nm, although the peptide contained only one aromatic residue, Tyr-52.

[c] pH 7.4.

cells [2,14] which is probably related to the neurotoxicity, and this function is not greatly diminished after modification with *p*-bromophenacyl bromide [15]. The loss of lethality attending the chemical modification might reflect a loss of specificity, possibly as a result of the impaired ability to bind calcium. The injected modified notexin might thus be diluted out by adsorption to other membranes and never reach the nerve terminal.

The slight residual phospholipase activity of the modified toxin is puzzling. It cannot be attributed to contamination by native notexin since it is not diminished by re-chromatography on Bio-Rex 70 and furthermore, the modification seems to be irreversible.

The phospholipase activity of notexin may be essential to its presynaptic blocking action, but the question of which structural features make this particular phospholipase highly neurotoxic whereas

the vast majority of phospholipases are non- or poorly toxic remains to be answered.

Acknowledgement

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References

[1] Karlsson, E., Eaker, D. and Rydén, L. (1972) Toxicon 10, 405–413.
[2] Harris, J. B., Karlsson, E. and Thesleff, S. (1973) Br. J. Pharmac. 47, 141–146.
[3] Halpert, J. and Eaker, D. (1975) J. Biol. Chem. 250, 6990–6997.
[4] Eaker, D., Halpert, J., Fohlman, J. and Karlsson, E. 4th Int. Symp. Plant, Animal and Microbial Toxins, Tokyo, September 1974.
[5] Eaker, D. (1975) Structure and Function of Snake Venom Toxins in: Peptides: Chemistry, Structure, and Biology (R. Walter and J. Meienhofer, eds.) (Proceedings of Fourth American Peptide Symposium), Ann Arbor Science Publishers.
[6] Wernicke, J. F., Vanker, A. D. and Howard, B. D. (1975) J. Neurochem. 25, 483–496.
[7] Volwerk, J. J., Pieterson, W. A. and Haas, G. H. de (1974) Biochemistry 13, 1446–1454.
[8] Haas, G. H. de, Postema, N. M., Nieuwenhuizen, W. and Deenen, L. L. M. van (1968) Biochim. Biophys. Acta 159, 103–117.
[9] Pieterson, W. A., Volwerk, J. J. and Haas, G. H. de (1974) Biochemistry 13, 1439–1445.
[10] Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660.
[11] Hirs, C. W. (1956) J. Biol. Chem. 219, 611–621.
[12] Pieterson, W. A., Vidal, J. C., Volwerk, J. J. and Haas, G. H. de (1974) Biochemistry 13, 1455–1460.
[13] S. Thesleff, personal communication.
[14] Harris, J. B., Johnson, M. A. and Karlsson, E., Br. J. Pharmac., in the press.
[15] J. Harris, personal communication.