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Immunological properties of notexin, a potent presynaptic and myotoxic component from venom of the Australian tiger snake *Notechis scutatus scutatus*

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Neutralizing antibodies were raised in mice against notexin, the most toxic phospholipase A_2 (PLA_2) from *Notechis scutatus scutatus* venom, without the necessity of detoxifying the toxin prior to immunization. Using a sensitive radioimmunoassay we demonstrated that anti-notexin antibodies recognized (i) the parent antigen, (ii) closely related isoforms of notexin and (iii) venoms from *Notechis* genus snakes. In contrast, they failed to recognize other purified PLA_2 or PLA_2-containing venoms from other origins. Substitutions or chemical modifications occurring in the C-terminal part of the polypeptide chain of notexin altered the binding affinity for antibodies, implying that this region constitutes an antigenic domain of notexin.

Snake venom; Notexin; Phospholipase A_2; RIA; Antibody

1. INTRODUCTION

Presynaptic phospholipases A_2 (PLA_2) from snake venoms block acetylcholine release from nerve endings, induce efficient flaccid paralysis [1] and thus provoke fatal respiratory failure. They are toxic in humans [2] and serotherapy constitutes the most efficient antidote treatment [3].

Notexin from the Australian tiger snake *Notechis scutatus scutatus* [4] is a monomeric PLA_2 with both presynaptic and myotoxic properties [1,5,6]. Despite the clinical importance of notexin, few studies have been devoted to its immunological properties [2,3,7]. The purpose of this study was (i) to examine the ability of anti-notexin antibodies to neutralize notexin lethality and enzymatic activity; (ii) to investigate the ability of antibodies to recognize a large range of purified PLA_2 and snake venoms containing PLA_2; and (iii) to identify antigenic domain(s) of notexin. The present data may be of importance for the design of more efficient protective antisera against notexin and related toxins.

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Abbreviations: PLA_2, phospholipase A_2; RP-HPLC, reverse-phase high-performance liquid chromatography; $O_2NPhSCl$, 2-nitrophenylsulfenyl chloride; PEG, poly(ethylene) glycol; LD_{50}, median lethal dose

2. MATERIALS AND METHODS

2.1. *Materials*

Notexin, notechis II-5 and notechis II-1 were purified from the venom of *N. scutatus scutatus* (Australian Reptile Park, Gosford, Australia), according to Karlsson et al. [4]. Notexin was further separated from an isoform called notechis N_S by reverse-phase high-performance liquid chromatography (RP-HPLC), as described [8]. Other PLA_2 were obtained as in [8].

2.2. *Methods*

Iodination with $Na^{125}I$ (Oris Industries, France) using Iodogen and modification by 2-nitrophenylsulfenyl chloride ($O_2NPhSCl$) were performed on notexin according to Fraker and Speck [9] and Scoffone et al. [10], respectively. Resulting

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derivatives of notexin were purified by RP-HPLC (Mollier et al., submitted).

For radioimmunoassays, 100 μl highly purified ^{125}I-labelled monoiodinated notexin (20000 cpm/assay, 3.7 TBq/mmol) was incubated overnight at 4°C with 100 μl antibody solution, 100 μl of purified unlabelled analogs or crude snake venoms dilutions, and 10 μl horse serum. 300 μl of a 20% poly(ethylene) glycol (PEG) 6000 solution was added and the mixture was centrifuged at 1000 × g for 30 min. Radioactivity in the pellets was measured in a gamma counter (LKB, Bromma, Sweden).

Immunized BALB/c mice received successively one intravenous injection of a sublethal dose of notexin (0.25 μg/20 g mouse) in physiological saline, three subcutaneous injections, one per week, of 0.30 μg/20 g mouse of notexin in physiological saline, and four subcutaneous injections, one per week, of increasing doses of notexin (from 10 to 75 μg) in physiological saline mixed with Freund's incomplete adjuvant (Difco, Detroit, MI) (5:3, v/v). Three days after the booster injection of notexin (75 μg), 3×10^5 Ehrlich tumor cells were injected intraperitoneally into the mice. Ascitic fluids containing polyclonal antibodies were precipitated with ammonium sulfate, then dialyzed three times for 24 h against 20 mM Tris-HCl (pH 7.7).

For toxicity measurements, native notexin was injected into the tail vein of four BALB/c mice IFFA CREDO (Lyon, France) (weight 20 ± 2 g), in the presence and absence of a 200-fold molar excess of antibodies. Mice were examined 24 h, 48 h and 1 week after injection.

Enzymatic activity was determined using a titrimetric method [11] with a Methrm pH-stat. Egg yolk phosphatidylcholine (type IX-E, Sigma) was sonicated under a nitrogen atmosphere for 30 min and then mixed with sodium deoxycholate in a molar ratio of 1:3.7. The final concentration of substrate was 45 mM in 20 mM $CaCl_2$, 120 mM NaCl and notexin concentrations varied between 0 and 9.2 nM. The assay volume was 4 ml and the reaction was monitored by addition of 10 mM NaOH, at pH 8.0 and 40°C. Antibodies used for enzymatic assays were dialysed three times for 24 h against 20 mM $CaCl_2$, 120 mM NaCl.

3. RESULTS AND DISCUSSION

3.1. *Radioimmunoassay and properties of anti-notexin antibodies*

Using Iodo-gen as a mild oxidizing agent [9], we prepared monoiodinated notexin with a specific activity equal to 1.8 or 3.7 TBq/mmol. We observed that native and mono-iodinated notexin (i) had virtually superimposable far-ultraviolet circular dichroic spectra (not shown) and (ii) had respective LD_{50} equal to 0.34 ± 0.03 and 0.38 ± 0.04 μg per 20 g mouse. Clearly, iodination had not altered the structural and basic biological properties of notexin.

In order not to bias the immunological response, we carefully avoided injecting detoxified notexin during the immunological procedure and therefore, mice received native notexin alone using low antigen doses. Nevertheless, antibody titers routinely reached 1/500–1/800, using ^{125}I-labelled notexin of specific activity 3.7 TBq/mmol (titer is defined as the serum dilution that binds half of total radioactivity). Precipitation of antigen-antibody complexes using poly(ethylene) glycol gave a mean standard variation equal to ± 3% and non-specific binding varied between 5 and 10% of the total radioactivity added in each assay.

Anti-notexin antibodies specifically abolished the lethal activity and greatly affected the enzymatic activity of notexin (table 1). However, enzymatic activity was never abolished completely, even in the presence of a large excess of antibodies. Approx. 4% of the original activity remained. Similarly, Caratsch et al. [12] reported that approx. 10% of the original enzymatic activity of β-bungarotoxin persisted in the presence of a large excess of polyclonal anti-β-bungarotoxin antibodies. The reason for this residual enzymatic activity is presently unclear.

3.2. *Competition data*

Natural isoforms as well as two chemically modified derivatives of notexin competed with ^{125}I-labelled notexin for binding to anti-notexin

Table 1

Effect of polyclonal antibodies on in vivo toxicity and enzymatic activity of notexin

Compound	Percent survival in mice	Enzymatic activity (μmol/min)
Notexin	0[a]	7360[e]
Notexin + polyclonal antibodies	100[b]	311[f]
Polyclonal antibodies	100[c]	N.D.
Notexin + $M\gamma_1$	N.D.	7360[g]
Tris buffer (20 mM, pH 7.7)	100[d]	N.D.

[a] 0.6 μg (44 pmol) notexin in 150 μl saline was injected intravenously in four BALB/c mice (LD_{100} value of notexin = 0.5 μg/20 g mouse). 8.8 nmol anti-notexin antibodies were incubated overnight in the presence ([b]) or absence ([c]) of notexin prior to injection; [d] the effect of the buffer was also examined; [e] the enzymatic activity was defined as the amount of substrate (in μmol) hydrolyzed per min and per μmol of enzyme at 40°C and pH 8.0; [f] polyclonal antibodies were used in a 200-fold molar excess over notexin. [g] $M\gamma_1$ is an unrelated monoclonal antibody; $M\gamma_1$ was used in a 200-fold molar excess over notexin. N.D., not done

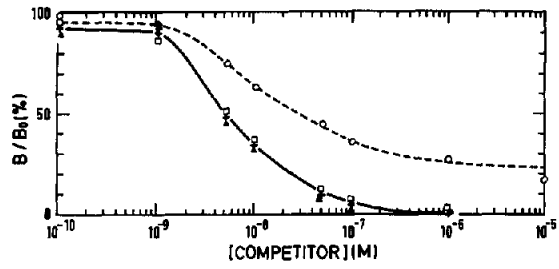

Fig.1. Experiments illustrating competition between ^{125}I-labelled notexin (3.7 TBq/mmol) and notexin (□), notechis N_s (+), notexin mono-NPhS (▲) and notexin di-NPhS-derivative (○) for binding to anti-notexin antibodies (1:500). B/B_0 was determined as the ratio (%) of radioactivity bound by antibodies in the presence (B) and absence (B_0) of competitor.

antibodies (fig.1). As shown in table 2 the binding affinities of notechis N_s and notechis II-5 were similar, if not identical to that of notexin, although they have one (position 16) and eight (positions 16, 20, 31, 47, 53, 54, 56 and 62) substitutions, respectively [13]. It is likely therefore that all these positions are excluded as major antigenic determinants of notexin. In contrast, concomitant incorporation of a nitrophenyl moiety via a thioether bond at the C-2 position of the indole ring of Trp-20 and Trp-110 led to a derivative, referred to as di-NPhS-notexin, which was recognized with weaker affinity. Previous work showed that modification of both tryptophans did not affect the overall architecture of notexin (Mollier et al., submitted). Since position 20 of notexin could be substituted in notechis II-5 (Trp is replaced by Arg) or modified with a nitrophenyl moiety without affecting binding affinity (table 2), we conclude that the alteration of antigenicity observed with di-NPhS-notexin was essentially due to modification of Trp 110. The data, therefore, strongly suggest that a critical antigenic area is located at, or in proximity to Trp 110, i.e. in the C-terminal region of the notexin polypeptide chain, a region which was proposed to be critical for the toxic activity in the case of both the PLA_2 from *Vipera ammodytes* [14] and notexin (Mollier et al., submitted). Notechis II-1 harbored as many as 40 substitutions [15] but nevertheless displayed substantial, though reduced, affinity for anti-notexin antibodies (table 2). Position 110 was substituted in notechis II-1, thus offering an explanation for its decrease in antigenicity.

Table 2

Competition between ^{125}I-notexin and different PLA_2 or venoms for anti-notexin polyclonal antibodies

	IC_{50}
Purified phospholipases A_2	(nM)
Notexin (*Notechis scutatus scutatus*)	5.0
Notechis N_s (*N. scutatus scutatus*)	5.0
Notexin modified at Trp-20	5.0
Notechis II-5 (*N. scutatus scutatus*)	7.0
Notechis II-1 (*N. scutatus scutatus*)	25.0
Notexin modified at Trp-20 and Trp-110	37.0
Nigexine (*Naja nigricollis*)	>1000
Na. nigricollis acidic PLA_2	>1000
Na. mossambica mossambica CM III	>1000
Na. mossambica mossambica CM II	>1000
β-Bungarotoxin (*Bungarus multicinctus*)	>1000
Crotoxin CA subunit (*Crotalus durissus terrificus*)	>1000
Crotoxin CB subunit (*C. durissus terrificus*)	>1000
C. durissus durissus PLA_2	>1000
C. adamanteus PLA_2	>1000
Bee venom PLA_2	>1000
Porcine pancreatic PLA_2	>1000
Crude venoms	(μg/ml)
N. scutatus scutatus	0.3
N. ater kangaroo	0.3
N. ater ater	0.4
N. ater serventyi	2.4
N. occidentalis	760
Pseudonaja nuchalis	>20000
Pa. butleri	>16000
Hoplocephalus stephensii	>20000
Acantophis antarticus	4000
A. barkley	>20000
Oxyuranus scutellatus	>20000
Na. melanoleuca melanoleuca	>20000
Na. haje (Mali)	>20000
Na. oxiana	>20000
Hemachatus haemachatus	>20000
Micrurus corallinus	>20000
Laticauda laticaudata	>20000
Aipysurus laevis	>20000
Vipera russelli	>20000
Agkistrodon rhodostoma	>20000
Bitis arietans	>20000
C. adamenteus	>20000

Specific radioactivity of ^{125}I-notexin was 3.7 TBq/mmol; polyclonal antibodies were used at a concentration corresponding to the titer (1:200); IC_{50}, concentration of competitor (purified proteins or crude venoms) which inhibits 50% of the binding of ^{125}I-notexin to polyclonal antibodies

As shown also in table 2, other purified PLA_2 from land and sea snakes venoms, bee venom and mammalian pancreas failed to compete with ^{125}I-

labelled notexin for binding to anti-notexin antibodies. Examination of amino acid sequences [16] revealed that these PLA_2 harbor more than 50 substitutions including some in the C-terminal area, but we do not yet know which particular substitution(s) render(s) the PLA_2 unrecognizable by anti-notexin antibodies.

To investigate the specificity of the anti-notexin antibodies further, we also examined their ability to recognize 22 different snake venoms (table 2). The few venoms that contained components antigenically related to notexin were from snakes of the *Notechis* genus and, to a substantially lesser extent, the venom of the Australian snake *Acantophis antarticus*. All other venoms, many of which are known to contain PLA_2, were unrecognized by anti-notexin antibodies, even when added in large excess.

The present paper conclusively demonstrates that anti-notexin antibodies from mice (i) are capable of neutralizing notexin activity; (ii) are highly specific for notexin and closely related homologs and (iii) recognize the highly variable C-terminal polypeptide chain as an antigenic region. Because of their narrow specificity, mouse anti-notexin antibodies are unlikely to offer significant protection against other presynaptic PLA_2.

In the course of the last decade, a number of studies have been devoted to the study of the immunological properties of various presynaptic PLA_2 [17]. These toxins include β-bungarotoxin from *Bungarus multicinctus* [18], crotoxin from *Crotalus durissus terrificus* [19,20] and taipoxin from *Oxyuranus scutellatus scutellatus* [21]. These studies indicated that poly- and monoclonal antibodies raised against these toxins are specific for the parental antigens. Our observations, therefore, are in support of this being a general property of anti-presynaptic PLA_2 antibodies. This conclusion is all the more interesting as Minton [22] reported, some years ago, that antiserum to tiger snake venom was exceptionally efficient at neutralizing other venoms. Conceivably, the broad specificity of antiserum against the venom of *N. scutatus scutatus* might be due to antibodies raised against notexin homologs present in the venom, such as notechis II-1 [15] as suggested by recent experiments of Nicholson et al. [23].

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