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Evidence that the anti-coagulant and lethal properties of a basic phospholipase A_2 from snake venom are unrelated

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The basic phospholipase A_2 (PLA_2) from venom of the African elapid *Naja nigricollis* was previously shown to have anti-coagulant and lethal properties, both of which were abolished by treatment with *p*-bromophenacyl bromide (pBP). In the present paper we first report that pBP-treated PLA_2 is capable of inhibiting the anti-coagulant activity but not the lethal activity of native PLA_2, thus suggesting that both properties might be independent. We then confirm this evidence using PLA_2-specific monoclonal immunoglobulins. One of these, called HSF, neutralized the lethal activity but not the anti-coagulant activity, whereas another antibody, called HSP2, inhibited the anti-coagulant activity but not the lethal activity of the PLA_2. The data presented in this paper are taken as evidence that the anti-coagulant activity is not implicated in the lethal effects of basic PLA_2 from *Naja nigricollis*.

Phospholipase A_2; Monoclonal antibody; Anti-coagulant activity; Toxicity

1. INTRODUCTION

Snake venom phospholipases A_2 (PLA_2) belong to a family of structurally homologous [1-3] proteins that exhibit esterase activity (EC 3.1.1.4). They display a variety of pharmacological effects which sometimes render them highly toxic. Snake venom PLA_2 can be classified into three groups, depending on their toxicity. The first group comprises acidic and neutral PLA_2 with little or no toxicity. The second group comprises highly toxic PLA_2 which block acetylcholine release from nerve endings [4,5] and/or provoke muscle damage [6]. The third group comprises relatively toxic and basic ($pI > 9$) PLA_2 with strong in vitro anti-coagulant activity associated with a marked ability to penetrate densely packed phospholipid monolayers [7]. Anti-coagulant PLA_2 have been isolated from *Crotalidae* [8], *Viperidae* [9] and *Elapidae* [10] venoms. The most active of them is the basic PLA_2 from the African spitting cobra *Naja nigricollis* [7].

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The strong anti-coagulant activity was attributed to the hydrophobic character of basic PLA_2 [11,12] and to the amino acid sequence between residues 54 and 77 [13]. In the present study we used (i) a chemical derivative modified with *p*-bromophenacyl bromide and (ii) specific monoclonal antibodies to demonstrate that the anti-coagulant and lethal activities of basic PLA_2 from *Naja nigricollis* are independent properties.

2. MATERIALS AND METHODS

Lyophilized *Naja nigricollis* venom was supplied by the Institut Pasteur (Paris, France). Porcine pancreatic PLA_2 was purchased from Boehringer Mannheim (Penzberg, FRG). All chemicals were of the purest grade commercially available.

Purification of basic PLA_2 from *Naja nigricollis* venom was performed as previously described [14]. The last step involved a reverse-phase high-performance liquid chromatography on a butyl large-pore column (4.6 × 250 mm) using an acetonitrile gradient in 0.1% trifluoroacetic acid: 28% to 32% CH_3CN for 120 min at a flow rate of 750 μl/min. The basic PLA_2 was previously designated as nigexine [14] and its primary sequence was recently elucidated (submitted for publication). Modification with *p*-bromophenacyl bromide (pBP) was carried out as previously described [15] and pBP-treated PLA_2 was purified by reverse-phase high-performance liquid chromatography on a butyl large-pore column (4.6 × 250 mm) using a 120 min linear

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acetonitrile gradient (29% to 34%) in 0.1% trifluoroacetic acid, at a flow rate of 750 μl/min [16]. Amino acid analysis of pBP-treated PLA_2 indicated the unique loss of one histidine residue per molecule and the molar extinction coefficient at 276.5 nm was determined as 45 500.

Immunization in BALB/c mice consisted of six subcutaneous injections, one every two weeks. Each animal was successively injected with: (1) 200 μg PLA_2 mixed with Freund's complete adjuvant (1:1; v/v); (2) 200 μg PLA_2 mixed with Freund's incomplete adjuvant; (3) 400 μg PLA_2 mixed with Freund's incomplete adjuvant; (4) 100 μg PLA_2 in physiological saline; (5 and 6) 200 μg PLA_2 in physiological saline. The fusion procedure was carried out according to Köhler and Milstein [17]. Hybrid-containing wells were tested by liquid-phase radioimmunoassay using ^{125}I-labelled PLA_2. Positive wells were cloned by limiting dilution. Monoclonal antibodies were produced in ascitic fluids in syngenic BALB/c mice. Antibodies (HSF, IgG1κ; HSP1, IgG1κ; HSP2, IgG2aκ) were purified in batches according to Reik et al. [18] using caprylic acid, followed by ammonium sulfate precipitation. The last purification step consisted of ion-exchange high-performance liquid chromatography on a column of polyvinylimidazole silica-bonded phase, a weak anion-exchange resin (SFCC, Neuilly-Plaisance, France). The purity of monoclonal antibody preparations was assessed by polyacrylamide gel electrophoresis in the presence of SDS (Phast System, Pharmacia).

Calcium clotting time assays [19] were carried out on platelet suspensions using standard platelet concentrates purchased from CNTS (les Ulis, France). Platelets ($3.5 \pm 0.2 \times 10^5$ cells per μl) were suspended in saline buffer containing 145 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 10 mM glucose and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid/NaOH buffer, pH 7.4 (Hepes-buffered saline). 200 μl of a mixture of 100 μl of the sample to be tested in Hepes-buffered saline and 100 μl of a solution containing 20 mM $CaCl_2$ and 0.14 M NaCl, were added to 200 μl of the platelet suspension in a 5-ml glass test-tube, all solutions being maintained at 37°C.

Median lethal dose (LD_{50}) determinations were carried out using the sequential up-and-down method of Dixon and Mood [20]. Female BALB/c mice (20 ± 1 g) were injected in the tail vein with various amounts of: (i) basic PLA_2; (ii) pBP-treated PLA_2; (iii) pBP-treated PLA_2 mixed with native PLA_2; or (iv) antibody-PLA_2 complex. Antibodies and pBP-treated PLA_2 were incubated with the basic PLA_2 for 4 h at 37°C. Eight mice were injected per dose and survivors were counted 48 h after injection.

3. RESULTS AND DISCUSSION

3.1. *pBP-treated PLA_2: an inhibitor of anti-coagulant activity*

The basic PLA_2 from *Naja nigricollis* venom is a potent in vitro anti-coagulant factor [7,10,21]. As illustrated in fig. 1A, the control clotting time of platelets was equal to 150 s and a concentration of 2×10^{-8} M PLA_2 was sufficient to double this time. Treatment with pBP, a reagent which modifies a critical histidine residue at the PLA_2 active site [15], resulted in a derivative with 1/750 of the anti-coagulant activity of native PLA_2 (fig. 1A). Two lines of evidence indicated that this residual activity was not due to traces of contaminating native PLA_2. Firstly, pBP-treated PLA_2 was highly pure as judged from several analytical criterions reported elsewhere [14]. Secondly, its enzymatic activity, if any, was less than 0.01% of that of native PLA_2 [16]. The residual anti-coagulant activity of pBP-treated PLA_2 is likely, therefore, to be an intrinsic property of the molecule. Decreases in anti-coagulant after treatment of PLA_2 with pBP has also been observed by other authors [7].

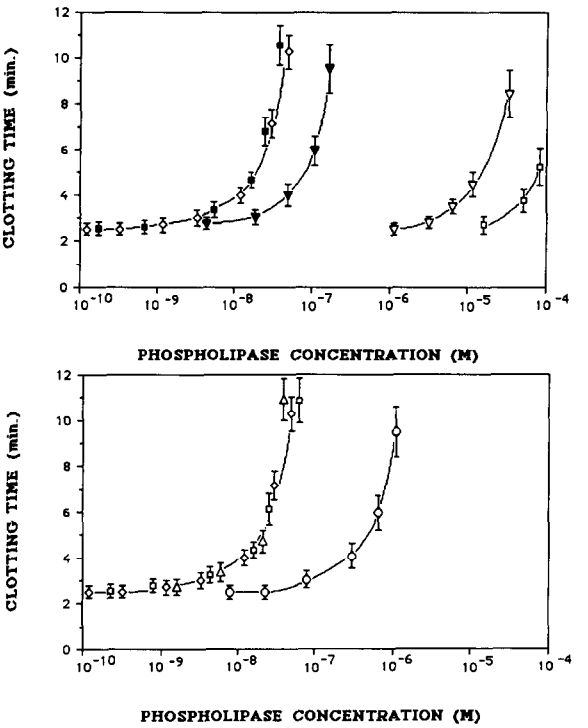

Fig.1.(A) Anti-coagulant activity of various PLA_2 and of a chemical derivative of PLA_2, alone or combined. Activities were determined as described in B: basic PLA_2 from *Naja nigricollis* venom (◇), pBP-treated PLA_2 (▽), porcine pancreatic PLA_2 (□), basic PLA_2 in the presence of 8×10^{-7} M pBP-treated PLA_2 (▼), and basic PLA_2 in the presence of 8×10^{-7} M porcine pancreatic PLA_2 (■). (B) Effect of different monoclonal antibodies on the anti-coagulant activity of basic PLA_2 from *Naja nigricollis* venom. The coagulation times for the platelet suspensions (1.7 ± 0.2 10^5 cells/μl, final concentration) after addition of calcium were measured (see section 2): in the absence of antibody (◇), in the presence of a 50-fold molar excess of HSP2 antibody (○), and in the presence of a 500-fold molar excess of HSF (□) or HSP1 (△) antibody.

Strikingly, we observed that pBP-treated PLA_2 inhibited the clotting effect of native PLA_2. In the presence of a non-anti-coagulant dose of 8×10^{-7} M pBP-treated PLA_2, a concentration of 8×10^{-8} M of native PLA_2 was required to double the coagulation time (fig. 1A), or, in other words, the activity of basic PLA_2 preincubated with pBP-treated PLA_2 decreased by 75%. This large change indicated that the inhibitory activity was not due to contaminating PLA_2 but was a genuine property of the derivative. We also observed that the anti-coagulant activity of native PLA_2 was unaffected by addition of 8×10^{-7} M porcine pancreatic PLA_2, a non-toxic PLA_2 with weak anti-coagulant activity (fig. 1A), indicating that inhibition of the anti-coagulant effect by pBP-treated PLA_2 was specific. Interestingly, it was previously shown that pBP-treated PLA_2 is still able to bind phospholipids [21]. Conceivably, the inhibitory property of pBP-treated PLA_2 was due to its ability to prevent native PLA_2 from binding to coagulation specifying phospholipids [22], perhaps by a competitive process.

Although the anti-coagulant properties of snake venom PLA_2 have been extensively studied, it is not yet clear whether this activity is related to the lethal activity of the molecule. We reasoned that if both properties are closely linked, pBP-treated PLA_2 might inhibit the lethal action of the native molecule, as it inhibited anti-coagulant activity. We therefore injected mice intravenously with various amounts of native PLA_2 mixed with a 100-fold molar excess of non-toxic pBP-treated PLA_2 (table 1) and found that the original toxicity of native PLA_2 remained unchanged (table 1). We considered this result as a preliminary indication that the lethal and anti-coagulant activities of basic PLA_2 might be unrelated properties. This possibility was subsequently confirmed.

3.2. *Monoclonal antibodies can discriminate between anti-coagulant and lethal activities of basic PLA_2*

Monoclonal antibodies bind to discrete areas which can incorporate as many as 16 amino acid residues [23], and constitute ideal tools for targetted probing of differend regions of protein surfaces. Using the procedure of Köhler and Milstein [17], we isolated three hybridomas secreting monoclonal antibodies designated HSF, HSP1, and HSP2 which all bind specifically to ^{125}I-labelled basic PLA_2 (not shown).

We first investigated the effect of each antibody on lethality (table 1). In the presence of a 50-fold molar excess of HSF, 5 LD_{50} of PLA_2 could be injected intravenously in mice without inducing any mortality. Clearly, a single monoclonal antibody was sufficient to neutralize lethality. This observation was all the more interesting as the other two antibodies did not exhibit this property since addition of a 50- to 500-fold molar excess of either HSP1 or HSP2 did not change the lethal potency of PLA_2 (table 1). The mechanisms associated with the neutralizing property of HSF are unknown.

We then investigated the effect of the antibodies on anti-coagulant activity. As shown in fig. 1B, this property remained unchanged when PLA_2 was incubated in the presence of a 500-fold molar excess of HSF or HSP1. The epitopes recognized by these two antibodies were clearly unrelated to the area associated with the anti-coagulant property of PLA_2. In sharp contrast, in the presence of a 50-fold molar excess of HSP2, anti-coagulant activity decreased dramatically: a concentration of 4×10^{-7} M of PLA_2 was required to double the coagulation time. In other words the residual coagulant activity of the PLA_2-HSP2 complex was approx. 5% of that of the PLA_2 alone. The mechanisms associated with the capacity of HSF to neutralize anti-coagulant activity are as yet unknown. However, it will be of interest, in the future, to examine whether the epitope recognized by this antibody is located within the region comprising residues 54–77, which has been suggested to be associated with the anti-coagulant activity of basic PLA_2 [13].

The data reported above clearly show that three PLA_2-specific monoclonal antibodies have different properties. Firstly, HSF neutralized lethality but not anti-coagulant activity of PLA_2. This result constituted in itself a strong indication that lethal and anti-coagulant activities were independent properties. Secondly, HSP2 was observed to be a potent inhibitor of anti-coagulant activity but did not neutralize the lethal potency of PLA_2. This result agreed with the previous conclusion that the two properties were independent, although it should be emphasized that the inability of HSP2 to neutralize in vivo lethal activity could not be interpreted completely satisfactorily, since the original

Table 1

In vivo toxicity of basic PLA_2 from *Naja nigricollis* venom in the presence of monoclonal antibodies or pBP-treated PLA_2

	Median lethal dose (in nmol phospholipase per kg bodyweight)
Basic phospholipase	30.2 ± 1.1
Basic phospholipase + HSF mAb	>180
Basic phospholipase + HSP1 mAb	30.3 ± 1.5
Basic phospholipase + HSP2 mAb	30.0 ± 2.0
pBP-phospholipase	>7100
Basic phospholipase + pBP-phospholipase	30.0 ± 1.9

The monoclonal antibodies were preincubated with basic PLA_2 for 4 h at 37°C. The HSF antibody was used at a 50-fold molar excess over PLA_2, and the HSP1 and HSP2 antibodies were used at a 500-fold molar excess. When mixed with native PLA_2, the amount of pBP-treated PLA_2 used was 3700 nmol per kg bodyweight. The preparations were injected intravenously in physiological solutions using female BALB/c mice weighing 20 ± 1 g

properties of HSP2 could be altered in the blood stream by a multiplicity of uncontrolled factors. Thirdly, HSP1 had no effect on either property of PLA_2 and could be regarded as a negative control.

4. CONCLUSION

The two following conclusions could be drawn from the present study. Firstly, pBP-treated PLA_2 inhibited the anti-coagulant activity but not the lethal property of native PLA_2. Secondly, monoclonal antibodies could selectively inhibit one of the two properties but not both concomitantly. These data indicate that the anti-coagulant activity is not implicated in the lethal activity of basic PLA_2 from *Naja nigricollis*. Further work is now required to investigate (i) the inhibitory activity of pBP-treated PLA_2 towards other anti-coagulant agents; (ii) the effect of the different antibodies on the other properties of PLA_2, including esterase activity, cytotoxicity [14,16] and hemolytic activity and (iii) the localization of the different epitopes recognized by the monoclonal antibodies.

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