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CHEMICAL MODIFICATION OF HISTIDINE AND LYSINE RESIDUES OF MYOTOXIC PHOSPHOLIPASES A_2 ISOLATED FROM *BOTHROPS ASPER* AND *BOTHROPS GODMANI* SNAKE VENOMS: EFFECTS ON ENZYMATIC AND PHARMACOLOGICAL PROPERTIES

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C. Díaz-Oreiro and J. M. Gutiérrez. Chemical modification of histidine and lysine residues of myotoxic phospholipases A_2 isolated from *Bothrops asper* and *Bothrops godmani* snake venoms: effects on enzymatic and pharmacological properties. *Toxicon* **35**, 241–252, 1997.—Lysine and histidine residues of two myotoxic phospholipases A_2, *Bothrops asper* myotoxin III and *Bothrops godmani* myotoxin I, were chemically modified in order to study the effects of these treatments on enzymatic and pharmacological properties. After lysine acetylation the overall basicity of these toxins was lost and their enzymatic activity was significantly reduced, although a residual effect remained, which corresponded to 25% of the activity of native toxins. This treatment abolished both myotoxic and anticoagulant effects, and partially reduced liposome-disrupting activity. Histidine alkylation with *p*-bromophenacyl bromide affected phospholipase A_2, myotoxic and anticoagulant effects in a parallel way. After 24 hr of incubation with the alkylating reagent, these three activities were totally inhibited, in contrast to the liposome-disrupting effect which was only partially affected by this treatment. It is suggested that: (1) catalytic activity plays a role in the pharmacological effects of these myotoxins; (2) lysine residues are relevant for the toxic effects induced by these phospholipases A_2; and (3) despite the apparent relevance of enzymatic activity to the pharmacological properties of these toxins, the dissociation observed in lysine acetylation experiments suggests that these myotoxins have a molecular region, different from the catalytic site, which might be also involved in the toxic effects observed. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Several basic phospholipases A_2 which induce myotoxicity have been purified from *Bothrops* snake venoms (Gutiérrez and Lomonte, 1995). Although they share common biochemical and immunochemical properties, marked differences in catalytic activity have been observed. Some myotoxic phospholipases have high enzymatic activity, whereas

others either lack or have extremely low phospholipase A_2 activity (Gutiérrez and Lomonte, 1995). Several catalytically inactive variants have lysine instead of aspartate in position 49, a modification that impairs the enzyme's ability to bind calcium, thereby affecting the catalytic mechanism (Francis *et al.*, 1991; Cintra *et al.*, 1993).

It has been proposed that Lys-49 myotoxic phospholipases A_2 induce myotoxicity *in vivo* and affect cells in culture by a mechanism independent of enzymatic phospholipid degradation (Gutiérrez *et al.*, 1989; Díaz *et al.*, 1991; Gutiérrez and Lomonte, 1995). It was hypothesized that enzymatically inactive myotoxic phospholipase-like variants have a molecular region that disrupts bilayers. It has been proposed that *Bothrops asper* myotoxin II, a Lys-49 variant (Francis *et al.*, 1991), has a stretch of residues located at the C-terminal region, rich in hydrophobic and basic amino acids, responsible for cytolysis (Lomonte *et al.*, 1994b).

In the case of Asp-49 enzymatically active *Bothrops* myotoxic phospholipases A_2, the role of catalysis in their pharmacological effects has not been established. A dissociation between enzymatic and myotoxic activities has been observed with *B. asper* myotoxin I using monoclonal antibodies (Lomonte *et al.*, 1992) and with *B. asper* myotoxin III in experiments with heparin (Lomonte *et al.*, 1994a), EDTA (Bultrón *et al.*, 1993a) and chemical modification with *p*-bromophenacyl bromide (Bultrón *et al.*, 1993b). Thus, it was suggested that Asp-49 *Bothrops* myotoxins have a region, distinct from the catalytic site, which plays a role in myotoxicity (Bultrón *et al.*, 1993b; Gutiérrez and Lomonte, 1995). This hypothesis needs to be tested using a variety of experimental protocols.

Chemical modification is a useful approach to study this issue. In the present work we have chemically modified *B. asper* myotoxin III and *Bothrops godmani* myotoxin I by two different procedures: alkylation of histidine residues with *p*-bromophenacyl bromide and modification of lysine residues by acetylation. The effects of these treatments on the enzymatic and pharmacological activities of these myotoxic phospholipases A_2 are described.

MATERIALS AND METHODS

Snake venom phospholipases

Bothrops asper myotoxin III (*B. asper* MT-III) and *B. godmani* myotoxin I (*B. godmani* MT-I) were purified by ion-exchange chromatography on CM-Sephadex C-25, as described by Lomonte and Gutiérrez (1989) and Díaz *et al.* (1992), respectively. Homogeneity was demonstrated by gel electrophoresis on two different systems: polyacrylamide gel electrophoresis (PAGE) at pH 4.5 (Reisfeld *et al.*, 1962) and sodium dodecyl sulfate (SDS)–PAGE (Laemmli, 1970).

Lysine acetylation with acetic anhydride

Modification of lysine residues was performed at a protein:reagent molar ratio of 1:50. Protein (3 mg) was dissolved in 1.5 ml of 0.2 M Tris–HCl buffer, pH 8.0, and 10 μl of acetic anhydride (Aldrich, U.S.A.) was added. The pH was adjusted again to 8.0 with NaOH after the addition of acetic anhydride. After 1 hr of incubation at 25°C, protein was separated from the free reagent by gel filtration on a Sephadex G-25 column equilibrated with 0.05 M ammonium acetate buffer, pH 6.8, then the protein was lyophilized. Control protein was subjected to gel filtration under the same conditions used for the protein incubated with acetic anhydride. PAGE was performed at pH 4.5 (Reisfeld *et al.*, 1962) and pH 8.6 (Davis, 1964) to determine the charge characteristics of modified toxins. In addition, both native and modified toxins were analysed by high-performance liquid chromatography (HPLC; Waters 600E chromatograph with automatic injector Waters 712 WISP) on a reverse-phase C_4 column (Vydac) of 1 cm diameter and 25 cm length. Chromatographic separation was performed using a flow rate of 2 ml/min and a pressure of 1200 p.s.i. Elution was carried out with a linear gradient from 0 to 60% acetonitrile in 0.1% trifluoroacetic acid.

Modification with p*-bromophenacyl bromide*

Modification of histidine residues was performed at protein:reagent molar ratio of 1:3. Protein (3 mg) was dissolved in 1.0 ml of 0.1 M Tris–HCl buffer, pH 8.0, containing 0.7 mM EDTA, and 125 μl of *p*-bromophenacyl bromide (pBPB) solution (1.5 mg/ml in ethanol) was added. Incubations were carried out for 1, 3 and 24 hr at 25°C. Protein was then separated from the free reagent by gel filtration on Sephadex G-25, as described for acetylation experiments. Control protein was incubated with 125 μl of ethanol and subjected to gel filtration under the same conditions. Proteins were then lyophilized. The protein concentration of dissolved samples was determined by recording the absorbance at 280 nm and by the method of Spector (1978). Amino acid analyses were performed in order to corroborate that histidine residues were modified. Protein samples (1–2 nmoles) were hydrolysed with 6 N HCl in sealed vials for 20 hr at 110°C under vacuum. Amino acid composition was then determined using a Beckman 6300 amino acid analyser.

Phospholipase A_2 activity

Enzymatic activity was tested using egg yolk phospholipids as substrate. Egg yolk (1 ml) diluted 1:5 with 0.1 M Tris, 10 mM $CaCl_2$ buffer, pH 8.5, in the presence of 1% Triton X-100, was incubated with various amounts (1–30 μg) of either native or modified toxins for 20 min at 37°C. After incubation, free fatty acids were extracted and titrated according to the method of Dole (1956). The activity of modified toxins was expressed as a percentage, taking as 100% the enzymatic activity of the corresponding native toxins.

Myotoxic activity

Swiss–Webster mice (18–20 g body weight) were injected i.m. in the right gastrocnemius with 80 μg of either native or modified myotoxins (dissolved in 80 μl of distilled water). Control mice received 80 μl of water. Animals were bled from the tail 3 hr after injection and blood was collected in heparinized capillary tubes and centrifuged to obtain the plasma. Creatine kinase (CK) activity in plasma was determined using the Sigma kit 520. Activity was expressed in units (U)/ml, 1 U resulting in the phosphorylation of 1 nmole of creatine per minute at 25°C.

Effect on negatively charged liposomes

Liposomes (L-α-phosphatidylcholine, 63 μmoles; dicetyl phosphate, 18 μmoles; cholesterol, 9 μmoles; Sigma kit L-4262) were prepared as described by Díaz *et al.* (1991). The effect of native and modified myotoxins on liposomes was tested in microtitre plates by incubating 20 μl of liposome suspension and 20 μl of solutions of varying concentration of myotoxins (8–500 μg/ml) for 30 min at 37°C. Then, 40 μl of peroxidase substrate (2.5 mM 5-aminosalycilic acid, 0.025% H_2O_2, pH 6.0) was added and the colour reaction was stopped with 6 N H_2SO_4. Absorbances were recorded at 492 nm. Absorbances of liposome samples incubated with only water were used as negative controls and subtracted from the absorbances of all experimental samples. Peroxidase release was expressed as a percentage, taking as 100% the absorbances of samples in which liposomes were incubated with 0.2% Triton X-100.

Anticoagulant effect

Sheep platelet-poor plasma was prepared by centrifugation of citrated blood twice at 1000 ***g***. Plasma (250 μl) was incubated with various concentrations of toxins (0.001–20 μg/ml), dissolved in 100 μl of distilled water. Incubations were carried out for 10 min at 37°C, 50 μl of 0.25 M $CaCl_2$ was added and clotting times were recorded. Observations were carried out for a maximum period of 45 min.

Statistical analysis

The Student's *t*-test was used to determine the significance of the differences between the mean values of two experimental groups.

RESULTS

Effects of lysine acetylation

The overall basicity of both toxins was lost after treatment with acetic anhydride, since neither of them entered into polyacrylamide gels run at pH 4.5, in contrast to native toxins

(results not shown). In agreement with these observations, acetylated toxins behaved as acidic proteins on PAGE at pH 8.6. When analysed by reverse-phase HPLC, both native and modified toxins gave single peaks, although modified toxins eluted with higher retention times.

Acetylation of lysine residues significantly reduced the enzymatic activity of both myotoxins (Table 1). However, a residual activity was detected, corresponding to 26% and 24% of the activities of native *B. asper* MT-III and *B. godmani* MT-I, respectively. Lysine modification abolished myotoxicity induced by both myotoxins (Fig. 1). There was no significant difference ($P > 0.1$) in the plasma CK levels between mice injected with modified toxins and those injected with distilled water (Fig. 1).

Liposome-disrupting activity of both myotoxins was significantly, but not totally, reduced after acetylation (Fig. 2). In the case of *B. godmani* MT-I this activity was significantly lowered at all toxin concentrations tested. However, in the case of *B. asper* MT-III liposome-disrupting activity was significantly reduced when tested at toxin concentrations of 0.125 mg/ml and 0.031 mg/ml, although no difference was observed between native and modified toxins at a concentration of 0.5 mg/ml (Fig. 2). At the concentrations tested, the anticoagulant effect of both myotoxins was eliminated as a consequence of lysine acetylation (Fig. 3).

Effects of histidine alkylation

Amino acid analysis of modified myotoxins indicated that treatment with pBPB resulted in the modification of one of the two histidine residues present in *B. godmani* MT-I and of the only histidine present in *B. asper* MT-III (results not shown).

A time-dependent reduction in enzymatic activity was observed (Table 1). After 24 hr of incubation with pBPB, both toxins were devoid of catalytic activity. However, when toxins incubated with pBPB for 1 and 3 hr were tested, the phospholipase A_2 activity of *B. asper* MT-III was reduced to 35% and 22%, respectively. In contrast, the enzymatic activity of *B. godmani* MT-I remained at higher levels after incubation for 1 hr (89% residual activity) and 3 hr (71% residual activity; Table 1).

Regarding myotoxic effect, *B. asper* MT-III was affected to a greater extent than *B. godmani* MT-I when treated with pBPB. After 1 hr of incubation with pBPB, the plasma CK activity of mice injected with modified *B. asper* MT-III corresponded to 18% of the CK levels of mice injected with native toxin (Fig. 4). Furthermore, when incubation with pBPB was extended to 24 hr, myotoxic activity of *B. asper* MT-III was abolished (Fig. 4). In the case of *B. godmani* MT-I, treatment with pBPB also affected myotoxicity, but to a lesser extent. After incubation times of 1 and 3 hr there was a reduced, but still significant, myotoxic effect (Fig. 4). However, when this myotoxin was treated with the alkylating reagent for 24 hr, myotoxicity was eliminated (Fig. 4).

Table 1. Enzymatic activity of *Bothrops* myotoxins after histidine alkylation and lysine acetylation

Myotoxin	Enzymatic activity after:			
	p-Bromophenacylation			Acetylation
	1 hr	3 hr	24 hr	1 hr
B. asper myotoxin III	35 ± 1.0%	22 ± 1.0%	0%	26 ± 1.0%
B. godmani myotoxin I	89 ± 1.0%	71 ± 2.2%	0%	24 ± 1.0%

Activity is expressed as a percentage, taking as 100% the enzymatic activity of native proteins. Results are presented as mean ± S.D. ($n = 3$).

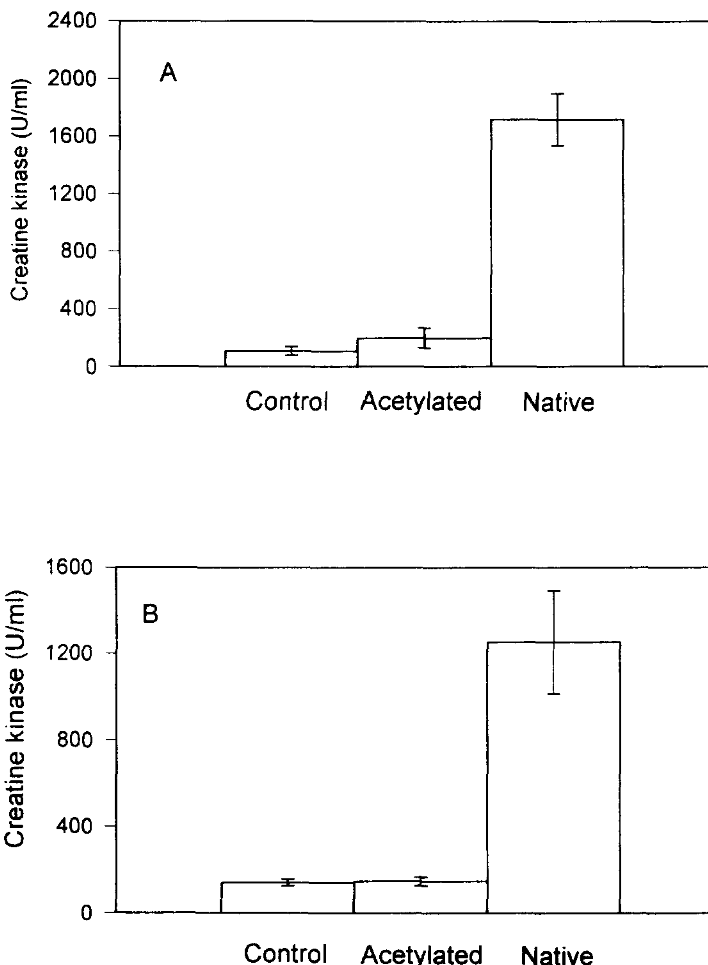

Fig. 1. Myotoxic effect of native and acetylated *B. asper* MT-III (A) and *B. godmani* MT-I (B), as judged by quantification of plasma CK levels.
Acetic anhydride treatment of toxins was carried out for 1 hr at 25°C. Mice were injected with 80 μg of each toxin and bled after 3 hr for determination of plasma CK levels. One CK unit results in the phosphorylation of 1 nmole of creatine per minute at 25°C. Results are presented as mean $\pm$ S.D. ($n = 6$).

Modification of histidine also affected the liposome-disrupting activity of myotoxins. As in the case of phospholipase A_2 and myotoxic activities, incubation with pBPB affected *B. asper* MT-III to a greater extent than *B. godmani* MT-I (Fig. 5). A significant liposome-disrupting effect was observed even in myotoxins treated for 24 hr, despite these modified toxins having lost their enzymatic and myotoxic activities. Regarding the anticoagulant effect, histidine modification drastically reduced the action of myotoxins on plasma recalcification times (Fig. 6). Again, as in the case of phospholipase, myotoxic and liposome-disrupting activities, *B. asper* MT-III was affected to a greater extent than *B. godmani* MT-I. However, the anticoagulant activity of both myotoxins was abolished when incubations with the alkylating reagent were carried out for 24 hr.

DISCUSSION

Various myotoxic phospholipases A_2 have been purified from *Bothrops* snake venoms (Gutiérrez and Lomonte, 1995). Some of them have high enzymatic activity, whereas others either lack or have extremely low catalytic effect, a finding that has been attributed to the presence of critical substitutions in residues forming the calcium-binding loop (Francis *et al.*, 1991; Cintra *et al.*, 1993). In this study we selected *B. asper* MT-III and *B. godmani* MT-I, two enzymatically active myotoxins, for chemical modification studies in order to investigate the role of lysine and histidine residues in their pharmacological and catalytic activities.

As expected, lysine acetylation resulted in drastic changes in charge, both toxins becoming acidic as judged by their electrophoretic behaviour. This modification reduced

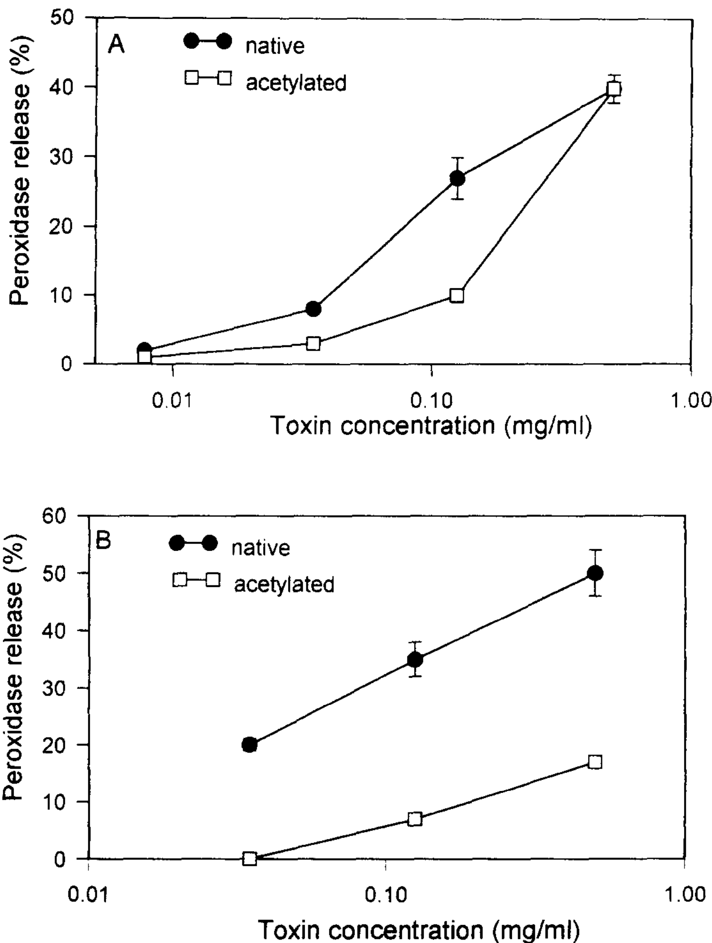

Fig. 2. Effect of native and acetylated *B. asper* MT-III (A) and *B. godmani* MT-I (B) on negatively charged liposomes.

Peroxidase-containing liposomes were incubated for 30 min at 37°C with either native or modified toxins, and peroxidase release was determined as detailed in Materials and Methods. Absorbance was recorded at 492 nm after addition of peroxidase substrate. Release is expressed as percentage, taking as 100% the peroxidase release from liposomes incubated with 0.2% Triton X-100. Results are presented as mean $\pm$ S.D. ($n = 6$).

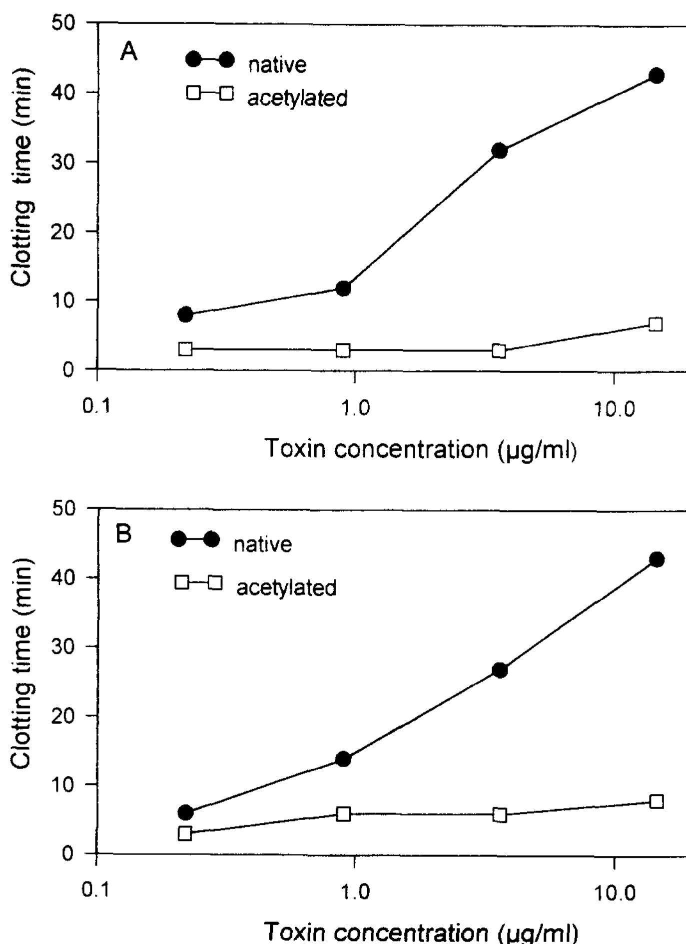

Fig. 3. Effect of native and acetylated *B. asper* MT-III (A) and *B. godmani* MT-I (B) on clotting time of sheep platelet-poor plasma.
Toxins were incubated with plasma for 10 min at 37°C, 0.25 M $CaCl_2$ was added and clotting times were recorded. Observations were carried out for a maximum period of 45 min. Results are presented as mean ± S.D. ($n = 6$).

enzymatic activity to a value of about 25% of the activity of native enzymes. In contrast, both myotoxic and anticoagulant effects were totally abolished, whereas a residual liposomal-disrupting effect remained. Our findings agree with previous studies in which the lysines of several phospholipases A_2 from the venoms of *Naja naja atra* and *Naja nigricollis* were modified by carbamylation (Condrea *et al.*, 1981), methylation (Ho *et al.*, 1986), ethoxyformylation and guanidination (Condrea *et al.*, 1983). These studies showed that such modifications had a greater effect on some pharmacological properties than on the enzymatic activity of the toxins, a conclusion also reached by Babu and Gowda (1994) after guanidination of lysine residues of a basic phospholipase A_2 from *Vipera russelli*. Thus, lysines seem to play a critical role in the toxicity of snake venom phospholipases A_2.

Acetylated myotoxins retained significant liposome-disrupting activity, indicating that these phospholipases can disrupt bilayers even after extensive lysine modification. The lack

of correlation between the ability of these toxins to induce liposome disruption and their myotoxicity agrees with previous observations of Díaz *et al.* (1991) and suggests that liposome and muscle membranes have different susceptibilities to these toxins. Furthermore, our results with acetylated myotoxins clearly show a dissociation between enzymatic and pharmacological (myotoxic and anticoagulant) activities, in agreement with previous studies with other phospholipases A_2 (Condrea *et al.*, 1981, 1983; Ho *et al.*, 1986; Babu and Gowda, 1994). These findings can be interpreted as evidence of the existence of molecular regions, distinct from the catalytic site, which may be responsible for at least some of the pharmacological properties of these toxins (Rosenberg, 1986; Kini and Evans, 1989; Bultrón *et al.*, 1993b). Our results also suggest that lysines play a role in the myotoxic and anticoagulant activities of these phospholipases A_2.

Alkylation of histidine residues with pBPB reduced the enzymatic activity of both myotoxins after incubations of 1 and 3 hr and, when treatment was extended to 24 hr, the catalytic effect was abolished. *Bothrops asper* MT-III was affected to a greater extent than *B. godmani* MT-I when incubations were performed for 1 and 3 hr. Interestingly, myotoxic

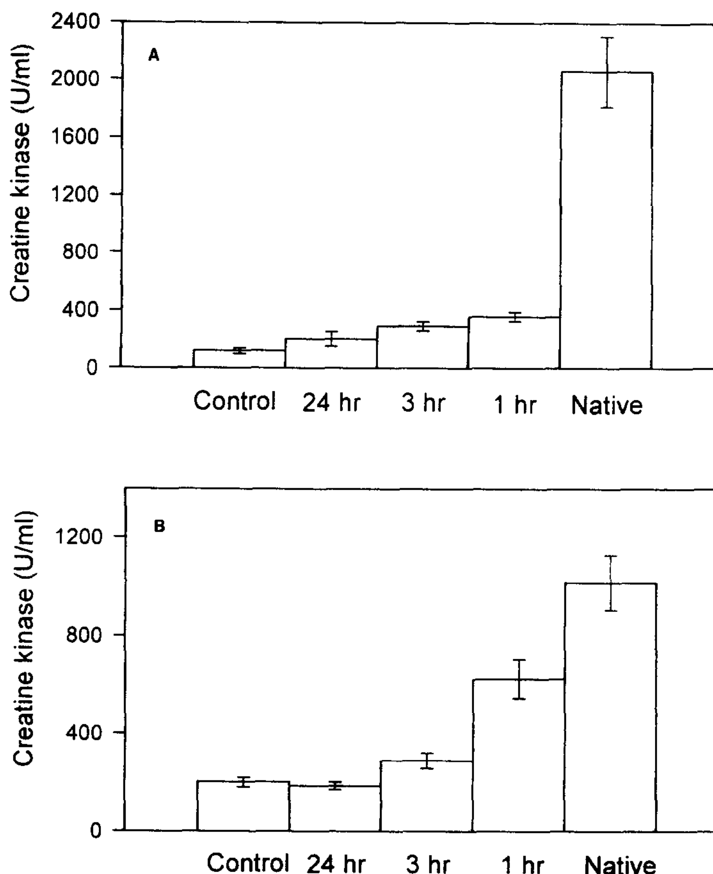

Fig. 4. Effect of *B. asper* MT-III (A) and *B. godmani* MT-I (B) treated with pBPB on plasma CK levels.

pBPB treatment of toxins was carried out for periods of 1, 3 and 24 hr at room temperature. Mice were injected i.m. with either native or modified toxins and CK levels were determined 3 hr after injection and expressed in U/ml. Results are presented as mean ± S.D. ($n = 6$).

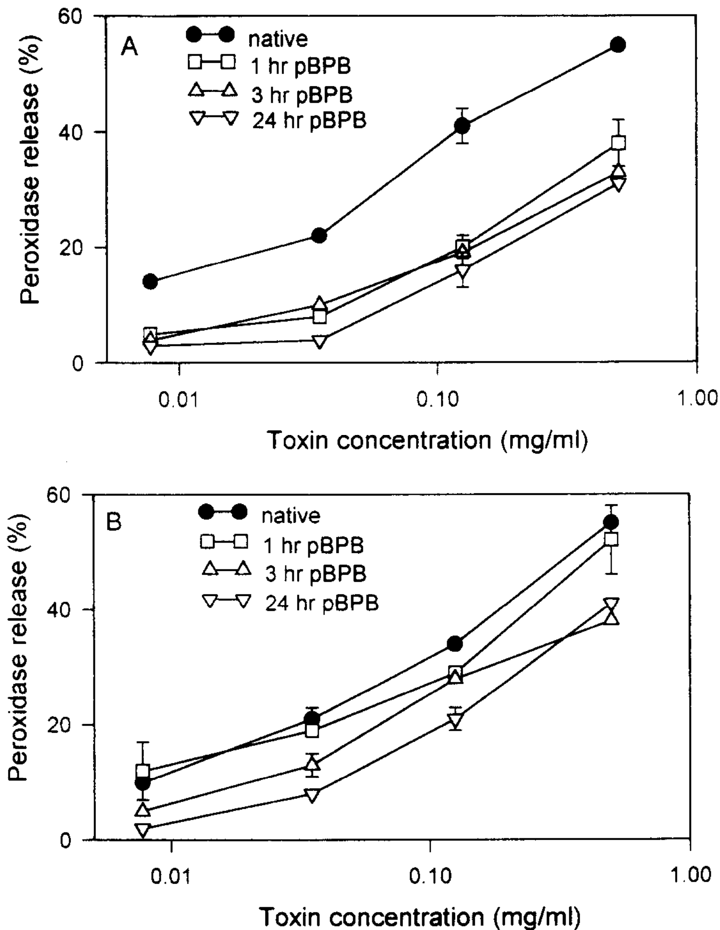

Fig. 5. Effect of *B. asper* MT-III (A) and *B. godmani* MT-I (B) treated with pBPB on negatively charged liposomes.
Results are expressed as a percentage, taking as 100% the peroxidase release from liposomes incubated with 0.2% Triton X-100. Results are presented as mean $\pm$ S.D. ($n = 6$).

and anticoagulant activities of both myotoxins were affected to a similar extent as enzymatic activity. After 24 hr of incubation with the alkylating reagent both activities were eliminated. As in the case of lysine acetylation, different results were obtained with the liposome-disrupting effect, since a significant activity was retained even when enzymatic activity had been abolished. It has been observed that *B. asper* myotoxin I and *B. atrox* myotoxin, two catalytically active variants, disrupt liposomes in conditions where enzymatic activity is inhibited, i.e. when calcium is eliminated and EDTA added (Díaz *et al.*, 1991).

In contrast to our results, earlier studies provided evidence of residual myotoxic and cytotoxic effects in *B. asper* MT-III even after inhibition of enzymatic activity by incubation with EDTA (Bultrón *et al.*, 1993a) or by pBPB treatment (Bultrón *et al.*, 1993b). The discrepancy between our data and these earlier studies may be due to the use of pharmacological and enzymatic assay systems of different sensitivities. In the case of *B. asper* MT-III and *B. godmani* MT-I, there was a parallelism between enzymatic activity

and myotoxic and anticoagulant effects after chemical modification with pBPB. Thus, pharmacological effects are likely to depend on the integrity of the catalytic active site and on enzymatic phospholipid hydrolysis. Alternatively, treatment with pBPB may induce conformational changes in other regions of the molecule responsible for the pharmacological effects induced by these toxins. Such conformational changes have been demonstrated by Renetseder *et al.* (1988) in the case of bovine pancreatic phospholipase A_2 after pBPB treatment.

In conclusion, our results suggest that the phospholipase A_2 activity of *B. asper* MT-III and *B. godmani* MT-I is relevant to their myotoxic and anticoagulant effects. In addition, lysine residues are critical for the induction of these pharmacological activities. The dissociation observed between the enzymatic and pharmacological effects after lysine modification suggests that, in addition to the catalytic site, these myotoxins have a molecular region involved in their toxic effects. Both the catalytic site and this

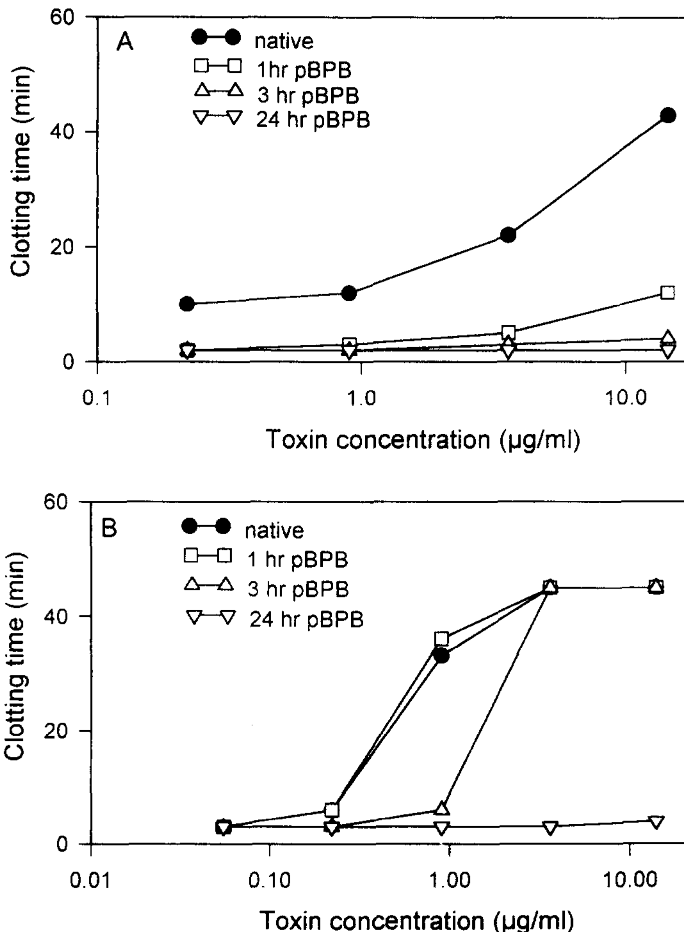

Fig. 6. Effect of *B. asper* MT-III (A) and *B. godmani* MT-I (B) treated with pBPB on clotting times of sheep platelet-poor plasma.

Toxins were incubated with plasma for 10 min at 37°C, 0.25 M $CaCl_2$ was added and clotting times were recorded. Observations were carried out for a maximum period of 45 min. Results are presented as mean ± S.D. ($n = 6$).

additional toxic region seem to be relevant in the pharmacological activities of these phospholipases A_2.

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