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Anticoagulant effect of myotoxic phospholipase A_2 isolated from the venom of the snake *Bothrops asper* (Viperidae)

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Resumen: La fosfolipasa A_2 miotóxica del veneno de *Bothrops asper* (terciopelo) prolonga el tiempo de recalcificación de plasmas de cinco especies de mamíferos. La fosfolipasa A_2 hidroliza los fosfolípidos del plasma, en tanto que su acción inhibitoria es revertida cuando se adicionan fosfolípidos plaquetarios. Estas dos observaciones sugieren que la acción anticoagulante se debe a una alteración de los fosfolípidos plasmáticos necesarios para la coagulación. Tanto la actividad enzimática como la anticoagulante se mantuvieron aún después de calentamiento a 95°C durante 10 min. Pese a su acción anticoagulante *in vitro*, la fosfolipasa A_2 no prolonga el tiempo de coagulación luego de inoculación intravenosa en ratones.

A basic monomeric phospholipase A_2 was isolated from *Bothrops asper* venom (Gutiérrez, Ownby & Odell 1984a, 1984b). This enzyme was initially described as a myotoxin due to its drastic effect on skeletal muscle cells, inducing extensive myonecrosis (Gutiérrez, Ownby & Odell 1984a). Other studies demonstrated that it also induces lethality, edema and anticoagulability (Gutiérrez *et al.* 1986). A variety of phospholipases A_2 are anticoagulant (Boffa *et al.* 1978; Verheij *et al.* 1980; Teng, Chang & Ouyang 1985). Verheij *et al.* (1980) observed that all potent anticoagulant phospholipases A_2 have a high isoelectric point and a high "penetrating power" on monomolecular phospholipid films. In this communication we report on the anticoagulant effect of *B. asper* myotoxic phospholipase A_2 as well as on some aspects related to the mechanism by which this toxin induces anticoagulability.

B. asper myotoxic phospholipase A_2 was isolated after two cycles of ion-exchange chromatography on CM-Sephadex C-25, as described by Gutiérrez *et al.* (1986). Homogeneity was demonstrated on disc-polyacrylamide gel electrophoresis (Reisfeld *et al.* 1962). The effect of the toxin on recalcification time was studied using platelet-poor plasma (Condrea *et al.* 1981) from five species; 0.5 ml of plasma were incubated for 10 min with different amounts of toxin (in 100μl). Then, 100 μl of 0.25 M $CaCl_2$ were added and clotting time was recorded. Observations were carried out for a maximum period of 45 min. In some experiments, platelet-rich plasma was used. The effects of addition of platelet phospholipids were also studied; briefly, a suspension of platelets was ultrasonicated and the phospholipid content of the homogenate was determined. Then, different amounts of platelet phospholipids were added to samples of 0.5 ml of platelet-poor plasma that had been incubated with 2 μg of myotoxin. After addition of $CaCl_2$, clotting times were recorded.

Phospholipase A_2 activity was assayed using egg yolk as source of lecithin. The phospholipolytic activity of the toxin was also assayed on plasma in order to correlate changes in recalcification time with plasma phospholipid hydrolysis. In these enzymatic determinations, released free fatty acids were extracted and titrated with 0.018 N NaOH according to Dole (1956). In some experiments, phospholipase

TABLE 1

Effect of Bothrops asper *myotoxic phospholipase A_2 on recalcification time of platelet-poor plasma form five mammalian species*

	Recalcification time (min)				
Phospholipase A_2 (μg)	Human	Horse	Sheep	Goat	Rabbit
0.0 (control)	10.10	17.35	5.05	10.37	11.30
0.05	10.00	19.00	6.35	13.00	11.40
0.1	10.20	28.00	10.50	>45.00	10.50
0.2	10.20	>45.00	>45.00	>45.00	11.50
0.4	10.50	>45.00	>54.00	>45.00	>45.00
0.8	30.00	>45.00	>45.00	>45.00	>45.00
1.6	>45.00	>45.00	>45.00	>45.00	>45.00

A_2 activity was quantified by using the agarose-egg yolk hemolytic test according to Gutiérrez *et al.* (1988). The heat stability of both anticoagulant and phospholipase A_2 activities was investigated by incubating samples of myotoxin (100 μg/ml) at 30°C, 50°C, 70°C and 95° C for 10 min before testing the two effects. Finally, in order to test the anticoagulant activity of this enzyme *in vivo*, groups of four mice (18-20 grams body weight) were injected intravenously with 30 μg of myotoxin. At various time intervals (15 min, 30 min, 1 hr and 2 hr) mice were anesthetized with ether and bled by cardiac puncture. Blood was placed in test tubes and coagulation times were recorded.

B. asper myotoxic phospholipase A_2 prolonged recalcification times in samples of platelet-poor plasma from the five species tested (Table 1). A similar effect was observed when toxin was added to platelet-rich sheep plasma; in this case, plasma did not clot after 45 min of incubation when toxin concentrations of 2 μg/ml were tested. When using platelet-poor plasma, the toxin hydrolyzed plasma phospholipids with an enzymatic activity of 10.8 ± 0.2 μEq/mg/min. When toxin concentration was lower than 0.25 μg/ml, phospholipid hydrolysis was negligible and recalcification times were similar to that of controls.

Addition of platelet-derived phospholipids after incubation of plasma and toxin reverted the inhibitory action of phospholipase A_2 on recalcification time (Table 2). On the other hand, both anticoagulant and phospholipase A_2 activities were thermoresistant since even after 10 min of incubation at 95°C neither of these activities was decreased. After intravenous injections of *B. asper* toxic phospholipase A_2 in mice there was not an anticoagulant effect, since blood collected at 15, 30, 60 and 120 min had clotting times of 65 ± 8 sec

TABLE 2

Effects of the addition of platelet extracts on the anticoagulant activity of B. asper *phospholipase A_2* *

	Recalcification time (min)	
Platelet phospholipids added (nanomoles)	No toxin	Toxin (2 μg)
Control	13.00	>45.00
6	6.10	>45.00
16	5.45	21.00
31	4.40	22.00
62	4.00	20.00
125	3.00	15.30

* Samples of sheep platelet-poor plasma were incubated with or without *B. asper* phospholipase A_2. Then, $CaCl_2$ 0.25M was added, followed by the addition of different amounts of plateled-derived phospholipids. Clotting times were recorded.

(n = 16), which are very similar to clotting times in mice injected with saline solution (61 ± 9 sec; n = 5).

Our results clearly demonstrate that *B. asper* myotoxic phospholipase A_2 prolongs recalcification times in plasma from several mammalian species. According to the classification of anticoagulant phospholipases A_2 proposed by Verheij *et al.* (1980), *B. asper* myotoxic phospholipase A_2 has strong anticoagulant activity since it prolongs recalcification time even at concentration of 0.2 μg/ml. Most of these highly active phospholipases are basic proteins, having isoelectric points higher than 9.0 (Verheij *et al.* 1980; Lomonte, Moreno & Gutiérrez 1987). *B. asper* toxic phospholipase A_2 probably affects coagulation due to its interaction with plasma phospholipids. Two experimental findings support this contention: (i) The toxin enzymatically degrades plasma phospholipids, and (ii) addition of platelet phospholipids reverts the inhibitory action of the enzyme on recalcification time. Therefore, it is very likely that *B. asper* toxic phospholipase A_2 affects coagulation by binding and disrupting plasma phospholipids required for the activation of protein coagulation factors.

Experiments *in vivo* clearly showed that *B. asper* phospholipase A_2 does not exert an anticoagulant effect after intravenous injection. Once inoculated, the toxin is probably diluted in the bloodstream where phospholipids are abundant. Moreover, Moreno & Gutiérrez (1987) demonstrated that a large fraction of this toxin is rapidly accumulated in liver and kidney after intravenous injection, thereby removing it from the circulation.

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