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A PHOSPHOLIPASE A_2 WITH ANTICOAGULANT ACTIVITY

II. INHIBITION OF THE PHOSPHOLIPID ACTIVITY IN COAGULATION

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Summary

An anticoagulant factor with phospholipase A_2 activity has been isolated from *Vipera berus* venom. Phospholipase activity was studied on platelet phospholipid and on brain cephalin. The venom factor showed a potent anticoagulant activity: 1 μg impaired the clotting of 1 ml of citrated recalcified platelet-poor plasma. The anticoagulant inhibited clotting by antagonism to phospholipid. The antagonism constant ($K_{an} = 6.8 \cdot 10^{-9}$ M) demonstrated the high affinity of the inhibitor for phospholipid.

As with other phospholipases A_2, the venom factor was thermoresistant but very sensitive to photo-oxidation. Both activities (anticoagulant activity and phospholipase activity) were not markedly dissociated by either denaturation or neutralization processes. Slightly different curves of photo-oxidative inactivation of both activities suggested the presence, on the molecule, of two very close sites responsible for phospholipase and anticoagulant activities.

The inhibitor effect on coagulation was independent of the hydrolysis process. In fact, lysoderivatives and fatty acids, resulting from complete hydrolysis with the venom factor, were as active as the native phospholipids. Moreover phospholipase A_2 from other *viperidae* venom, which did not have anticoagulant activity, produced similarly active lysoderivatives. This showed that the cleavage of the β-acyl bond does not interfere with the activity of phospholipid.

A possible mechanism of clotting inhibition by the venom factor was proposed. Owing to its high affinity for phospholipid, the inhibitor would complex phospholipid at its protein binding site impairing the normal arrangement of coagulation protein factors and, consequently, their activation. The positive charges of the inhibitor ($pI = 9.2$) could bind with phosphoryl or carboxyl groups of phospholipid, making them unavailable for protein binding. The complex formation involves a loss of dissociating capacity of the enzyme to-

wards its substrate. This required an additional interaction of the inhibitor with a coagulation protein factor.

The inhibitor could be removed from the complex by specific antibodies, permitting recovery of normal phospholipid-protein interaction. The role of calcium in the complex has not yet been elucidated.

This venom factor affords a useful tool for investigating the phospholipid-clotting protein interaction.

Introduction

An inhibitor of blood coagulation has been purified from the venom of *Vipera berus orientale* [1]. It showed phospholipase A_2 activity. The anticoagulant activity had been investigated previously [2]. *Berus* inhibitor was shown to impair clot formation of recalcified citrated plasma without any proteolytic effect on plasma clotting proteins. Its action on human plasma is closely related to the presence of phospholipid components and could be explained by the formation of a complex between the inhibitor and the phospholipids interfering in clot formation. The anticoagulant effect was reversible when antivenom immunoglobulins were added.

The anticoagulant and phospholipase activities have not been dissociated during purification processes or physicochemical studies, suggesting the presence of one protein, carrier of both activities. Thereafter different denaturation or inactivation procedures were used to investigate the possible presence of different active sites on the molecule.

These further studies have been carried out to explore the mode of inhibition of the phospholipid site active in clotting and to determine if the anticoagulant activity could be dependent on phospholipase A_2 activity.

Experimental procedures

1. Vipera berus inhibitor. Vipera berus inhibitor was isolated and purified by chromatographic procedures [1]. After lyophilization, it was solubilized in water at the concentration of 1 mg/ml. 1 ml samples could be stored at −20°C for 12 months without loss of activity.

2. Vipera aspis phospholipase. Several phospholipases A_2 were purified from the venom of *Vipera aspis* [3]. Phospholipase 1 was obtained in the crystallized form. It was diluted in the veronal buffer (pH 7.3) before clotting assays.

3. Anti-inhibitor immunoglobulins. Anti-inhibitor immunoglobulins were prepared by action of caprylic acid [4] on non-immunized rabbit control serum and on serum from rabbits immunized against the purified inhibitor, by repeated intradermal injection of 100 μg of inhibitor with incomplete Freund's adjuvant. Repeated dialysis in water and saline buffered at pH 7.3 was an essential step in the preparation of immunoglobulins devoid of any effect in clotting assays. Protein content was determined by the method of Lowry [5] or by spectrophotometric adsorption in neutral buffer. The extinction coefficient ($E^{1\%}_{280\ nm}$) value of 14 was used [6]. Precipitating antibodies against inhibitor were revealed by immunodiffusion.

4. Phospholipids. Phospholipids were suspended homogeneously by mixing on a Whirlmixer with a teflon stirring rod in the tube. This method gave more reproducible results than ultrasonication in non-sealed containers. Ovolecithin (40 μg of phosphorus/mg) was from Merck. Soybean phospholipids (azolectin) was from Associated Concentrates Inc. Partially purified phospholipids from bovine brain (Fractions I, III, V, VI of Folch) were from Sigma. The crude cephalin used in clotting assays was a chloroform extract from human thromboplastin [7] with a phosphorus content of 300 μg/ml of suspension. Total phospholipids were extracted from blood platelets: pooled platelets were obtained from 50 donors. Platelets were washed twice in Tyrode solution (without Ca^{2+} and Mg^{2+}), pH 6.6, and , following the technique described by Renaud [8], phospholipids were extracted by chloroform/methanol mixture (2 : 1, v/v) [9]. The phosphorus content was 5.5 mg/ml of suspension.

5. Phosphorus determination and phospholipase assay. These were performed as previously described [1].

6. Thin-layer chromatography. Two dimensional thin-layer chromatography [10] was performed both for purification of phospholipid and for the identification of the phospholipids present in the platelet, brain or egg extract. It was carried out with a stationary phase of Silica gel HF 254 type 60 in borate buffer prepared "in vacuo". Chromoplates (thickness : 0.4 mm) were silica gel coated using a Desaga applicator. For preparative thin-layer chromatography, the first solvent was chloroform/methanol/25% ammonia (35 : 60 : 5, by vol.) [10]. The second solvent was chloroform/methanol/ammonia (60 : 35 : 5, by vol.) [11]. Powder elution was performed under nitrogen by chloroform/methanol (1 : 1, v/v) with vigorous stirring and several washings. Hydrolysis of platelet phospholipid by the enzyme was studied as described previously [1]. Ninhydrin coloration was performed according to Skipski [12].

7. Blood clotting assays. Citrated platelet-poor plasma [2] was obtained from blood donors, by centrifugating twice for 15 min at 2500 × *g* at 10°C in 50-ml centrifugation plastic tubes. For very platelet-poor plasma, successive centrifugations were performed: 1300 × *g*, then 3000 × *g* (15 min, 10°C), 6000 × *g* and 15 000 × *g* (1 h, 4°C). Crude cephalin was used as previously described [2]. Plasma deprived of calcium was prepared by collecting blood on ion-exchange resin (JB-2 blood pack-Fenwal) and filtering it once again on the resin. Calcium was found to be absent by complexometry.

Veronal buffer (0.026 M barbital sodium/0.026 M sodium acetate/0.1 M NaCl, pH 7.3) was used for controls and for diluting the venom inhibitor. Recalcification times were measured at 37°C with a ratio of 0.025 M $CaCl_2$/plasma of 1 : 1, v/v.

8. Photo-oxidation technique. Photo-oxidation was performed according to the general procedure described by Ray and Koshland [13] and Semeriva et al. [14], with the following modifications. The chamber was a glass vessel (10 ml) with magnetic stirrer used for pH-stat titration, jacketed with black paper. 13°C was maintained by the use of a stream of cold water, provided by a Cryostat and a thermoregulator unit (Haake). A ventilated 500 W Mazda 32, 125 V spotlight with silver reflector was fixed above the reaction chamber. The distance from the spotlight filament to the reaction surface was 20 cm. Pure oxygen was supplied by a N45 apparatus, at a flow rate of 22 ml/min.

Results

Anticoagulant activity

The anticoagulant unit of inhibitor has been defined as the minimal amount of inhibitor necessary to maintain 1 ml of recalcified human plasma incoagulable for 1 h. The plasma was obtained after centrifugation (1800 × *g* 15 min) of a citrated blood (1 : 9, v/v) at room temperature within 3 h of blood collection in polystyrene tubes. In these conditions 1 unit corresponded to 1 μg ± 0.25 of inhibitor (1 mg = 1000 units), disregarding minimal individual variations related to platelets. Marked variations were observed when conditions of centrifugation were modified. Lyophilization did not alter the specific anticoagulant activity of the venom inhibitor.

Procoagulant phospholipids such as cephalin, azolectin, or native or damaged platelets can restore normal clotting of the recalcified unclottable plasma. As seen in Fig 1, the velocity of clot formation of human plasma in the presence of progressive concentrations of cephalin can be summarized in a S-shaped curve (curve c). In the presence of inhibitor, clotting velocity was decreased as illustrated by the spacing out of the curves b and a from the control curve c. However, the clotting reaction was not apparently modified by the inhibitor, when considering the similarity between control and inhibitor curves. It was noticeable that the measurements of highly delayed recalcification times in the presence of inhibitor were precise enough to give quite reliable results. This could result from the fact that the inhibitor dealyed the clot formation but did not alter it.

The study of inhibition had to be performed within the limits in which the

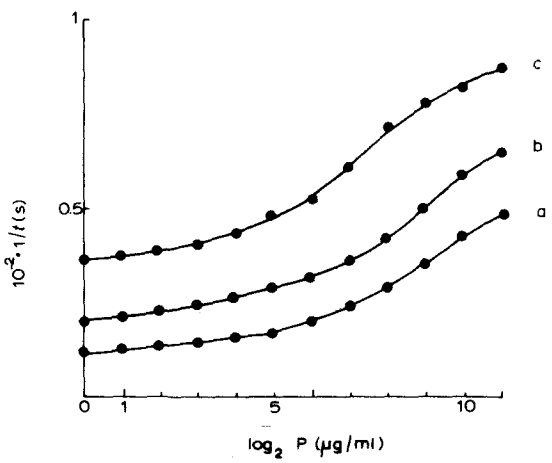

Fig. 1. Effect of the inhibitor on the clotting velocity of plasma in function of phospholipid concentration. *T*, recalcification time expressed in seconds. Phospholipids were measured by the phosphorus determination of the cephalin suspensions. In abcissa, serial concentration ($\log_2$) of phosphorus from 0.585 ng ($\log_1$) to 600 ng/ml ($\log_{11}$). Clotting system: 0.3 ml of platelet poor plasma + 0.05 ml of phospholipid (cephalin) at various concentrations + 0.1 ml of inhibitor or buffer + 0.1 ml of kaolin (2.5 mg/ml) + 0.1 ml of 0.075 M $CaCl_2$. Kaolin has been previously tested toward the inhibitor and no interaction has been found. The a and b curves correspond respectively to 1.25 and 0.625 μg/ml of inhibitor, control curve c without inhibitor.

anticoagulant effect of inhibitor could be neutralized by addition of phospholipids, without large excess of inhibitor. The inverse of the velocity ($1/V$) was expressed as a function of the inhibitor concentration for a series of phospholipid concentrations. Each step of the series represented a two-fold dilution. The graph obtained (Fig. 2A) appeared related to what is found in competitive inhibition [15—16]. However, "it differed from it by the spacing observed between the lines of the $\log_2$ serial dilution" (Dixon, M., personal communication). We think that differences come from the fact that, in our experiment, the system used is far more complex than that of the model (Fig. 2B) and that phospholipid interferes as a cofactor of the reaction. This appears clearly when looking at both figures ordinates established without inhibitor. In Fig. 2B the spacing changes rapidly over the series, reaction time is only limited by $1/V$ downwards. In the case of the clotting system (Fig. 2A) the accelerating effect of phospholipid on the reaction time could be measured only in a very restricted time space: upwards by clotting time without phospholipid (220 seconds), downwards the addition of cephalin will shorten the time up to $1/V$ obtained without inhibitor. The spacing changed slowly over the series in spite of logarithmic dilution of cephalin. These differences are found again when adding inhibitor. The inhibition constant given by the meeting of all the lines was found to equal to $6.8 \cdot 10^{-9}$ M. To avoid a direct reference to competitive inhibition this constant was called "antagonism constant" (K_{an}).

In the clotting system, if inhibitor was added, more phospholipids were necessary to obtain similar clotting times and reciprocally. In our experimental conditons, 1.5 μg of phospholipid (cephalin) will shorten the recalcification time of plasma from 4 min 30 s to 2 min 45 s; if 0.125 μg of inhibitor is added, 4 min 30 sec will be obtained again. 0.125 μg of inhibitor inhibited the activity in clotting of 1.5 μg of phospholipid. The weight ratio used for phospholipid

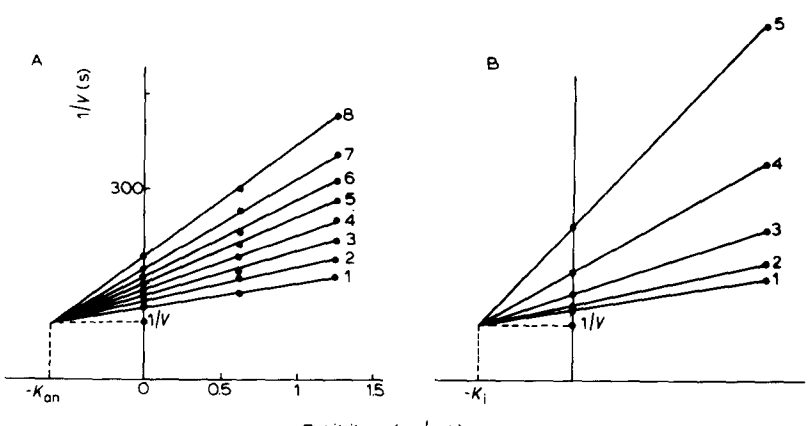

Fig. 2. (A) Inhibition of phospholipid activity by *berus* inhibitor. $1/V$ in which time was measured in seconds by the recalcification of the mixture. 1 to 8 represent the serial dilution ($\log_2$) of phospholipids suspended in buffer from 600 (1/100 diluted cephalin) to 4.685 ng of phosphorus. Three recalcification times were measured for each phospholipid concentration: one without inhibitor, the others with 0.625 and 1.25 μg/ml of inhibitor. The K_{an} was measured by the projection of the meeting point of all the curves on the abcissa. The inverse of the maximum velocity of the reaction ($1/V$) was calculated by projecting the same point on the ordinate. Clotting system as in Fig. 1. (B) Corresponding theoretical diagram of competitive inhibition according to Dixon.

activity in clotting/inhibitor necessary to inhibit it, was approx. 12 : 1. One molecule of inhibitor (13 400) inhibited the activity of approximately 200 molecules of phospholipids (≃800).

Phospholipase A_2 activity

The phospholipase activity of *berus* inhibitor on platelet phospholipids was compared to that measured on other phospholipid substrates (Table I). The specific activity of the enzyme was higher on purified phospholipids from platelets than on ovolecithin. The hydrolysis of the different platelet phospholipids to their corresponding lysoderivatives appeared clearly on thin-layer chromatography on silica gel (Fig. 3). The inhibitor, in the experimental conditions, hydrolyzed almost completely the phosphatidylcholine and phosphatidylserine present in the platelet, to a lesser extent the phosphatidyl ethanolamine.

The effect of various cations on phospholipase activity differed markedly with the cations used. Phospholipase activity of *berus* inhibitor did not appear in EDTA buffer deprived of cation. As seen in Table II, calcium was necessary to promote high phospholipase activity. Mg^{2+} or Mn^{2+} did not show a similar role of cofactor in the enzymatic reaction.

Attempts to separate phospholipase and anticoagulant activities by inhibition or denaturation processes

1. Effect of strontium. The effect of strontium was studied, since this cation permitted the clot formation of a citrated plasma while, in its presence, the phospholipase activity of the inhibitor was not promoted (Table II). However, it appeared that Sr^{2+} could suitably displace Ca^{2+} from citrated plasma, making it clot, but it could not bring about the coagulation of calcium-free resin plasma.

2. Temperature and pH changes. Temperature and pH were used to denature *berus* inhibitor. Phospholipase activity from different venoms is known to be

TABLE I

PHOSPHOLIPASE ACTIVITY OF *BERUS* INHIBITOR

a, On 5.5 ml of ovolecithin (phosphatidylcholine) and semi-purified brain extracts: fraction III (phosphatidylserine), fraction V (phosphatidylethanolamine), fraction VI (phosphatidylethanolamine, sphingomyelin, cerebrosides). b, On ovolecithin, "cephalin" and platelets phospholipids at equal phosphorus content each sample containing 70 μg of phosphorus per ml of suspension. Titration as in Experimental procedures with NaOH 0.01 M. *Berus* inhibitor 2 μg (20 μl of a 100 μg/ml solution. Final volume in a and b: 3 ml with 0.02 M sodium cholate/0.001 M EDTA/0.02 M $CaCl_2$ (pH 8.0, 37°C). Results are expressed in nmol of NaOH consumed per min.

Phospholipids		Phosphorus content (μg)	Phospholipase activity	Phospholipids	Phospholipase activity
Ovolecithin		220	500	Ovolecithin	500 ± 40
Bovine	Fraction III	210	340	"Cephalin"	590 ± 20
Brain	Fraction V	225	420	Platelet	
Extracts				phospholipids	653 ± 10
	Fraction VI	205,5	215		

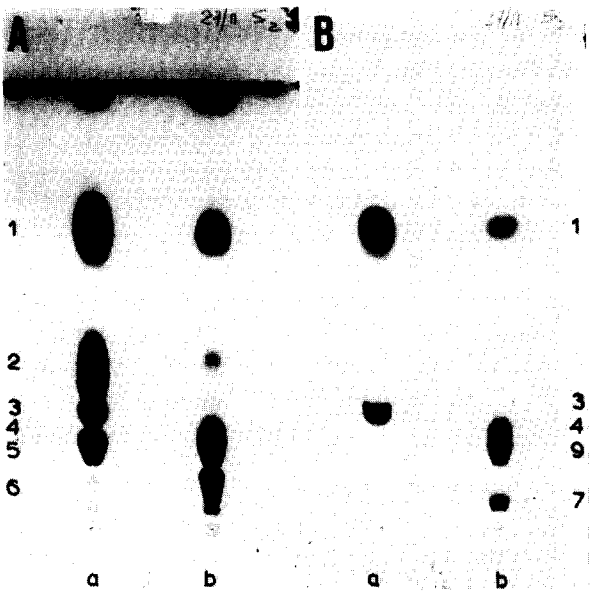

Fig. 3. Chromatograms of platelet phospholipids hydrolysed by *berus* inhibitor. Thin-layer chromatography was performed on Silica gel plates (10 cm X 20 cm) coloured (A) by iodine vapors and (B) by ninhydrin. Total phospholipid extracts 0.5 mg (10 μl) without (a) and with (b) *berus* inhibitor 10 μg (10 μl) were incubated in solvents as described in Experimental procedures. At the bottom of each silica gel plate, 10 μl of the phospholipids hydrolyzed or not by the inhibitor were incorporated. Developing solvent: chloroform/methanol/acetone/acetic acid/water (10 : 2 : 4 : 2 : 1, by vol). (1) Phosphatidylethanolamine; (2) phosphatidylcholine; (3) phosphatidylserine; (4) lysophosphatidylethanolamine; (5) sphingomyelin; (6) lysophosphatidylcholine; (7) lysophosphatidylserine; (8) fatty acids and residual lipids; (9) undetermined derivative.

thermoresistant. Indeed, pH and temperature changes had to be combined to denature the phospholipase. When *berus* inhibitor was heated for 10 min at 100°C in a boiling water bath at pH 8, phospholipase activity decreased from 100% to 20% and anticoagulant activity from 10 min 30 s to 3 min 30 s. These two activities appeared, therefore, to be related. However, the method was inconvenient for adjustment of the pH in the titrimetric studies and was not studied further.

3. Photo-oxidation. Photo-oxidation appeared to be a more suitable denaturation procedure. Preliminary pH adjustments were unnecessary, and very small amounts of enzymes could be used. This allowed very precise study of

TABLE II

EFFECTS OF DIVALENT CATIONS ON PHOSPHOLIPASE ACTIVITY

Phospholipase activity was measured as in Fig. 1 in the presence of 0.001 M EDTA without ion or in the presence of different cations (45 μmol in the 3 ml ovolecithin/sodium cholate suspension). *Berus* inhibitor 2 μg. Results are expressed in percentage of the maximum activity obtained with Ca^{2+}.

Cations	None	Ca^{2+}	Zn^{2+}	Fe^{2+}	Pb^{2+}	Mg^{2+}	Mn^{2+}	Cu^{2+}	Sr^{2+}	Ba^{2+}	Cs^{2+}	Hg^{2+}
Relative activity (%)	0	100	62	32	26	14	7	6	4.5	0	0	0

the kinetics of denaturation. The inactivation of anticoagulant and phospholipase A_2 activities were measured in the presence of $25 \cdot 10^{-6}$ M methylene blue and pure oxygen. During 10 min of photo-oxidation, both phospholipase and anticoagulant inactivations were found to be a first-order reaction up to 80 and 95%, respectively (Fig. 4). Photo-oxidation led to a rapid decrease of both anticoagulant and phospholipase activities. According to the comparative units of both activities, their rate of inactivation was slightly different. Anticoagulant activity was inhibited more rapidly ($K_{a'} = 0.376$ min^{-1}) than phospholipase activity ($K_a = 0.230$ min^{-1}).

4. Neutralization of phospholipase and of anticoagulant activities by anti-inhibitor immunoglobulins. The neutralization curves of the two activities as a function of the concentration of anti-inhibitor rabbit immunoglobulins were established (Fig. 5). They did not differ significantly one from the other, but, as for photo-oxidation, anticoagulant activity was inhibited more rapidly.

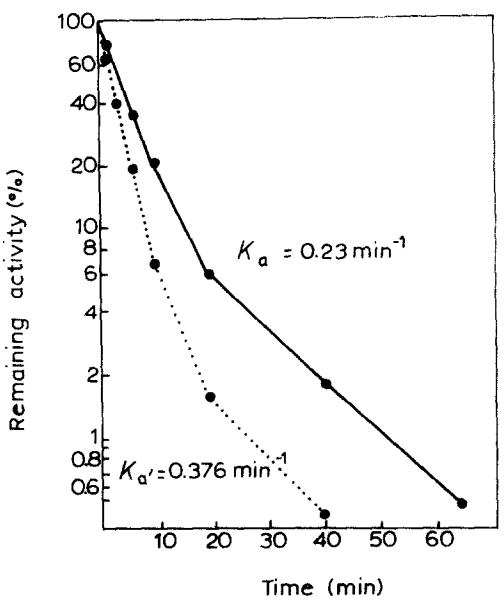

Fig. 4. Inactivation of phospholipase (———) and anticoagulant (· · · · · ·) activities of *berus* inhibitor by photo-oxidation. Photo-oxidation was performed with 150 µg of *berus* inhibitor in 3 ml aqueous solution with 25 µM methylene blue. Aliquots of 0.15 ml were removed successively during 40 min and kept at +2°C in the dark before measurements of both activities. Specific phospholipase activity measured on a 3 ml mixture of 0.01 M ovolecithin, 0.02 M sodium cholate, 0.001 M EDTA and 0.02 M $CaCl_2$, pH 8.0. Titration in µmol of NaOH M/100 for 5 µg of the inhibitor per min. Results expressed in percentage of activity (100% = 1.65 ± 0.20 µmol of liberated fatty acid). Specific anticoagulant activity was determined by recalcification time in the presence of *berus* inhibitor before and after photo-oxidation. Clotting system: 0.2 ml of platelet poor plasma + 0.025 ml of 1/5th diluted photo-oxidated mixture + 0.3 ml of 0.025 M $CaCl_2$. Results are expressed in percentage of anticoagulant activity calculated on a reference curve. Before photo-oxidation a reference curve was established, giving for decreasing concentrations of *berus* inhibitor the corresponding plasma recalcification time. 100% of anticoagulant activity corresponded to 1 unit or 1 µg of *berus* inhibitor per ml of plasma, 50% to 0.5 µg, etc. The recalcification time of the mixture before photo-oxidation corresponded to 100% anticoagulant activity or 1 unit. The percentage of anticoagulant activity obtained during photo-oxidation are plotted on the curve (· · · · · ·). K_a corresponds to $1/T$, time necessary to give an inhibition of 50% of activity, K_a for phospholipase activity, $K_{a'}$ for anticoagulant activity.

TABLE III

EFFECT OF *ASPIS* PHOSPHOLIPASE A_2 ON RECALCIFICATION TIME

Recalcification times with 0.05 M $CaCl_2$ were measured after an incubation of 120 s at 37°C which permitted in the presence of Ca^{2+}, a complete hydrolysis of cephalin by the phospholipase. Three series of recalcification times were performed with and without *aspis* phospholipase at the concentration of 10 μg/ml. The ratio phospholipase/phospholipid, 0.5/1.5 (w/w in μg) was primarily determined by pH-stat titration to obtain a complete hydrolysis of the cephalin in 2 min at 37°C.

Vol. (ml)	Experiment					
	a	b	c	d	e	f
0.05	phospholipase	buffer	phospholipase	buffer	phospholipase	buffer
0.1	buffer	buffer	6.5 ng cephalin/ml	6.5 ng cephalin/ml	13 ng cephalin/ml	13 ng sephalin/ml
0.03	$CaCl_2$	$CaCl_2$	$CaCl_2$	$CaCl_2$	$CaCl_2$	$CaCl_2$
Incubation 120 s						
0.3	plasma	plasma	plasma	plasma	plasma	plasma
0.13	$CaCl_2$	$CaCl_2$	$CaCl_2$	$CaCl_2$	$CaCl_2$	$CaCl_2$
	6 min 30 s	6 min 34 s	2 min 39 s	2 min 42 s	2 min 07 s	2 min 06 s

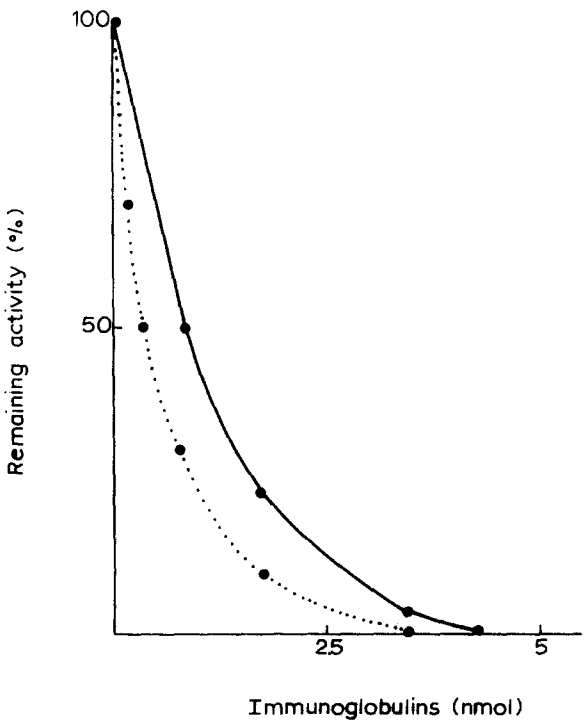

Fig. 5. Inactivation of phospholipase and anticoagulant activities by antiinhibitor immunoglobulins (55 mg/ml). Phospholipase activity (———) 0.2 ml of increasing concentrations of immunoglobulins was added to 0.5 μg of *berus* inhibitor and the mixture incubated 2 min at 37°C. Residual phospholipase activity was measured on ovolecithin in cholate/EDTA/Cl_2Ca as in Fig. 4. It is expressed in percentage 100% represents the activity of the inhibitor without immunoglobulins. Anticoagulant activity (· · · · · ·) was measured by recalcification time on platelet poor plasma as in Fig. 4. Clotting system: after an incubation 2 min at 37°C of 50 μl of *berus* inhibitor (4 μg/ml) with 0.2 ml of immunoglobulins, 0.2 ml of plasma was added. Time was recorded when adding 0.2 ml of 0.025 M $CaCl_2$. Results are expressed in percentage of activity calculated on a reference curve with 1 anticoagulant unit of *berus* inhibitor.

Effect on clotting of a phospholipase A_2 purified from the venom of Vipera aspis

This experiment was performed to determine if phospholipase from another viper would display an anticoagulant effect similar to that of *berus*. The weight ratio *aspis* phospholipase/cephalin necessary to obtain about 100% hydrolysis in 2 min was determined and used in the following experiments. The recalcification time of platelet poor plasma was measured in the presence and the absence of the phospholipase. Similar results were obtained in both cases (Table III). *Aspis* phospholipase A_2 was devoid of clotting inhibitor effect and lyso derivatives from cephalin had the same procoagulant activity as native phospholipids.

Discussion

Relationship between phospholipase A_2 and inhibitor activities

Vipera berus inhibitor has been shown to possess a phospholipase A_2 activity on phosphatidylcholine [1]. This activity was found also on phospholipid

extracts from platelets and on crude cephalin with hydrolysis of phosphatidylethanolamine, phosphatidylcholine and phosphatidylserine in both substrates.

The isolated inhibitor, carrier of both anticoagulant and phospholipase activity, responded to the usual criteria of homogeneity [1], which cannot exclude the possibility of a very small weight percentage of heterogeneous material, not detectable by currently available techniques of analysis. Therefore a trial of dissociation of these two activities was carried out by denaturation and inhibition techniques. In no case was there a marked dissociation of the two activities. A unique molecule appeared to be responsible for both activities. However, photo-oxidation showed some difference in the velocity of inactivation, which probably meant the presence of two very close sites on the same molecule, each one responsible for each activity.

The anticoagulant and the phospholipase activities of snake venoms are well established. Kruse and Dam [17] showed that the strong anticoagulant action of *Naja* venom was not related to the lecithinase A (phospholipase A) of the venom. The anticoagulant effect was impaired by heat, while the phospholipase A activity remained, which was not the case for purified *berus* inhibitor.

The anticoagulant activity on plasma has been shown to be reversible in the presence of antibody to the inhibitor [2]. This suggested that inhibitory activity was independent of the enzymatic activity. In fact, no anticoagulant effect of lyso derivatives had been reported: lysophosphatidylcholine and lyso derivatives from natural or synthetic phosphatidylethanolamine have been described as inactive in clotting by different authors [21,22]. Lysophosphatidylcholine was shown not to shorten the Stypven time as much as phosphatidylethanolamine, phosphatidylserine or phosphatidylethanolamine/phosphatidylserine [23]. As seen in Table III, cephalin hydrolysed by *Vipera aspis* phospholipase A_2 [3,18] kept the same clot accelerating activity as native phospholipids. It can be concluded that the site of phospholipid involved in clotting is not modified by the cleavage of the β-acyl bond. It there is a rearrangement of the molecule of phospholipid after release of fatty acid chains, it does not interfere with the clotting protein—phospholipid interaction. More probably, lyso products and free fatty acids remain associated in the interface as it was reported for aggregated phospholipid substrates [24] and for myelin [25]. Such a mechanism would imply the absence of conformational change in the active structure. According to Wells [24], after phospholipid hydrolysis by phospholipase A_2, Ca^{2+} is involved in ionic binding of the free fatty acid.

Inhibition of the active site of phospholipid

In the enzymatic sequence of clot formation, phospholipids provide the structure to which clotting proteins adhere and settle in such a reciprocal arrangement that activation is accelerated [26—29]. Maximum thrombin formation will occur if the prothombinase—prothrombin complex, phospholipid + calcium + factor Xa + factor V — factor II (prothrombin) is realized [30—33] and if it is realized on the same phospholipid micell [34]. At an earlier step in the activation sequence of clotting factors (factor IXa, factor VIII), a similar complex appears responsible for the activation of factor X [35—38]. Factor V [27,30—32,34,39], factor Xa [27,30,31,34] and factor II [27—29,37,40] bind to phospholipids and to each other: factor Va to factor II

[41] factor V to factor Xa [27—30]. Jackson, Esmon and coworkers have identified the activation of factor II by factor Xa in the presence of phospholipids [43]. Calcium is necessary for the binding of factor II and factor Xa on the phospholipid vesicle [27,30,34,35,40,44—46]. The binding of factor V to phospholipid is not completely elucidated. Calcium in excess dissociates its complex with phospholipid [34]. The presence of lipid in its composition [47] supports the hypothesis of a hydrophobic binding to the apolar part of phospholipid, proposed by Vroman [39] and by Kahn [48].

Surfaces charges are of prime importance for protein binding [49—51,40,52]. However, several works show that final thrombin generation depends also on the nature of the phospholipid [40,52,53], on their concentration [54], on the presence or the absence of carboxyl groups, and on the conformation arrangement between their polar heads [55]. The transition temperature of different phospholipids, the degree of dispersion and the size of aqueous compartments between them are all relevant for these interactions [53,55].

The activities of the inhibitor were studied on the phospholipids particularly involved in clot formation, i.e. platelet phospholipids. This was extended to crude "cephalin" from human brain: both mixtures present similar high clot accelerating activities. They were chosen also because of their extensive use in hemostasis laboratories.

Vipera berus inhibitor behaved as an antagonist of phospholipid activity. Competitive inhibition appeared as the nearest theoretical model applicable to phospholipid inhibition in a system as complex as plasma coagulation. This model permitted us to measure the "antagonism constant" (K_{an}). Equal to $6.8 \cdot 10^{-9}$ M this constant explained the potent anticoagulant activity of the inhibitor. K_{an} expressed the very distinctive property of this anticoagulant phospholipase A_2, showing its high affinity for the procoagulant site of phospholipid. On the other hand, the affinity of *berus* inhibitor for phospholipid, defined by dissociation constant (K_m) and related to the enzymatic site, was similar to that observed for other phospholipase A_2 (about 10^{-3} M).

The inhibition was produced by the formation of a complex between the inhibitor and the phospholipid at their clotting protein binding site, rendering them unavailable for the clotting enzymes. Calcium did not seem to interfere directly, since addition of Ca^{2+} did not fully restore normal clotting (unpublished results), as far as this could be measured by recalcification times. It cannot be told from the experiments if it prevented factor II-Ca^{2+} and/or factor Xa-Ca^{2+} and/or factor V binding to phospholipid, or if it accted at the level of factors VIII, IXa and X. In contrast to enzymatic behaviour, in the presence of plasma, the phospholipase (inhibitor) formed a stable complex with its substrate. The loss of dissociation between the enzyme and its substrate suggested an additional interaction of the inhibitor with a coagulation protein.

The complex with phospholipid might be favoured by their surface charge. Electropositively charged (pI = 9.2), the inhibitor would bind to the phosphoryl and carboxyl groups of phospholipid. This hypothesis was also supported by the fact that a phospholipase A_2 such as *aspis* phospholipase, devoid of anticoagulant property, does not possess a basic isoelectric point. When specific antibody was added, the negative charges of the phospholipid could become again available for the clotting proteins.

The role of the inhibitor can be imagined taking into account its compact structure [1]. It may have modified the malleable micellar surface: the contact of the apolar head of phospholipid would then take place around the molecule of inhibitor, leaving intact its antigenic site. This conformation would increase the steric bulkiness in the competition with proteins.

It has been possible to define a unit of active phospholipid able to be inhibited by an equivalent functional unit of inhibitor. In our experimental conditions, using human brain phospholipid, the molar inhibitor/inhibited phospholipid ratio was about 1 : 200. This ratio gave the smaller functional structure of phospholipid in which phospholipid molecules forming the active site had to be present. Such a molecular pattern could be applied for platelet membrane receptor.

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