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THE SYNERGISM OF CARDIOTOXIN AND PHOSPHOLIPASE A_2 IN HEMOLYSIS

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Summary

The synergistic effect of exogenous cobra phospholipase A_2 on the hemolysis rate of guinea pig erythrocytes by highly purified snake venom cardiotoxins was investigated. In the presence of phospholipase A_2 the reaction was not only faster and had a lower activation energy but followed a sigmoidal instead of a linear time course. Similar results were obtained using porcine pancreatic phospholipase A_2. Significantly, addition of even a trace of cobra phospholipase A_2 (approx. 0.1%, w/w) was sufficient to bring about the full synergistic effect, emphasizing the stringent purity requirements for any meaningful investigation of cardiotoxin's own action. The possibility that the action of cardiotoxin on its own may involve the stimulation of an endogenous phospholipase is discussed in the light of the results obtained with exogenous cobra enzyme.

Introduction

Cardiotoxins represent the principal protein component of cobra venoms (up to 50%, on a weight basis). The toxicity of this group of low molecular weight proteins is closely related to the marked permeability changes that they induce in the membranes of a variety of cell types [1—6], which in the case of heart muscle result in cardiac arrest. It is also well documented that phospholipases A_2 can act synergistically with cardiotoxins to enhance these effects, e.g. in the hemolysis of erythrocytes [7,8] and the depolarization of skeletal muscle [9]. These observations raised several questions about the mechanism by which phospholipases exert a synergistic effect on cardiotoxin action on the one hand and about the possibility, on the other hand, that cardiotoxins may stimulate an endogenous membrane phospholipase to facilitate their in vivo action.

Hemolysis studies by Condrea et al. [2,7] with viper venoms led them to believe that phospholipase A_2 can only hydrolyze erythrocyte membrane phospholipids if cardiotoxin were present. The lyso-products of the enzyme's action were presumed to be lytic by themselves under such conditions and it was proposed that their formation promotes cardiotoxin-induced hemolysis in an autocatalytic fashion. This proposal, however, requires reexamination in view of recent evidence that cobra phospholipases A_2 can hydrolyze the outer layer of membrane phospholipids in the absence of cardiotoxins, and furthermore, that the reaction products are not released from the membrane matrix [10,11].

Vogt et al. [12,13] originally suggested that cardiotoxin action may involve the stimulation of an endogenous membrane phospholipase but several attempts at demonstrating such an enzyme in human erythrocytes failed [14–19]. However, Paysant et al. [20] later succeeded in showing up a low endogenous phospholipase A_2 activity which could be increased by trypsinization of human erythrocyte membranes. The known effect of cardiotoxins to unmask or release membrane enzymes [21] could also facilitate expression of a dormant phospholipase activity.

We have consequently investigated various properties of the combined action of cardiotoxin and exogenous cobra venom phospholipase A_2 on guinea pig erythrocytes. The results are discussed here in relation to our earlier work [3] on the separate action of cardiotoxin.

Materials and Methods

The sources and purification procedures of cardiotoxins and phospholipases A_2 from the venoms of *Naja mossambica mossambica* and *Hemachatus haemachatus* have been described previously [3]. Residual traces (approx. 0.2%, w/w) of phospholipase A_2 were removed from the cardiotoxins by a gel chromatographic method [22], as confirmed by radio-chemical assay [23]. Pig pancreas phospholipase A_2 was obtained from Boehringer, Mannheim, desalted on Sephadex G-25 and freeze-dried. Lipids were purchased from P-L Biochemicals, Inc., and Sigma Chemical Co. and used without further purification.

Blood was collected from adult guinea pigs, centrifuged, washed and finally resuspended in phosphate-buffered saline and hemolysis was estimated from changes in turbidity at 700 nm at 37°C [3]. Assays were carried out in small volume (1.25 ml) fused quartz 1 cm pathlength cuvettes containing the following medium: 4.9 mM Veronal/145 mM NaCl/2.5% glucose/0.2% gelatin (Difco)/0.6 mM $CaCl_2$, adjusted to pH 7.4 with HCl. Where appropriate, cardiotoxin and/or phospholipase A_2, dissolved in 154 mM NaCl, was added and the reaction started by the addition of 50 μl of a 1.25% (v/v) erythrocyte suspension (0.05%, v/v final erythrocyte concentration). Measurements were made in a thermostated Unicam SP-800 spectrophotometer with automatic sample changer against air as reference. One cuvette containing assay medium and erythrocytes, but no lytic agent, served as a control. In order to obtain reproducible results we found it necessary to wash the cuvettes successively with distilled water, 95% ethanol, 1 M NaOH, distilled water, 1 M HCl and distilled water to remove material which adheres to the quartz surfaces and interferes with subsequent assays.

The lipids of erythrocyte membranes were extracted according to the method of Broekhuyse [24]. The extracts were taken to dryness under N_2 and dissolved in 50 μl chloroform. Phospholipids (20 μl) were separated via two-dimensional chromatography on silica gel 60 thin-layer plates (Merck) using chloroform/methanol/acetic acid/water (50 : 30 : 8 : 3, v/v) as developing medium [25] in the first direction. The plates were dried in vacuo for 2.5 h and developed in the second direction with chloroform/methanol/25% ammonia (13 : 5 : 1, v/v) [26]. Phospholipids were localized with iodine [27] and analyzed for inorganic phosphorous [28].

Rates of hemolysis are expressed either as the inverse of the time, in minutes, that is required for the test substance to lyse 50% of the available erythrocytes ($1/t_{50}$ min^{-1}) at a particular temperature, or as the maximal change in $A_{700\,nm}$ per unit time (slope).

Results

Time course of hemolysis. The progress curves of hemolysis by cardiotoxin on its own or in combination with phospholipase A_2 are compared in Fig. 1. The linear time-dependence of hemolysis by cardiotoxin (curve A) became sigmoidal in the presence of 0.2% (w/w) phospholipase A_2 (curve B). The rate ($1/t_{50}$) of hemolysis of an 0.05% (v/v) erythrocyte suspension in the presence of phospholipase A_2 was approximately three times faster than for cardiotoxin alone (0.11 vs. 0.04 min^{-1}).

Effect of erythrocyte concentration on the synergistic rate of hemolysis. The rate of hemolysis by a mixture of cardiotoxin and phospholipase A_2 (60 : 1, weight basis) increased relatively slowly with erythrocyte concentration up to 0.05% (v/v), after which there was a marked linear increase that did not yet level off at 0.5% (Fig. 2, curve A). At any particular erythrocyte concentration the synergistic rate was nonetheless, much faster than hemolysis by

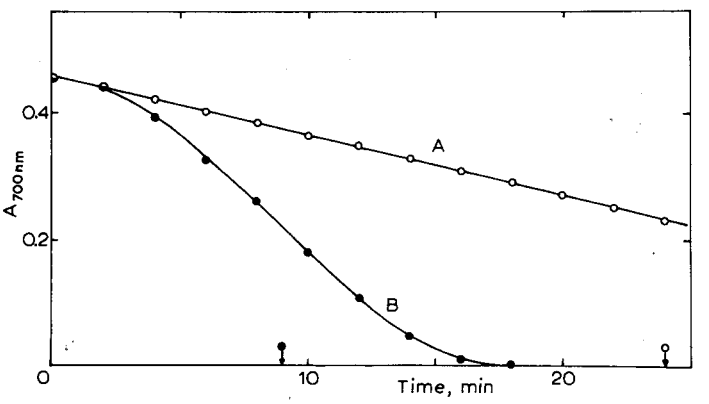

Fig. 1. Time course of hemolysis. The final erythrocyte concentration was 0.05% (v/v). The concentrations of *H. hemachatus* direct lytic factor and *N. mossambica mossambica* phospholipase A_2 (fraction CM-II, ref. 42) were 35.5 μg/ml ($E^{1\%}_{280} = 2.91$) and 0.08 μg/ml ($E^{1\%}_{280} = 28.1$), respectively. Values are expressed as a decrease of absorbance at 700 nm with time for cardiotoxin alone (o———o) and in the presence of phospholipase A_2 (●———●). Arrows indicate respective t_{50}-values.

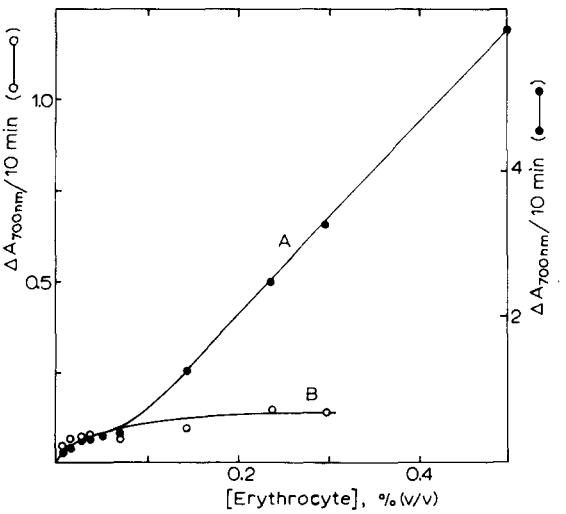

Fig. 2. Effect of erythrocyte concentration on hemolysis rates. The final concentration of erythrocytes was varied as indicated at a constant *N. mossambica mosaambica* cardiotoxin $V^{II}2$ concentration of 92 μg/ml (o——o), and 1.53 μg/ml phospholipase A_2 (fraction CM-III, ref. 42) was added for the synergistic reaction (•——•). Rates are given as the maximum decrease in $A_{700\,nm}$ per 10 min. Note the differences in ordinate scale for the respective reactions.

cardiotoxin alone (cf. the different ordinate scales in Fig. 2). The rate curve for cardiotoxin alone apparently obeyed saturation kinetics, being linear with erythrocyte concentration up to 0.025% (curve B) and reaching a plateau at approximately 0.3%.

Effect of relative concentrations of cardiotoxin and phospholipase A_2 on synergism. The synergistic effect of a fixed amount of added exogenous phospholipase A_2 on the rate of hemolysis of an 0.05% erythrocyte suspension * by increasing concentrations of cardiotoxin is depicted in Fig. 3. In the absence of phospholipase A_2 the maximum rate of hemolysis of a 0.05% (v/v) erythrocyte suspension was reached at a cardiotoxin concentration of approx. $20 \cdot 10^{-6}$ M (curve A). The maximum for the faster synergistic rate occurred at the same concentration of cardiotoxin (curve B). Similar results were obtained when mixtures with different fixed concentrations of phospholipase A_2 were assayed. These more comprehensive data are expressed in tabular form in Table I, which shows that the synergistic rate of hemolysis relative to that by cardiotoxin alone varied remarkably little (between 2.1 and 3.0) over a 135-fold range of molar ratios. Significantly, the same absolute increase in the hemolysis rate was observed whether phospholipase A_2 was added at $1 \cdot 10^{-8}$ M (0.156 μg/ml) or $2.2 \cdot 10^{-7}$ M (2.96 μg/ml) concentration to $20 \cdot 10^{-6}$ M (137 μg/ml) cardiotoxin.

The order of addition of cardiotoxin and phospholipase A_2. The effect on

* Larger synergistic effects could be obtained at erythrocyte concentrations higher than 0.05% (cf. Fig. 2), but the latter concentration was employed in these experiments for reasons of practical convenience: measurements could be made in a region where turbidity changes recorded on the Unicam instrument were linearly related to the extent of hemolysis [3] and t_{50} values could be obtained in a reasonable time period where low amounts of cardiotoxin had to be assayed separately.

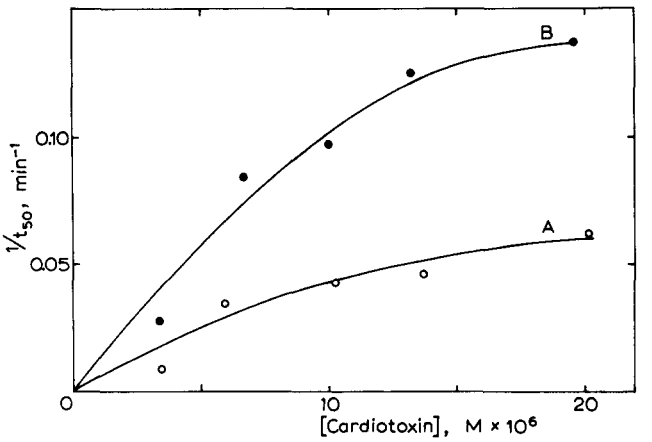

Fig. 3. Effect of relative concentrations of cardiotoxin and phospholipase A_2 on synergism. The final concentration of cardiotoxin $V^{II}2$ was varied as indicated. Rates are expressed as $1/t_{50}$ for cardiotoxin alone (o———o) and in the presence of 3 μg/ml phospholipase A_2 (fraction CM-III) for the synergistic (•———•) reaction. The final erythrocyte concentration was 0.05% (v/v).

the synergistic rate of incubating erythrocytes with either cardiotoxin or cobra venom phospholipase A_2 prior to addition of the respective second component is compared in Table II with the situation where both were added simultaneously. Exposure to cardiotoxin before phospholipase A_2 resulted in a synergistic rate which was within experimental error the same as when they were initially added together. Preincubation with phospholipase A_2, however, subsequently gave rates which were between 40 and 60% faster than the control synergistic rate. In no instance did preincubation with phospholipase A_2 alone result in any measurable hemolysis. However, such an incubation caused the

TABLE I

INFLUENCE OF PHOSPHOLIPASE A_2 CONCENTRATION ON HEMOLYSIS BY CARDIOTOXIN

Hemolysis was followed turbidimetrically as described under Materials and Methods. Rates of hemolysis ($1/t_{50}$ min^{-1}) were determined for cardiotoxin alone and in the presence of phospholipase A_2. The final concentration of erythrocyte suspensions was 0.05% (v/v).

Molar concentration (X10^6) *		Molar ratio	% Phospholipase (molar basis)	Relative rate
Phospholipase A_2	Cardiotoxin	Phospholipase A_2 : Cardiotoxin		
0.23	3.4	1 : 15	6.8	3.0
0.23	6.7	1 : 29	3.4	2.4
0.23	10.0	1 : 44	2.3	2.2
0.22	13.2	1 : 60	1.7	2.7
0.22	19.6	1 : 89	1.1	2.2
0.03	3.5	1 : 116	0.86	2.1
0.06	10.3	1 : 172	0.58	2.1
0.06	13.6	1 : 227	0.44	2.4
0.06	20.1	1 : 334	0.30	2.2
0.03	20.1	1 : 670	0.15	2.4
0.01	20.2	1 : 2020	0.05	2.3

* Molecular weights: phospholipase A_2, 13 400; cardiotoxin, 6800.

** Expressed as the ratio of the rate of hemolysis ($1/t_{50}$) by cardiotoxin in the presence of phospholipase A_2 to the rate of hemolysis by cardiotoxin alone.

TABLE II

EFFECT OF THE ORDER OF ADDITION OF CARDIOTOXIN AND PHOSPHOLIPASE A_2 ON THE SYNERGISTIC REACTION RATE

Rates of hemolysis were determined as described under Materials and Methods. The final concentration of erythrocyte suspensions was 0.3% (v/v). The erythrocyte suspensions were preincubated with either cardiotoxin or snake venom phospholipase A_2 at 37°C for non-lytic periods of 10—20 minutes. The rate of the reaction was measured after the subsequent addition of phospholipase A_2 or cardiotoxin, respectively. Values are given as M × 10^6.

Pre-incubation with		Addition of		Rate ($1/t_{50}$ min^{-1})
Cardiotoxin $V^{II}2$	Phospholipase A_2	Cardiotoxin $V^{II}2$	Phospholipase A_2	
—	—	13.5	0.11	0.17
13.5	—	—	0.11	0.20
—	0.11	13.5	—	0.24
—	—	3.5	0.03	0.05
3.5	—	—	0.03	0.05
—	0.03	3.5	—	0.08

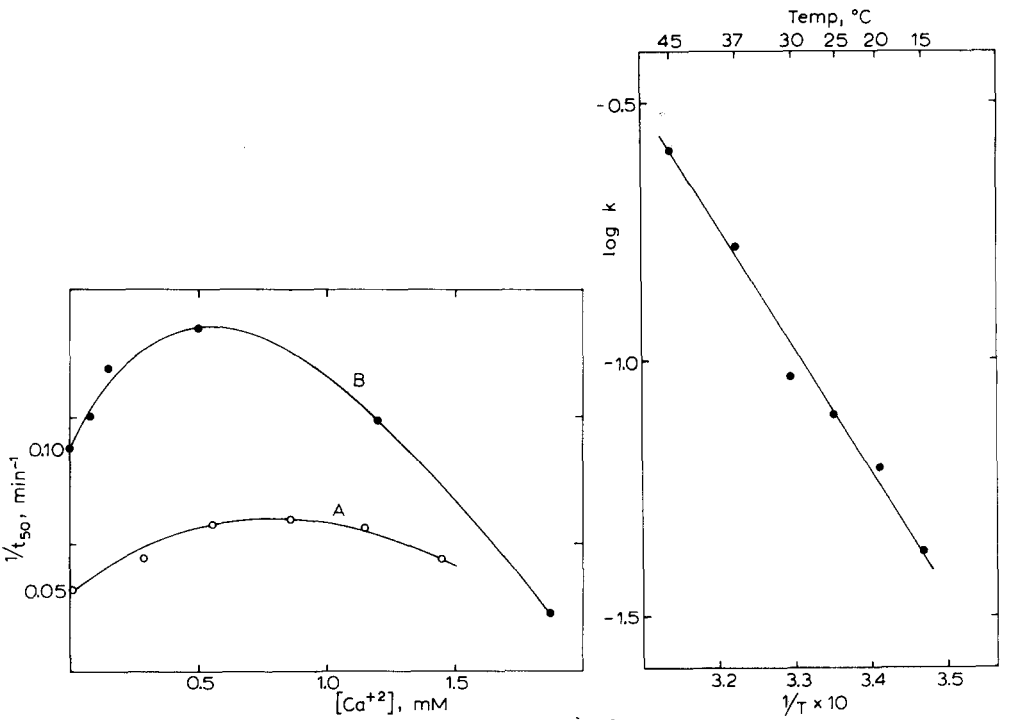

Fig. 4. Effect of Ca^{2+} concentration on rates of hemolysis. Ca^{2+} as $CaCl_2$ was added to the incubation buffer at the final concentrations indicated. The final concentration of cardiotoxin $V^{II}2$ was 115 μg/ml and erythrocyte concentration was 0.0125% (v/v) for hemolysis by cardiotoxin alone (○———○). For the synergistic reaction (●———●) the final erythrocyte concentration was 0.05% (v/v), cardiotoxin $V^{II}2$ concentration was 60 μg/ml and phospholipase A_2 (fraction CM-III) concentration was 1.2 μg/ml.

Fig. 5. Effect of temperature on the synergistic rate of hemolysis. The final concentrations of cardiotoxin $V^{II}2$ was 60 μg/ml and of phospholipase A_2 (fraction CM-III) 1.2 μg/ml. Final erythrocyte concentration was 0.05% (v/v). In the Arrhenius plot k represents $1/t_{50}$ values.

hydrolysis of membrane phosphatidylcholine to an extent of 68—71% as determined from the distribution of inorganic phosphorous after thin-layer chromatography of lipid extracts from untreated and phospholipase A_2-treated guinea pig erythrocytes (see Materials and Methods).

Effect of calcium ions on hemolysis. Shown in Fig. 4 is the influence of Ca^{2+} on the rate of hemolysis by cardiotoxin alone (curve A) and in the presence of phospholipase A_2 (curve B). Whereas maximum stimulation of hemolysis by cardiotoxin occurred at approximately 0.9 mM Ca^{2+} and only slight inhibition was evident at higher concentrations, the rate of hemolysis in the presence of phospholipase A_2 reached a maximum at 0.6 mM Ca^{2+} and was severely inhibited at concentrations higher than 1.2 mM Ca^{2+}.

Effect of temperature on the synergistic rate of hemolysis. The Arrhenius plot for the temperature dependence of hemolysis by cardiotoxin plus phospholipase A_2 between 15 and 45°C is given in Fig. 5. From the slope of the linear plot an activation energy of 11 kcal/mol was calculated for the synergistic reaction.

Discussion

The kinetic parameters of the synergistic reaction by cardiotoxin and cobra phospholipase A_2 differ in several respects from those observed for hemolysis induced by cardiotoxin itself.

The synergistic reaction is not only much faster with an appreciably lower activation energy (11 vs. 14 kcal/mol; ref. 3) but has a characteristic sigmoidal time course (Fig. 1) reminiscent of the autocatalytic effect described for complement-induced hemolysis [29]. This clear distinction from the linear pattern given by cardiotoxin on its own could serve as a useful indicator for the presence or absence of a phospholipase contaminant in any preparation of cardiotoxin from snake venoms. Attention has previously been drawn to the significance of such knowledge in studies on cardiotoxin action [3], and the fact that the full synergistic effect was observed in the present study at phospholipase A_2 levels as low as 0.1% (on weight basis; Table I) is further cogent reason to suspect mechanisms of cardiotoxin action based on evidence obtained with preparations not rigorously proved to be free of phospholipase.

It would furthermore appear that, over the 20-fold concentration range of cobra phospholipase A_2 tested at a particular erythrocyte concentration, the increase in the synergistic rate was only affected by the amount of cardiotoxin present. This is seen in Fig. 3 and in the fairly constant relative rates given in Table I. Phospholipase A_2 therefore acts as a catalyst of high specific activity in enhancing the hemolysis rate in the presence of cardiotoxin. This was also found to be true for pancreatic phospholipase A_2 (Louw, A.I. and Visser, L., unpublished) and *Vipera palistinae* [7,8] which are normally unable to hydrolyze erythrocyte membrane phospholipids. Monolayer studies have shown that pancreatic phospholipase A_2 only hydrolyzes phospholipids at lateral surface pressures well below those found in erythrocyte membranes [30], suggesting that the interaction of cardiotoxin with erythrocytes leads to a lowering of membrane surface pressure. We could demonstrate that phospholipase A_2 from *N. mossambica mossambica* hydrolyzes guinea-pig erythrocyte membrane phos-

pholipids without causing hemolysis, as has been found to be the case for phospholipases A_2 from other *Naja* speices acting on human [31] and rat [32] erythrocytes. The synergistic effect of the enzyme on cardiotoxin-induced hemolysis may, therefore, depend on the intrinsic lytic properties of the lysophosphatidylcholine formed [11], or on a general weakening of the osmotic stability of enzyme-treated membranes [10]. Whatever the case may be, synergism does not depend on the release of lysoproducts from the membrane, since it has been shown by others that these remain embedded in erythrocyte membranes after phospholipase A_2 treatment [33], and neither could we detect any lyso-derivatives by thin-layer chromatography in the supernatant of such hemolysates (Visser, L. and Louw, A.I., unpublished).

The reason for the biphasic dependence of the synergistic rate on the erythrocyte concentration (Fig. 2) is unclear. At low concentrations, a relative excess of cardiotoxin could be bound to the membrane, thereby limiting access of the enzyme or resulting in appreciable surface dilution [24] of the phospholipid substrate in the bilayer, particularly since we have found that cardiotoxins do lyse synthetic liposomes (Harris, Louw and Visser, unpublished).

The stimulation of the synergistic reaction by low Ca^{2+} concentrations (Fig. 4) is reminiscent of a similar stimulation of cardiotoxin-induced hemolysis [3]. Salach et al. [35] found that cobra venom phospholipase A_2, in contrast to its action on phospholipid-detergent mixed micelles, does not require any Ca^{2+}, nor is it inhibited by EDTA in its action on membrane phospholipids. The significant inhibition of the synergistic reaction by higher Ca^{2+} concentrations probably stems from a comparable effect on cardiotoxin action. Since it has been shown (Fig. 3) that the synergistic reaction is dependent on the concentration of cardiotoxin, inhibition of cardiotoxin-induced hemolysis by progressively higher concentrations of Ca^{2+} should, therefore, be reflected in a slower rate of hemolysis for the synergistic reaction.

The kinetic results do not provide an unequivocal answer to the question of whether the hemolytic action of cardiotoxin on its own involves the stimulation of an endogenous membrane phospholipase. The differences in shape of the progress curves, reaction rates and energies of activation given by cardiotoxin, as opposed to cardiotoxin plus exogenous phospholipase A_2 (Figs. 1 and 5), do not favour such a mechanism. However, it is noteworthy that while cardiotoxin has been found to inhibit the $(Na^+ + K^+)$-dependent membrane ATPase of brain cells, axons and erythrocytes [6,36,37], activity was also lost by treatment with either phospholipase C from *Clostridium welchii* [38] or snake venom phospholipase A_2 [39—41]. Activity could be restored to the latter treated ATPase preparations by the addition of phosphatidylserine [40], a phospholipid which has been shown to inhibit cardiotoxin-induced lysis of Yoshida sarcoma cells [37].

The possibility of an endogenous phospholipase activity cannot, therefore, be completely ruled out in the absence of a detailed analysis of membrane phospholipid patterns after exposure of erythrocytes to highly purified cardiotoxins.

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