Lipid monolayers: action of phospholipase A of *Crotalus atrox* and *Naja naja* venoms on phosphatidyl choline and phosphatidal choline

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ABSTRACT The activity of phospholipase A on phosphatidyl choline and phosphatidal choline spread as monolayers on phosphate buffers containing snake venom (*Crotalus atrox* or *Naja naja*) was studied by measuring the fall of surface potential as a function of time, pH, film pressure, temperature, and concentrations of phosphate and venom.

At 25°C, pH 7.0, and 0.2 μg of venom per ml, optimal activity was observed with both venoms on both substrates at 12 dynes/cm film pressure on 0.04 M phosphate. Under these conditions, the pH optimum for *C. atrox* was broad (6.6–7.4) and that for *N. naja* was sharp (8.0) for the action on phosphatidyl choline, whereas both venoms had a sharp optimum at pH 8.0 in their action on phosphatidal choline. The optimal temperature with phosphatidyl choline was 27.5°C for *N. naja* and 40°C for *C. atrox*.

In line with studies of phospholipase A activity in bulk phase in ether, phosphatidal choline was attacked much more slowly than phosphatidyl choline by *C. atrox*. Under conditions where both venoms had equal activity on phosphatidyl choline, *C. atrox* was only half as active as *N. naja* on phosphatidal choline. The studies suggest that the linkage of the hydrophobic chains in glycerophosphatides may affect their interaction with proteins.

KEY WORDS monolayers · snake venoms · phospholipase A · phosphatidyl choline · phosphatidal choline · surface potential · substrate structure · enzyme activity

ALTHOUGH THE ACTION of snake venoms on phospholipids has been studied extensively in bulk phase reactions in both aqueous and ether media, no studies have been reported using phospholipid monolayers as substrates since Hughes first published his experiments in 1935 (1). Since it is known that the first step in enzymic catalysis is the combination of enzyme and substrate molecules, this reaction may be considered as a model of the more general problem of lipid–protein interaction. It can, of course, be readily appreciated that in the study of lipid–protein interactions, the monolayer technique offers certain advantages over bulk phase methods, since it deals with molecules that have been oriented by the air–liquid interface to provide a degree of organization resembling that found in biological membranes. Hughes showed that snake venoms produced a fall in surface potential of monolayers of lecithin since the surface potential of the lysolecithin product was 150 mv less than that of the lecithin substrate (1). Using *Notechis scutatus* (black tiger), *Denisonia superba* (copperhead) and *Naja naja* (cobra) venoms, he studied this phenomenon as a function of pH, venom concentration, and film pressure.

Recently Gottfried and Rapport (2) showed that two closely related phospholipid molecules, phosphatidyl choline (lecithin) and phosphatidal choline, behave differently with respect to rattlesnake venom (*Crotalus atrox*) and cobra venom (*Naja naja*). These studies were carried out in ether, which for this reaction is an ill-defined and very unphysiological medium, and it was therefore of considerable interest to determine whether similar findings could be established using monolayers as substrates. In this report, the studies of Hughes are extended by describing the action of phospholipase A on phosphatidyl choline monolayers as a function of venom concentration, phosphate (buffer) concentration, pH, temperature, and film pressure, and a comparison is then made of the activity of *C. atrox* and *N. naja* venoms on phosphatidyl choline and phosphatidal choline. It should be borne in mind that these studies are different from those of Bangham and Dawson (3, 4) who studied phospholipases B and C using other techniques.

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MATERIALS AND METHODS

Lipids

Three different preparations of phosphatidyl choline (egg lecithin; Sylvana Chemical Company, Millburn, N. J.) and three of phosphatidal choline, isolated from bovine heart lecithin according to Gottfried and Rapport (2), were studied. The lipid preparations were homogeneous as judged by thin-layer chromatography, and gave satisfactory analyses for phosphorus, ester, and unsaturated ether groups (2).

Venoms

Two different preparations of *C. atrox* venom and two of *N. naja* venom were obtained from Ross Allen Reptile Institute, Silver Springs, Fla. Solutions of venom were prepared as follows. The venom (3.5 mg) was dissolved in 10 ml of a cold (2°C) solution of 0.1 M $CaCl_2$. After 1 hr at 2–5°C an aliquot of the venom solution was diluted 10-fold with cold 0.01 M $CaCl_2$, and stored in the cold for not less than 12 nor more than 48 hr before use. We observed that the activity of the venom increased in the first 12 hr, was stationary between 12 and 48 hr, and then decreased after 48 hr. Two milliliters of the cold dilute venom solution was added to 350 ml of phosphate buffer (which thus contained 0.2 μg venom per ml) and the solution was used immediately to fill the trough. The enzyme solution containing 350 μg of venom per ml was prepared fresh for each series of experiments. On several occasions, tests of this stock solution kept at 5°C indicated that the enzyme was stable for 5 days.

Water

Low conductivity, salt-free water was obtained by glass distillation of water distilled once. Water of 1.1 microreciprocal ohm/cm conductivity was collected in polyethylene bottles. The phosphate buffer solutions, prepared with this water, were foamed in order to remove long-chain surfactants probably present in the salts and in the water in variable quantities on different days. The technique, suggested by Dr. Henri L. Rosano, was as follows. A 2 liter, medium porosity sintered glass funnel was filled to the rim with buffer solution; a stream of nitrogen was bubbled through the sintered glass at moderate rates for 10 min (disappearance of stable foam). During this process, foam was removed continuously by sweeping the surface, first with a glass barrier, then by aspiration with a capillary. Potassium phosphates of reagent grade were used without further purification.

Apparatus and Procedure

The experiments were performed by means of the Wilhelmy plate modification of the Langmuir film balance (5). A trough of Lucite (19 mm in depth) was filled to the rim with phosphate buffer, the aqueous subphase. The edges of the trough and a glass barrier were made hydrophobic by a uniform coat of pure paraffin (mp 48°C; Fisher Scientific Co., New York, N. Y.) in order to contain the film. Two measurements were made: surface tension and surface potential. The surface tension was measured by a sandblasted platinum blade (the Wilhelmy plate) suspended from a torsion balance (Federal Pacific Co., Newark, N. J.). Surface pressure was calculated: $\pi = \gamma_{aqueous} - \gamma_{film}$. For the measurement of surface potential, a radioactive (^{226}Ra) air electrode (6) was held in the air 4 mm above the aqueous surface. The radioactive electrode and saturated calomel electrode with 1 M KCl salt bridges connected the air gap above the film and the aqueous subphase under the film to the positive and negative poles of a high impedance electrometer, Keithley Model 610 A (Keithley Instruments, Inc., Cleveland, Ohio). Surface potential was obtained ($\Delta V = V_{film} - V_{solution}$) as the difference between two electrometer readings corresponding to surfaces with and without the film. The temperature under the film was kept constant by circulating water through the aqueous subphase in a multiple coil glass tubing, which was connected to a circulating pump. Trough, electrodes, and film-compressing mechanism were housed in an electrostatically shielded box. The assembly and an example of surface pressure-area and surface potential-area curves are illustrated in Fig. 1.

The film was formed by injecting with a microsyringe 10 μg of lecithin dissolved in 25 μl of hexane–ethanol 98:2 at the center of a perfectly clean 7.6 × 20 cm surface, which corresponded to an area of 200 A^2/molecule

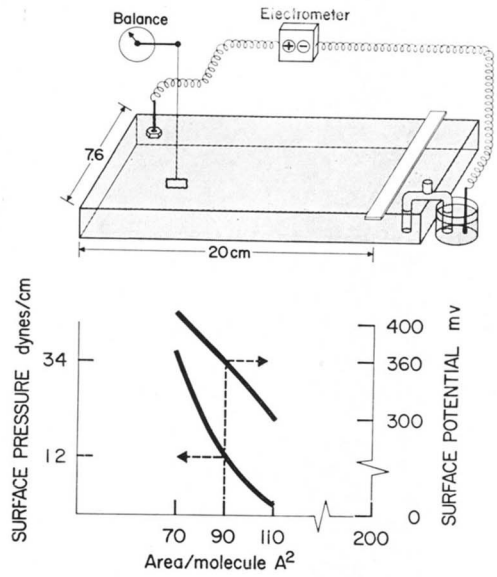

FIG. 1. Schematic diagram of apparatus. Force–area and surface potential–area curves of phosphatidyl choline on 0.04 M phosphate buffer, pH 7.0, 25°C.

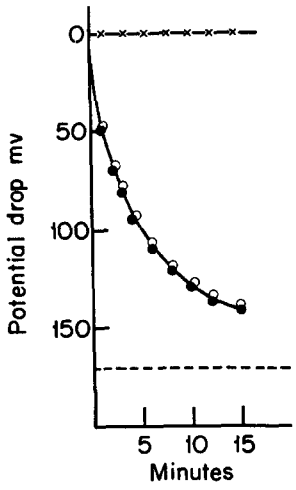

FIG. 2. Typical kinetic curves of action of *C. atrox* and *N. naja* venoms on phosphatidyl choline (0.04 M phosphate buffer, pH 7.0, 25 °C, 12 dynes/cm initial film pressure): ×, no venom; ●, *C. atrox*, 0.2 μg/ml; ○, *N. naja*, 0.1 μg/ml. Dashed line represents 100% reaction (170 mv drop in potential).

(calculated according to the phosphorus analysis of the lecithin). Under these conditions, at 25°C, the pressure of the film was zero and the surface potential was also zero. As the film is compressed from right to left in Fig. 1, the area per molecule decreases, the surface pressure (on the left axis) increases and the surface potential (on the right axis) increases. In going from 2 to 34 dynes/cm film pressure, the area goes from 110 to 70 A^2/molecule and the surface potential changes from 300 to 410 mv. Attention is called to the pressure of 12 dynes/cm, corresponding to an area of 90 A^2/molecule and surface potential of 360 mv; most of the studies described were done at this film pressure, at which both venoms displayed optimal activity on lecithin.

For the study of snake venom action, the cold venom solution was mixed with the phosphate buffer, which was at the required temperature, and the mixture was transferred immediately to the trough. After 1 min the surface was cleaned by sweeping twice in each direction, an operation lasting a total of 3 min. One minute later the lipid was spread. After another minute the film was compressed rapidly (10 sec) to the desired pressure, and the fall in surface potential with time (at constant area) was recorded every 30 sec.

RESULTS

In the absence of venom, both surface pressure and surface potential of phosphatidyl choline and phosphatidal choline monolayers remained constant over several hours. In the presence of venom (Fig. 2) a fall in surface potential took place which varied between zero (no activity) and 175 ± 5 mv, depending on the various conditions of venom concentration, pH, film pressure, temperature, and phosphate concentration. The fall in surface potential, which is assumed (1) to be proportional to the number of molecules of lecithin being transformed into lysolecithin with the release of fatty acid, can be used to express the activity of the venom. The fall of potential stopped at 175 ± 5 mv, which corresponded to complete reaction. In a typical activity curve (Fig. 2) the surface potential of a lecithin film compressed to 12 dynes/cm dropped 59 mv in the first minute and 140 mv in 15 min under the action of either 0.2 μg/ml of *C. atrox* or 0.1 μg/ml of *N. naja* venom in 0.04 M phosphate buffer, pH 7.0, at 25°C. Under these conditions the activity of the cobra venom was twice that of rattlesnake venom and the two curves were superimposable within ± 2 mv.

For convenience in presentation the activity is expressed as the drop in potential during the first minute. This activity increased linearly with venom concentration up to 0.2 μg/ml (Fig. 3).

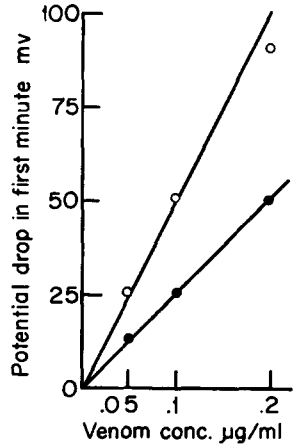

FIG. 3. Effect of venom concentration on hydrolysis of phosphatidyl choline (12 dynes/cm initial film pressure, 0.04 M phosphate buffer, pH 7.0, 25 °C): ●, *C. atrox;* ○, *N. naja.*

With both venoms at 0.2 μg/ml, 25°C, and pH 7.0, the optimal conditions for their action on egg lecithin were 0.04 M phosphate (Fig. 4) and 12 dynes/cm film pressure (Fig. 5). The two venoms differed markedly with regard to pH dependence (Fig. 6) and the effect of temperature (Fig. 7). At 25°C the pH optimum of *C. atrox* was broad (6.6–7.4), whereas that of *N. naja* was sharp at pH 8.0. At pH 7.0, *N. naja* venom had a temperature optimum at 27.5°C, where its activity was more than double that of *C. atrox*. In contrast, the temperature optimum of *C. atrox* was at 40°C, where its activity was more than twice that of cobra venom. The two venoms had the same activity at 34°C.

The activities of the two venoms are compared on the two different substrates at 25°C, 12 dynes/cm film pres-

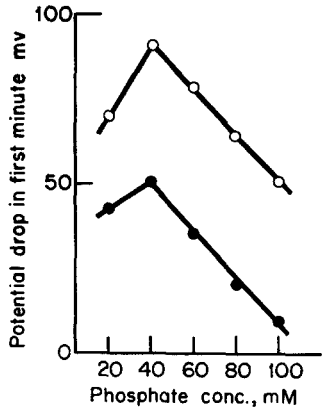

FIG. 4. Effect of phosphate concentration on hydrolysis of phosphatidyl choline (0.2 μg of venom per ml.; 12 dynes/cm initial film pressure; pH 7.0; 25 °C): ●, *C. atrox;* ○, *N. naja.*

sure, 0.04 M phosphate buffer, and pH 7.0 in Fig. 8. Under conditions in which *N. naja* and *C. atrox* venoms had the same activity on phosphatidyl choline (lowest curve, 140 mv drop in 15 min), the activity of *C. atrox* venom on phosphatidal choline was only one-half of that on phosphatidyl choline over the entire time interval. Using different samples of venom and different samples of phosphatidyl choline and phosphatidal choline, these results were reproduced within ± 2 mv.

DISCUSSION

For studies of phospholipase A activity, the monolayer technique offers certain advantages over bulk phase studies in either ether or water (2, 7), namely, the reproducibility of the results and the rapidity of the reaction. The lag phase, frequently seen with some venoms in bulk phase studies, is absent, so that reliable kinetic comparisons can be made.

Because of the many technical factors that introduce variation in monolayer studies and the emphasis we wish to place on reproducibility, it will be necessary to discuss the technique in some detail.

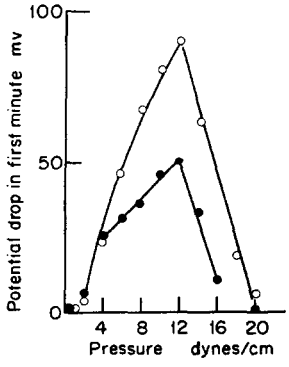

FIG. 5. Effect of initial film pressure on hydrolysis of phosphatidyl choline (0.2 μg of venom per ml; 0.04 M phosphate buffer; pH 7.0; 25 °C): ●, *C. atrox;* ○, *N. naja.*

Water

Although water distilled over alkaline permanganate is probably best, water of satisfactory quality can be obtained by foaming after double distillation in glass. The criteria we used were (*a*) that the specific resistance was greater than 900,000 ohms, and (*b*) that, in the trough, after the surface had been swept clean four times, the initial surface tension ($\gamma_{aqueous}$) and initial potential ($V_{solution}$) remained constant for a minimum of 10 min, even under repeated compression and decompression. These values also remained constant when venom and phosphate were present.

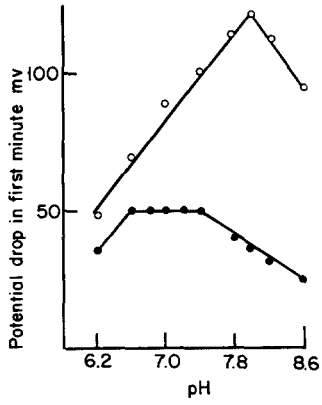

FIG. 6. Effect of pH on enzymic hydrolysis of phosphatidyl choline (0.2 μg of venom per ml; 12 dynes/cm initial film pressure; 0.04 M phosphate buffer; 25 °C): ●, *C. atrox:* ○, *N. naja.*

Addition of Venom

Spreading the lipid on the venom solution at zero pressure was preferred to injecting the venom under the lipid film (at a given pressure) because it simplified the operation and yielded more reproducible results. Both techniques were used by Hughes (1). This method eliminates the need for mixing the aqueous solution in the trough and is permissible because enzyme activity is nil at very low film pressure. The critical factors are the quality of the water, the timing of the individual operations, and the electrical connections. In the presence of sufficiently pure water, surface denaturation of the venom protein was extremely slow as judged by the constancy of the values of γ_o and V_o. This stability was achieved by preparing all solutions with water that had been distilled, subjected to foaming within 24 hr, and then used within 72 hr. Erratic behavior (unstable γ_o and V_o values after surface cleaning) was observed when water or solutions were stored for longer than 1 wk in polyethylene or pyrex glass bottles. This may result from the extraction

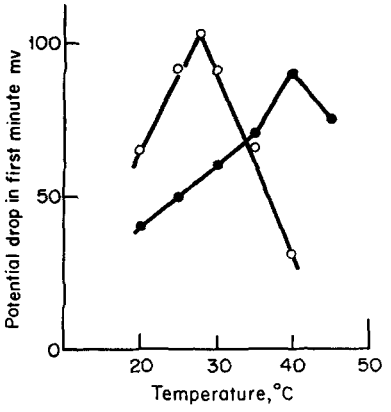

FIG. 7. Effect of temperature on enzymic hydrolysis of phosphatidyl choline (0.2 μg of venom per ml; 12 dynes/cm initial film pressure; 0.04 M phosphate buffer, pH 7.0): ●, *C. atrox;* ○, *N. naja.*

of substances (polymers or silicates) which facilitate the accumulation of protein at the interface. When this happens, errors may be introduced directly either by inhibition or stimulation of enzyme activity by the foreign materials, or indirectly by decreasing the concentration of enzyme during the cleaning of the surface.

Electrodes

The saturated calomel electrode (8) was preferred to the Ag/AgCl electrode, used both by Hughes (1) and by Shah and Schulman (9), in order to avoid errors introduced by the reaction of the unprotected Ag/AgCl system either with protein or phosphate in the aqueous subphase. Protection of the electrode with bridges of concentrated KCl is advisable. However, with such bridges, erratic results may result from leaks in them or by the accumulation, with time, of substances which produce fluctuating boundary potentials.

Standardization of Surface Potential Measurements

Periodically a standard film of stearic acid on 0.01 N HCl was examined. A surface potential of 388–394 mv at an area of 20.5 A^2/molecule was obtained, in agreement with the literature (10). The surface potential values of egg lecithin that we obtained were about 30 mv higher than those reported by Hughes (1) and by Shah and Schulman (9). It was found that surface potentials as well as apparent enzyme activity became progressively lower with aging of the electrical connections between the air electrode and the electrometer. Deterioration was more rapid if the connections were not soldered. Reconstitution of the terminals or replacement of the cables restored the surface potentials to their correct values.

Preparation of Trough and Barrier

Heavy paraffin coating of the trough edges and the glass barrier was required for good performance. A lightly coated barrier, recommended for monolayer work and satisfactory for making a single isotherm of a lipid film, was found to be unsuitable for experiments with venoms. A heavy paraffin coating permitted the use of a barrier for as many as 50 experiments and the trough for 500. At temperatures above 35°C, where paraffined apparatus was not practical, a Teflon trough and a heavy Teflon barrier were used.

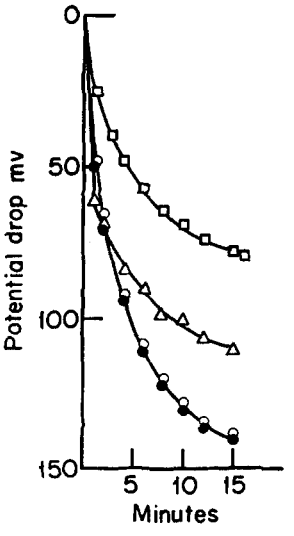

FIG. 8. Kinetic comparison of action of *C. atrox* and *N. naja* venoms on films of phosphatidyl choline and phosphatidal choline (12 dynes/cm initial film pressure, 0.04 M phosphate buffer; pH 7.0; 25°C): ●, 0.2 μg/ml *C. atrox* on phosphatidyl choline; ○, 0.1 μg/ml *N. naja* on phosphatidyl choline; △, 0.1 μg/ml *N. naja* on phosphatidal choline; □, 0.2 μg/ml *C. atrox* on phosphatidal choline.

Extent and Course of the Reaction

In numerous experiments the largest fall in surface potential was between 170 and 180 mv, and this value was taken to represent complete reaction. Two facts support the assumption that both products of the action of phospholipase A on egg lecithin, namely, fatty acid and lysolecithin, remain in the film. The first is that a mixed film of equimolar quantities of stearic acid and lysolecithin at an area of 90 A^2 (simulating 100% reaction) had a surface potential 175 ± 5 mv less than that of lecithin (0.04 M phosphate buffer, pH 7, 25°C). The second is that no loss of film pressure took place during the action of the venoms on either phosphatidyl choline (egg) or phosphatidal choline, and the loss of either product from the film would be expected to produce a loss of film pressure.

The film was collected on a wire gauze and analyzed by thin-layer chromatography at intervals corresponding to no reaction, 50% reaction (90 mv drop), and com-

plete reaction (175 mv drop). The appearance of fatty acid and lysolecithin and the disappearance of lecithin were in accord with expectations. At zero time, lecithin only was recovered. At 50% reaction, half of the lecithin was recovered together with corresponding quantities of lysolecithin and fatty acid. At complete reaction, only lysolecithin and fatty acid were found.

The lipid–protein interaction per se involves little change in surface pressure and surface potential at these low protein concentrations. No change in pressure was seen with nonspecific proteins such as rabbit serum albumin and γ-globulin at 0.2 μg of protein per ml, or even with venom at a pH below 5, where it is inactive. The change in surface potential with serum albumin and γ-globulin was nil. With phosphatidyl choline and either venom the surface pressure rose 2–3 dynes in the first minute, 1–2 dynes in the next 5 min and then fell slowly (0–1 dyne) during the following 10 min. This rise may indicate some specific enzyme–substrate interaction. The small initial rise in surface pressure may serve as a control: if it did not occur, then the drop in surface potential was low, indicating either a leak or an inactive enzyme.

Comparison of Venoms

Crotalus atrox and *Naja naja* venoms were similar in showing optimal activity on films of either phosphatidyl choline or phosphatidal choline at the same film pressure and concentration of phosphate. The venoms were quite different, however, with respect to the effects of pH (Fig. 6) and temperature (Fig. 7) on their activity on phosphatidyl choline. These differences may reflect differences in the protein structure of the enzymic component of the *C. atrox* and *N. naja* venoms.

Comparison of Substrates

With phosphatidal choline, optimal temperatures were slightly lower than with phosphatidyl choline (35°C vs. 40°C for *C. atrox;* 25°C vs. 27.5°C for *N. naja*).

The broad pH optimum obtained for the action of *C. atrox* on egg phosphatidyl choline is unusual. The activities of *C. atrox* on phosphatidal choline and of *N. naja* and *C. helleri* on phosphatidyl choline and phosphatidal choline all showed sharp optima at pH 8.0. Hughes reported that the pH optimum of black tiger snake (*N. scutatus*) venom on phosphatidyl choline was 7.3. The dependence of the pH effect on the nature of the substrate requires further investigation.

Both *C. atrox* and *N. naja* venoms were able to distinguish between phosphatidyl choline and phosphatidal choline, but the difference was much larger with *C. atrox* than with *N. naja*. These studies suggest that the linkage of the hydrophobic chains in glycerophosphatides may affect their interaction with proteins in biological membranes, and differences in structure may reflect some specificity of interaction between lipid and protein. Replacement of an acyl ester linkage of phosphatidyl choline by an α, β-unsaturated ether in phosphatidal choline may effect a change not only in steric configuration but also in dielectric properties. Differences in the latter among phospholipids are suggested by the finding that the surface potential of phosphatidal choline is considerably smaller than that of phosphatidyl choline (9).

Our observations at the air–water interface confirm those in bulk phase in ether reported by Gottfried and Rapport (2), and thus the two independent methods provide harmonious information. It is conceivable that the orientations of the lipid–protein structures at both the ether–water interface (which is probably present in bulk phase) and at the air–water interface are similar at these temperatures.

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