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Interaction of *Staphylococcus aureus* δ-Lysin with Phospholipid Monolayers†

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ABSTRACT: The interactions of purified staphylococcal δ-lysin and melittin with various phospholipid monolayers containing different polar head groups and fatty acid moieties and with monolayers of cod and sheep erythrocyte lipids at various initial film pressures (π_i) were studied by using the Wilhelmy plate method. In each case the final increase in surface pressure ($\Delta\pi$) was a linear function of π_i. In the case of δ-lysin, the critical pressures (π_c, the extrapolated values of π_i at $\Delta\pi$ = 0) for phosphatidylcholines with different fatty acid chain length, dipalmitoylphospholipids with different polar head groups, and cod or sheep erythrocyte total lipids fell within a relatively narrow range whereas melittin showed a much wider range. The collapse pressures of the δ-lysin and melittin films at the air–water interface when adsorbed from the hypophase were very similar. δ-Lysin showed little or no specificity in its interactions with all types of lipid films studied, whereas melittin showed preferential interaction with films of acidic lipid, similar to the specificity reported for cardiotoxins of *Naja mossambica mossambica* described by other workers.

δ-Lysin of *Staphylococcus aureus* is one of four principal extracellular proteins produced by numerous strains of the bacterium (Freer & Arbuthnott, 1976). It is a 26 amino acid polypeptide (Fitton et al., 1980) containing 14 hydrophobic residues and a high percentage of nonionizable side chain amino acids. Properties of the lysin such as solubility in chloroform–methanol (2:1 v/v), ethanol, and water, inactivation by phospholipids, and strong surface activity suggest an amphiphilic structure. It forms an unusually stable monolayer film at air–water interfaces similar to a lipid film, the collapse pressure depending on the solvent utilized (Colacicco et al., 1977).

Like δ-lysin, bee venom melittin, again a 26 amino acid amphiphilic polypeptide, displays strong surface activity and interacts with membranes and phospholipids. Melittin is predominantly hydrophobic in the first 20 residues (Habermann & Jentsch, 1967), but unlike δ-lysin the charge distribution is highly asymmetric with a cluster of four ionizable groups near the C terminus (residues 21–24, Lys-Arg-Lys-Arg) and single charges at the N terminus (Met) and lysine-7. Dawson et al. (1978) suggested a secondary structure for melittin consisting of two short α-helical sequences separated by a flexible "hinge" region centred on Pro-14. The basic C-terminal portion of the molecule is thought to interact with acidic groups on biomembranes, leading to planar orientation on the membrane surface. Subsequent conformational changes associated with hydrophobic interactions between the polypeptide and membrane lipids may result in a "wedgelike" insertion of melittin into the membrane and subsequent membrane destabilization. Fitton (1981) has suggested by the predictive method of Chou & Fasman (1974) that δ-lysin has a secondary structure very similar to that of melittin, with two α-helical domains separated by a flexible hinge region. A considerable α-helical content in aqueous solution is evident from the circular dichroism studies of Colacicco et al. (1977) and Fitton (1981). Partly on the basis of these similarities, a common mechanism for membrane penetration has been

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suggested for δ-lysin and melittin (Fitton, 1981).

Recently, the nature of the interactions of monolayers of phospholipids with melittin and with various cardiotoxins and neurotoxins from *Naja mossambica mossambica* have reported by Bougis et al. (1981). Melittin exhibited preference for negatively charged phospholipids although it also penetrated zwitterionic phospholipid monolayers (Bougis et al., 1981). Cardiotoxins, which are polycationic polypeptides with several intrachain disulfide bonds, display preferential penetration of negatively charged phospholipid monolayers.

To investigate the degree of similarity between the interactions of δ-lysin and melittin with phospholipids, we have compared their behavior at an air–water interface and their interaction with different phospholipid monolayers. This was done at various initial pressures to determine the extent of "penetration" and the degree of specificity involved in peptide–phospholipid interactions.

Materials and Methods

δ-Lysin. Staphylococcal δ-lysin was prepared from *Staphylococcus aureus* strain NCTC 10345 and purified according to Kreger et al. (1971) followed by precipitation with 1 N Cl_3AcOH, washing in 1 N Cl_3AcOH, and lyophilization (T. H. Birkbeck, unpublished data). ^3H-Labeled δ-lysin was similarly prepared from cultures grown in the presence of [^3H]lysine.

Melittin. Melittin, free of detectable phospholipase activity, was a gift from Dr. A. J. Lawrence, University of Glasgow.

Phospholipids. Chromatographically pure dilauroylphosphatidylcholine (PC12:0,12:0), dimyristoylphosphatidylcholine (PC14:0,14:0), dipalmitoylphosphatidylcholine (PC16:0,16:0), 1-palmitoyl-2-oleoylphosphatidylcholine (PC16:0,18:1), distearoylphosphatidylcholine (PC18:0,18:0), dioleoylphosphatidylcholine (PC18:1,18:1), dibehenoylphosphatidylcholine (PC22:0,22:0), dipalmitoylphosphatidic acid (PA16:0,16:0), dipalmitoylphosphatidylethanolamine (PE16:0,16:0), and dipalmitoylphosphatidylglycerol (PG16:0,16:0) were obtained from Sigma (St. Louis, MO) at 99% purity. This was confirmed by gas–liquid chromatographic analysis at 175 °C on a polar column (10% diethylene glycol adipate; DEGA) in a Packard 428 gas chromatograph (Bhakoo & Herbert, 1979).

Solvents. Analar chloroform, ethanol, and methanol (BDH, Poole, England) were redistilled before use. All phospholipids and lipid mixtures used in monolayer studies were dissolved in chloroform at fixed concentrations except PE16:0,16:0, which was dissolved in chloroform–ethanol (7:3 v/v).

Sheep and Cod Erythrocyte Lipids. Sheep erythrocyte lipids were extracted from freshly drawn sheep blood as described by Nelson (1967). Fresh cod blood was washed according to Chao & Birkbeck (1978) and erythrocyte lipids were extracted as described by Addison & Ackman (1971). The fatty acids were methylated with methanol–6 M HCl (14:1 v/v) and were analyzed by GLC as described by Bhakoo & Herbert (1979) using a glass column packed with 10% DEGA on Chromosorb W.

Monolayer Techniques. Water (pH 6.8) was glass distilled and deionized by using a Milli-Q purification system (Millipore Ltd., London) until high resistivity was achieved and was collected in pristine glassware. Surface tension (γ) was measured by the Wilhelmy hanging-plate method with a roughened platinum plate (perimeter 2.876 cm) to maintain a zero contact angle. The plate was lowered carefully until it just touched the water surface in order to minimize the effect of buoyancy on the readings, although the term cancels out when differences in γ are measured.

A circular glass crystallizing dish, 23.5 cm in diameter, was used as a trough for spreading phospholipid monolayers at the air–water interface. A small glass ring covered with cleaned paraffin wax (BDH, Poole, England) was sited at the interface to allow injection of δ-lysin into the subphase. The Wilhelmy plate was attached to a Beckman LM800 microbalance (Beckman-RIIC Ltd., Glenrothes, Scotland) connected to a Bryans 29000 X-Y recorder (Bryans Southern Instruments, Ltd., Mitcham, Surrey, England) for measurement of the change in surface tension with time. Surface pressure (π) equals $\gamma_f - \gamma_i$ where γ_f and γ_i are surface tension measurements of the film and of clean water, respectively.

The whole apparatus was enclosed within a thermally insulated chamber thermostatically controlled at 22 ± 0.2 °C and with a nitrogen atmosphere. Phospholipid monolayers were formed at the required surface pressure by intermittent application of phospholipid solutions with a micrometer-controlled Agla syringe (Burroughs-Wellcome Laboratories, England). Before spreading a monolayer the surface-active impurities were removed by simultaneous sweeping and suction of the interface; the absolute surface tension value of water was used to indicate surface-active impurity. A solution of δ-lysin was injected to give a final concentration of 1 μg/mL in the subphase and was dispersed with a magnetic stirrer and circular follower magnet which did not disturb the stability of the surface monolayer. The change in surface pressure ($\Delta\pi$) of the monolayer was measured for various values of π_i after the addition of δ-lysin. Each different determination required a separate experiment. Force–area curves were determined in order to validate the monolayer technique and to establish the purity of phospholipid films; the purity in terms of fatty acid moieties was confirmed by GLC.

Procedure for [^3H]Lysin. In experiments involving δ-[^{3}H]lysin, the lipid film was removed by simultaneous suction and sweeping at the interface to collect the film in a scintillation vial. The lipid film was assumed to have a negligible volume, and a correction for the bulk-phase lysin, unavoidably collected during recovery of the film, was made by subtracting the counts present in a volume of bulk-phase liquid equal to that of the liquid harvested with the film.

Results

Figure 1 and Table I show the effect of δ-lysin and melittin on the surface pressure of films of phospholipids with different head groups or fatty acyl chains spread at different initial surface pressures. Each point represents the final increase in π ($\Delta\pi$) after injection of δ-lysin or melittin into the subphase. The values plotted are for separate monolayers of different π_i obtained by the addition of appropriate quantities of individual phospholipids to the air–water interface. For each phospholipid the maximum value of $\Delta\pi$ is a function of π_i.

The interaction of δ-lysin or melittin with the phospholipid monolayers resulted in a marked increase in surface pressure. As $\Delta\pi$ was greater at lower π_i values, one may conclude that a greater area/molecule favored interaction.

The observed kinetics were nonlinear and $\Delta\pi$ reached a maximum within a few minutes, in agreement with data on the penetration of lipid monolayer films by various proteins (MacRitchie, 1978; Kimelberg & Paphadjopoulos, 1971). The critical pressure, π_c, which represents the extrapolated values of π_i at which there was no penetration by δ-lysin of the monolayers with different fatty acid chain lengths or head groups, ranged from 23.6 to 37.3 mN m^{-1}. For melittin, a wider range of 19.6–41 mN m^{-1} was observed. The values for maximum film penetration by δ-lysin and melittin at zero initial film pressure were derived by extrapolation (Table I).

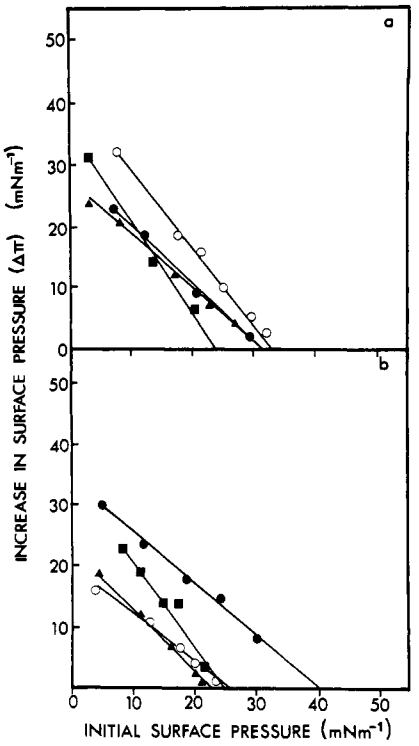

FIGURE 1: Increase in maximum value of surface pressure as a function of initial film pressure. (a) δ-Lysin and (b) melittin in various phospholipid films. Peptide present at 3.4×10^{-7} M. PG16:0,16:0 (●), PE16:0,16:0 (▲), PA16:0,16:0 (■), PC16:0,16:0 (○); temperature 22 °C and pH 6.8.

Table I: Extrapolated Film Pressure at Zero Initial Film Pressure and Critical Pressure for Various Phospholipids

type of monolayer	film penetration pressure at $\pi_i = 0$ (mN m^{-1})		critical pressure, π_c (mN m^{-1})	
	δ-lysin	melittin	δ-lysin	melittin
PC12:0,12:0[a]	37.7	23.0	35.2	41.0
PC14:0,14:0	33.5	n.t.[b]	36.5	n.t.
PC16:0,16:0	42.2	19.2	32.6	26.0
PC16:0,18:1	34.0	20.4	36.0	24.8
PC18:0,18:0	32.8	n.t.	34.6	n.t.
PC18:1,18:1	38.5	22.0	33.8	24.6
PC22:0,22:0	31.2	n.t.	37.3	n.t.
PA16:0,16:0	35.5	32.0	23.6	25.2
PE12:0,12:0	32.6	26.7	33.6	19.6
PE16:0,16:0	28.0	21.4	31.4	23.0
PG16:0,16:0	29.0	33.0	31.4	40.0
cod erythrocyte lipids	32.9	21.8	32.0	48.0
sheep erythrocyte lipids	30.3	25.7	33.2	43.4

[a] PC, phosphatidylcholine; PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol. The number before the colon gives the carbon chain length of the fatty acid and the number after the colon gives the number of double bonds. [b] n.t. = not tested.

No appreciable difference in π_c was observed for interaction of δ-lysin with monolayers of phosphatidylcholines carrying saturated or unsaturated fatty acyl chains.

Also, the relationship of $\Delta\pi$ to π_i during the interaction of δ-lysin or melittin with monolayers formed from total extractable lipids of either cod or sheep erythrocytes was investigated. The π_c values, which represent the monolayer pressure at or above which no "interaction" or "penetration" by the peptides was evident, were very similar to each other with values of 32.0 and 33.2 mN m^{-1} for cod and sheep lipid films, respectively, for δ-lysin and 48.0 and 43.4 mN m^{-1}, respectively, for melittin.

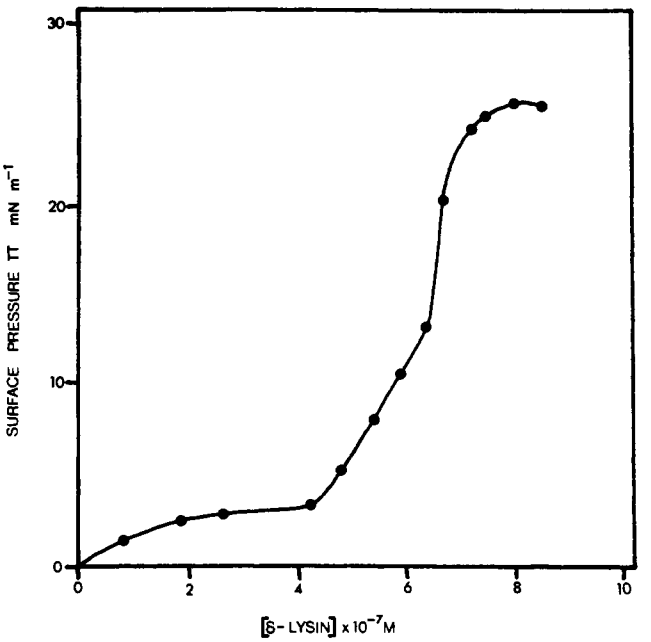

FIGURE 2: Surface pressure of δ-lysin film as a function of δ-lysin concentration in the subphase; temperature 22 °C and pH 6.8.

In spite of the similarity in the π_c values for δ-lysin interaction with cod and sheep lipid films, the fatty acid composition of the two lipid extracts varied markedly. The major fatty acids in cod erythrocytes were, in order of abundance, C22:6, C18:1, C16:0, C20:5, C20:1, C16:1, C18:0, C14:0, and C20:4. Unsaturated fatty acids accounted for approximately 75% of the total. In sheep erythrocytes the major fatty acids were, in order of abundance, C18:1, C20:4, C14:0, C18:2, and C18:0.

Formation of a film at the air–water interface by δ-lysin or melittin was very rapid and was dependent on concentration below 2 μg/mL. As shown in Figure 2 a sigmoid curve was obtained when π was plotted against δ-lysin concentration. The collapse pressure of 25.5 mN m^{-1} was similar to the value of 24.5 mN m^{-1} obtained for melittin.

Radiolabeled δ-lysin gave force–area curves which were identical with those obtained with unlabeled lysin. The amount of lysin associated with the lipid was similar in films of dilauroyl-, dipalmitoyl-, and dioleoylphosphatidylcholine and amounted to approximately 3% of added lysin in all cases. For dipalmitoylphosphatidylcholine this gives a lysin:phospholipid ratio of approximately 1:27.

Discussion

The collapse pressure of 25.5 mN m^{-1} for δ-lysin is very similar to that obtained for melittin in the present study and by Sessa et al. (1969) (24.5 mN m^{-1}) and considerably higher than that obtained for cardiotoxins (10–12 mN m^{-1}) by Bougis et al. (1981). Any increase in π above the collapse pressure of δ-lysin can be considered to involve "penetration" or "deformation" of the phospholipid monolayer (Kimelberg & Paphadjopoulos, 1971), but no differentiation between these two possible mechanisms was attempted.

When surface-active peptides interact with phospholipid films, comparison of the plots of $\Delta\pi$ values against π_i may reveal any preferential interaction of the peptide with particular phospholipid types. Such plots, when a constant amount of δ-lysin or melittin was introduced into the subphase of monolayers of phospholipids at different initial surface pressures, yielded π_c values (the intercept on the abscissa) above which

there was no detectable interaction between lysin and phospholipid film. The π_c values for monolayers of phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, and phosphatidic acid, all containing two palmitic acid moieties, showed differences which reflected the size and charge of head groups. Melittin exhibited a marked preference for films composed of phosphatidylglycerol (π_c = 40 mN m^{-1}) an acidic phospholipid, compared with films of the zwitterionic lipids phosphatidylcholine and phosphatidylethanolamine (π_c = 26 and 23 mN m^{-1}, respectively). The above π_c values confirm the earlier observations of Bougis et al. (1981) that melittin exhibits a marked preference for acidic lipids and surfaces. With films of phosphatidic acid, a π_c value (25.2 mN m^{-1}) similar to that obtained with phosphatidylcholine and phosphatidylethanolamine was found in spite of the acidic head group. However, the compact head group of phosphatidic acid would yield a less expanded film than does phosphatidylglycerol, allowing van der Waals forces between paraffin chains to predominate in the interaction between adjacent molecules. From the resultant condensed film a low value of π_c, compared to the higher value of π_c for a more expanded yet similarly charged film of phosphatidylglycerol, would be expected.

δ-Lysin exhibited little preference for films of phospholipids differing in size or charge of head group, except for phosphatidic acid for the reasons discussed above. The marked preference of melittin for acidic phospholipids is acknowledged in the proposed mechanism of membrane perturbation which involves initial binding via ionic interactions followed by conformational changes resulting in membrane perturbation (Dawson et al., 1978). In contrast, as δ-lysin shows no such preference for specific phospholipid head groups, it is unlikely that initial ionic interactions are a requirement for membrane perturbation.

Differences in the phospholipid head group had little effect on the degree of interaction between δ-lysin and the phospholipid film, and similarly, variation of the fatty acid chain length (phosphatidylcholine; C12 to C22) had no significant influence on the π_c values. Thus, it seems that the interaction of δ-lysin with phospholipids shows little or no specificity with regard to structure. This is borne out by the very similar critical pressures and similar slopes for plots of $\Delta\pi$ vs. π_i for cod and sheep erythrocyte lipid monolayers although their lipid composition appears to differ significantly. Phospholipids account for 75% of total lipid in cod erythrocytes, approximately 56% of the total phospholipid being phosphatidylcholine with 30% being noncholine-containing phospholipids (Addison & Ackman, 1971). Sheep erythrocyte lipids consist of 26.5% cholesterol and 63% phospholipid, 26% being phosphatidylethanolamine, 51% sphingomyelin, and 14% phosphatidylserine (Nelson, 1967). Demel et al. (1975) deduced that the surface pressure at the human erythrocyte membrane was approximately 31 mN m^{-1}. If this pressure is typical of other membranes, it may explain the wide lytic spectrum of δ-lysin since π_c was >31.4 mN m^{-1} for all single or mixed lipid monolayers tested. Whereas the π_c values for the interaction of δ-lysin with films of total lipids of cod and sheep erythrocytes were not distinguishable from those obtained with films of single phospholipids, this was not the case for melittin. The π_c values for the interaction between melittin and erythrocyte lipid films were markedly higher than those obtained from films of single phospholipids. Such high values may result from a combination of negatively charged phospholipids and an expanded film due to the high content of polyunsaturated fatty acids in the phospholipids, especially in the case of cod lipids.

Bougis et al. (1981) have performed a similar study of the interactions of melittin and cardiotoxins of *N. mossambica mossambica* with phospholipid monolayers. Cardiotoxins in general interacted with all phospholipid films though the interactions were much stronger with negatively charged molecules. Due to the low surface activity of cardiotoxins at the air–water interface and much greater activity in the presence of a phospholipid monolayer, a more specific type of interaction than that with melittin appears to be involved. For cardiotoxins an initial electrostatic binding is thought to occur followed by hydrophobic interactions. The degree of specificity for penetration of the various lytic agents into acidic phospholipids is in the order cardiotoxins > melittin > δ-lysin. Thus, cardiotoxins and melittin both exhibit a preference for negatively charged phospholipids, but to different degrees, whereas δ-lysin shows no such preference.

An α-helical configuration of δ-lysin would result in a rod-shaped molecule with lateral amphipathic properties (Freer & Birkbeck, 1982) with distinct hydrophilic and hydrophobic faces on the long axis. This would favor self-association of monomers in an aqueous environment and may also explain the sigmoidal shape of the curve when π is plotted against δ-lysin concentration. Nonspecific hydrophobic interactions between acyl chains of phospholipid and the hydrophobic faces of the rod-shaped toxin monomers may be associated with penetration of lipid monolayers and bilayers by δ-lysin. The lateral amphipathic properties of such rodlike structures would favor aggregation of toxin in the plane of a fluid membrane, possibly resulting in transmembrane channels which initiate the loss of selective membrane permeability. Evidence for the formation of ion-selective transmembrane pores in black lipid membranes by melittin has been presented by Tosteson & Tosteson (1981). In a study of the changes in electrical properties of bilayers induced by melittin, they concluded that melittin forms tetrameric complexes specifically orientated in the lipid bilayer.

In order to further investigate the biological significance of the relatively nonspecific interactions of δ-lysin with the single and mixed lipid monolayers, it will be necessary to study changes in permeability induced in similarly composed bilayer membranes. Such permeability studies may indicate whether interactions involving penetration of toxin into the lipid domain result in the loss of selective permeability by formation of ion-selective transmembrane pores similar to those already described for melittin.

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Novel Intestinal Phospholipase A_2: Purification and Some Molecular Characteristics†

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ABSTRACT: We purified to homogeneity a new phospholipase A_2 from pig ileum which hydrolyzes phosphatidylglycerol at least 200 times more rapidly than phosphatidylcholine. The method involved the following steps: (1) complete delipidation of ileal homogenates by solvent extraction; (2) fractionation and partition between *n*-butanol and $(NH_4)_2SO_4$ solution; (3) hydrophobic affinity chromatography on octyl-Sepharose; (4) adsorption chromatography on hydroxylapatite; (5) ion-exchange chromatography on carboxymethyl-Sepharose. Amino acid composition, molecular weight (15 000–16 000), *N*-terminal amino acid sequence to residue 48, and enzymatic activity on phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylglycerol were determined.

Lipolytic enzymes are present in nearly all tissues which have been investigated (Brockerhoff & Jensen, 1974; van den Bosch, 1980). However, the amount of enzyme activity can vary by a factor of 10^6 as, for example, between pancreatic exocrine glands and hepatocytes.

Phospholipases in intestinal mucosa were first described in 1931 by several laboratories (King, 1931; Epstein & Shapiro, 1957, 1959; Schmidt et al., 1957; Ottolenghi, 1964, 1967; Gallai-Hatchard & Thompson, 1965; Sarzala, 1969; Subbaiah & Ganguly, 1970; Bonnefis et al., 1975, 1977, 1978; Takagi & Sasaki, 1979) by using either endogenous phospholipids, exogenous phosphatidylcholine (PC),[1] or phosphatidylethanolamine (PE) as substrate. Unfortunately, these investigations were carried out with different types of crude intestinal preparations, and therefore contamination with enzymes from other origins (pancreas) cannot be ruled out. In 1974, when the first preparations of closed intestinal brush border vesicles became available in our laboratory (Louvard et al., 1973), we detected hydrolytic activity using monomolecular phospholipid films of phosphatidylglycerol (PG) that was 200 times greater than when PC was used. This initial observation prompted us to trace and isolate the enzyme responsible for this unusually selective phospholipase activity of the A type.

In a preliminary work, Mansbach et al. (1982) evaluated phospholipase activity in the intestine of rats and other species. In pancreatic juice diverted rats, mucosal and gut luminal phospholipase specific activity was greater than controls, thus assuring that enzyme activity was not due to pancreatic phospholipase. A bacterial origin of phospholipase activity was also eliminated by finding phospholipase activity in germ-free rats.

In the present paper, we describe the purification from porcine intestine of an acid-stable, small molecular weight phospholipase A_2 which hydrolyzes PG at least 200 times more rapidly than PC. We also detail several physicochemical and molecular characteristics of this enzyme.

Materials and Methods

Determination of Phospholipase Activity. When using the classical potentiometric egg yolk assay, designed for pancreatic phospholipases A_2 (Nieuwenhuizen et al., 1974), we could not detect any enzymatic activity due to the intestinal phospholipase using different taurodeoxycholate (0–6 mM) or calcium (0–15 mM) concentrations. Similarly, no measurable hydrolysis was found at pH 8.0 when short-chain dioctanoyl-PC was used at different NaCl concentrations. Due to the sharp substrate specificity of the intestinal phospholipase, we tried to set up a pH stat titration assay using several tissue homogenates or lipid extracts naturally rich in PG or acidic phospholipids.

We first checked, without success, *Escherichia coli* or lamb brain homogenates at pH 8.0 in 21 mM $CaCl_2$ and 1.2 mM taurodeoxycholate. Then we tried, also unsuccessfully, chloroform/methanol (2:1) extracts of *E. coli*, spinach leaves, and lamb brain as phospholipase substrates as well as purified *E. coli* PG in the presence of Triton X-100 (1%) or deoxycholate

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[1] Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PG, phosphatidylglycerol; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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