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Activation of Histamine Secretion from Rat Mast Cells by Aqueous Dispersions of Phosphatidylserine†

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ABSTRACT: Aqueous dispersions were generated from bovine brain phosphatidylserine (PS) and three synthetic derivatives: 1,2-dimyristoyl-, 1,2-dipalmitoyl-, and 1,2-distearoyl-*sn*-3-glycerophosphoserine (DSPS). The gel–liquid-crystalline phase transition temperatures of the dispersions were determined with a fluorescence probe and by 90° light-scattering measurements. The effectiveness of the dispersions in activating histamine secretion from mast cells stimulated with concanavalin A was examined, and the results were evaluated in terms of the differences in acyl chain composition and phase transition temperature. At 22 °C, fluid-phase bovine brain PS dispersions were more active on a molecular basis than solid-phase DSPS dispersions, whereas at 37 °C, the solid-phase DSPS dispersions were more active. When the relative activities of three different solid-phase PS dispersions were compared, activity was found to be dependent on the length of the acyl chains of the PS molecule with longer chains being more effective. Since the binding isotherms for the interaction of all three solid-phase PS dispersions with mast cells were virtually indistinguishable, the greater activity of PS molecules with longer acyl chains could not be explained on the basis of an enhanced binding capability. The mechanism by which PS vesicles interact with mast cells was further studied by trapping the hydrophilic fluorescent dye carboxyfluorescein within the aqueous compartment of the vesicles. Incubation of such dye-loaded vesicles with cells resulted in considerable release of the dye from the vesicles into the extracellular medium with little transfer of dye from the vesicles to the cells. The rate of extracellular dye release was saturable in the concentration range over which the vesicles were found to activate secretion. PS vesicles but not phosphatidylcholine vesicles afforded competitive inhibition of extracellular dye release. On the basis of these results and previous observations on the specificity and selectivity of the effect of PS on mast cell secretion, a tentative model is presented to explain the mechanism of PS action.

The mast cell is a highly specialized secretory cell ubiquitous in the connective tissue of man and other vertebrates. Although a detailed understanding of the function of the mast cell is lacking, the demonstration of histamine and other inflammatory mediators within its secretory granules and their release upon immunologic challenge (Becker & Henson, 1973) serve as the basis for association of this cell with the pathophysiology of immediate hypersensitivity and anaphylaxis (Lewis & Austen, 1977). In conjunction with its immunopathologic relevance, the mechanism of histamine secretion from mast cells has been the focus of numerous experimental investigations as a system for correlative study of the bio-

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chemistry and morphology of the secretory process.

It is now well established that histamine is liberated from its storage sites within the secretory granules of the mast cell by an exocytotic process which involves fusion of the perigranule membrane with the cell surface plasma membrane (Röhlich et al., 1971; Lagunoff, 1973; Anderson et al., 1973). The events which regulate membrane fusion in the mast cell and other cells are presently unknown, although it may be anticipated that these events are vitally important to the control of such diverse cellular processes as secretion, phagocytosis, mitosis, and fertilization.

Exogenous phosphatidylserine (PS)[1] potentiates the secretory response of the mast cell to a variety of secretagogues (Goth et al., 1971). The effect of PS on mast cells is highly specific. No other phospholipids can substitute for PS (Goth et al., 1971; Mongar & Svec, 1972; Baxter & Adamik, 1976), and several inactive N-substituted derivatives serve as competitive antagonists of the effect of PS (Martin & Lagunoff, 1979a). While membrane fusion is essential for exocytosis and secretion of histamine from mast cells regardless of the nature of the initial secretory stimulus, PS selectively potentiates the action of IgE-dependent releasing agents and has no stimulatory effect on other secretagogues such as polymyxin B (Read et al., 1977), compound 48/80, or chymotrypsin (Goth et al., 1971). The specificity and selectivity in the action of PS provide the basis for our continued investigation of the mechanism by which exogenous PS activates mast cell secretion.

In a prior study, we demonstrated that bovine brain PS acts in a micellar state, presumably as bilayer vesicles, to activate the secretory response of rat mast cells to Con A (Martin & Lagunoff, 1978). The effect of PS on secretion was associated with the binding of 3.7×10^9 molecules of PS to the cell. The present study was initiated in an effort to further characterize the interaction between PS and mast cells. Experiments were designed to examine the effect of the gel–liquid-crystalline phase transition temperature and the length of the hydrocarbon chains on the ability of PS to activate Con A induced secretion. Con A was chosen as the primary secretagogue in this study because its saccharide binding specificity is known with some certainty (Sharon & Lis, 1972), it is capable of initiating noncytotoxic histamine secretion at the cell surface (Lawson et al., 1978) through an interaction with specific saccharide components (Sullivan et al., 1975), and the response of rat mast cells to this secretagogue has an absolute dependence on PS (Martin & Lagunoff, 1978).

Materials and Methods

Phospholipid Dispersions. PS was isolated from bovine brain by the method of Sanders (1967). Radiolabeled DMPS, DPPS, and DSPS were synthesized from the corresponding 1,2-diacyl-*sn*-PC's by a procedure based on that of Ito et al. (1975). The PC's were synthesized by acylation of 3-*sn*-glycerophosphocholine (Brockerhoff & Yurkowski, 1965) using the method of Cubero Robles & Van den Berg (1969). Fatty acid anhydrides were prepared from myristic, palmitic, and stearic acids (Supelco, Inc., 99+%) by the procedure of Selinger & Lapidot (1966). L-[G-^{3}H]Serine (New England Nuclear) was introduced into the PS's by the action of PS synthetase (Raetz & Kennedy, 1974). An enzyme preparation devoid of significant PS decarboxylase activity was prepared from a frozen cell paste of *Escherichia coli* (Miles Laboratories, Inc.) by repeated precipitation of the ribosomes as described by Raetz & Kennedy (1972). Cytidine 5′-monophosphomorpholidate used for the synthesis of the cytidine 5′-diphospho-1,2-diacyl-*sn*-glycerols (Agranoff & Suomi, 1963) was supplied by Sigma Chemical Co. The reaction conditions of Kornberg & McConnell (1971) were used for hydrolysis of the PC's catalyzed by phospholipase D (Yang, 1969).

The PS's were purified by column chromatography on DEAE-cellulose (Whatman, DE-52) using the solvent system and elution protocol of Raetz & Kennedy (1973). The purity of the final products was established by thin-layer chromatography on silica gel H plates (Analtech) developed in diisobutyl ketone–acetic acid–H_2O (40:30:7 by volume). A single spot with an R_f value of 0.35–0.40 was obtained after exposure of the dried chromatograms to either a phosphorus or a ninhydrin spray reagent (Kates, 1972). The radiochemical purity determined by radioassay of 5-mm strips of silica gel scraped from the plates was 98 to 99% for each derivative. The specific radioactivity based on phosphorus analysis (Fiske & Subbarow, 1925) after perchloric acid digestion was 1 to 2 Ci/mole. The disaturated PS's were only slightly soluble in chloroform or mixtures of chloroform and methanol. They were stored for up to 1 year as suspensions in chloroform under nitrogen at −20 °C without detectable degradation.

The fatty acid composition of the PS's was verified by gas chromatography of the methyl esters after transesterification (Kates, 1972). The methyl esters were chromatographed at 175 °C on a Barber-Colman Model 5001 gas chromatograph. The column was packed with 60–80 mesh Chromosorb W with ethylene glycol succinate (HI-EFF-2BP, Applied Science) as the stationary phase. The retention times were compared with a series of methyl ester standards purchased from Supelco, Inc. The analyses were performed in triplicate and revealed that DMPS, DPPS, and DSPS contained 96% myristic acid, 97% palmitic acid, and 95% stearic acid, respectively.

PS was dispersed in BSS (4 mM Na_2HPO_4, 2.7 mM KH_2PO_4, 166 mM NaCl, and 2.7 mM KCl, pH 7.2) or Hepes buffer (2 mM Hepes, 100 mM NaCl, and 0.1 mM EDTA, pH 7.4) at a temperature 10 °C above T_c (see below) by a procedure based on that of Bangham et al. (1965). Unsuspended PS was removed by a 20-min centrifugation at 220*g*, and the concentration of the remaining suspension was determined by an assay of lipid phosphorus (Fiske & Subbarow, 1925). Where indicated, bovine brain PS dispersions (27 mM) were sonicated under nitrogen with a 100-W MSE ultrasonic disintegrator. Unilamellar vesicles were separated from multilamellar vesicles by gel filtration on a calibrated column (2.5 × 37 cm) of Sepharose 4B (Huang, 1969). Standards used for calibration were rabbit IgG, equine ferritin, and unilamellar PC vesicles (Huang, 1969). The included volume of the gel was determined with dinitrophenyllysine.

The T_c of the unsonicated dispersions was measured in BSS with the uncharged, polarity-dependent fluorescence probe NPN (Aldrich Chemical Co., Inc.) and by 90° light-scattering measurements (Overath & Träuble, 1973).

Purification of Mast Cells and Assay of Histamine Release. Mast cells were obtained from the peritoneal cavities of outbred adult albino male rats (Tyler Laboratories, Bellevue, WA) and purified to greater than 90% homogeneity by albumin density gradient centrifugation (Lagunoff, 1975).

[1] Abbreviations used: BSS, balanced salt solution; CF, carboxyfluorescein; Con A, concanavalin A; DMPS, 1,2-dimyristoyl-*sn*-3-glycerophosphoserine; DPPS, 1,2-dipalmitoyl-*sn*-3-glycerophosphoserine; DSPS, 1,2-distearoyl-*sn*-3-glycerophosphoserine; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; NPN, *N*-phenyl-1-naphthylamine; PC, phosphatidylcholine; PS, phosphatidylserine; T_c, gel–liquid-crystalline phase transition temperature.

Purified mast cells were suspended in BSS, and the cell concentration was determined in a hemocytometer. Histamine release experiments were performed in replicate by incubating 2×10^5 mast cells in 0.75 mL of BSS containing varying concentrations of PS for a specified time and temperature. Con A (Miles-Yeda Ltd.) (100 μg/mL) and Ca^{2+} (0.68 mM) were then added, and the incubation was continued at 37 °C for 1 min. The incubation was terminated by centrifugation of the cells at 4 °C for 6 min at 220g. Histamine release was calculated as a mean percentage of total cell histamine after assay of supernatants and perchloric acid extracts of cell pellets by a modification of the *o*-phthalaldehyde fluorometric method (Kremzner & Wilson, 1961). Histamine release values obtained in controls without added PS were subtracted from the values obtained in the presence of PS.

Binding of Radiolabeled PS Dispersions to Mast Cells. Binding of radiolabeled PS to mast cells was determined by a procedure described previously (Martin & Lagunoff, 1978). Replicate samples of cells [$(2-4) \times 10^5$ cells/mL] were incubated in disposable plastic Falcon test tubes with the labeled PS dispersions in a final volume of 0.2 mL of BSS for 60 min at 22 °C. The cells were centrifuged at 220g for 6 min, and unbound PS was determined by radioassay. The cell pellet together with the remaining supernatant was dissolved in 0.05 mL of 1% Triton X-100 by sonication. The solubilized pellet was then transferred to a scintillation vial and assayed for radioactivity. Cell-associated PS was calculated after correcting for radioactive carry-over from the residual supernatant and nonspecific binding to the incubation tubes. Saturation binding was estimated by using a least-squares analysis of a double-reciprocal plot (Edsall & Wyman, 1958) of the data obtained 1 log unit on either side of the binding midpoint. The complete data were then evaluated by using a least-squares analysis of the logarithmic plot of Hill (1910).

Interaction of CF-Loaded PS Vesicles with Mast Cells. A methodology based on that originally devised by Weinstein et al. (1977) was used to investigate the mode of interaction of bovine brain PS vesicles with mast cells. CF (Eastman) was trapped within the vesicles at a concentration of 100 mM by sonication. Free CF was separated from vesicle-associated dye by Sephadex G-200 column chromatography. Fractions eluting in the void volume were pooled and analyzed for phosphorus and CF content. The latter assay was performed by lysing the vesicles in 0.25% Triton X-100 and measuring the fluorescence emission at 520 nm with excitation at 490 nm. Since Triton X-100 has no effect on the fluorescence of the free dye, CF concentrations were determined from a standard curve. The mean (±SEM) molecular ratio of PS to CF in eight preparations was 47 ± 5.3. The dye trapped within the vesicles was largely self-quenched so that addition of Triton X-100 resulted in a 20–30-fold fluorescence enhancement.

Mast cells [$(0.5-1.0) \times 10^6$] were incubated with freshly prepared CF-loaded PS vesicles in a final volume of 1.0 mL of BSS for 10 min at 37 °C. The cells were centrifuged at 220g for 6 min, and the supernatant was decanted into a test tube for subsequent fluorescence measurements. The cell pellet was washed twice with 2 mL of BSS at 4 °C, and the washed cells were resuspended in 1.0 mL of BSS. Controls containing dye-loaded vesicles but lacking cells were processed in an identical manner. Fluorescence emission at 520 nm with excitation at 490 nm was measured in the original cell supernatant and the final cell suspension prior to and after addition of 0.25% Triton X-100. The latter measurements were corrected for light scattering by subtracting the values obtained in controls containing cells and no dye.

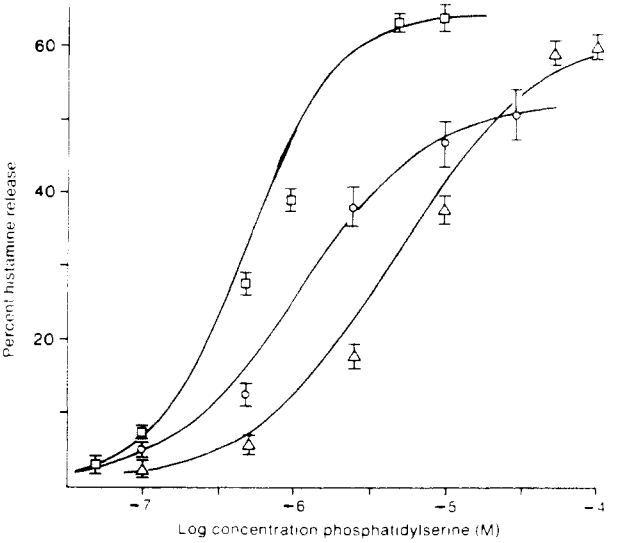

FIGURE 1: Effect of fatty acid composition on the ability of PS to activate secretion at 37 °C. After equilibration of 2×10^5 mast cells with DSPS (□), bovine brain PS (○), or DMPS (△) for 10 min at 37 °C, the cells were exposed to Con A (100 μg/mL) and Ca^{2+} (0.68 mM) for an additional 1 min at the same temperature. Histamine in the supernatant and cell pellet after centrifugation was measured, and release is expressed as a percent of total cell histamine (bar = SEM, $n = 3$). The curves shown are semilog transformations of the lines obtained by least-squares analyses of log plots (Hill plots).

The fraction of total CF released from PS vesicles after their exposure to cells, denoted P, can be calculated from the fluorescence measurements by using the equation

$$P = \frac{F_c + F_e - F_o}{F_t - F_o}$$

F_o is the fluorescence of the dye-loaded vesicles in controls in which the dye is maximally self-quenched, F_t is the fluorescence when all of the dye is released from the vesicles, F_e is the fluorescence of the initial supernatant after interaction with cells, and F_c is the fluorescence of cell-associated CF. The absolute quantity of CF released is then simply $P \times [CF]_v$, where $[CF]_v$ is the amount of CF trapped within the vesicles. Since addition of Triton X-100 failed to enhance the fluorescence of the cell suspension, it can be assumed that all of the cell-associated dye was unquenched within the cell interior. This permits a direct calculation of $[CF]_c$, the quantity of cell-associated CF from F_c. The quantity of CF released into the medium, $[CF]_e$, was calculated from the expression $P \times [CF]_v = [CF]_c + [CF]_e$.

Results

Effect of Fatty Acid Composition on Activity of PS Dispersions. The fluidity of bovine brain PS, DMPS, and DSPS dispersions is markedly different when equilibrated at 37 °C. Bovine brain PS is well above its T_c (8 °C), DSPS is well below its T_c (≥70 °C), and DMPS is in the region of its T_c (36 °C). Dose–response curves obtained with these different PS dispersions in the histamine secretion assay performed at 37 °C are presented for comparison in Figure 1. The logarithms of the activation midpoints and cooperativity coefficients (in parentheses) were determined from Hill plots of these data to be −5.97 (1.02) for bovine brain PS, −5.32 (0.92) for DMPS, and −6.33 (1.46) for DSPS.

The effect of the hydrocarbon chain composition of PS on its ability to activate histamine secretion independent of any effect of fluidity was investigated after equilibration of mast cells with aqueous dispersions of DMPS, DPPS (T_c = 53 °C), and DSPS at 22 °C. All three PS dispersions are in a similar

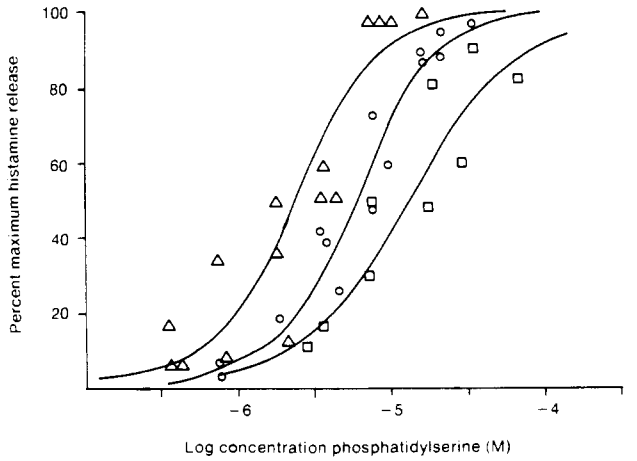

FIGURE 2: Effect of fatty acid composition on the ability of PS to activate secretion after equilibration at 22 °C. Purified mast cells (2×10^5) were equilibrated with DSPS (△), DPPS (○), and DMPS (□) for 20 min at 22 °C. Con A (100 μg/mL) and Ca^{2+} (0.68 mM) were then added, and the cells were further incubated at 37 °C for 1 min. Histamine release was determined as described in Figure 1. The data were normalized by setting the maximum observed histamine release in a given experiment equal to 100%. Maximum release ranged between 30 and 50% of the total cell histamine and was virtually identical for each derivative. The curves drawn through the data points are semilog transformations of the lines obtained by least-squares analyses of linear log (Hill) plots. Each point shown represents the average of duplicate determinations. The unbound PS concentration was measured by radioassay in each experimental determination.

physical state at this temperature because each is well below its T_c. Dose–response curves obtained under these conditions are presented in Figure 2. Hill plots of these data indicated activation midpoints (log values) and cooperativity coefficients (in parentheses) of −4.85 (1.24), −5.23 (1.64), and −5.63 (1.65) for DMPS, DPPS, and DSPS, respectively.

Binding experiments were performed with mast cells and DMPS, DPPS, and DSPS dispersions at 22 °C. The binding isotherms are presented in Figure 3. For each PS derivative, saturation binding was estimated by a double-reciprocal plot to be 28 nmol of PS per 10^6 mast cells. The midpoints of the three isotherms and their cooperativity coefficients were determined by Hill plots of log $\theta/(1-\theta)$ vs. log unbound PS concentration, where θ is the fractional saturation. Values of −4.89 (0.93), −4.84 (0.80), and −4.76 (0.92) were obtained for the logarithms of the midpoints and the cooperativity coefficients (in parentheses) of the isotherms of DMPS, DPPS, and DSPS, respectively.

Interaction of Bovine Brain PS Vesicles with Mast Cells. When bovine brain PS was suspended in Hepes buffer and subjected to gel filtration in the absence of sonication, the majority of the phospholipid eluted as expected in the void volume of a Sepharose 4B column, indicating the predominance of micellar particles with a radius in excess of 500 Å. After extensive sonication, however, the phospholipid eluted from the Sepharose 4B column in two peaks with ~75% of the total PS applied to the column recovered in a fraction included in the gel. Comparison of the elution volume of this fraction with the elution volumes of standards of known Stokes' radius (Siegel & Monty, 1966) provided an estimate of 150 Å for the radius of the small PS vesicles. Refiltration of samples taken from the void volume or the included fraction resulted in an elution profile nearly identical with the original, supporting the notion that these fractions represent relatively stable populations of PS vesicles of different size. Freeze-fracture electron microscopy of a sample taken from the fraction eluting in the void volume revealed the presence of

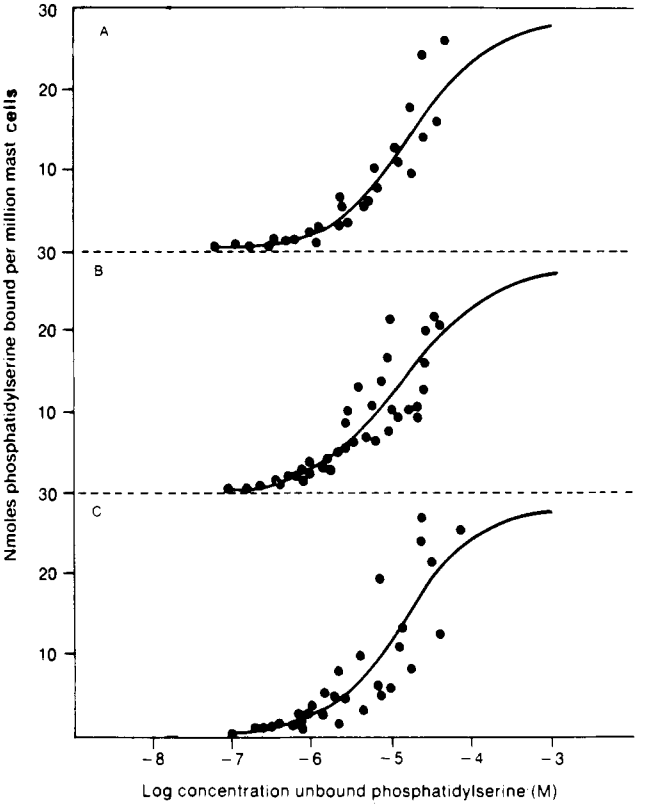

FIGURE 3: Binding isotherms for the interaction of synthetic PS's with mast cells. Equilibrium binding experiments were performed with purified mast cells and DSPS (A), DPPS (B), and DMPS (C) at 22 °C as described in detail under Materials and Methods. The curves drawn through the individual data points are transformations of the lines obtained by least-squares analyses of Hill plots of log $\theta/(1-\theta)$ vs. log unbound PS concentration, where θ is the fractional saturation. Each point shown represents the average of replicate determinations. Saturation binding for all three derivatives was estimated by a double-reciprocal plot to be 28 nmol of PS per million mast cells.

a rather heterogeneous population of uni- and multilamellar vesicles.

The experiment presented in Figure 4 was performed to assess whether or not the PS vesicles which eluted in the two different fractions after Sepharose 4B chromatography differed in their capacity to activate Con A induced histamine secretion. The dose–response curves indicated that both populations of vesicles were capable of eliciting the same maximum level of activation; however, the small PS vesicles included in the Sepharose 4B column were ~3 times more effective based on PS concentration.

The mode by which sonicated PS vesicles interact with mast cells was investigated by incubating mast cells with vesicles containing CF at concentrations at which the fluorescence of the dye is largely self-quenched. Release of the dye from the vesicles results in a 20–30-fold enhancement in fluorescence and provides a sensitive method for assessing the mode of interaction of dye-loaded vesicles with cells (Blumenthal et al., 1977; Szoka et al., 1979).

The initial rate of dye release from dye-loaded PS vesicles incubated with mast cells was measured as a function of vesicle concentration (Figure 5). These data represent a single experiment that was repeated 7 times with essentially the same result. A comparison of the values on the ordinates of the two curves shown in Figure 5 illustrates the far greater tendency for dye molecules to be released from the vesicles into the surrounding medium rather than into the cell interior. A plot of the initial rate of extracellular dye release vs. the vesicle concentration yields a rectangular hyperbola indicative of a

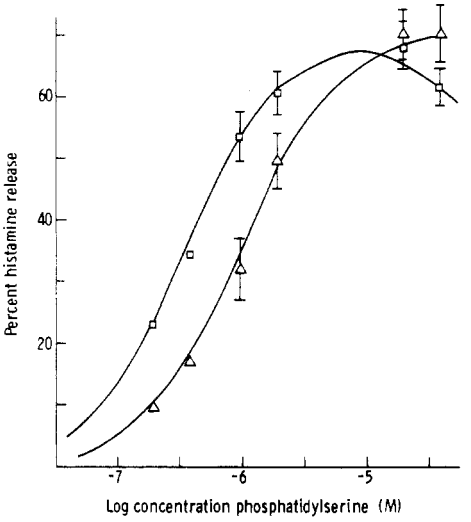

FIGURE 4: Effect of size and lamellar structure on the ability of bovine brain PS vesicles to activate Con A induced histamine secretion. Mast cells (2×10^5) were incubated in a final volume of 1.0 mL of BSS containing 100 μg/mL Con A, 0.68 mM Ca^{2+}, and PS at 37 °C for 15 min: (□) small, unilamellar PS vesicles (r = 150 Å); (△) large, multilamellar PS vesicles eluting in the void volume of a Sepharose 4B column. Histamine release is expressed as a percent of total cell histamine. The bars indicate SEM in experiments repeated 3 or more times. Each data point represents at least two separate experiments with duplicate determinations in each individual experiment.

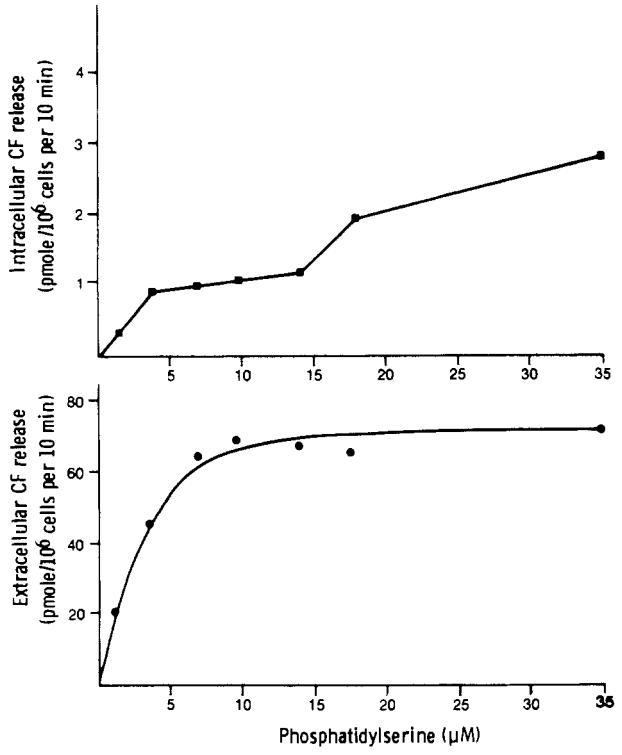

FIGURE 5: Release of CF from sonicated PS vesicles exposed to mast cells. Purified mast cells were incubated with CF-loaded bovine brain PS vesicles at 37 °C for 10 min. The amount of CF transferred from the vesicles to the cells (upper panel) and the amount of CF released into the extracellular medium (lower panel) were quantitated as described under Materials and Methods as a function of initial phospholipid concentration.

saturable process. In contrast, a maximum rate of dye transfer from the vesicles into the cell interior was not obtained in the concentration range investigated and a plot of these data yields a biphasic curve (Figure 5).

The kinetic data obtained for extracellular dye release were subjected to analysis by using the Michaelis–Menton equation.

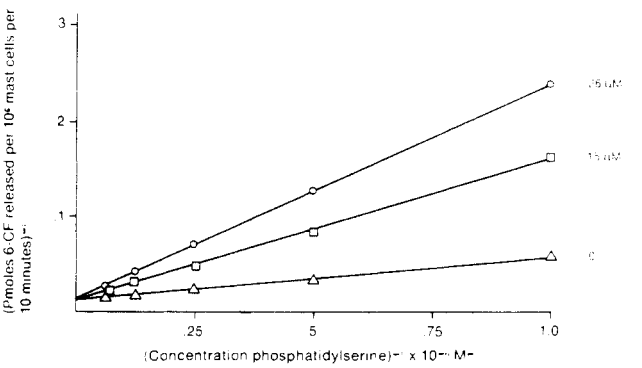

FIGURE 6: Lineweaver–Burk plots of the rate of extracellular CF release from PS vesicles. The data obtained from experiments performed as in Figure 5 were analyzed by using the Lineweaver–Burk plot to determine the Michaelis constant (K_m) and V_{max}. Unloaded PS vesicles were tested as potential inhibitors of the rate of dye efflux. The fixed concentration of unloaded PS vesicles is indicated in the right margin. The slopes of the reciprocal plots were increased by addition of unloaded vesicles and the intersection on the ordinate was unchanged, indicative of competitive-type inhibition. The K_i for unloaded vesicle inhibition was determined from the dependence of the slope change on the concentration of competitor.

Implicit is the assumption that extracellular dye release is mediated via formation of a vesicle–cell intermediate. A Lineweaver–Burk plot constructed from the data presented in Figure 5 indicates a V_{max} for extracellular dye release of 74.5 pmol of CF per 10^6 mast cells per 10 min at 37 °C and a K_m of 3.3 μM based on total phospholipid (Figure 6). By expressing V_{max} in terms of PS equivalents using the PS/CF molecular ratio of the vesicle preparation, the data obtained in eight individual experiments could be normalized. The mean V_{max} obtained in the eight experiments was 3.69 ± 0.34 nmol of PS equivalents per 10^6 cells per 10 min, and the mean K_m was 2.8 ± 1.2 μM.

Unloaded PS vesicles were tested for their ability to provide competitive inhibition of the rate of extracellular dye release from dye-loaded vesicles incubated with mast cells. An incremental increase in the slope of the plots presented in Figure 6 as the concentration of unloaded PS vesicles included in the incubations is increased is supportive of a mechanism in which these vesicles compete with dye-loaded vesicles for sites on the mast cell which mediate extracellular dye release. The mean K_i calculated from two experiments performed as in Figure 6 was 5.5 μM. PC vesicles failed to inhibit the rate of extracellular dye release from dye-loaded PS vesicles.

Discussion

Role of Bilayer Fluidity and Acyl Chain Length in PS Action. The primary purpose of the present investigation has been to characterize the interaction of PS dispersions with mast cells in hopes of obtaining information regarding the mode and site by which they activate histamine secretion. A major question concerns the relative effectiveness in activating secretion of PS dispersions in solid vs. fluid states. Dose–response curves were initially performed at 37 °C with unsonicated dispersions of bovine brain PS (fluid), DSPS (solid), and DMPS (coexisting fluid and solid domains). The data presented in Figure 1 indicate that all three dispersions are capable of activating secretion, but it is evident that there are marked differences in the concentration dependence of activation. One explanation for this result might be that PS in a solid state is preferable to PS in a fluid state and either is more favorable than PS in an equilibrium mixture of solid and fluid states. This interpretation, however, omits the possibility that factors

other than fluidity might contribute to the observed differences. One such factor is the length of the PS acyl chains.

The experiment presented in Figure 2 indicates that the length of the acyl chains of PS determines the relative activity of the dispersions in the absence of any difference in fluidity. At 22 °C, all three synthetic PS dispersions are in a solid state. When secretion is assayed after equilibration of the cells with the PS dispersions at 22 °C, there is a linear relationship between the length of the acyl chains of PS and the logarithm of the midpoint of the activation curves. For each additional methylene group, there is an incremental −0.19 log unit shift in the midpoint of the curves in Figure 2, indicating that PS molecules with longer acyl chains are more effective in activating secretion. The data presented in Figure 1 are also consistent with this interpretation as DSPS is 1 log unit more effective in activating secretion than DMPS.

The binding isotherms for the interaction of DMPS, DPPS, and DSPS with mast cells at 22 °C are virtually indistinguishable. Thus, cells have equivalent amounts of PS bound after equilibration with the same initial concentration of any of the three synthetic PS's. When the cells are subsequently exposed to Con A and Ca^{2+}, the magnitude of the secretory response is greater for cells equilibrated with dispersions generated from PS molecules with longer acyl chains. This establishes that the greater activity of PS dispersions with longer hydrocarbon chains cannot be attributed to an increased binding of micellar aggregates to the cell.

Data obtained with dispersions generated from DSPS and bovine brain PS were compared in an effort to directly examine the effect of fluidity on secretion in the absence of a difference in acyl chain length.[2] At 22 °C, the dose–response curves for DSPS (solid) and bovine brain PS (fluid) have identical midpoints [Figure 2 and Figure 4 in Martin & Lagunoff (1978)]. It can be calculated that at this concentration there are 1.6 times the number of DSPS molecules associated with the cell, suggesting that on a molecular basis PS in a fluid state is more effective than PS in a solid state. If the same calculation is performed with the data obtained at 37 °C, however, this relationship is reversed as there are approximately twice as many bovine brain PS molecules associated with the mast cell at the midpoint of the activation curves.

The difference in preference for fluid- and solid-phase PS dispersions at different temperatures is a function of a greater dependence on temperature of the activation by solid-phase dispersions. This is evident from an examination of the midpoints of the activation dose–response curves for bovine brain PS, DMPS, and DSPS at 22 and 37 °C. For each dispersion, the activation midpoint is shifted to a lower value at 37 °C. The magnitude of the shift is fivefold for solid DSPS, threefold for DMPS which changes from a solid to a mixed state as the temperature is increased, and twofold for fluid bovine brain PS.

The data in Figure 4 establish that a homogeneous population of 150-Å radius unilamellar PS vesicles is 3 times more potent in activating secretion than a heterogeneous population of multilamellar vesicles. In addition to size, unilamellar vesicles differ from multilamellar vesicles in the amount of exposed surface area per mole of phospholipid, the molecular packing density, and the motional freedom of individual molecules (Sheetz & Chan, 1972; Huang & Mason, 1978). We cannot at present distinguish among these as the basis for the observed difference in potency.

[2] Bovine brain PS is a heterogeneous mixture of 1,2-diacyl-*sn*-3-glycerophosphoserines; however, greater than 80% of the esterified fatty acids are either stearic or oleic acid (Silver et al., 1963).

Interaction of PS Vesicles with Mast Cells. CF has been used previously as an aqueous space marker to assess the mode of interaction of phospholipid vesicles with cells (Blumenthal et al., 1977; Szoka et al., 1979). Attempts to entrap CF within dispersions formed from the synthetic PS's were unsuccessful, precluding the use of this dye in a comparative study of the interaction of disaturated PS dispersions with mast cells. Since CF has been successfully trapped within bovine brain PS vesicles (Portis et al., 1979), we examined the mode of interaction of PS dispersions with mast cells by using PS isolated from bovine brain.

In the concentration range over which sonicated bovine brain PS vesicles activate histamine secretion from mast cells (Figure 4), the cells induce considerable release of CF from dye-loaded vesicles with 30–40 times more dye released into the medium than into the cell interior (Figure 5). The rate of extracellular dye release is saturable and competitively inhibited by unloaded PS vesicles. The K_m for extracellular dye release (2.8 μM) is not very different than the K_i for inhibition by unloaded vesicles (5.5 μM). The apparent dissociation constant for binding of sonicated PS vesicles to the mast cell determined from equilibrium binding experiments at 22 °C with radiolabeled PS is 4.5 μM (Martin & Lagunoff, 1978). On the assumption that each 150-Å radius vesicle contains ~6000 PS molecules,[3] the binding experiments indicate the existence of 6.2×10^5 vesicle binding sites/cell. The V_{max} for extracellular dye release corresponds to 3.69 nmol of PS equivalents per 10^6 mast cells per 10 min or 3.7×10^4 vesicles/(cell min). If the number of sites per cell determined in the equilibrium binding study is used as an estimate for the number of sites which mediate the process of extracellular dye release, then the turnover rate for the sites is relatively low (6.0×10^{-2} min^{-1}) at 37 °C.

PC vesicles do not inhibit dye release from CF-loaded PS vesicles incubated with mast cells in the concentration range between 10 and 50 μM (data not shown). The inability of PC vesicles to inhibit dye release from PS vesicles indicates specificity in the interaction between PS vesicles and cells and provides support for the existence of a vesicle–cell complex which mediates the process of extracellular dye release.

Blumenthal et al. (1977) studied the transfer of CF from PC vesicles to lymphocytes. Leakage of dye from neutral PC vesicles into the extracellular medium was not detectable in their experiments. Szoka et al. (1979) studied the interaction of CF-loaded phospholipid vesicles composed of mixtures of PC and PS with lymphocytes and L1210 cells. Their results indicate that as much as 90% of the entrapped CF initially cell associated leaks out of the vesicles after interaction with cells. They suggest the possibility that insertion of cell surface proteins into the vesicles may cause the vesicles to become leaky. Additional experiments are necessary to distinguish among the several plausible mechanisms by which mast cells could induce leakage of CF from PS vesicles. Recent observations that lyso-PS is a much more effective activator of mast cell secretion than PS (Martin & Lagunoff, 1979b; Smith et

[3] The number of PS molecules in a PS vesicle of known outer radius can be estimated by assuming that the vesicle is spherical and the individual PS molecules are arranged in a single bilayer. The available surface area for packing of PS head groups in such a vesicle will be $4\pi(r_1^2 + r_2^2)$, where r_1 and r_2 are the outer and inner radii of the bilayer, respectively. For a PS vesicle of r_1 = 150 Å, r_2 = 150 Å − W, where W is the width of the bilayer. Since W is in the range of 50 Å, r_2 = 100 Å. The total available surface area will be $4\pi[(150\text{ Å})^2 + (100\text{ Å})^2] = 4.08 \times 10^5$ Å^2. The best value for the molecular area occupied by an individual PS molecule above T_c is 70 Å^2, corresponding to 5830 molecules/vesicle.

al., 1979) raise the possibility that leakage of CF may result from the action of a phospholipase in the cell membrane.

Two-Step Model for the Action of PS. Although binding of PS to the mast cell is apparently required for activation of secretion, binding can be distinguished from activation on the basis of two criteria. (1) For each PS dispersion investigated, the binding curves are noncooperative while the activation curves display significant cooperativity. Hill plots of the binding data at 22 °C yield slopes which range from 0.80 to 0.93, whereas Hill plots of the activation data have slopes which range from 1.24 to 1.65. (2) Binding of the PS dispersions is not a function of their hydrocarbon chain length while activation is dependent on this property (Figures 2 and 3). Our interpretation is that the binding of PS dispersions is mediated primarily by electrostatic interactions between the surface of the mast cell and PS bilayer membranes and is thus relatively insensitive to changes in the length of the PS acyl chains. The observed dependence of activation on acyl chain length is not easily explained if activation is envisioned as strictly a micellar surface phenomenon. The dependence of activation of purified membrane enzymes by amphiphiles on hydrocarbon chain length (Grover et al., 1975; Blake et al., 1978) has been interpreted as evidence for the existence of hydrophobic binding domains in the enzymes with greater affinities for molecules with longer hydrocarbon chains. By analogy, we propose that the acyl chain length dependence observed in the action of PS is representative of a second reaction step subsequent to the binding of micellar aggregates in which bound molecules insert into the mast cell plasma membrane perhaps associating with a specific membrane component possessing a hydrophobic domain. The second step could involve fusion of cell-bound vesicles with the mast cell plasma membrane or might result from a molecular transfer analogous to that described for phospholipid exchange proteins (Wirtz, 1974).

Although there is as yet no direct evidence for the existence of a specific PS receptor in the mast cell, several characteristics in addition to the acyl chain length dependence of activation are those expected of a receptor-mediated response. Activation displays a remarkable degree of chemical specificity (Martin & Lagunoff, 1979a,b); it is associated with a finite number of relatively high-affinity vesicle binding sites (Martin & Lagunoff, 1978), and it is competitively inhibited by N-substituted PS analogues (Martin & Lagunoff, 1979a).

The proposed second step in the activation process would require a major alteration in the structure of bound micellar aggregates of PS. Analysis of the CF release data and the radiolabeled PS binding data indicates that at the midpoint of the activation curve for sonicated PS vesicles, 80% of the bound vesicles totally lyse upon interaction with mast cells. Regardless of the mechanism responsible for release of CF from the vesicles, these data are consistent with a gross alteration in their structural integrity and support the notion of a multiple reaction sequence in the activation process. This model further provides a plausible explanation for the observation that fluid-phase PS dispersions are preferred over solid-phase dispersions at lower incubation temperatures. If activation is dependent upon insertion of PS molecules into the mast cell plasma membrane, then it seems reasonable that this event would become less favorable for solid-phase dispersions as compared to fluid-phase dispersions when the temperature is progressively decreased.

Evidence implicating the insertion of PS molecules into the mast cell membrane during activation of Con A stimulated histamine secretion has recently been presented by Hirata et al. (1979). Exposure of Con A to mast cells in the presence of exogenous PS increased the incorporation of PS into the cells with a portion of the PS subsequently decarboxylated to phosphatidylethanolamine which in turn was N-methylated to form mono-, di-, and trimethylated derivatives. The quantity of PS undergoing metabolic conversion was not measured. In view of these results and our evidence in support of a second step in the activation process, it will be necessary to quantitate the proportion of total cell-associated PS that inserts into the membrane and its distribution and subsequent metabolic processing.

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Characterization of the Plant Nicotinamide Adenine Dinucleotide Kinase Activator Protein and Its Identification as Calmodulin†

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ABSTRACT: A protein activator of plant NAD kinase has been extracted from plant sources (peanuts and peas), purified to homogeneity, characterized, and identified as calmodulin. A comparison of the properties of calmodulin isolated from either plant or animal sources shows that they are strikingly similar proteins. The similarities include molecular weight, Stokes radii, amino acid composition, Ca^{2+}-dependent enhancement of tyrosine fluorescence, Ca^{2+}-dependent interaction with troponin I, equal abilities to activate cyclic nucleotide phosphodiesterase, Ca^{2+}-dependent inhibition of calmodulin action by the phenothiazine drugs, and electrophoretic mobility. We discuss the possibility that plant cells may undergo Ca^{2+}-dependent regulatory events that are mediated by calmodulin in a manner similar to those found in animals.

Calmodulin, a low molecular weight Ca^{2+}-binding protein, has been isolated from a variety of animal sources including coelenterates, annelids, and mammals (Teo et al., 1973; Teo & Wang, 1973; Lin et al., 1974; Wolff & Brostrom, 1974; Childers & Siegel, 1975; Dedman et al., 1977a; Yagi et al., 1978; Dabrowska et al., 1978; Waisman et al., 1978a; Jones et al., 1979; Head et al., 1979). Similarities in the physicochemical properties of calmodulin isolated from these sources, along with recent sequence data (Watterson et al., 1976; Vanaman et al., 1977; Dedman et al., 1978), suggest that the primary structure of calmodulin has been highly conserved during evolution. A high degree of conservation has been shown recently by a comparison of the amino acid sequences of coelenterate and mammalian calmodulin (F. Sharief, H. P. Jones, M. J. Cormier, and T. C. Vanaman, unpublished experiments). Such data suggest one or more fundamental roles for calmodulin in Ca^{2+}-dependent regulatory processes. Indeed, calmodulin has been found to activate a number of enzymes in vitro, including cyclic nucleotide phosphodiesterase (Cheung, 1970; Kakiuchi et al., 1970), brain adenylate cyclase (Brostrom et al., 1975; Cheung et al., 1975), (Ca^{2+} + Mg^{2+})-ATPase (Jarrett & Penniston, 1977, 1978; Gopinath & Vincenzi, 1977), several protein kinases (Yagi et al., 1978; Dabrowska et al., 1978; Waisman et al., 1978a,b), plant NAD kinase (Anderson & Cormier, 1978), phosphorylase kinase (Cohen et al., 1978), and phospholipase A_2 (Wong & Cheung, 1979). Calmodulin has also been found to be involved in regulating cellular processes such as the phosphorylation of synaptic vesicle proteins with subsequent release of neurotransmitter (Schulman & Greengard, 1978; DeLorenzo et al., 1979) and the disassembly of microtubules (Marcum et al., 1978).

Preliminary studies in this laboratory have shown that a heat-stable, Ca^{2+}-dependent activator of partially purified plant NAD kinase exists in extracts of higher plants, and that this activator protein has many properties in common with those observed for calmodulin isolated from animal sources (Anderson & Cormier, 1978). It was also shown that mammalian brain calmodulin will replace the plant protein activator in the Ca^{2+}-dependent activation of this plant NAD^+ kinase preparation. We report here the characteristics of this plant ac-

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