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Rat Mast Cell Phospholipase A_2: Activity toward Exogenous Phosphatidylserine and Inhibition by *N*-(7-Nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylserine†

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ABSTRACT: The presence of phospholipase A_2 in intact rat peritoneal mast cells was investigated by using two synthetic radiolabeled phosphatidylserine (PS) substrates. Incubation of intact cells with 1-oleoyl-2-[^{3}H]oleoyl-PS resulted in the release of a considerable quantity of [^{3}H]oleic acid from the substrate. To establish that [^{3}H]oleic acid release was mediated via direct enzymatic attack at the *sn*-2 position, we measured release of the [^{3}H]serine moiety from the glycerol backbone of 1,2-dimyristoylphosphatidyl[^{3}H]serine. This activity, which represents the combined actions of phospholipases C and D, was 10-fold lower than [^{3}H]oleic acid release, indicating that neither of these enzymes is required for the release of the preponderance of [^{3}H]oleic acid. These results establish the existence in intact rat mast cells of a phospholipase A_2 active toward exogenous PS. Over the concentration range at which exogenous PS activates mast cell secretion, intact mast cells and broken cells possessed nearly equal levels of phospholipase A_2 activity, and enzyme activity was 3–4-fold higher toward PS than phosphatidylcholine. Several agents were tested for their ability to inhibit phospholipase A_2 in intact mast cells. Of the agents tested, an N-substituted derivative of PS previously identified as an inhibitor of mast cell secretion was shown to be a particularly potent and efficacious inhibitor of mast cell phospholipase A_2. The concentration dependence of enzyme inhibition paralleled inhibition of histamine secretion, providing a strong positive correlation between the level of phospholipase A_2 in mast cells and the capacity for secretion.

Of the many agents known to induce rat mast cells to secrete histamine, a subset is distinguished by dependence on exogenous phosphatidylserine (PS)[1] for a maximal response (Goth et al., 1971; Baxter & Adamik, 1978). Included in this group

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[1] Abbreviations: Con A, concanavalin A; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; NBD-PS, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylserine; PC, phosphatidylcholine; PS, phosphatidylserine.

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are IgE-dependent systems (Goth et al., 1971; Stechschulte & Austen, 1974), the anaphylatoxins C3a and C5a (Johnson et al., 1975), and dextran (Goth et al., 1971; Chakravarty et al., 1973). Histamine secretion induced by these agents is markedly potentiated by the presence in the medium of exogenous PS. Under certain conditions, histamine secretion from rat mast cells exposed to concanavalin A (Con A) (Sullivan et al., 1975; Martin & Lagunoff, 1978) or dextran (Foreman & Mongar, 1972; Garland & Mongar, 1974) is absolutely dependent on exogenous PS, making this phospholipid, in effect, an obligatory cofactor for secretion. The specificity of the effect of PS on histamine secretion is considerable. Other diacylphospholipids tested are completely inactive (Goth et al., 1971; Mongar & Svec, 1972; Sugiyama et al., 1975; Baxter & Adamik, 1976). A requirement for the unblocked free amino group of PS is indicated by the failure of several N-substituted PS derivatives to activate secretion (Martin & Lagunoff, 1979a).

The observation that exogenous lyso-PS is an effective activator of histamine secretion, 50–100-fold more potent than PS (Martin & Lagunoff, 1979b; Smith et al., 1979), may be a clue to the mechanism of action of PS. Of several lysophospholipids tested, only lyso-PS possessed activity (Martin & Lagunoff, 1979b), and, as with the parent compound, N-substitution with methyl acetimidate completely inactivated lyso-PS (Martin & Lagunoff, 1979b). On the basis of common characteristics in the actions of PS and lyso-PS and the far greater potency of the lyso compound, we suggested that the action of PS may involve its conversion to lyso-PS by a mast cell phospholipase A_2 (Martin & Lagunoff, 1979b).

This communication describes experiments performed with two synthetic radiolabeled PS derivatives which demonstrate the presence in intact rat mast cells of a phospholipase A_2 active toward exogenous PS. Evidence for the involvement of phospholipase A_2 in mast cell secretion is supplied by the parallelism observed between inhibition of this enzyme and inhibition of mast cell secretion induced by agents representative of two distinct secretagogue classes.

Materials and Methods

Materials. Materials were obtained from the suppliers indicated: L-serine, quinacrine dihydrochloride, and phospholipases A_2 from *Naja naja* and *Crotalus adamanteus* (Sigma Chemical Co.), *p*-bromophenacyl bromide, dicyclohexylcarbodiimide, and carbonyldiimidazole (Aldrich Chemical Co.), [9,10(N)-^{3}H]oleic acid (5 Ci/mmol) (Amersham Corp.), 3A70 scintillation fluid (Research Products International Corp.), concanavalin A and Patho-O-Cyte 4 (Miles Laboratories, Inc.), Triton X-100 (Eastman Kodak Co.), and oleic acid (Supelco, Inc.). A23187 was generously provided by Dr. R. Hamill of Lilly Research Laboratories, Indianapolis, IN.

Procedures for the synthesis of NBD-PS (Martin & Lagunoff, 1979a) and 1,2-dimyristoylphosphatidyl[^{3}H]serine (Martin & Lagunoff, 1980) and their chemical characterization have been published previously.

Synthesis of 1-Oleoyl-2-[^{3}H]oleoyl-PS. Egg yolk PC was isolated by the procedure of Singleton et al. (1965). L-α-Glycerophosphocholine prepared from egg yolk PC (Brockerhoff & Yurkowski, 1965) was acylated with oleoyl anhydride (Selinger & Lapidot, 1966) to give 1,2-dioleoyl-PC (Cubero Robles & Van den Berg, 1969). Treatment of 1,2-dioleoyl-PC with *C. adamanteus* phospholipase A_2 (Wells & Hanahan, 1969) yielded 1-oleoyl-2-lyso-PC which was purified by silicic acid column chromatography and used for acylation with [^{3}H]oleoylimidazolide. [^{3}H]Oleic acid, purchased from Amersham Corp. (5 Ci/mmol), was mixed with cold oleic acid to give a specific activity of 0.15 Ci/mmol. The [^{3}H]oleic acid (66 μmol) was dried by lyophilization from benzene and reacted with 89 μmol of carbonyldiimidazole in 1.0 mL of anhydrous chloroform under nitrogen for 45 min at 22 °C. The reaction was terminated by evaporating the chloroform under nitrogen, and the residue was then lyophilized from benzene. 1-Oleoyl-2-lyso-PC (52 μmol) was dried prior to acylation by lyophilization from benzene. Acylation of lyso-PC was performed in dimethyl sulfoxide with sodium methylsulfinylmethide as the catalyst according to a modification of the procedure described by Warner & Benson (1977). The lyophilized [^{3}H]oleoylimidazolide was dissolved in 1.5 mL of dry dimethyl sulfoxide and transferred to a reaction vessel containing the lyophilized 1-oleoyl-2-lyso-PC. An additional 1.5 mL of solvent was used to ensure complete transfer of [^{3}H]-oleoylimidazolide, giving a final volume of 3.0 mL. The reactants were completely dissolved prior to the addition of 0.37 mL of sodium methylsulfinylmethide which was freshly prepared by reacting 25 mg of sodium metal with 1.8 mL of dimethyl sulfoxide under nitrogen for 2 h.

With the addition of the catalyst, the reaction mixture turned yellow. The reaction vessel was evacuated with nitrogen and stoppered immediately, and the reaction was allowed to proceed for 13 min at 22 °C. The reaction was terminated by the addition of 0.82 mL of 1.2 N HCl in methanol. Thin-layer chromatography of an aliquot of the reaction mixture on a silica gel G plate (Redi-Coat, Supelco, Inc.) developed in chloroform–methanol–water (65:25:4 v/v) indicated that acylation of 1-oleoyl-2-lyso-PC was greater than 80%. The reaction mixture was extracted by adding 6 mL of chloroform, 2.5 mL of water, and 1.5 mL of methanol. After vortexing and centrifugation at 200*g* for 6 min, the chloroform layer was collected, and the aqueous phase was extracted twice more with 8 mL of chloroform–methanol–water (4:1:1 v/v). The pooled chloroform phase was then extracted twice with 10-mL volumes of methanol–water (1:1 v/v), and the final chloroform phase was evaporated to a residue in vacuo and then dried from benzene. The dry residue was dissolved in 5 mL of chloroform and applied to a 15-mL column of heat-activated silicic acid (Mallinckrodt SilicAR CC-7; 100–200 mesh) poured and washed with chloroform. The column was eluted with a sequential solvent system consisting of 10 column volumes each of chloroform–methanol (8:2 v/v), chloroform–methanol (1:1 v/v) and chloroform–methanol (1:2 v/v). Over 90% of the 1-oleoyl-2-[^{3}H]oleoyl-PC eluted with the second solvent free of contaminating oleic acid and residual dimethyl sulfoxide. The final yield was slightly greater than 60% based on analysis of the phosphorus content of initial reactant and final product (Fiske & Subbarow, 1925). 1-Oleoyl-2-[^{3}H]oleoyl-PS was prepared from 1-oleoyl-2-[^{3}H]-oleoyl-PC by transphosphatidylation catalyzed by phospholipase D in the presence of L-serine (Comfurius & Zwaal, 1977). Phospholipase D was obtained from Savoy cabbage and used as the acetone-precipitated powder (Yang, 1969). 1-Oleoyl-2-[^{3}H]oleoyl-PS was freed of contaminating PC and phosphatidic acid by DEAE-cellulose column chromatography using a solvent system and elution protocol previously described (Raetz & Kennedy, 1973). The yield of *sn*-2-[^{3}H]oleoyl-PS following transphosphatidylation was 20%. The final product migrated as a single ninhydrin- and phosphorus-positive spot after thin-layer chromatography in several solvent systems (Kates, 1972). The radiochemical purity determined by radioassay of 5-mm strips scraped from the plates was greater than 99%. For verification that the [^{3}H]oleic acid label was

incorporated into the *sn*-2 position of the product, an aliquot was mixed with a 100-fold excess of egg yolk lecithin and treated with phospholipase A_2 from *C. adamanteus* for 48 h at 22 °C. Analysis of the digestion products indicated that greater than 98% of the ^3H label was released from 1-oleoyl-2-[^3H]oleoyl-PS as oleic acid.

Assay of Phospholipase Activity in Mast Cells. Peritoneal cells were obtained from adult male Sprague-Dawley rats (Hilltop Animal Laboratory, Scotdale, PA), and the mast cells were purified to greater than 90% homogeneity by centrifugation through concentrated albumin as previously described (Lagunoff, 1975). The final cell pellet was suspended in a small volume of Hepes-buffered saline (166 mM NaCl and 10 mM Hepes, pH 7.5), and the cells were counted in a hemocytometer. The radiolabeled PS substrates 1-oleoyl-2-[^3H]oleoyl-PS or 1,2-dimyristoylphosphatidyl[^3H]serine which were stored in chloroform were taken to dryness under a nitrogen stream and suspended in Hepes-buffered saline by brief sonication in a small bath-type sonicator. For routine assays, mast cells [(2–6) × 10^6] were suspended in fresh Hepes-buffered saline and aliquots of the [^3H]PS suspensions added. $CaCl_2$ was then added separately from a stock solution in distilled water to a final concentration of 1 mM. The final cell concentrations were maintained at 2 × 10^6 cells/mL. The reactions were carried out at 37 °C for 30 min and terminated by the addition of 2.4 volumes of chloroform–methanol (5:1 v/v) which was 0.006 N in HCl. The resulting mixture was vortexed thoroughly for 30 s, and the phases were separated completely by centrifugation at 200*g* for 6 min. The chloroform phase was removed with a Pasteur pipet, and the aqueous phase was extracted 2 additional times with 2 volumes each of chloroform. The pooled chloroform phases were then taken to dryness under nitrogen and redissolved in a small volume of chloroform containing the appropriate lipid standards (see below). In each series of experiments, duplicate control reactions containing radiolabeled substrate but without cells were processed in an identical manner to measure the amount of spontaneous hydrolysis. This value, which never exceeded 0.5% of the total initial substrate, was subtracted from the values obtained in reactions containing mast cells prior to calculation of the results.

Thin-Layer Chromatography of Chloroform-Extracted Reaction Mixtures. Chloroform extracts from reactions performed with 1-oleoyl-2-[^3H]oleoyl-PS were combined with authentic diacylglycerol and oleic acid prior to thin-layer chromatography. The entire samples were then applied to silica gel G thin-layer sheets (Eastman Kodak Co., No. 13179) and chromatographed in a double-development system. The first solvent employed was diethyl ether–benzene–ethanol–triethylamine (40:50:2:1 v/v), and the sheets were developed to a height of 13 cm from the origin. After thorough drying, the sheets were chromatographed in the same dimension in chloroform–methanol (9:1 v/v) to a distance of 7 cm from the origin. Diacylglycerol migrated at the solvent front in the first solvent, whereas oleic acid migrated with an R_f of 0.35 with slight trailing. Oleic acid migrated at the solvent front in the second solvent system which was employed to concentrate the oleic acid spot well separated from diacylglycerol and 1-oleoyl-2-[^3H]oleoyl-PS, the latter remaining at the origin in both solvent systems. The dried chromatograms were briefly exposed to iodine vapor, and the spots corresponding to diacylglycerol and oleic acid were marked. After complete evaporation of the iodine, the marked spots were scraped directly into 10 mL of 3A70 scintillant and radioassayed.

In experiments performed with 1,2-dimyristoylphosphatidyl[^3H]serine, the entire reaction mixture was subjected to thin-layer chromatography in order to identify and quantitate all of the ^3H-labeled products. After extraction of the reaction mixture with chloroform, the extract was combined with bovine brain PS (Sigma Chemical Co.) and lyso-PS (Supelco, Inc.) standards and applied to a Whatman LK5D preabsorbent thin-layer plate. The aqueous phase from the original reaction mixture was taken to dryness under nitrogen at 60 °C and redissolved in 0.2% Triton X-100 containing a serine standard. The aqueous sample was then layered over the corresponding chloroform extract in the same lane of the Whatman LK5D plate, and the plate was chromatographed in diisobutyl ketone–acetic acid–water (40:30:7 v/v). After being dried, the plate was sprayed with a ninhydrin reagent (Kates, 1972), and the spots corresponding to PS, lyso-PS, and serine were scraped directly into 10 mL of 3A70 scintillant and radioassayed. The data obtained were corrected for quenching which differed for each of the three spots and for the percent recovery which was calculated following quantitation of the recovery of a known amount of standard. This procedure yielded a high recovery and complete separation of PS, lyso-PS, and aqueous-soluble products which migrated with the chromatographic behavior of serine or phosphoserine. Extraction alone could not completely separate lyso-PS from the other aqueous-soluble products.

Assay of Histamine Secretion. Purified mast cells were obtained as described previously (Lagunoff, 1975). Each assay was performed with 2.5 × 10^5 cells in a final volume of 1.0 mL of balanced salt solution (4 mM Na_2HPO_4, 2.7 mM KH_2PO_4, 166 mM NaCl, and 2.7 mM KCl, pH 7.2). For experiments performed with PS and Con A, each reagent was added independently to the cell suspension, and the reaction was initiated by the addition of $CaCl_2$ to a final concentration of 0.68 mM. In experiments performed with A23187, $CaCl_2$ (0.68 mM) was included in the original balanced salt solution. The cells were incubated at 37 °C for 15 min, and the reaction was terminated by centrifugation of the cell suspension at 200*g* for 10 min. Histamine release was calculated as a mean percentage of total cell histamine after assay of supernatants and trichloroacetic acid extracts of cell pellets by a modification of the *o*-phthalaldehyde fluorometric method (Kremzner & Wilson, 1961). The histamine release data are expressed as a percentage of total cell histamine after subtraction of values obtained in the absence of any secretagogue. In the inhibition experiments, the results are presented as a percent of control histamine release.

Results

Intact rat peritoneal mast cells were incubated independently with phospholipid dispersions generated from 1-oleoyl-2-[^3H]oleoyl-PS and 1,2-dimyristoylphosphatidyl[^3H]serine. Following extraction of the reaction mixtures and analysis by thin-layer chromatography, the conversion of these compounds to specific labeled products was quantitated. The results are presented in Table I. A considerable amount of [^3H]oleic acid was released from 1-oleoyl-2-[^3H]oleoyl-PS after interaction with intact mast cells. In contrast, there was comparatively little formation of 1-oleoyl-2-[^3H]oleoyl-*sn*-glycerol, the product of phospholipase C action.

Release of [^3H]oleic acid from the substrate could result from either the direct action of phospholipase A_2 or the sequential actions of phospholipase D and phospholipase A_2 or phospholipase C and diglyceride lipase. Either of the latter two pathways would release the radiolabeled serine moiety from the glycerol backbone of the substrate. The data obtained

Table I: Phospholipase Activity in Intact Rat Mast Cells[a]

substrate	product	activity
1-oleoyl-2-[^{3}H]oleoyl-PS (5)	[^{3}H]oleic acid	537 ± 99
	[^{3}H]diacylglycerol	16 ± 5
1,2-dimyristoylphosphatidyl[^{3}H]serine (3)	[^{3}H]lyso-PS	403 ± 43
	[^{3}H]serine–phosphoserine	37 ± 12

[a] Purified intact rat mast cells were incubated with synthetic radiolabeled PS substrates at a final concentration of 30 μM in Hepes-buffered saline, pH 7.5, containing 1 mM $CaCl_2$ for 30 min at 37 °C. After extraction and thin-layer chromatography, products of the action of phospholipases were quantitated. Activity is expressed in picomoles per 10^6 cells per 30 min ± SEM. The number in parentheses indicates the number of individual experiments performed. Serine and phosphoserine comigrated in the solvent system employed for thin-layer chromatography so that the value given represents the sum of these two compounds.

with 1,2-dimyristoylphosphatidyl[^{3}H]serine indicated that only a very small amount of the ^{3}H label was recovered in product(s) which migrated on thin-layer chromatography with the properties expected of serine or phosphoserine derived from enzymatic attack by phospholipases C and D. These data are consistent with the low level of [^{3}H]diacylglycerol generated by cells exposed to 1-oleoyl-2-[^{3}H]oleoyl-PS and indicate that phospholipase D activity under these conditions is quite low. On the other hand, a substantial amount of lyso-phosphatidyl[^{3}H]serine was formed following interaction of the [^{3}H]serine–PS substrate with mast cells. Taken together, the pooled data obtained with the two PS substrates indicate that the major pathway for release of [^{3}H]oleic acid from the *sn*-2 position of PS involves the direct action of a mast cell phospholipase A_2. This conclusion follows from the observation that the combined products of the action of phospholipases C and D on exogenous PS can account for no more than 8% of the [^{3}H]oleic acid released from the *sn*-2 position of PS.

If all of the [^{3}H]oleic acid released from 1-oleoyl-2-[^{3}H]-oleoyl-PS was derived from the action of phospholipase A_2, then a corresponding amount of lyso-PS would be generated, unless the enzyme acted as or was associated with a transacylase. Using the dimyristoyl-PS substrate labeled with [^{3}H]serine, it was possible to isolate [^{3}H]lyso-PS in an amount corresponding to 75% of the amount predicted from the [^{3}H]oleic acid release data. The failure to isolate an equivalent amount of lyso-PS may be attributable to a slight difference in activity of the enzyme toward the two PS substrates which differed in fatty acid composition. Alternatively, a portion of the 1-oleoyl-2-lyso-PS generated following release of [^{3}H]oleic acid could have undergone reacylation to form diacyl-PS. The observation that the amount of [^{3}H]lyso-PS isolated was somewhat less than the amount of [^{3}H]oleic acid release makes it unlikely that lyso-PS is derived from the action of a phospholipase A_1, but additional experiments are necessary to rigorously eliminate this possibility. Nevertheless, the results do establish that most of the [^{3}H]oleic acid release from *sn*-2-[^{3}H]oleoyl-PS is a direct consequence of enzymatic attack at the *sn*-2 position and is not dependent upon any mechanism involving either phospholipase C or D. Additional experiments were performed with this substrate to assay phospholipase A_2 in mast cells.

The experiments presented in Figure 1 were performed to measure the activity of phospholipase A_2 in mast cells exposed to exogenous dioleoyl-PS over the concentration range at which activation of histamine secretion is observed (0.5–10 μM). Intact mast cells and mast cells disrupted by sonication expressed similar levels of enzyme activity throughout this range. Phospholipase A_2 activity of intact cells was some 3-fold less

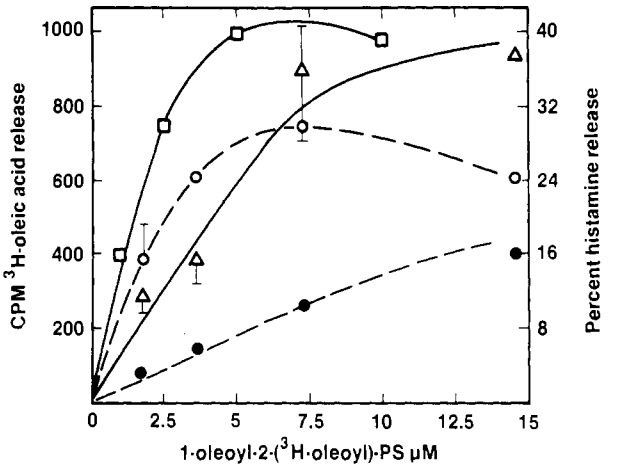

FIGURE 1: Relationship between phospholipase A_2 activity and PS activation of mast cell secretion induced by Con A. Mast cells were incubated with Con A (100 μg/mL) and Ca^{2+} (0.68 mM) in the presence of increasing concentrations of 1-oleoyl-2-[^{3}H]oleoyl-PS for 15 min at 37 °C, and histamine secretion was measured (□). In separate reactions, release of [^{3}H]oleic acid from this substrate was quantitated in intact (○) and broken (△) cell suspensions. [^{3}H]Oleic acid release from 1-oleoyl-2-[^{3}H]oleoyl-PC incubated with intact mast cells under the same conditions is shown for comparison (●). Phospholipase A_2 activity is expressed in cpm of [^{3}H]oleic acid release per 10^6 cells per 30 min. The specific activity of each substrate is 2160 cpm/nmol. Each point shown is the average of at least two separate experiments, and the bars represent SEM in experiments performed 3 or more times.

Table II: Inhibition of Phospholipase A_2 Activity in Unstimulated Intact Rat Mast Cells[a]

experimental conditions	% control	no. of expts
control	100	19
100 μM *p*-bromophenacyl bromide	100 ± 7	6
1 mM quinacrine	62 ± 10	11
25 μM NBD-PS	12 ± 3	8
1 mM EDTA	39 ± 8	6

[a] Purified intact rat mast cells were incubated with the agents listed above and 36 μM 1-oleoyl-2-[^{3}H]oleoyl-PS in Hepes-buffered saline, pH 7.5, containing 1 mM $CaCl_2$ for 30 min at 37 °C, and [^{3}H]oleic acid release was quantitated. The results are presented as the percent ± SEM of control [^{3}H]oleic acid release which was measured in the absence of the specified agent. Control activity was 611 ± 69 pmol $(10^6 \text{ cells})^{-1}$ $(30 \text{ min})^{-1}$. The concentration of EDTA shown indicates that in excess of added $CaCl_2$.

when 1-oleoyl-2-[^{3}H]oleoyl-PC was used as the substrate instead of dioleoyl-PS.

Several agents were tested for their ability to inhibit phospholipase A_2 activity in intact mast cells, and the results are presented in Table II. *p*-Bromophenacyl bromide, an alkylating agent previously shown to inhibit phospholipase A_2 from several sources (Volwerk et al., 1974; Roberts et al., 1977), possessed no inhibitory effect on the mast cell enzyme when tested at 100 μM. Quinacrine, another commonly employed phospholipase A_2 inhibitor (Flower & Blackwell, 1976; Blackwell et al., 1978; Billah et al., 1981), was found to be a relatively poor inhibitor of the enzyme in mast cells. NBD-PS, however, was found to be a potent inhibitor of mast cell phospholipase A_2. While Ca^{2+} was required for optimal phospholipase A_2 activity, removal of free Ca^{2+} from the medium with 1 mM EDTA only partially inhibited enzyme activity.

Previous experiments demonstrated that NBD-PS was a potent inhibitor of histamine secretion from mast cells stimulated with several secretagogues (Martin & Lagunoff,

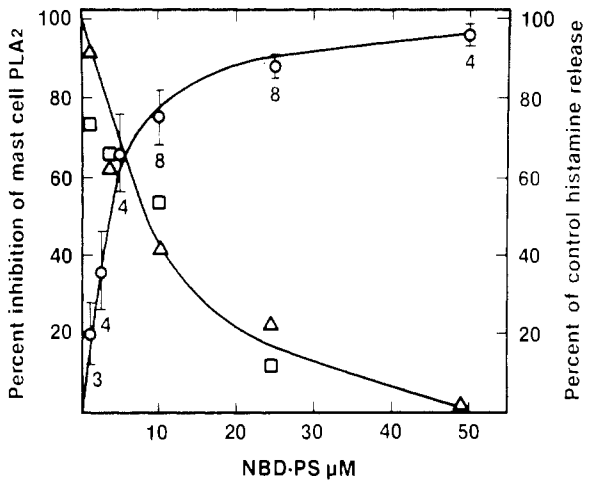

FIGURE 2: Parallel inhibition of mast cell phospholipase A_2 and histamine secretion by NBD-PS. Phospholipase A_2 was assayed in intact mast cells by measuring the release of [^{3}H]oleic acid from 1-oleoyl-2-[^{3}H]oleoyl-PS as described in Table II. The results are presented as the mean ± SEM percent inhibition of control activity (○), and the number of individual determinations is indicated below the error bars. Histamine secretion induced by 100 μg/mL Con A plus 30 μM PS (□) and 1 μM A23187 (△) in the presence of NBD-PS was assayed in separate reactions. These data are expressed as the mean percentage of control histamine secretion induced by each secretagogue in the absence of added NBD-PS. Each point represents the average of two separate experiments with duplicate determinations in each experiment.

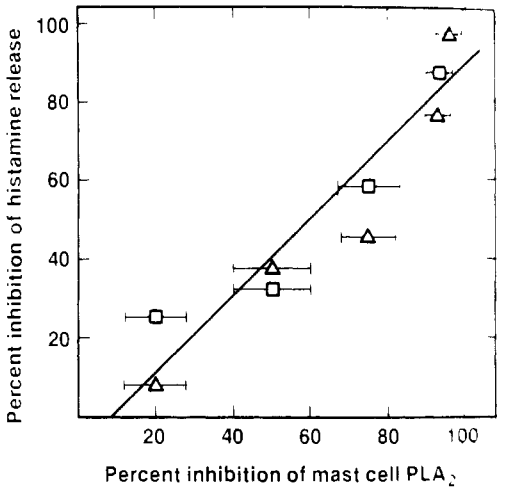

FIGURE 3: Correlation between inhibition of mast cell phospholipase A_2 and histamine secretion. The data presented in Figure 2 were subjected to regression analysis by using the least-squares method to evaluate the strength of the correlation between inhibition of mast cell phospholipase A_2 and inhibition of histamine secretion. The correlation coefficient is 0.93. The secretion data obtained with Con A plus PS (□) and A23187 (△) were pooled.

1979a). To investigate the relationship between mast cell phospholipase A_2 and mast cell secretion, we compared the concentration dependence of the inhibitory effect of NBD-PS on phospholipase A_2 and histamine secretion. The results presented in Figure 2 illustrate the inverse relationship between inhibition of phospholipase A_2 and the capacity of mast cells to respond to two stimulating agents, Con A plus PS and the calcium ionophore A23187. The I_{50} calculated for inhibition of phospholipase A_2 is 5 μM which corresponds closely with the I_{50} obtained for inhibition of histamine secretion (10 μM) induced by the two secretagogues. These values are close to the dissociation constant of 7 μM determined for the binding of NBD-PS to mast cells (Martin & Lagunoff, 1979a).

The strength of the correlation between inhibition of histamine secretion and inhibition of phospholipase A_2 is indicated by a correlation coefficient of 0.93 obtained from the regression analysis depicted in Figure 3. Quinacrine was also found to be an effective inhibitor of mast cell histamine secretion, but inhibition occurred at concentrations nearly 2 orders of magnitude below those required for comparable levels of inhibition of phospholipase A_2, suggesting an alternative site of action for this inhibitor unrelated to phospholipase A_2.

Discussion

Exogenous PS activates histamine secretion from rat mast cells stimulated with Con A (Sullivan et al., 1975; Martin & Lagunoff, 1978) and several other secretagogues (Goth et al., 1971; Johnson et al., 1975). Lyso-PS is as effective as PS and is 50–100-fold more potent (Martin & Lagunoff, 1979b; Smith et al., 1979). We previously proposed that the action of PS involves its conversion to lyso-PS (Martin & Lagunoff, 1979b). The results presented in Table I and Figure 1 establish that intact mast cells are in fact capable of converting exogenous PS to lyso-PS. The data further indicate that lyso-PS production is attributable to direct enzymatic attack of PS at the *sn*-2 position by a mast cell phospholipase A_2. This follows from the observation that [^{3}H]oleic acid release from 1-oleoyl-2-[^{3}H]oleoyl-PS is more than sufficient to account for all of the lyso-PS produced. Justification for using [^{3}H]oleic acid release from 1-oleoyl-2-[^{3}H]oleoyl-PS as a measure of phospholipase A_2 activity is derived from the observation that [^{3}H]oleic acid release is more than 10-fold greater than the combined activities of phospholipases C and D toward exogenous 1,2-dimyristoylphosphatidyl[^{3}H]serine. Thus, no more than 8% of the [^{3}H]oleic acid release from 1-oleoyl-2-[^{3}H]-oleoyl-PS can be attributed to any mechanism other than direct attack at the *sn*-2 position.

The experiments in Table I were performed with cell suspensions in which mast cells comprised 85–95% of the total cell population. The remaining cells were exclusively monocyte–macrophages. When purified free of mast cells and assayed independently, these cells express no phospholipase A_2 activity toward exogenous phospholipids unless disrupted by sonication. This observation eliminates the possibility that the data presented in Table I were altered by the presence of contaminating cells.

Since intact mast cells were found to possess substantial phospholipase A_2 activity, we examined the level of this activity in the substrate concentration range over which exogenous PS activates histamine secretion. The data presented in Figure 1 demonstrate that the concentration-dependent increase in activation is accompanied by a corresponding increase in [^{3}H]oleic acid release from the *sn*-2 position of PS. At substrate concentrations over which PS activates secretion, mast cells disrupted by sonication expressed no greater phospholipase A_2 activity than intact cells. For both preparations, enzyme activity over this substrate range corresponds to 90–370 pmol $(10^6 \text{ cells})^{-1}$ $(30 \text{ min})^{-1}$. In view of this finding, it is tempting to speculate that phospholipase A_2 activity is associated with the mast cell plasma membrane, but it is also possible that exogenous PS distributes within the interior of the cell.

Although we have not as yet examined the substrate specificity of mast cell phospholipase A_2 in detail, it is of interest that enzyme activity toward 1-oleoyl-2-[^{3}H]oleoyl-PS is 3–4-fold greater than toward 1-oleoyl-2-[^{3}H]oleoyl-PC. The specificity of the effect of PS on secretion cannot be explained by this relative substrate preference, however, because neither PC (Goth et al., 1971; T. W. Martin and D. Lagunoff, unpublished experiments) nor lyso-PC (Martin & Lagunoff, 1979b) enhances histamine secretion when added to mast cells

over a wide concentration range.

We have previously used bovine brain PS vesicles with carboxyfluorescein entrapped within the aqueous compartment to investigate the interaction of PS vesicles with mast cells (Martin & Lagunoff, 1980). The results indicated that virtually all of the vesicles that became cell associated lysed upon interaction with cells with rapid release of the entrapped dye from the vesicles into the extracellular medium. In view of the present results, it is likely that dye release, which was saturable with respect to vesicle concentration, was mediated by the action of phospholipase A_2. Many investigators have studied the interaction of phospholipid vesicles with eukaryotic cells (Pagano & Weinstein, 1978), but we are unaware of any prior report describing a phospholipase in intact cells active toward exogenous phospholipid vesicles.

In order to more directly examine the role of phospholipase A_2 in mast cell secretion, we tested the ability of several agents to inhibit this enzyme in intact mast cells. Of the several agents tested, NBD-PS alone was found to be a potent and effective inhibitor. Since we had previously shown that NBD-PS inhibits mast cell secretion (Martin & Lagunoff, 1979a), we investigated the concentration dependence of the effect of this agent on phospholipase A_2 and histamine secretion. The results presented in Figures 2 and 3 establish a strong positive correlation between inhibition of phospholipase A_2 and inhibition of histamine secretion.

While NBD-PS inhibits phospholipase A_2 and histamine secretion in parallel, it does not necessarily follow that inhibition of this enzyme is the mechanism by which NBD-PS acts to inhibit secretion. Inhibitory effects of this agent on another step in the secretory process are possible. The ability of NBD-PS to inhibit secretion induced by Con A and exogenous PS might result from inhibition of the conversion of PS to lyso-PS and thus lend support to our hypothesis that this conversion is essential to the action of PS. Following this line of reasoning, alternative explanations are required for the observation that NBD-PS also inhibits secretion induced by A23187 and Ca^{2+} since exogenous PS has no effect on this secretagogue (Martin & Lagunoff, 1979a). In addition to the possibility of a site of action unrelated to any effect on phospholipase A_2, it is conceivable that this enzyme participates in another step in the secretory pathway common to all stimulating agents, for example, by serving to release arachidonic acid or generate fusogenic lysophospholipids from membrane phospholipids. The data presented in Figure 2 indicate that the concentration dependence observed for NBD-PS inhibition is nearly identical for secretion induced by two different secretagogues, consistent with a common site of inhibition.

Although the data presented here do not establish phospholipase A_2 as the site at which NBD-PS inhibits mast cell secretion, it is evident that NBD-PS is nevertheless a potent inhibitor of this enzyme in mast cells. Since there are few agents available which are known to be effective phospholipase A_2 inhibitors, NBD-PS may prove useful in studies of phospholipases A_2 in other cells. In this regard, we have recently demonstrated that NBD-PS inhibits phospholipases A_2 present in *N. naja* venom and in disrupted suspensions of rat peritoneal monocytes and rat basophilic leukemia cells (T. W. Martin and D. Lagunoff, unpublished experiments).

Many cells are known to generate arachidonic acid metabolites following activation by a variety of stimuli, and it has been thought that these agents, perhaps via an increase in cytoplasmic Ca^{2+}, activate phospholipase A_2 or other enzymes necessary for releasing the precursor arachidonic acid (Vogt, 1978). Within this scheme, enzyme activation represents the point of regulatory control for arachidonic acid release. Our demonstration of substantial phospholipase A_2 activity in intact unstimulated mast cells raises the possibility that substrate activation may in some cases be an important control point. Perhaps in some cells, stimulation by certain agents changes the substrate specificity of an already active enzyme or alters the properties of the membrane in such a way as to render certain local phospholipid domains preferential sites for enzymatic attack.

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Assignment of the Carbon-13 Nuclear Magnetic Resonance Spectra of Gangliosides G_{M4}, G_{M3}, G_{M2}, G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b}†

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ABSTRACT: Complete ^{13}C nuclear magnetic resonance assignments are presented for gangliosides in the series G_{M4}, G_{M3}, G_{M2}, G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b}. The gangliosides studied are related by the sequential addition of single saccharide residues. The structural relationships among these molecules were confirmed and subsequently utilized to provide the basis for a detailed investigation of ^{13}C NMR oligomer–monomer shielding differences accompanying increasing oligosaccharide complexity. This gradual increase in complexity was reflected in the ^{13}C NMR spectra and proved to be of significant value in the assignment task, resulting in the reassignment of four G_{M1} resonances from our previous work [Sillerud, L. O., Prestegard, J. H., Yu, R. K., Schafer, D. E., & Konigsberg, W. H. (1978) *Biochemistry 17*, 2619–2628]. The carboxyl-containing sialic acids in gangliosides have glycosidic linkage resonance shifts only ~30% as large as those found for neutral hexopyranosides; thus, care must be used in interpreting the ^{13}C spectra of charged oligosaccharides. Secondary structural effects are also found to produce shifts in the resonances of the sialic acid adjacent to the GalNAc residue of G_{M2} and the more complex gangliosides, leading to inequivalence of the sialic acids in G_{D1a}, G_{D1b}, and G_{T1b}.

The gangliosides, glycosphingolipids containing sialic acid, have long been recognized as major membrane components of the cells of nervous tissue (Ledeen & Yu, 1973). More recently, gangliosides have been implicated as participants in important membrane functions of other cell types, where their concentration is far lower than in nervous tissue (Fishman & Brady, 1976). The most widely studied example is the interaction of the monosialoganglioside G_{M1} (II^3NeuAcGgOse$_4$Cer*)[1] (Figure 1) with the protein toxin produced by *Vibrio cholerae* (Cuatrecasas, 1973; Holmgren et al., 1975; Sattler et al., 1977). It has been suggested that specific ganglioside receptors exist in target cells for all of the glycoprotein hormones (Mullin et al., 1977), as well as for many bacterial toxins, interferon (Fishman & Brady, 1976), and lectins (Maget-Dana et al., 1979). Interesting correlations have been reported between ganglioside concentration or complexity and cell differentiation (Dreyfus et al., 1979), cell senescence (Rahmann & Hilbig, 1979), cell transformation (Fishman & Brady, 1976), tumorigenic progression (Morre et al., 1979), and the phylogenetic development of the central nervous system (Hilbig & Rahmann, 1979). Ganglioside G_{D1a} of the thyrocyte specifically binds TSH (Mullin et al., 1976), and it has recently been shown that the binding of FSH to G_{M1} in model membranes specifically alters membrane conductance (Deleers et al., 1979).

In a previous paper, we reported the first complete assignments for the ^{13}C NMR spectrum of ganglioside G_{M1} and demonstrated that ^{13}C NMR is useful for studying the subtle changes in ganglioside structure induced by environmental perturbations such as binding to ligands (Sillerud et al., 1978). We have now extended these results to include G_{M4}, G_{M3}, G_{M2}, G_{D1a}, G_{D1b}, and G_{T1b}. These studies, together with our earlier paper and that of Harris & Thornton (1978) on G_{M1}, G_{M3}, and G_{D1a}, permit a detailed comparison of the complete ^{13}C NMR spectra of seven of the major known gangliosides. Such a comparison confirms most of the earlier assignments, and

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[1] Abbreviations: G_{M1}, II^3NeuAcGgOse$_4$Cer* = Galβ1→3GalNAcβ1→4Gal[3←2αNeuAc]β1→4Glcβ1→1Cer; G_{M2}, II^3NeuAcGgOse$_3$Cer* = GalNAcβ1→4Gal[3←2αNeuAc]β1→4Glcβ1→1Cer; G_{M3}, II^3NeuAcLacCer* = NeuAcα2→3Galβ1→4Glcβ1→1Cer; G_{M4}, I^3NeuAcGalCer* = NeuAcα2→3Galβ1→1Cer; G_{D1a}, IV^3NeuAc,II^3NeuAcGgOse$_4$Cer* = NeuAcα2→3Galβ1→3GalNAcβ1→4Gal[3←2αNeuAc]β1→4Glcβ1→1Cer; G_{D1b}, II^3(NeuAc)$_2$GgOse$_4$Cer* = Galβ1→3GalNAcβ1→4Gal[3←2αNeuAc8←2αNeuAc]β1→4Glcβ1→1Cer; G_{T1b}, IV^3NeuAc, II^3(NeuAc)$_2$GgOse$_4$Cer* = NeuAcα2→3Galβ1→3GalNAcβ1→4Gal[3←2αNeuAc8←2αNeuAc]β1→4Glcβ1→1Cer [forms marked with an asterisk follow the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature (1977)]; Me_4Si, tetramethylsilane.

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