Biochimica et Biophysica Acta, 298 (1973) 562–576

BBA 76261

PHOSPHOLIPID HYDROLYSIS BY PHOSPHOLIPASES A_1 AND A_2 IN PLASMA MEMBRANES AND MICROSOMES OF RAT LIVER

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(Received September 25th, 1972)

SUMMARY

1. Plasma membranes and microsomes isolated as described previously (Newkirk, J. D. and Waite, M. (1971) *Biochim. Biophys. Acta* 225, 224–233), were assayed for enzymes characteristic of each organelle. These assays indicated that plasma membranes were contaminated with microsomes to as much as 20%. Microsomes were contaminated with plasma membranes to as much as 5%.

2. The membrane preparations were assayed for phospholipase A with phosphatidylethanolamine and with phosphatidylglycerol. Plasma membranes and microsomes each contain a phospholipase A_1 and a phospholipase A_2; both enzymes require Ca^{2+}. The A_1 predominates in each fraction when phosphatidylethanolamine is substrate. The A_2 predominates in each fraction when phosphatidylglycerol is substrate.

3. Plasma membranes and microsomes deacylate phosphatidylserine and phosphatidylinositol at low rates; hydrolysis of these substrates is stimulated by a cationic detergent. Phosphatidylserine is hydrolyzed more rapidly than phosphatidylinositol. In the plasma membranes phosphatidylinositol is deacylated at about the same rate as phosphatidylcholine. In microsomes, phosphatidylserine is mainly deacylated by the phospholipase A_1.

4. Phospholipases A_1 and A_2 of plasma membranes and microsomes were extracted by treating the fractions with ammoniacal acetone and were separated from each other by gel filtration. Phospholipases A_1 and A_2 of plasma membranes had similar elution characteristics, respectively, to the phospholipases A_1 and A_2 of microsomes.

INTRODUCTION

In an earlier report[1] we demonstrated that a phospholipase A_1 (specific for the 1-acyl ester of the substrate) exists in plasma membranes of rat liver. This enyzme had an alkaline pH optimum, required Ca^{2+} for activity, and deacylated exogenous

Abbreviations: monoacyl-GPC, monoacyl glycerylphosphorylcholine; monoacyl-GPE, monoacyl glycerylphosphorylethanolamine; monoacyl-GPG, monoacyl glycerylphosphorylglycerol; monoacyl-GPI, monoacyl glycerylphosphorylinositol; monoacyl-GPS, monoacyl glycerylphosphorylserine.

phosphatidylethanolamine. While that report was in press, Torquebiau-Colard *et al.*[2] showed that plasma membranes could deacylate phosphatidylethanolamine and phosphatidylglycerol, but they did not determine which acyl ester of their substrates was hydrolyzed. Victoria *et al.*[3] have shown that there is a phospholipase A_2 in plasma membranes which is active on phosphatidylglycerol and phosphatidylethanolamine, and that plasma membranes can synthesize phosphatidylglycerol from CDP-diglyceride and glycerol 3-phosphate.

We have compared the phospholipases of plasma membranes and microsomes. The enzymes from each source have been partially purified. This paper presents data that demonstrate both phospholipases A_1 and A_2 are in plasma membranes and in microsomes.

MATERIALS

Radioisotopes, standard phospholipids, and chromatography materials

DL-[1-^{14}C]Serine, [2-^{3}H]inositol, [1-^{14}C]oleic acid, and [1-^{14}C]linoleic acid were from New England Nuclear (Boston, Mass.). Silica gel G and H was from Merck (Brinkman Instruments, Inc., Westbury, N.Y.). Phosphatidylserine, and CDP-diglyceride were from Serdary Chemical Co. (London, Ontario). Phosphatidylglycerol was from Supelco (Bellefonte, Penn.). *p*-Nitrophenyl-5′-thymidilic acid and phospholipase D were purchased from Sigma Chemical Co. (St. Louis, Mo.). Venom from *Crotalus adamanteus* was from Ross Allen's Reptile Institute (Silver Springs, Fla.).

Scintillation fluid

We counted aqueous samples in Aquasol (New England Nuclear). To count ^{3}H or ^{14}C on silica gel, we used 0.4% (w/v) Omnifluor (New England Nuclear) in toluene mixed with Triton X-100 (Rohm and Haas) and water in a ratio of 2:1:0.2, (v/v/v).

METHODS

Isolation of plasma membranes and microsomes

Plasma membranes were isolated by rate-isopycnic zonal centrifugation as reported earlier[1] except that the gradient contained 300 ml of 0.59 M sucrose and 400 ml of 1.02 M sucrose, rather than 200 ml and 300 ml, respectively. Gradients were loaded and unloaded from the rotor at a rate of 60–70 ml/min. Microsomes were prepared by homogenizing minced rat liver in 0.25 M sucrose with a Potter–Elvehjem homogenizer. The homogenate was centrifuged at $15000 \times g$ for 15 min. The supernatant fraction was then centrifuged at $100000 \times g$ for 60 min. Pellets (the microsomes) were suspended in 10 mM $NaHCO_3$ (pH 7.6).

Extraction procedure for plasma membranes and microsomes

Suspensions of plasma membranes and microsomes in 10 mM $NaHCO_3$ (pH 7.6) were dripped into 9 vol. of ice-cold acetone containing 0.0125 ml conc. NH_4OH per 100 ml[9]. The mixture was centrifuged at $15000 \times g$ for 10 min. The supernatant fraction was discarded. The precipitate was resuspended with a Potter–Elvehjem homogenizer in 0.05 M glycine–NaOH (pH 9) and centrifuged at $27000 \times g$ for 20

min. The supernatant fraction was applied to a 2.5 cm × 50 cm column of Sepharose 4B equilibrated with 0.05 M glycine–NaOH (pH 9), and was filtered through the column with the same buffer. 9.0–10.5-ml fractions were collected.

Marker enzyme assays, lipid phosphorus determination, chromatography and miscellaneous methods

The assays for marker enzymes were performed as noted elsewhere[1,5]. Occasionally we used phosphodiesterase I (EC 3.1.4.1) with *p*-nitrophenyl-5′-thymidilic acid as substrate[6] as an additional marker for plasma membranes. Lipid phosphorus determinations were carried out by the method of Chalvardjian and Rudnicki[7]; protein was measured as described earlier[1] or by absorbance at 260 nm and 280 nm[8]. All chromatography of [^{14}C]phosphatidylglycerol, [^{14}C]phosphatidylserine, and [^{3}H]phosphatidylinositol was carried out on 20 cm × 20 cm plates of silica gel H made in 1 mM Na_2CO_3 (40 g silica gel per 100 ml 1 mM Na_2CO_3). Chromatography of [^{14}C]phosphatidylethanolamine and [^{14}C]phosphatidylcholine was performed on silica gel G.

In this study we used four solvents, all of which were made by adding volumes of each component: Solvent 1, chloroform–methanol–conc. NH_4OH–water (65:35:3:2, by vol.); Solvent 2, chloroform–methanol–acetic acid–water (50:30:8:4, by vol.); Solvent 3, chloroform–light petroleum (b.p. 63–75 °C)–acetic acid (70:30:2, by vol.); Solvent 4, chloroform–methanol–water (70:30:4, by vol.). The products of the syntheses of radiolabelled lipids were identified on the thin-layer plates by spraying the plates with 0.2% (w/v) 2′,7′-dichlorofluorescein in 95% ethanol. The products were then extracted from the silica gel by adding in equal volumes, first methanol, then chloroform, then water. The chloroform layer contained the eluted product.

Synthesis of 1,2-dioleoyl-3-sn-phosphatidyl-1′-sn-[^{14}C]serine

Phosphatidylserine was synthesized using the CDP-diglyceride seryl transferase of *E. coli* B described by Kanfer and Kennedy[10] and modified as follows: 130 mg of the fraction that precipitated between 0.4 and 0.6 satn with $(NH_4)_2SO_4$ were incubated for 30 min at 37 °C in a 20-ml volume that contained 2.0 mmoles Na_2SO_4, 0.2 mmole 2-mercaptoethanol, 0.8 mmole Tris–HCl (pH 8), 40 μmoles EDTA, 0.1 ml 1-octanol, 6.0 μmoles DL-[1-^{14}C]serine (spec. act. = 8.2 Ci/mole) and 18 μmoles CDP-diglyceride which was added to the mixture as an ultrasonicated suspension in water. [^{14}C]phosphatidylserine was extracted from the reaction by the method of Bligh and Dyer[11], and was purified by chromatography in Solvent 1.

The [^{14}C]phosphatidylserine was identified by chromatography with standard phosphatidylserine in Solvent 1 and in Solvent 2, and by a positive reaction when sprayed with ninhydrin. 40% of the L-[1-^{14}C]serine was converted to [^{14}C]phosphatidylserine. Specific activity of the compound was $7.4 \cdot 10^6$ dpm/μmole lipid phosphorus.

Synthesis of 1-acyl-2-[1′-^{14}C]oleoyl-3-sn-phosphatidyl-1′-sn-glycerol

[^{14}C]Phosphatidylglycerol was isolated from the lipids of *Mycoplasma laidlawaii* B grown in 2 l of lipid-poor medium[12] that contained 50 μCi [1-^{14}C]oleate (54 Ci/mole). After 24 h, the culture was centrifuged at 10000 × *g* for 15 min. Pellets were resuspended in 40 ml water and extracted according to the procedure of Bligh and Dyer[11]. The product was taken up in chloroform and eluted through a column

of 7 g of SIL-R (Sigma) equilibrated with chloroform with mixtures of chloroform and methanol[13]. Phosphatidylglycerol was further purified by chromatography on silica gel first in Solvent 3, then in Solvent 4.

The 1-acyl-2-[1′-^{14}C]oleoyl phosphatidylglycerol was identified as follows: it cochromatographed with standard phosphatidylglycerol in Solvents 1 and 2; it was degraded by phospholipase A, purified from the venom of *C. adamanteus*[14] to produce radiolabelled free fatty acid; it was degraded by phospholipase D to produce radiolabelled phosphatidic acid, and by purified phospholipase C from *Bacillus cereus*[15] to produce radiolabelled diacylglycerol. When the substrate was degraded by phospholipase A from venom, more than 95% of the [1-^{14}C]oleate was found to be esterified at the 2-acyl position of the glycerol moiety. About 15% of the [1-^{14}C]oleate was incorporated into [^{14}C]phosphatidylglycerol; the spec. act. of the phosphatidylglycerol was $5.7 \cdot 10^6$ dpm/μmole lipid phosphorus.

Synthesis of 1,2-dioleoyl-3-sn-phosphatidyl-2′-sn-[^{3}H]inositol

[^{3}H]Phosphatidylinositol was synthesized by the CDP-diglyceride inositol transferase in microsomes of rat liver prepared as described by Paulus and Kennedy[16]. 200 mg microsomal protein were incubated in 20 ml that contained 1 mmole Tris–HCl (pH 7.4), 40 μmoles $MnCl_2$, 80 nmoles [2-^{3}H]*myo*-inositol (spec. act. = 3.09 Ci/mmole), and 20 μmoles CDP-diglyceride, which was added as an ultrasonicated suspension in water. The mixture was incubated 60 min at 40 °C. [^{3}H]Phosphatidylinositol was extracted from the reaction mixture[11] and purified using Solvent 1.

[^{3}H]Phosphatidylinositol was identified by co-chromatography with standard phosphatidylinositol in Solvents 1 and 2. About 15% of the [2-^{3}H]inositol was converted into [^{3}H]phosphatidylinositol; spec. act. was $5.4 \cdot 10^6$ dpm/μmole lipid phosphorus.

1-Acyl-2-sn-[1′-^{14}C]linoleoyl phosphatidylethanolamine and 1-acyl-2-sn-[1′-^{14}C]linoleoyl phosphatidylcholine

These compounds were prepared as described previously[17].

Incubation techniques

1,2-Dioleoyl-3-sn-phosphatidyl-1′-sn[^{14}C]serine. We used the following conditions to assay hydrolysis of [^{14}C]phosphatidylserine by microsomes and plasma membranes. Incubation mixtures (1.0 ml) contained 10 μmoles $CaCl_2$ and 100 μmoles glycine–NaOH (pH 9) for microsomes or 100 μmoles glycine–NaOH (pH 8.5) for plasma membranes. To this was added up to 160 μg protein, 24 nmoles [^{14}C]phosphatidylserine (750 cpm/nmole) and 24 nmoles decyltrimethylammonium bromide (Eastman). The decyltrimethylammonium bromide and [^{14}C]phosphatidylserine together were added to the mixture as an ultrasonicated suspension in water. Reaction mixtures were incubated for 15 min at 37 °C and then partitioned into a water–methanol and a chloroform phase by the method of Bligh and Dyer[11]. A 1.0-ml aliquot of the water–methanol phase of each tube was added to 10 ml Aquasol and counted.

This assay depends on the distribution coefficients of phosphatidylserine and monoacyl-[^{14}C]GPS between the chloroform phase and the methanol–water phase.

Under these conditions of assay more than 90% of the monoacyl-[^{14}C]GPS formed in the reaction is partitioned into the water–methanol phase; only 2% to 5% of the [^{14}C]phosphatidylserine is partitioned into that phase. To insure that the radioactivity in the upper phase was monoacyl-[^{14}C]GPS, samples of this phase were chromatographed on silica gel in Solvent 2. The radioactivity cochromatographed with monoacyl-GPS made by treating standard phosphatidylserine with the venom of *C. adamanteus* in the presence of detergent.

1-Acyl-2-[1′-^{14}C]oleoyl-3-sn-phosphatidyl-1′-sn-glycerol. The enzymatic hydrolysis of this substrate was measured in a 1.0-ml mixture that contained 10 μmoles $CaCl_2$, 100 μmoles Tris–maleate (pH 8) for both plasma membranes and microsomes, 9 nmoles [^{14}C]phosphatidylglycerol (1700 cpm/nmole) added as an ultrasonicated suspension in water, and up to 100 μg plasma membrane or microsomal protein. Tubes were incubated 15 min at 37 °C; reactions were stopped by adding 3 ml chloroform–methanol (1:2 v/v). 0.10 ml 1 M acetic acid was added to each tube. The single phase was broken by adding 1.0 ml water, followed by 2.0 ml chloroform.

To measure phospholipase A_1 activity on this substrate, we counted a 1.0-ml aliquot of the water–methanol phase. We confirmed Scandella and Kornberg's observation[18] that monoacyl-GPG is partitioned into the water–methanol phase under these extraction conditions. To assay phospholipase A_2 activity on this substrate, the chloroform extract was chromatographed with phosphatidylglycerol standards first in Solvent 3, then in Solvent 4.

Areas of the silica gel that contained free fatty acid and phosphatidylglycerol were scraped and counted.

1,2-Dioleoyl-3-sn-phosphatidyl-1′-sn-2′-[^{3}H]inositol. [^{3}H]Phosphatidylinositol was assayed in a total volume of 1.0 ml which contained 10 μmoles $CaCl_2$, 100 μmoles Tris–maleate (pH 8.5), up to 200 μg plasma membrane or microsomal protein, 24 nmoles of [^{3}H]phosphatidylinositol (750 cpm/nmole) and 24 nmoles decyltrimethylammonium bromide. Substrate and detergent were added as an ultrasonicated suspension in water. Reaction mixtures were incubated 15 min at 37 °C, and were quick frozen and lyophilized. The residue from each tube was dissolved in 2.0 ml chloroform–methanol–water (60:30:4.5, by vol.), and eluted through a 1-g column of Sephadex G-25 equilibrated with the same solvent, after the procedure of Wells and Dittmer[19]. The column was eluted with a total of 5.0 ml chloroform–methanol–water, followed by 5.0 ml chloroform–methanol (2:1, v/v). The eluates were concentrated, and chromatographed with standards of phosphatidylinositol and monoacyl-GPI in Solvent 2. Portions of silicic acid containing phosphatidylinositol and monoacyl-GPI were scraped and counted.

1-Acyl-2-sn-[1′-^{14}C]linoleoyl-3-sn-phosphatidylcholine. This substrate was assayed in a 1.0-ml incubation volume that contained 2 μmoles $CaCl_2$, 100 μmoles glycine–NaOH (pH 9), 60 nmoles [^{14}C]phosphatidylcholine (300 cpm/nmole) and up to 150 μg plasma membrane protein. The assays were incubated 15 min at 37 °C. The products of the reaction were extracted by the method of Bligh and Dyer[11] and were chromatographed with standard monoacyl-GPC, fatty acids, and phosphatidylcholine, first in Solvent 3, then in Solvent 4.

1-Acyl-2-sn-[1′-^{14}C]linoleoyl-3-sn-phosphatidylethanolamine. This substrate was assayed as described previously.[1]

RESULTS

When plasma membranes and microsomes were incubated with [^{14}C]phosphatidylethanolamine, each fraction produced more [^{14}C]monoacyl-GPE than [1-^{14}C]-linoleic acid at all pH values (Fig. 1). Phospholipase A_1 was the predominant activity in each fraction, although each also contained a lesser amount of activity directed at the 2-acyl ester of the substrate. Phospholipases A_1 both deacylate the most substrate near pH 9. Each enzyme requires Ca^{2+} for activity; the concentration of Ca^{2+} that gave optimal activity varied from 0.5 mM to 2.0 mM.

Since phospholipase A_1 from plasma membranes and microsomes had similar properties, it was necessary to determine the amount of microsomes the fractions of plasma membranes contained. Table I shows results of marker enzyme and phospholipase assays on plasma membranes and microsomes of rat liver. 5'-Nucleotidase

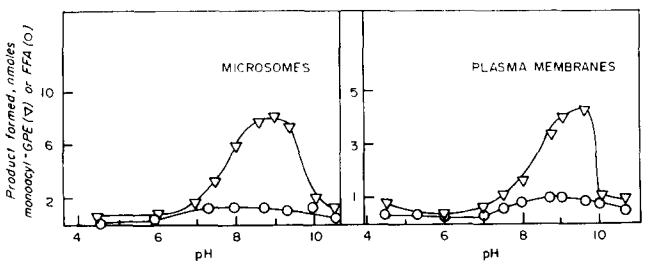

Fig. 1. Phospholipase A_1 and phospholipase A_2 activity assayed as a function of pH. Procedures are listed in Methods. 90 μg microsomal or 60 μg plasma membrane protein and 100 μmoles of each buffer was used (sodium acetate between pH 4.5 and pH 6.0, glycylglycine from pH 6.0 to pH 8.6, glycine–NaOH from pH 8.6 to pH 10.5). FFA, free fatty acids.

TABLE I

SPECIFIC ACTIVITIES OF MARKER ENZYMES AND PHOSPHOLIPASES A IN PLASMA MEMBRANES AND MICROSOMES

The results are given as nmoles substrate transformed/min per mg protein. The numbers in parentheses are the number of preparations of which the specific activity is an average. Substrate for phospholipase reactions was 2-[1'-^{14}C]linoleoylphosphatidylethanolamine.

Marker enzymes	*Homogenate**	*Plasma membranes**	*Plasma membranes***	*Plasma membranes****	*Plasma membranes* §	*Microsomes**
5'-Nucleotidase	39 (7)	944 (7)	686	528	–	48 (5)
Phosphodiesterase I	25 (2)	418 (2)	–	–	–	–
NADPH–cytochrome *c* reductase	28 (5)	19.0 (5)	3.8	4.0	–	108 (5)
Phospholipase A_1	–	8.6 (14)	2.7	5.5	2.0	5.2 (10)
Phospholipase A_2	–	3.4 (14)	0.3	2.0	0.3	3.4 (10)

* Fractions produced in this laboratory.
** Membranes provided by Dr Victoria.
*** Membranes prepared by C. T. Rankin, Jr, in the laboratory of Dr Anderson.
§ Membranes provided by Dr Fleischer.

(EC 3.1.3.5) and phosphodiesterase I (EC 3.1.4.1) are enzymes believed to be located mainly, but not exclusively[20], in plasma membranes; NADPH–cytochrome *c* reductase (EC 1.6.99.1) is located in microsomes[21]. On the average there was a 24-fold increase in specific activity of 5′-nucleotidase in our fractions of plasma membranes over the activity of the homogenate. On two occasions we used phosphodiesterase I as well as 5′-nucleotidase to indicate purity of our plasma membranes. With these fractions of membranes there was a 15-fold increase in specific activity with both enzymes. Specific activity of NADPH–cytochrome *c* reductase in plasma membranes compared to that found in microsomes indicates that on an average about 20% of our plasma membrane fractions is microsomes. Measured in this way, microsomes contaminated our fractions of plasma membranes from about 10% to about 30%; plasma membranes contaminate microsomes by about 5%.

Table I shows specific activities of phospholipases A_1 and A_2 in microsomes and in plasma membranes isolated in this laboratory. We also assayed plasma membranes donated by Dr S. Fleischer (Dept of Molecular Biology, Vanderbilt University, Nashville), Dr E. J. Victoria (National Institutes of Health, Bethesda) and Dr N. Anderson (Oak Ridge National Laboratory, Oak Ridge, Tenn.). The ratio of A_1 to A_2 activity varied from about 2:1 to 8:1 in plasma membranes, independent of the laboratory in which they were prepared. The specific activity of the phospholipase A_1 in plasma membranes was roughly proportional to the specific activity of 5′-nucleotidase rather than to the specific activity of the NADPH–cytochrome *c* reductase in these preparations. Table I also shows that there is more phospholipase A_1 than A_2 activity in the microsomes, as previously reported[17]. Data of Table I indicate that phospholipase A_1 in both microsomes and plasma membranes cannot be accounted for by cross contamination of the two fractions. These studies also suggested that there were both phospholipases A_1 and A_2 in plasma membranes and in microsomes, a possibility also indicated by the data of Victoria *et al.*[3]

To determine if a second phospholipase was present, we examined the deacylation of other radiolabelled phospholipids by plasma membranes and microsomes. Fig. 2 shows that as a function of protein, time, and substrate concentration, plasma membranes and microsomes produced from [^{14}C]phosphatidylglycerol more [1-^{14}C]-oleic acid than [^{14}C]monoacyl-GPG. In an earlier experiment we found that the optimum pH to assay both tissue fractions was at pH 8 with Tris–maleate buffer. The production of [1-^{14}C]oleic acid indicates that these membranes contain a phospholipase A_2 which acts preferentially on phosphatidylglycerol. The hydrolysis of substrate (Figs 2A, 2D) increased with increased protein up to at least 60 μg/ml. Hydrolysis of substrate was proportional to time for about 15 min for plasma membranes and microsomes (Figs 2B, 2E). Substrate concentration was rate-limiting below 6 μM (Figs 2C, 2F) for each fraction. Activity of these enzymes towards [^{14}C]phosphatidylglycerol was not stimulated by preparing the substrate as a sonicate with decyltrimethylammonium bromide, a cationic detergent. The average of five experiments (Table II) demonstrates that the phospholipase A_2 is three to four times as active as the phospholipase A_1 on phosphatidylglycerol in both plasma membranes and microsomes.

Fig. 3 shows that the hydrolysis of [^{14}C]phosphatidylserine was maximal with glycine–NaOH buffer at pH 8.5 with plasma membranes and pH 9.0 with microsomes. Fig. 4 depicts hydrolysis of [^{14}C]phosphatidylserine by plasma membranes as a

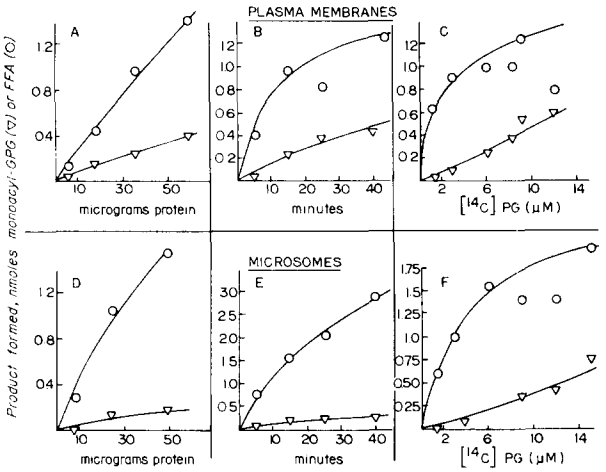

Fig. 2. Optimal assay conditions of phospholipase A activity of plasma membrane and microsomal fractions with [^{14}C]phosphatidylglycerol ([^{14}C]PG) as substrate. Formation of monoacyl-[^{14}C]-GPG and [1-^{14}C]oleic acid (^{14}C-labelled free fatty acids, FFA) are expressed as nmoles product recovered. (A), (D); $CaCl_2$, buffer, and reaction volume were as described in Methods. Each assay contained 6 nmoles [^{14}C]phosphatidylglycerol (2500 cpm/nmole), and up to 60 μg plasma membrane or microsomal protein. (B), (E); conditions here were the same as in (A), (D) and the tubes were incubated up to 40 min at 37 °C. (C), (F); conditions were the same as in (A), (D) with up to 14 nmoles [^{14}C]phosphatidylglycerol. Specific activities for phospholipase A_1 and A_2 of plasma membranes and microsomes with [^{14}C]phosphatidylglycerol were 0.45 and 1.8, and 0.36 and 2.5 nmoles/min per mg protein, respectively. Specific activity of 5′-nucleotidase of plasma membranes was 795 nmoles/min per mg protein. Specific activity of NADPH–cytochrome *c* reductase in plasma membranes was 25.8, in microsomes it was 100.4 nmoles/min per mg protein.

TABLE II

SPECIFIC ACTIVITIES OF PLASMA MEMBRANES AND MICROSOMES TOWARDS [^{14}C]PHOSPHATIDYLETHANOLAMINE, [^{14}C]PHOSPHATIDYLGLYCEROL, [^{14}C]-PHOSPHATIDYLCHOLINE, [^{14}C]PHOSPHATIDYLSERINE AND [^{3}H]PHOSPHATIDYL-INOSITOL

Figures above are specific activities in nmole product recovered/min per mg protein. Figures in parentheses are the number of preparations of which the specific activity is an average. Enzyme assays were performed as described in Methods. In the assay with [^{14}C]phosphatidylcholine, plasma membranes were prepared by the method of Neville[22]. [^{14}C]PE, [^{14}C]phosphatidylethanolamine; [^{14}C]PG, [^{14}C]phosphatidyl-glycerol; [^{14}C]PC, [^{14}C]phosphatidylcholine; [^{14}C]PS, [^{14}C] phosphatidylserine; [^{3}H]PI, [^{3}H]phosphatidylinositol; PLA_1, phospholipase A_1; PLA_2, phospholipase A_2.

Substrate	[^{14}C]*PE*		[^{14}C]*PG*		[^{14}C]*PC*		[^{14}C]*PS*	[^{3}H]*PI*
	PLA$_1$	*PLA*$_2$	*PLA*$_1$	*PLA*$_2$	*PLA*$_1$	*PLA*$_2$	*PLA*	*PLA*
Plasma membranes	8.6 (14)	3.4 (14)	0.47 (5)	1.8 (5)	0.25	0.19	0.90 (2)	0.13 (2)
Microsomes	5.2 (10)	3.4 (10)	0.77 (5)	2.2 (5)	–	–	0.76 (2)	0.05 (2)

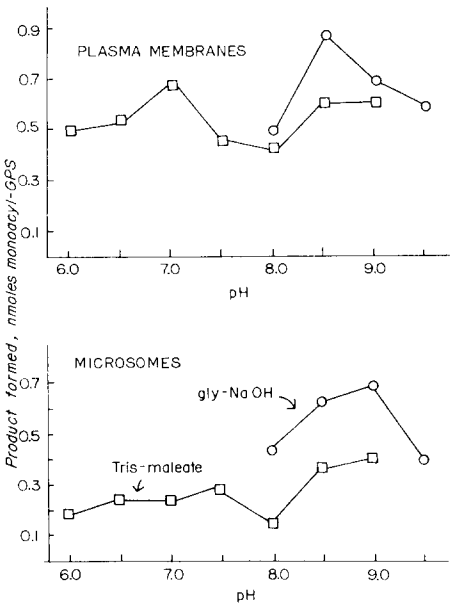

Fig. 3. pH optima of plasma membranes and microsomes with [^{14}C]phosphatidylserine as substrate. Incubations were in a final volume of 1.0 ml and contained 10 μmoles $CaCl_2$, 100 μmoles buffer, 5 nmoles [^{14}C]phosphatidylserine and 5 nmoles decyltrimethylammonium bromide. Tubes were incubated 15 min at 37 °C and contained either 68 μg microsomal protein or 170 μg plasma membrane protein. The product, monoacyl-[^{14}C]GPS is expressed as nmoles product recovered.

function of protein, time, and substrate. When sonicated suspensions of [^{14}C]phosphatidylserine were made with an equimolar amount of decyltrimethylammonium bromide, a cationic detergent, hydrolysis of substrate was stimulated as much as 5-fold. Detergent to substrate ratios greater or less than one did not stimulate as well. Fig. 4A shows that hydrolysis of substrate increased with increased plasma membrane protein up to 160 μg. Substrate was rate-limiting below 20 μM (Fig. 4B). Reactions were linear with time for as long as 40 min. Similar results were found with microsomes.

Fig. 5 shows relationship of protein, time and substrate to enzymatic deacylation of [^{3}H]phosphatidylinositol by plasma membranes. Fig. 5A shows that the rate of hydrolysis of [^{3}H]phosphatidylinositol was stimulated about 2-fold when substrate was prepared as a sonicated suspension with an equimolar quantity of decyltrimethylammonium bromide. Fig. 5B indicates that with detergent, substrate concentration was rate-limiting below about 20 μM. Reactions were over after 15 min (Fig. 5C).

Table II compares rates of hydrolysis of [^{14}C]phosphatidylethanolamine, [^{14}C]phosphatidylglycerol, [^{14}C]phosphatidylserine and [^{3}H]phosphatidylinositol by plasma membranes and microsomes. Also hydrolysis of [^{14}C]phosphatidylethanolamine and [^{14}C]phosphatidylcholine was compared with plasma membranes prepared by the method of Neville[22]. Intact plasma membranes and microsomes deacylate phosphatidylcholine and phosphatidylinositol at low rates even when phosphatidylinositol is prepared with detergent. Phosphatidylserine is deacylated by plasma

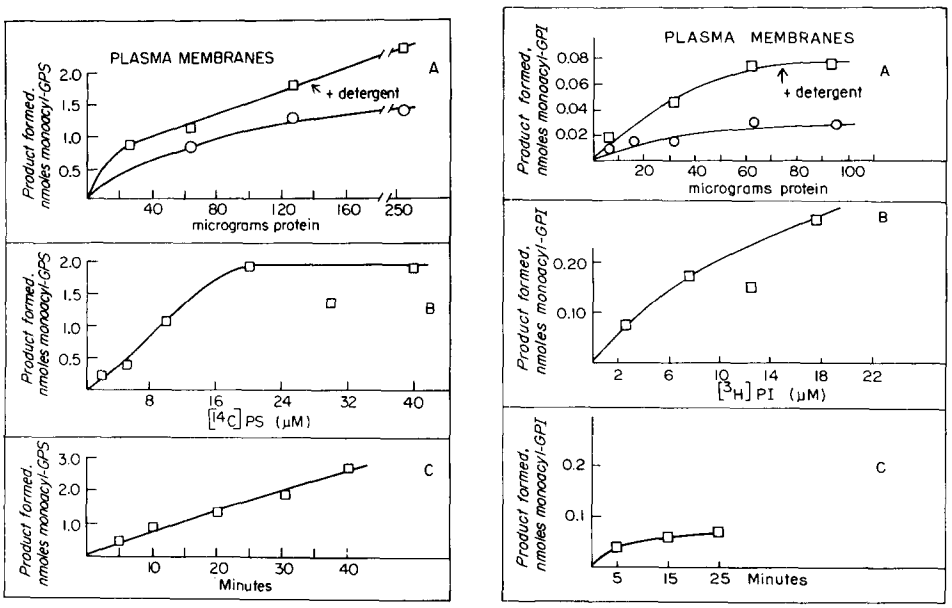

Fig. 4. Optimal assay conditions for the hydrolysis of [^{14}C]phosphatidylserine by plasma membranes. Formation of monoacyl-[^{14}C]GPS as nmoles product recovered. (A); $CaCl_2$, buffer concentration and incubation time were as indicated in Methods. 20 nmoles [^{14}C]phosphatidylserine (1000 cpm/nmole) with or without 20 nmoles detergent and up to 250 μg plasma membranes were in each reaction mixture. (B); $CaCl_2$, buffer and time of reaction were as in (A) with 130 μg plasma membrane protein and up to 40 nmoles [^{14}C]phosphatidylserine ([^{14}C]PS). (C); 130 μg plasma membranes used, $CaCl_2$, buffer, and reaction conditions were as in (A). Tubes were incubated up to 40 min. Plasma membranes in this experiment were as prepared by Neville[22]. When assayed with [^{14}C]phosphatidylethanolamine, the specific activities of the phospholipase A_1 and A_2 were 4.3 and 1.2 nmoles/min per mg protein, respectively. The specific activity of 5′-nucleotidase was 430 nmoles/min per mg protein.

Fig. 5. Optimal conditions for assay of phospholipase A activity towards [^{3}H]phosphatidylinositol ([^{3}H]PI) with plasma membranes. (A); tubes were incubated for 15 min at 37 °C, and contained 1.9 nmoles [^{3}H]phosphatidylinositol, with or without 2.0 nmoles decyltrimethylammonium bromide and up to 95 μg plasma membrane protein. Formation of monoacyl-[^{3}H]GPI is expressed as nmoles product recovered per 15 min incubation. (B); tubes contained 140 μg protein and up to 18 nmoles detergent. (C); tubes contained 140 μg protein, 5 nmoles [^{3}H]phosphatidylinositol, 5 nmoles detergent, and were incubated up to 25 min at 37 °C.

membranes at a slower rate than phosphatidylglycerol, but at a faster rate than either phosphatidylinositol or phosphatidylcholine. Victoria *et al.*[3] also found that phosphatidylcholine was a poor substrate for the phospholipase A_2 of plasma membranes. Similarly the phospholipase of microsomes does not hydrolyze phosphatidylcholine as well as phosphatidylethanolamine[17,23].

We were able to separate the phospholipase A_1 from the phospholipase A_2 after treatment of plasma membranes and microsomes with acetone. 10–30% of the phospholipase A activity of each fraction was solubilized by this procedure. The extracts were filtered through Sepharose 4B and assayed with [^{14}C]phosphatidylethanolamine, [^{14}C]phosphatidylglycerol, and [^{14}C]phosphatidylserine. Results of

these studies are depicted in Fig. 6 (elution diagram of solubilized microsomes) and in Fig. 7 (elution diagram of solubilized plasma membranes). Protein distribution in the fractions is shown in Fig. 6A and Fig. 7A, phospholipases A_1 (monoacyl-GPE) and A_2 (free fatty acid) activities with [^{14}C]phosphatidylethanolamine in Fig. 6B and Fig. 7B, phospholipases A_1 (monoacyl-GPG) and A_2 (free fatty acid) activities with [^{14}C]phosphatidylglycerol in Fig. 6C and Fig. 7C, and phospholipase A activity with [^{14}C]phosphatidylserine on solubilized microsomes is depicted in Fig. 6D.

When extracts obtained by acetone treatment were filtered through Sepharose 4B, phospholipase A_1 from both plasma membranes and microsomes eluted from the column with about 120 ml buffer, about one-half the total volume of the column. Phospholipase A_2 of each fraction eluted from the column with about 50 ml buffer,

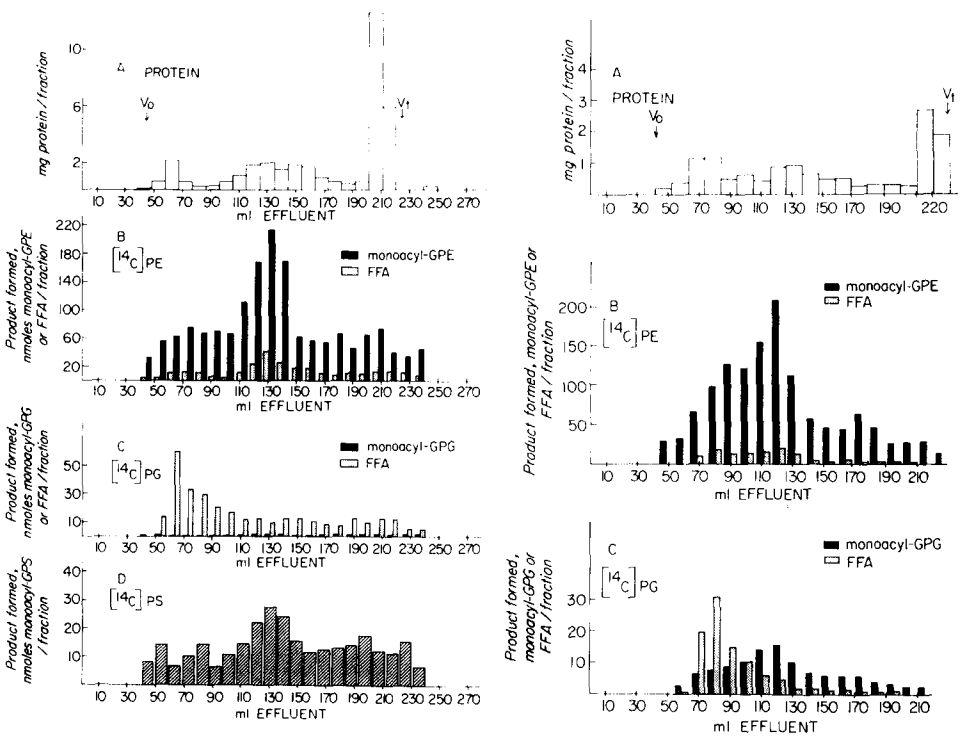

Fig. 6. Phospholipases of microsomes eluted through Sepharose 4B and assayed with [^{14}C]phosphatidylethanolamine ([^{14}C]PE), [^{14}C]phosphatidylglycerol ([^{14}C]PG) and [^{14}C]phosphatidylserine ([^{14}C]PS). (A); protein recovered per fraction. (B); 0.4 ml of a 9.6 ml fraction was assayed with [^{14}C]phosphatidylethanolamine as indicated in Methods. (C); same amount of samples as in (B) was incubated as in Methods. (D); same amount of samples as in (B) was assayed with 20 nmoles [^{14}C]phosphatidylserine and 20 nmoles of detergent. Recovery of activities are expressed as total nmoles product recovered per fraction per 15 min at 37 °C. The void volume of the column was 50 ml, the total volume was 240 ml. FFA, free fatty acids.

Fig. 7. Phospholipases of plasma membranes eluted through Sepharose 4B and assayed with [^{14}C]phosphatidylethanolamine ([^{14}C]PE) and [^{14}C]phosphatidylglycerol ([^{14}C]PG). (A); protein recovered per fraction. (B); 0.50 ml of 10.5 ml fractions were assayed with [^{14}C]phosphatidylethanolamine as indicated in Methods. Recoveries of activities are expressed as total nmoles product recovered per fraction per 15 min at 37 °C. FFA, free fatty acids.

near the void volumn. Fig. 6D indicates that the column fraction with the most phospholipase A activity towards [^{14}C]phosphatidylserine was also the fraction with the most phospholipase A_1 activity towards [^{14}C]phosphatidylethanolamine. This suggests that the fatty acid hydrolyzed from [^{14}C]phosphatidylserine was at the 1-acyl position of that substrate. Phospholipase A_1 of microsomes and plasma membranes deacylated little [^{14}C]phosphatidylglycerol. The A_1 of plasma membranes hydrolyzed less than one-tenth as much [^{14}C]phosphatidylglycerol as [^{14}C]phosphatidylethanolamine (*cf.* Figs 7B and 7C).

Phospholipase A_2 activity of microsomes towards [^{14}C]phosphatidylglycerol does not co-elute with phospholipase A_2 activity of microsomes towards [^{14}C]phosphatidylethanolamine. Fig. 6B shows that most [^{14}C]phosphatidylethanolamine was hydrolyzed to produce [1-^{14}C]linoleic acid in the fraction that eluted with 130 ml buffer. In contrast, phospholipase A_2 activity towards [^{14}C]phosphatidylglycerol from the microsomal extract came off the column with 60 ml buffer (Fig. 6C). The fraction with the most phospholipase A_2 activity towards [^{14}C]phosphatidylethanolamine is also the fraction with the most phospholipase A_1 activity towards that substrate (Fig. 6B). This may mean there is either a phospholipase A_2 or a lysophospholipase that co-elutes with the phospholipase A_1 of microsomes, or that the phospholipase A_1 of microsomes may deacylate the 2-acyl ester of [^{14}C]phosphatidylethanolamine to a small degree.

Sepharose separates substances of large molecular weight. We calculated K_a values, the partition coefficient between gel and liquid phase[24], for the A_2 activities with [^{14}C]phosphatidylglycerol and for A_1 activities with [^{14}C]phosphatidylethanolamine. K_a for phospholipase A_2 extracted from plasma membranes and microsomes was 0.1, whereas the K_a for phospholipase A_1 for each fraction was 0.5. These K_a values indicate that these enzymes are not in the monomeric units, rather they are in some aggregated form.

Column fractions containing the enzymes were extracted by the procedure of Bligh and Dyer[11] and the amount of lipid phosphorus in the chloroform layer was determined. Phospholipase A_2 from plasma membranes had a ratio of mg protein/μmole lipid phosphorus of 2:1, which is close to the value obtained with intact plasma membranes[25]. The phospholipase A_2 from microsomes had a ratio of 9:1; it also eluted near the void volume. These results suggest that the phospholipase A_2 is a lipoprotein aggregate. On the other hand, phospholipases A_1 of plasma membranes and microsomes had protein to lipid phosphorus ratios of 60:1 and 20:1, respectively. Hence, these extracted enzymes probably are not lipoproteins but may exist in polymeric forms or as aggregates with other proteins of the membranes. This tendency for phospholipases to aggregate has been observed with the phospholipase A_2 of rat liver mitochondria[9].

DISCUSSION

In an earlier report[1] we identified a phospholipase A_1 in plasma membranes of rat liver. This enzyme had an alkaline pH optimum and required Ca^{2+} for activity, similar to the phospholipase A_1 found in microsomes[17]. Since microsomes were the main contaminant in our plasma membranes, it was necessary to insure that A_1 activity in our preparations was not due to microsomal contamination. We have

estimated contamination by microsomes in this study by comparing the specific activity of NADPH–cytochrome *c* reductase in plasma membranes relative to that in microsomes. This method indicates that our plasma membranes were contaminated to a greater degree than we had previously estimated by a different method. However, data of Table I show that phospholipase A_1 activity in plasma membranes is not caused by contaminating microsomes, although phospholipases of microsomes undoubtedly contribute to the activities that we observe in these preparations. In addition, the A_1 in plasma membranes is not caused by nonspecific binding of positively charged proteins to the negatively charged membrane[26] since the enzyme is not solubilized when plasma membranes are incubated in isotonic saline. Only drastic treatment with organic solvent removes the phospholipase from the plasma membrane. The phospholipase A_1 is therefore functionally a part of the plasma membrane like 5′-nucleotidase, Mg^{2+}-ATPase, (Na^+–K^+–Mg^{2+})-ATPase[26,27] and the glycosidase of bovine liver membranes[27].

Shortly after our first report was published, Victoria *et al.*[3] found that plasma membranes contain a phospholipase A_2 that deacylates [^{14}C]phosphatidylethanolamine and [^{14}C]phosphatidylglycerol. When they assayed plasma membranes with [^{14}C]phosphatidylethanolamine, they found about three times as much A_2 as A_1 activity. From their data it can be calculated that the specific activity of the phospholipase A_1 in these plasma membranes was about 2 nmoles/min per mg protein, about the same value we found with this same preparation. The difference appears to be that we found much lower phospholipase A_2 activity on [^{14}C]phosphatidylethanolamine in plasma membranes than did Victoria *et al.*[3].

Some of the differences in the results of the two laboratories may be caused by the methods of assay. In this laboratory 0.5 mM to 2.0 mM is the optimal Ca^{2+} concentration to measure phospholipase A_1 activity with [^{14}C]phosphatidylethanolamine. We find that Ca^{2+} concentrations as high as 10 mM, as used by Victoria *et al.*[3] often, but not always, inhibited the hydrolysis by the phospholipase A_1. There are also differences in the way products are extracted after the enzymatic assay. Scandella and Kornberg[18] showed that monoacyl-GPG partitioned into the methanol–water phase. We also found that up to 20% of the [^{14}C]monoacyl-GPE and [1-^{14}C]-linoleic acid remained in the methanol–water phase. Because of this, specific activities reported here are somewhat low. Since the amounts of [^{14}C]monoacyl-GPE and [1-^{14}C]linoleic acid that remained in the methanol–water phase were about the same relative to each other, the ratio of A_1 to A_2 activity in these assays would not be greatly altered. Victoria *et al.*[3] acidified their reaction mixtures before extraction, which caused greater than 99% extraction of the fatty acid into chloroform. Since the distribution of [^{14}C]monoacyl-GPE is not appreciably altered by acidification, such treatment will alter the A_2 to A_1 ratio.

We also observed that the microsomal phospholipase A_1 required Ca^{2+} for activity; this is contrary to the earlier findings of Waite and van Deenen[17]. This may be due to the higher amount of protein used in those studies; perhaps not all endogenous Ca^{2+} was chelated by EDTA.

The ratio of A_1 to A_2 activity with [^{14}C]phosphatidylethanolamine varies from preparation to preparation of plasma membranes, possibly a reflection of the physiological state of the animal. Vogel and Zieve[28] have shown that phospholipase A_1 is released into plasma after an animal is treated with heparin. Zieve and Zieve[29] have

shown that this enzyme is not found in plasma of heparin-treated hepatectomized rats, which suggests that liver is the source of the phospholipase A_1. Under certain physiological states, phospholipase A_1 might be released into the plasma, and one would, therefore, find a decreased specific activity in plasma membrane. If this were the case, it would be consistent with our finding that when phospholipases A_1 and A_2 of plasma membranes are extracted with acetone, the A_1, unlike the A_2, is virtually lipid-free. Hence, it appears as though the A_1 is more easily released from its association with lipids, or that it exists in a less lipophilic region of the membrane than the phospholipase A_2.

[^{14}C]Phosphatidylserine was mainly hydrolyzed by the phospholipase A_1 of microsomes. We have some evidence to indicate that this is also true for plasma membranes. Enzymatic hydrolysis of [^{14}C]phosphatidylserine and [^{3}H]phosphatidylinositol was stimulated by preparing ultrasonicated suspensions of these substrates with an equimolar quantity of decyltrimethylammonium bromide, a cationic detergent. Micelles of [^{14}C]phosphatidylserine and [^{3}H]phosphatidylinositol are negatively charged at the pH at which they were assayed. The cationic detergent presumably reduces the negative charge of the micelles and makes substrate more accessible to enzyme binding and hydrolysis. A similar phenomenon was observed by van Deenen and de Haas[30] in studies of deacylation of 1,2-diacylglycerol 3-phosphate by the phospholipase A_2 in venom of *C. adamanteus*. [^{14}C]Phosphatidylglycerol is also a negatively-charged phospholipid. Hydrolysis of this lipid by the phospholipase A_2 of plasma membranes and microsomes was slightly inhibited when [^{14}C]-phosphatidylglycerol was prepared as a sonicate with the detergent. Before final assignment of the function of the base group charge on the enzymatic activity can be made, further information on the physical–chemical state of the different substrates must be obtained[31,32].

Phospholipids have been shown to be important to the activity of several membrane-bound systems, for example, the (Na^+–K^+–Mg^{2+})-ATPase[27], and in the hormone-sensitive adenyl cyclase systems[33,34] of plasma membranes. Pohl *et al.*[33] found that when plasma membranes were treated with phospholipase A or digitonin, the hormone receptor for glucagon was modified so that it could no longer stimulate adenyl cyclase, although adenyl cyclase sensitivity to glucagon could be restored if phospholipids were added to the system. Of the phospholipids added back, phosphatidylserine restored the most activity. Hence, the activity of the phospholipases of plasma membranes may play a physiological role by altering hormone receptors.

ACKNOWLEDGEMENTS

We are indebted to Dr Paul F. Smith of the Department of Microbiology, University of South Dakota School of Medicine, for helping us to identify the phospholipids of *Mycoplasma*. We wish to thank Miss Patricia Sisson for excellent technical assistance. This research was supported by the U.S. Public Health Service (Grant AM11799) of the National Institutes of Health. This paper and the study on the phospholipase A_1 in plasma membranes of rat liver[1] were submitted to the Department of Biochemistry of Bowman Gray School of Medicine, Wake Forest University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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