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PHOSPHATIDYLINOSITOL DISTRIBUTION AND TRANSLOCATION IN SONICATED VESICLES

A STUDY WITH EXCHANGE PROTEIN AND PHOSPHOLIPASE C

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Summary

The distribution of phosphatidylinositol and phosphatidylcholine in sonicated phospholipid vesicles (phosphatidylcholine : diphosphatidylglycerol : phosphatidylinositol, 90 : 5 : 5 mol%) has been determined by the use of exchange protein from beef heart and phosphatidylinositol-specific phospholipase C from *Staphylococcus aureus*. Approximately 70% of the phosphatidylinositol in the sonicated vesicles was accessible to the exchange protein and 70—75% was accessible to the phospholipase C. A similar proportion (65%) of the phosphatidylcholine was accessible to the exchange protein suggesting that phosphatidylinositol was not preferentially located in either surface of the phospholipid bilayer. The rate of translocation of both phospholipids was very slow but the rate for phosphatidylcholine ($t_{1/2}$ = 4—7 days) appeared to be greater than that for phosphatidylinositol ($t_{1/2}$ = 8—60 days). Production of asymmetric vesicles by removing phosphatidylinositol from the outer surface with either exchange protein or phospholipase C did not induce rapid phospholipid translocation.

Introduction

The distribution and rate of translocation of phosphatidylcholine in sonicated vesicles has been extensively studied with exchange proteins or NMR

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

techniques [1—4]. The rates of phosphatidylcholine translocation in sonicated vesicles and influenza virus [5] were found to be extremely low in contrast to the higher rates observed in various other biological membranes [6—9]. The distribution of phosphatidylinositol in sonicated vesicles has only been studied to a limited extent [10,11] and no reports on the rate of translocation of this phospholipid have appeared. The distribution and translocation of phosphatidylinositol in rat liver microsomes and in mammalian erythrocytes has been studied but conflicting results have been obtained [8,12—17].

In this paper we have investigated the distribution and rate of translocation of phosphatidylinositol in sonicated vesicles by measuring its accessibility to beef heart exchange protein and to the phosphatidylinositol-specific phospholipase C from *Staphylococcus aureus*.

Materials and Methods

Egg yolk phosphatidylcholine, ox heart diphosphatidylglycerol, and wheat germ phosphatidylinositol were obtained from Lipid Products (Redhill, Surrey, U.K.). All the phospholipids gave a single spot after thin-layer chromatography on silica gel H in $CHCl_3/CH_3OH$/acetic acid/H_2O (25 : 15 : 4 : 2, v/v/v/v).

[^{32}P]Phosphatidylcholine was purified from livers of rats injected with 10 mCi/100 g body weight of $^{32}P_i$ (New England Nuclear, Boston, MA) 16 h before killing [18]. Phosphatidyl[^{3}H]inositol was prepared from rat liver microsomes labeled in vitro with [^{3}H]inositol. To 9 ml of rat liver microsomes [8] suspended in 0.25 M sucrose, 0.5 mM EDTA, 5 mM Tris/acetate, pH 7.4, at a concentration of 10 μmol phospholipid/ml, was added 200 μCi of [^{3}H]-inositol (New England Nuclear; 12.5 Ci/mmol), 0.1 ml 100 mM $MnCl_2$, and 1 ml 100 mM Hepes/NaOH buffer, pH 7.4. After incubation under N_2 for 90 min at 37° C, the lipids were extracted in 20 vols. of $CHCl_3/CH_3OH$ (2 : 1, v/v) and phosphatidylinositol was isolated by chromatography on Alumina [18]. In early experiments the phosphatidyl[^{3}H]inositol was further purified either by silicic acid column chromatography or preparative thin-layer chromatography on silica gel. However, the phosphatidyl[^{3}H]inositol prepared by these methods was found to be relatively unstable. After several weeks in $CHCl_3/CH_3OH$ (99 : 1, v/v) at −20° C, thin-layer chromatography showed a significant proportion (up to 5%) of the radioactivity to be present as a slower moving contaminant. For this reason all the experiments reported in this paper were done with phosphatidyl[^{3}H]inositol eluted from the alumina column without further purification. This preparation, which appeared to be quite stable, contained approximately 10% of other phospholipids (mostly phosphatidylcholine), but 98—99% of the ^{3}H moved with phosphatidylinositol on thin-layer chromatography. No significant differences in subsequent exchange experiments were observed between this partially purified phosphatidylinositol and freshly prepared phosphatidylinositol from silicic acid columns. The specific activity of the phosphatidyl[^{3}H]inositol was approximately 20 Ci/mol.

Beef heart exchange protein was purified as described by DiCorleto and Zilversmit [19]. The pooled fractions from the CM-cellulose column were

adjusted to pH 7.8 (at 0°C) with 0.5 M Tris-HCl, pH 8.5, prior to use in exchange experiments.

Phosphatidylinositol-specific phospholipase C from *S. aureus* was purified as described by Low and Finean [17] except for a few modifications. 4 l culture supernatant was loaded onto a column of Amberlite CG-50 and eluted with 1 M trisodium citrate. The eluates from two Amberlite CG-50 columns were combined and dialyzed against three times 5 vols. of 50 mM Tris/acetate 0.02% NaN_3, pH 7.35 (at 4°C). The dialyzed material was concentrated by ultrafiltration (Amicon PM 10) to approximately 80 ml, loaded onto a column (5 × 200 cm) of Sephadex G-75 equilibrated in the same buffer and eluted at 35 ml/h. The pooled fractions were adjusted to pH 7.8 (at 0°C) with 0.5 M Tris/HCl, pH 8.5, prior to use.

Small unilamellar vesicles were prepared as described previously [20]. 2.7 μmol phosphatidylcholine, 0.15 μmol diphosphatidylglycerol, 0.15 μmol phosphatidyl[^{3}H]inositol, 2.5 nmol [^{14}C]triolein (New England Nuclear; 60 Ci/mol), and 9 nmol butylated hydroxytoluene (Nutritional Biochemicals Co.) dissolved in $CHCl_3$ were mixed and dried under a stream of nitrogen. The last traces of solvent were removed in a rotary evaporator at 30°C in vacuo for 30 min. The phospholipids were allowed to swell at 20°C for 15 min in 0.5 ml of 50 mM Tris-HCl, 5 mM EDTA, 1 mM dithiothreitol, 0.02% NaN_3 (pH 7.4), before sonicating for 30 min under N_2 in a bath-type sonifier. Sonicated vesicles were prepared the day before chromatography on Sepharose 4B, i.e., 2 days before the start of the incubations. Thin-layer chromatography of sonicated vesicles showed no significant change in radiopurity of the phosphatidyl[^{3}H]inositol. In some experiments sonicated vesicles were prepared with rat liver [^{32}P]phosphatidylcholine, in others egg phosphatidylcholine was used.

0.2 ml sonicated vesicles were loaded onto a column (1 × 46 cm) of Sepharose 4B, equilibrated in 50 mM Tris-HCl, 5 mM EDTA, 0.02% NaN_3, 0.1 mM dithiothreitol, pH 7.4. The column was eluted at 10 ml/h (20°C) and 1.2-ml fractions were collected. The Sepharose column used in these experiments had been pre-eluted with non-radioactive sonicated phosphatidylcholine vesicles (12 μmol) to reduce adsorption of radioactive vesicles to the column. The void volume of the column, assessed with blue dextran, was 12 ml.

Multilamellar vesicles were prepared essentially as described by DiCorleto and Zilversmit [20]. 72 μmol phosphatidylcholine, 4 μmol diphosphatidylglycerol, 4 μmol phosphatidylinositol and 0.34 μmol butylated hydroxytoluene, dissolved in $CHCl_3$, were mixed and dried in vacuo at 30°C for 30 min. The phospholipids were allowed to swell overnight at 20°C in 6 ml 5 mM Tris-HCl, 5 mM EDTA, 0.02% NaN_3, buffer pH 7.4 (Tris/EDTA buffer). The multilamellar vesicles were then centrifuged at 40 000 × *g* for 20 min and the pellet was resuspended with 0.8 ml buffer. Multilamellar vesicles, which contained no phosphatidylinositol, were also prepared for use in some experiments but in general the same phospholipid composition as the unilamellar vesicles was used.

Phospholipid exchange incubations contained 1.6 ml purified beef heart exchange protein (6700 units; [21]), 0.1 ml multilamellar vesicles (approximately 6 μmol phospholipid) and 0.3 ml fractionated unilamellar vesicles

(approximately 50 nmol phospholipid). The mixture was incubated at 37°C in a shaking water bath and after various time intervals (3, 7, 12 and 24 h) was centrifuged at 40 000 × *g* for 20 min at 20°C. Aliquots (0.2 ml) of the supernatant were taken for counting radioactivity while the remainder of the supernatant was mixed with fresh multilamellar vesicles (3 μmol/ml) and the incubation was continued. After 24 h incubation fresh exchange protein (1700 units) was added as well as multilamellar vesicles. For the last two time points (30 and 48 h), 0.3 ml aliquots were taken from the incubation mixture, centrifuged and 0.2 ml of the supernatant was counted (see below). Recovery of unilamellar vesicles in these incubations, calculated from the recovery of the non exchangeable marker [^{14}C]triolein, was generally 95—99%. Phosphatidyl[^{3}H]inositol exchange in the absence of exchange protein was less than 2% after 3 h.

Phosphatidylinositol hydrolysis was determined in incubations containing 0.2 ml purified phosphatidylinositol-specific phospholipase C (3.75—15 μg protein), 2.35 ml 40 mM Tris/acetate, 0.02% NaN_3, pH 7.8 (at 4°C), and 0.45 ml fractionated unilamellar vesicles (approximately 75 nmol phospholipid). The mixture was incubated at 37°C and after various time intervals, 0.2 ml aliquots were added to 4 ml ice-cold $CHCl_3$/CH_3OH/conc. HCl (100 : 50 : 1, v/v/v) containing 90 nmol phosphatidylcholine, 5 nmol diphosphatidylglycerol and 5 nmol phosphatidylinositol as non-radioactive carriers. The extract was allowed to stand for 5 min at 0°C, then 2 ml 0.1 M HCl and 1.3 ml CH_3OH were added and the phases separated by centrifugation. Approximately 5% of the phosphatidyl[^{3}H]inositol was lost into the upper phase. 2 ml of the lower phase was evaporated to dryness in a scintillation vial and the lipids were resuspended in 1 ml of Tris/EDTA buffer and analyzed for radioactivity (see below). Additional phospholipase C (1.25—5 μg/ml) was added to the incubation mixture after 24 h incubation. In all the experiments the extent of hydrolysis was also determined by dialyzing the hydrolyzed vesicles against a large volume of buffer to remove [^{3}H]inositol phosphate. 0.2 ml aliquots were taken after 3, 25, and 50 h incubation and dialyzed against 250 vols. of Tris/EDTA buffer at 37°C for 5 h. The dialyzed vesicles were removed from the dialysis tubing and radioactivity was determined. In this procedure the recovery of vesicles, calculated from the recovery of [^{14}C]-triolein, was 70—80%.

Samples (final volume of 1 ml in Tris/EDTA buffer) from both phospholipase C and exchange experiments were mixed with 10 ml of water-miscible counting scintillant (Liquiscint; National Diagnostics, Parsippany, NJ) and radioactivity determined. The percentage of labelled phospholipid remaining was determined by comparing the ^{32}P/^{14}C or ^{3}H/^{14}C ratio in incubated vesicles with that in the original vesicles (prepared for counting by the same method).

Lipid phosphorus was determined according to Bartlett [22] and protein by a slightly modified Coomassie blue procedure with crystallized bovine serum albumin (Pentex, Miles Laboratories, Inc.) as a standard [23].

Results

Chromatography of sonicated vesicles on Sepharose 4B

The vesicles used in these studies were first fractionated on a column of

Sepharose 4B. A typical elution profile for sonicated phosphatidylcholine/diphosphatidylglycerol/phosphatidyl[^{3}H]inositol (90 : 5 : 5 mol%) vesicles containing [^{14}C]triolein is shown in Fig. 1 (only ^{14}C activity shown). Generally, less than 5% of the eluted activity was excluded from the column. Recovery of eluted ^{3}H and ^{14}C activity was in the range 75—90%. The ^{3}H/^{14}C ratio (Fig. 1) either remained constant or increased slightly (10—20%) over the whole elution profile indicating that neither phosphatidyl[^{3}H]inositol nor [^{14}C]triolein was preferentially located in vesicles of a given size. Sonicated vesicles, which contained [^{32}P]phoshatidylcholine in addition to phosphatidyl[^{3}H]inositol and [^{14}C]triolein, also showed constant ratios of all three isotopes throughout the elution profile.

The three central fractions (elution volume 21—24 ml) were pooled and used in experiments to determine the distribution of phosphatidylinositol in the unilamellar vesicles (see below). These three fractions contained approximately 40% of the radioactivity applied to the column.

Accessibility of phosphatidyl[^{3}H]inositol in unilamellar vesicles to beef heart exchange protein

The exchange of phosphatidyl[^{3}H]inositol between fractionated unilamellar vesicles and multilamellar vesicles in the presence of exchange protein was studied in order to determine the accessibility of phosphatidylinositol in the unilamellar vesicles (Fig. 2). Approximately 70% of the phosphatidyl[^{3}H]-inositol in the unilamellar vesicles exchanged rapidly ($t_{1/2} < 3$ h) whereas the remainder exchanged only slowly ($t_{1/2} > 2$ days). The apparent inaccessibility of a portion of the phosphatidyl[^{3}H]inositol was not due to insufficient multilamellar vesicles or exchange protein, as extra additions of these after 24 h incubation did not significantly increase the rate of exchange of the remaining phosphatidyl[^{3}H]inositol.

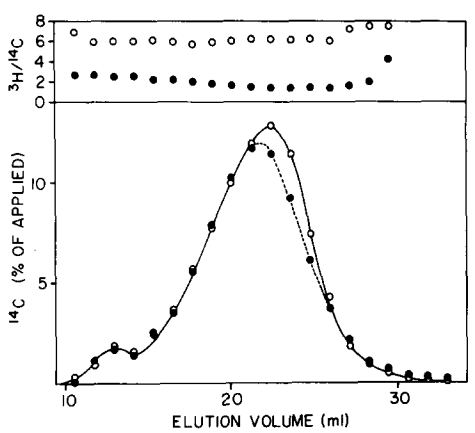

Fig. 1. Gel filtration of sonicated phosphatidylcholine : diphosphatidylglycerol : [^{3}H]phosphatidylinositol (90 : 5 : 5 mol%) vesicles. Sonicated vesicles containing [^{14}C]triolein were applied to a Sepharose 4B column and 1.2-ml fractions collected. The ^{14}C (lower figure) and the phosphatidyl[^{3}H]inositol/[^{14}C]triolein ratio (upper figure) in each fraction was determined. ○, control sonicated vesicles one day after preparation; ●, control sonicated vesicles incubated at 37°C for 48 h with phosphatidylinositol-specific phospholipase C (35 μg/ml) prior to fractionation.

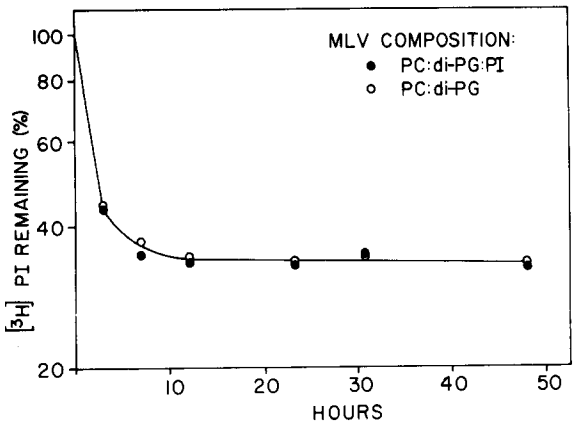

Fig. 2. Exchange of phosphatidyl[^{3}H]inositol from fractionated unilamellar vesicles (phosphatidylcholine : diphosphatidylglycerol : phosphatidyl[^{3}H]inositol, 90 : 5 : 5 mol%) to multilamellar vesicles (MLV) by beef heart exchange protein. Multilamellar vesicles contained either phosphatidylcholine (PC) : diphosphatidylglycerol (di-PG) : phosphatidylinositol (PI) (90 : 5 : 5 mol%), or phosphatidylcholine : diphosphatidyglycerol (90 : 5 mol%). The percentage of labeled phospholipid remaining in the unilamellar vesicle was determined by comparing the $^3H/^{14}C$ ratio in the exchanged unilamellar vesicles with that in the original unilamellar vesicles. Each point represents the mean of duplicate incubations.

The proportion of phosphatidyl[^{3}H]inositol which was inaccessible could be estimated by fitting a two-pool model [7] to these data. The rate at which the inaccessible phosphatidylinositol becomes accessible to the exchange protein can be estimated from the slope of the curve after 12 h of incubation (Fig. 2). These values were determined in several experiments and are summarized in Table I. The results indicate that approximately 65—73% of the phosphatidyl[^{3}H]inositol in the unilamellar vesicle is accessible to the exchange protein and therefore, probably at the outer surface of the bilayer. The remainder is inaccessible, presumably at the inner surface, and only redistributes across the bilayer at a very slow rate ($t_{1/2}$ = 8—60 days, as determined from the final slope of the curves).

In several experiments, unilamellar vesicles which contained [^{32}P]phosphatidylcholine as well as phosphatidyl[^{3}H]inositol were used and, as both these phospholipids are exchanged by the beef heart protein, it was possible to compare their distribution in the unilamellar vesicle (Fig. 3). In these experiments it was found that approximately 64—68% of the [^{32}P]phosphatidylcholine was readily exchanged as compared to approximately 68—72% of the phosphatidyl[^{3}H]inositol. The rate of the slow phase of phospholipid exchange appeared to be faster for [^{32}P]phosphatidylcholine than for phosphatidyl-[^{3}H]inositol, suggesting a higher rate of translocation for [^{32}P]phosphatidylcholine (Fig. 3 and Table I).

Also shown in Table I and Fig. 2 are data obtained in experiments in which the multilamellar vesicle acceptor contained no phosphatidylinositol. In this situation asymmetric unilamellar vesicles are produced with no phosphatidylinositol in the outer monolayer of the phospholipid bilayer. However, it can be seen that such an asymmetric distribution does not significantly change the

TABLE I

DISTRIBUTION AND TRANSLOCATION OF PHOSPHATIDYLINOSITOL AND PHOSPHATIDYLCHOLINE IN SMALL UNILAMELLAR VESICLES

The proportion of phosphatidylinositol (PI) or phosphatidylcholine (PC) inaccessible to exchange protein or phospholipase C was determined with the two-pool model described by Bloj and Zilversmit [7]. $t_{1/2}$ was calculated from the slope of the curves after 12 h (for exchange) or 7 h (for hydrolysis). Each experiment was done with a different preparation of unilamellar vesicles fractionated on Sepharose 4B.

Experiment	Exchange protein				Phospholipase C	
	Inaccessible phospholipid (%)		$t_{1/2}$ (days)		Inaccessible PI (%)	$t_{1/2}$ (days)
	PI	PC	PI	PC		
1	34.1	—	20.0	—	26.9	8.9
2	26.6	—	7.6	—	24.1	7.4
3 **	29.7	35.7	>60	4.9	—	—
4 **	28.3	33.7	12.1	4.9	25.3	17.2
	29.0 *	32.1 *	16.3 *	5.8 *	—	—
5 **	30.2	34.7	10.8	4.4	26.5	15.2
	31.7 *	36.4 *	34.7 *	4.7 *	—	—
6	34.4	—	33.8	—	28.0	13.9
	34.9 *	—	28.4 *	—	—	—

* Multilamellar vesicle composition: phosphatidylcholine:diphosphatidylglycerol (90:5 mol%).

** Unilamellar vesicle composition: [^{32}P]phosphatidylcholine:diphosphatidylglycerol:phosphatidyl[^{3}H]-inositol (90:5:5 mol%).

size of the inaccessible phosphatidylinositol pool or the rate of its translocation across the bilayer (Fig. 2 and Table I).

Accessibility of vesicular phosphatidyl[^{3}H]inositol to phospholipase C

Incubation of fractionated unilamellar vesicles with the phosphatidylinositol specific phospholipase C from *S. aureus* resulted in a rapid hydrolysis of

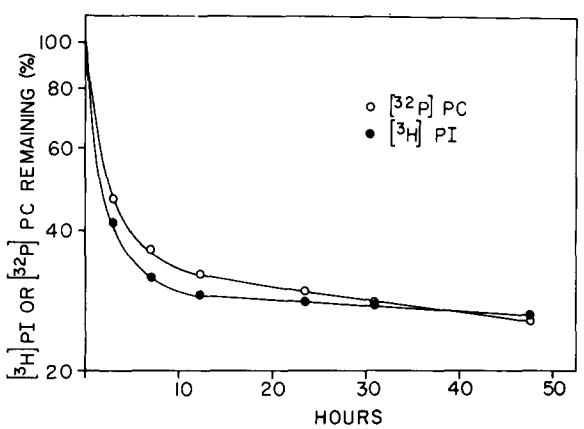

Fig. 3. Exchange of phosphatidyl[^{3}H]inositol ([^{3}H]PI) and [^{32}P]phosphatidylcholine ([^{32}P]PC) from fractionated unilamellar vesicles ([^{32}P]phosphatidylcholine : diphosphatidylglycerol : phosphatidyl[^{3}H]-inositol, 90 : 5 : 5 mol%) to multilamellar vesicles (phosphatidylcholine : diphosphatidylglycerol : phosphatidylinositol, 90 : 5 : 5 mol%). The percentage of labeled phospholipid remaining was calculated as for Fig. 2. Each point represents the mean of duplicate incubations.

70—75% of the phosphatidyl[^{3}H]inositol in the unilamellar vesicles (Fig. 4). The rate of this initial phase of hydrolysis was dependent on the amount of phospholipase C present; at the highest amount used (5 μg/ml) half of the hydrolysis took place in approximately 10 min. The remaining 25—30% of the phosphatidyl[^{3}H]inositol was hydrolyzed quite slowly, at a rate which was independent of the amount (up to 30 μg/ml) of phospholipase C added. The slow rate of hydrolysis was not due to inactivation of the phospholipase C as addition of extra enzyme after 24 h incubation did not significantly increase the rate of hydrolysis (Fig. 4).

In unilamellar vesicles which also contained [^{32}P]phosphatidylcholine, less than 5% hydrolysis of this phospholipid was detected after 48 h incubation with phospholipase C (5 μg/ml) which confirms the previously reported specificity of this enzyme [24].

In these experiments the unhydrolyzed phosphatidyl[^{3}H]inositol was separated from the [^{3}H]inositol phosphate by lipid extraction. These results were confirmed in all of the experiments by removing the [^{3}H]inositol phosphate by dialysis against a large volume of buffer. The amounts of unhydrolyzed phosphatidyl[^{3}H]inositol obtained by this method were very close (within 10%) to those obtained by lipid extraction. Similar extents of hydrolysis were also obtained with unfractionated sonicated vesicles when the [^{3}H]-inositol phosphate was removed by gel filtration on Sepharose 4B (see below).

The proportion of phosphatidyl[^{3}H]inositol inaccessible to the phospholipase C and the rate at which it becomes accessible were determined in several experiments and are summarized in Table I. In all experiments the accessible pool was slightly greater when determined with the phospholipase C than with the exchange protein. This difference, amounting to about 10—25% of the inaccessible phosphatidylinositol was also observed when unilamellar vesicles, which had previously been extensively exchanged with multilamellar vesicles

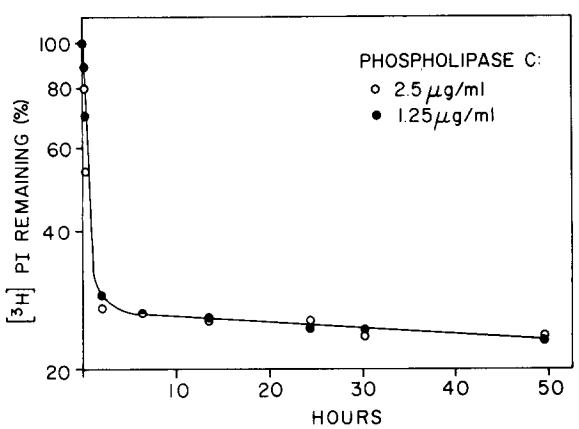

Fig. 4. Hydrolysis of phosphatidyl[^{3}H]inositol in fractionated unilamellar vesicles (phosphatidylcholine : diphosphatidylglycerol : phosphatidyl[^{3}H]inositol, 90 : 5 : 5 mol%) by phosphatidylinositol-specific phospholipase C. The first three time points were taken at 10 s, 4 and 12 min. For clarity, the curve was only drawn connecting the filled symbols. Phosphatidyl[^{3}H]inositol remaining after hydrolysis was calculated as for Fig. 2.

containing phosphatidylinositol, were treated with phospholipase C. A small portion (10—25%) of the phosphatidyl[^{3}H]inositol which had been inaccessible to the exchange protein was accessible to the phospholipase C and was subsequently hydrolyzed by it.

Chromatography of phospholipase C-treated unilamellar vesicles on Sepharose 4B

Chromatography on Sepharose 4B was used to determine the effect of phospholipase C treatment on the size of the vesicles. Sonicated vesicles (nonfractionated) were incubated with phospholipase C for 48 h and then applied to the Sepharose column. The elution profile of the phospholipase C-treated vesicles does not differ substantially from that of untreated vesicles (Fig. 1). Although the major peak of the phospholipase C-treated vesicles often appeared one fraction earlier, there was no significant change in the amount of [^{14}C]triolein excluded from the column. This suggests that phospholipase C treatment does not aggregate or fragment an appreciable fraction of the vesicles.

The total amount of either phosphatidyl[^{3}H]inositol or [^{14}C]triolein recovered from the column was not substantially affected by phospholipase C treatment. The ^{3}H-labeled headgroup of phosphatidylinositol ([^{3}H]inositol phosphate), which is removed by phospholipase C treatment, was eluted as a sharp peak (31—38 ml eluted; data not shown). After phospholipase C treatment, approximately 70—75% of the applied ^{3}H is found in this peak. The separation of the [^{3}H]inositol phosphate from the phosphatidyl[^{3}H]inositol is also reflected in the decrease in the ^{3}H/^{14}C ratio of the vesicles after phospholipase C treatment. However, the ^{3}H/^{14}C ratio after phospholipase C treatment is not constant throughout the elution profile (Fig. 1). The ratio is highest for fractions eluted in the void volume. This is probably due to the fact that the larger unilamellar vesicles and multilamellar vesicles, which are excluded from the column, have larger proportions of their phospholipid at their inner surface, than the smaller unilamellar vesicles. Comparison of the ratios before and after phospholipase C treatment, for the larger vesicles (12 ml eluted) indicate that only 60—65% of the phosphatidylinositol was hydrolyzed, while in the smallest vesicles (26 ml eluted), the amount of phosphatidylinositol hydrolysis was 75—80%.

Discussion

We have shown that exchange protein can be used to study the distribution of phosphatidylinositol in mixed phospholipid vesicles. For the verification of this procedure we have used a phosphatidylinositol-specific phospholipase C. This enzyme acts very slowly on phosphatidylcholine/phosphatidylinositol vesicles with low phosphatidylinositol content. At higher phosphatidylinositol concentration rapid rates of hydrolysis are attainable but the vesicles appear to aggregate during long-term incubations. In vesicles which contained phosphatidylcholine/diphosphatidylglycerol/phosphatidylinositol (90 : 5 : 5 mol%) it was possible to avoid aggregation while maintaining high rates of phosphatidylinositol hydrolysis. For the purpose of comparing accessibility of phos-

phatidylinositol to exchange protein or phospholipase C we have, therefore, confined the studies to this particular composition.

The evidence presented in this paper shows that approximately 30% of the phosphatidylinositol and 35% of the phosphatidylcholine in sonicated vesicles is inaccessible to phospholipid exchange protein and is presumably located at the inner surface of the phospholipid bilayer. From the value for phosphatidylcholine it can be calculated that the outside/inside ratio for this phospholipid is 1.86 which is close to the ratio obtained by other techniques [2,10]. This unequal distribution is thought to be a consequence of the small radius of the sonicated vesicles compared to the thickness of the bilayer. The value of 2.33 for the outside/inside ratio of phosphatidylinositol suggests that it has a similar distribution to phosphatidylcholine. There may be slight preference for the outer surface but there is no evidence for a markedly asymmetric distribution of this phospholipid.

The size of the inaccessible phosphatidylinositol pool was always found to be 10—25% smaller when determined with phospholipase C rather than with exchange protein. That this difference is a real one is suggested by the observation that 10—25% of the phosphatidylinositol, which was inaccessible to exchange protein, can be hydrolyzed in a subsequent treatment of the unilamellar vesicles with phospholipase C. The reasons for this difference are unknown at present and we have no basis for deciding which of the two techniques gives the most accurate relative sizes of the inside and outside monolayers. Incomplete exchange of phosphatidyl[^{3}H]inositol from the outer surface of the vesicles appears particularly unlikely because addition of fresh acceptor plus exchange protein after 24 h of incubation did not significantly increase the amount of phospholipid exchange. Additional phosphatidylinositol hydrolysis in vesicles disrupted by a lytic contaminant in the phospholipase C preparation also appears unlikely, as the extent of hydrolysis was independent of phospholipase C concentration.

The accessibility of phosphatidylinositol in unfractionated sonicated vesicles has also been studied with the phosphatidylinositol-specific phospholipase C from *Bacillus cereus* [25]. They observed that at high levels of phosphatidylinositol (greater than 20 mol%), essentially complete hydrolysis occurs, presumably as a consequence of the disruption of vesicles by accumulated diacylglycerol. However, when the phosphatidylinositol content was reduced to 20 mol% and lower, a maximum of 70—75% hydrolysis was observed, a result that is in close agreement with the values obtained in the present study.

Previous studies of the distribution of anionic phospholipids in sonicated phosphatidylcholine vesicles with NMR or dye-binding techniques have indicated that they may be preferentially located either at the inner [10,26] or outer [11,27] surface of the bilayer. However, the experiments were done under a variety of conditions of pH and vesicle composition which are known to affect the distribution of anionic phospholipids [10,11,26,28]. The discrepancy between our results and those of Berden et al. [10] could not be accounted for by the presence of cardiolipin [19]. Under the conditions which most closely approximate those used in our present study more than 80% of the phosphatidylinositol was found to be located at the outer surface of small unilamellar vesicles [11] compared to the 70—75% figure in our studies.

The rate of translocation of phosphatidylcholine and phosphatidylinositol in sonicated vesicles was estimated in several experiments from the rate of exchange of the inaccessible phospholipid pool over a 36 h period. The $t_{1/2}$ for the translocation of phosphatidylinositol was rather variable (8—60 days) and although the rate of translocation of this phospholipid in sonicated vesicles has not previously been reported it is of a similar order of magnitude to the values obtained for phosphatidylcholine. It was observed, however, that in all the experiments where phosphatidylcholine and phosphatidylinositol exchange were studied simultaneously, the rate constant of translocation of phosphatidylcholine was at least twice as fast as for phosphatidylinositol.

The rate of translocation of phosphatidylinositol (or phosphatidylcholine) was also very low in asymmetric vesicles which contained no phosphatidylinositol on the outer surface. These vesicles were produced by using as acceptors multilamellar vesicles without phosphatidylinositol. Similarly, the asymmetric vesicles generated by phospholipase C treatment gave rates of phosphatidylinositol translocation in the same ranges as those obtained by exchange experiments. It appears, therefore, that the deliberate generation of a phosphatidylinositol 'gradient' across the bilayer does not lead to rapid movement of phospholipids across the membrane. This conclusion is in agreement with the results of Johnson et al. [1] who found that phosphatidylcholine located at the inner surface of the vesicle remained inaccessible after extensive hydrolysis of the outer surface by phospholipase D. However, Sundler et al. [25] and de Kruijff and Baken [29] concluded that phospholipid at the inner surface of the bilayer was accessible to externally located phospholipase D.

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