Lipid vesicle fusion induced by phospholipase C activity in model bile

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Abstract Using a system of phosphatidylcholine-cholesterol vesicles to model the vesicle phase of mammalian bile (1:1 molar ratio) we evaluated whether very small amounts of C. perfringens phospholipase C activity (0.5–6.5 nmol/min per ml) could lead to vesicle fusion, a precursor step for cholesterol precipitation in gallbladder bile. Quasielastic light scattering spectroscopy (QLS) was used to monitor vesicle growth and aggregation in model bile (0.89 mM total lipid) in the presence of phospholipase C. Vesicle growth over 2 h could be detected with phospholipase activity as little as 0.5 nmol/min per ml. Vesicle growth was sustainable over days in the absence of Ca2+ once as little as 3–7 mol% diacylglycerol had been generated as a result of the initial phospholipase C treatment. The presence of fusion intermediates was confirmed using transmission electron microscopy. In addition, kinetically slow vesicle fusion with intravesicle content mixing and minimal leakage was also confirmed by fluorescence spectroscopy using two populations of vesicles containing 5 mM TbCl3 or 50 mM dipicolinic acid. Efficient fusion (40% maximum fluorescence) was obtained at 30 min at 25°C with phospholipase C activity. This level of enzyme activity approximates that found in human gallbladder bile (1.2 nmol/min per ml). We conclude that the hydrolysis products of phospholipase C activity can, in very small amounts (3–7 mol% diacylglycerol), lead to destabilization and fusion of cholesterol-saturated biliary vesicles. A reappraisal of the importance of phospholipase C hydrolysis products in the pathogenesis of cholesterol gallstones is warranted based on these observations.

Phospholipase C has recently been demonstrated to be present in human bile (1) and has been shown to shorten the nucleation time of supersaturated model bile (1, 2). Phospholipase C may, therefore, be potentially important in the process of cholesterol gallstone formation. Small (600 Å) unilamellar vesicles are important carriers of cholesterol in bile (3–10) and serve as substrate for phospholipase C as well as a major source of cholesterol for nucleation and precipitation in gallstones (9, 11, 12).

Cholesterol nucleation in bile involves a number of lipid structural transitions probably beginning with vesicle aggregation and fusion, formation of liquid crystals, and ultimately precipitation of solid cholesterol crystals (7). Phospholipase C is known to alter vesicle membrane behavior in other lipid systems leading to membrane fusion (13). We explored the possibility that phospholipase C could lead to the important phase transitions in model bile vesicles that are known to be preliminary steps in cholesterol nucleation. In the present study quasielastic light scattering spectroscopy (QLS), transmission electron microscopy (EM), and fluorescence spectroscopy were used to characterize phospholipase C-induced fusion in cholesterol-saturated model bile vesicles.

MATERIALS AND METHODS

Phospholipase C [EC 3.1.4.31] from Clostridium perfringens was obtained from Sigma Chemical Co. (St. Louis, MO). The purity of the preparation was evaluated by polyacrylamide gel (12%) electrophoresis with SDS showing a major band at 50,000 daltons and a faint band at 200,000 daltons. The enzyme was not further purified. Cholesterol (96% pure by GC), lecithin (egg yolk, 99% pure by TLC), and dipicolinic acid from Sigma Chemical Co. were used in these experiments without further purification. Radiolabeled phospholipid (dipalmitoyl-sn-glycero-phospho[H-methyl]choline) was obtained from New England Nuclear, Boston, MA. Glycerol determinations were done using a kit from Boehringer Mannheim, Cat. No. 148-270 (Indianapolis, IN).

Supplementary key words biliary vesicles • liposomes • quasielastic light scattering spectroscopy • membrane fusion • diacylglycerol • fluorescence spectroscopy • gallstones

Abbreviations: QLS, quasielastic light scattering spectroscopy; EM, transmission electron microscopy; SDS, sodium dodecyl sulfate; Tes, N-Tris(hydroxymethyl)methyl-2-amino-ethanesulfonic acid; GC, gas chromatography; TLC, thin-layer chromatography.
Preparation of model bile vesicles

Vesicles composed of cholesterol and phospholipids were prepared using the method of Newman and Huang (14) with minor modification. Cholesterol and lecithin mixtures in a molar ratio of 1:1 were prepared by coprecipitation from methanol–chloroform 1:2 (v/v). The solvent was evaporated by a stream of nitrogen and the residue was thoroughly dried in a vacuum desiccator. The dried material was resuspended in 0.15 M NaCl, Tris-HCl buffer (50 mM, pH 7.5) containing 5 mM CaCl2. Hydrated lipid solutions were sonicated indirectly using a Heat Systems Ultrasonics Model W-225 in a cup sonicator with external circulation of 10°C ethylene glycol as coolant. A scintillation vial, containing 5 ml of a 1% lipid solution (by wt) that had been sparged with nitrogen, was placed in the sonicator cup. Continuous sonication at 30% power for 16–20 h was sufficient to produce small unilamellar vesicles (R = 500 Å). Sonicated dispersions were centrifuged for 30 min at 50,000 g to remove unsuspended lipid. Maximum levels of diacylglycerol in prepared model bile vesicles were always less than 2 mol%.

Determination of phospholipase C activity

Radiolabeled substrate was prepared by adding the vesicle solution to 1 μCi of the labeled phospholipid previously taken to dryness in a glass tube. The mixture was incubated for at least 1 h prior to the enzyme activity experiment. The molar ratio of labeled lipid to total phosphatidylcholine was 1:5 × 106. Phospholipase C activity was measured by the release of water-soluble [3H]phosphocholine as described by Pattinson (1) and was expressed as nanomoles of hydrolyzed phosphatidylcholine per minute per milliliter of reaction solution (nmol/min per ml). Because the activity of phospholipase C was critically dependent on the form and composition of the lipid phase that contains the monomeric substrate, the same model bile vesicle system was used in all experiments. The reaction was terminated by adding 500 μl of chloroform–methanol 2:1 (v/v) and 200 μl of 0.1 N HCl to 25 μl of the vesicle–enzyme solution. The separated aqueous phase containing the hydrolyzed [3H]phosphocholine was recovered by careful removal with a syringe and [3H]phosphocholine content was then determined by scintillation counting. The lipid extract in the organic phase was concentrated under a stream of nitrogen and analyzed for radioactivity to determine the concentration of unreacted [3H]phosphatidylcholine. All experiments were carried out at 25°C.

In separate experiments, the accuracy of the radiolabeled phosphatidylcholine assay for phospholipase C was validated by simultaneous biochemical determination of diacylglycerol using selective alkaline hydrolysis to form free glycerol as described by Chernick (15). Glycerol was then determined by coupled enzymatic reactions monitoring NADH oxidation after the method of Eggstein and Kuhlmann (16). Correlation between phospholipase C activity as measured by radiolabeled phosphocholine release and, as measured by analytic determination of diacylglycerol, was high (R2 = 0.99). The high correlation, together with a regression slope close to unity (slope = 0.91; standard error = 0.028), confirmed the validity of the labeled lipid assay.

Studies using QLS

Phospholipase C was added to the sonicated vesicles and their mean hydrodynamic radii, Rn, were measured with QLS. To avoid dust contamination, the scattering cells, 1 cm diameter borosilicate tubes, were acid-washed and rinsed with deionized water. All samples were held at room temperature and the QLS measurements were done at a 90° scattering angle. The diffusion coefficient of the vesicles was obtained from a single exponential fit to the measured intensity autocorrelation function. The effect of interparticle interactions on the QLS signal was assumed to be negligible. In this case the diffusion coefficient, D, of a particle was related to its hydrodynamic radius (Rn) through the Stokes-Einstein equation, D = kT/6πηRn, where η is the solvent viscosity, T the absolute temperature, and k the Boltzmann constant.

Studies using electron microscopy

Vesicular aggregates of cholesterol and phospholipids were also examined by transmission electron microscopy after addition of enzyme. Formvar-coated grids were dipped in the reaction mixture of vesicles and phospholipase C, air-dried, fixed with 3% glutaraldehyde followed by 1% osmium tetroxide, and then stained with 1% phosphotungstic acid. The grids were examined with a Phillips 100S microscope. Twenty vesicles in each electron micrograph were randomly assigned, and then measured to derive a mean vesicle radius.

Fluorescence monitoring of vesicular fusion

Phospholipase C-induced fusion-related aqueous mixing of vesicle contents was determined by a modification of the method of Wilschut et al. (17). A coprecipitate of 25 μmol cholesterol and 25 μmol phosphatidylcholine was prepared as described above with the following modifications. Vesicles were prepared by probe sonication of the coprecipitate in 3 ml of 5 mM TbCl3, 50 mM citrate, 2 mM Tris, and 2 mM histidine. After centrifugation the extravesicular aqueous phase was replaced by terbium-free 50 mM Tris, 0.15 M NaCl buffer, pH 7.4, by chromatography over Sephadex G-75 in 1 × 20 cm columns. Dipicolinic acid was encapsulated in a similar fashion by sonication of the same lipid coprecipitate over 50 mM dipicolinic acid, 2 mM Tris, 2 mM histidine followed by centrifugation and G-75 chromatography.

The assay of aqueous mixing of vesicle contents was performed by the addition of phospholipase C, (ac-
tivity = 1.2 nmol/min per ml) to a 1:1 mixture of terbium and dipicolinic acid-containing vesicles (total lipid 1.25 mM) in the presence of 1 mM CaCl₂. The reaction was allowed to proceed with gentle mixing. Fluorescence was measured by scanning the 520-590 nm emission band using a Perkin-Elmer LS-5B Spectrophotometer with excitation at 276 nm. Second order excitation wavelengths were filtered by a Melles Griot 03FCGI77 filter and the emission spectra were filtered by a Wratten #8 filter. One hundred percent fluorescence was determined by replacing the dipicolinic acid vesicle fraction with the same volume of unencapsulated 50 μM dipicolinic acid and 1% w/v sodium cholate. Suppression of leakage-related fluorescence (as well as reaction termination) was achieved by the addition of EDTA to aliquots of the reaction mixture to a final concentration of 2 mM.

RESULTS

Calcium was required for phospholipase C enzyme activity in this system and EDTA inhibited the hydrolysis reaction. Fig. 1 shows the radioactivity recovered in the aqueous phase over time representing the [3H]phosphocholine produced by phospholipase C action at both 0 and 10 mM EDTA. At 10 mM EDTA the percent activity in the aqueous phase did not change over 2 h reaction time and increased only slightly (10%) 1 day later. With no EDTA present the reaction neared completion at 90 min at room temperature with no radiolabel remaining in the organic phase. In this case (Fig. 1) the phospholipase C activity was calculated from the slope of the line to be 4.3 nmol/min per ml.

The effect of phospholipase C on vesicle mean hydrodynamic radius using QLS was then evaluated. Vesicle size was measured as a function of time after addition of increasing concentrations of phospholipase C to a 890 μM solution of sonicated lipid vesicles in the presence of 5 mM Ca²⁺. The rate and extent of aggregate growth increased with enzyme concentration (Fig. 2). Aggregates continued to grow for hours after addition of the enzyme even though the hydrolysis reaction had reached its end point. Lipid aggregates grew from an initial radius of 500 Å to as large as 6500 Å. With the growth of vesicles, liquid crystals were seen to form and settle. The kinetics of crystal formation were not determined in the present experiments, although cholesterol monohydrate crystals with the typical notched rhomboïdal plate configuration were observed with polarizing microscopy in samples containing phospholipase C. On the other hand, vesicles in the control solution were stable and no cholesterol crystals or liquid crystals were observed for several weeks.

Vesicle size was also measured by QLS after adding the enzyme to a solution that did not contain any calcium. At low phospholipase C concentrations (activity 4.3 nmol/min per ml) there was no change in vesicle radius for several days, but when Ca²⁺ was present with phospholipase C, the same vesicles had aggregated to a size > 2000 Å within an hour. Vesicles were stable in the presence of 5 mM Ca²⁺ alone for at least 4 days. At excess

![Fig. 1. Phospholipase C activity using cholesterol-phosphatidylcholine model bile vesicles (50:50) as substrate. Phospholipase C activity measured as liberation of phosphocholine at 0 and 10 mM EDTA. The total lipid concentration of model bile vesicles was 890 μM. No detectable phospholipase C activity was apparent in the presence of 10 mM EDTA at 150 min. In the absence of EDTA reaction completion was at 90 min. Calculated phospholipase C activity is 4.3 nmol/min per ml.](image)

![Fig. 2. Quasielastic light scattering (QLS) determination of cholesterol-phosphatidylcholine vesicle growth induced by phospholipase C as a function of time. Phospholipase C activities are given in the insets (nmol/min per ml). Total lipid, 890 μM. [Ca²⁺] = 5 mM.](image)
phospholipase C concentrations (activity > 450 nmol/min per ml) in the absence of Ca$^{2+}$, vesicle size increased by 10 Å immediately after addition of the enzyme and remained at that size for several days. These results are consistent with rapid, calcium-independent, binding of enzyme to vesicles.

Vesicle growth, after partial phosphatidylcholine hydrolysis was evaluated as a function of time (Fig. 3). Stepwise increases in partial phosphatidylcholine hydrolysis were achieved by addition of 6.5 nmol/min per ml phospholipase C to vesicles followed by the addition at regular intervals of 10 mM EDTA to aliquots of the reaction mixture. Vesicle growth, subsequent to phospholipase inhibition by EDTA, correlated directly with the time of prior uninhibited phospholipase C reaction time. The earliest growth that could be detected after addition of EDTA (> 10% increase in size from 833 Å to 956 Å) occurred after 2 min of incubation with phospholipase C with a final calculated diacylglycerol content of 3 mol% (not apparent in Fig. 3 due to scale). More complete hydrolysis of phosphatidylcholine led to more rapid vesicle growth after inhibition of phospholipase C by EDTA. Notably, continued vesicle growth occurred in partially hydrolyzed vesicle dispersions in the absence of free ionized Ca$^{2+}$. This demonstrated that in this system vesicle growth was dependent only on the generation of diacylglycerol and/or phosphatidylcholine, and was not merely due to the presence of Ca$^{2+}$-induced fusion.

QLS data gives highly accurate information about unperturbed aggregate size changes (as exemplified by the measurements of phospholipase C binding to vesicles) but gives little information about structural detail. Therefore, we additionally characterized the evolution of size and structure of phospholipase C-treated model bile with standard transmission electron microscopy. The vesicles had an initial mean radius of 200 Å. Electron micrographs taken at different reaction times showed that vesicles formed progressively larger unilamellar vesicle-like structures that appeared to fuse together (Fig. 4). Initially there was no evidence of fusion (Fig. 4A); but within 15 min of incubation with phospholipase C (4.3 nmol/min per ml) evidence of vesicle growth and fusion was apparent (Fig. 4B). Some binary and tertiary vesicle structures were noted to have a common unilamellar-like interface separating the aqueous contents.

Because EM morphology can be complicated by the introduction of artifacts during fixation and staining of lipids, fluorescence labeling of vesicles was performed to confirm that fusion was taking place with intravesicular content mixing. The addition of 1 mM CaCl$_2$ to cholesterol-phosphatidylcholine (1:1 molar ratio) vesicles containing either terbium or dipicolinic acid did not result in any detectable fusion. Phospholipase C (1.2 nmol/min per ml), however, readily induced fusion of 1.25 mM cholesterol-phosphatidylcholine vesicles with mixing of intravesicular contents in the presence of 1 mM CaCl$_2$ (Fig. 5). Under the conditions used in these experiments, the reaction kinetics were slow (minutes) with peak fluorescence occurring at 30 min of reaction time. Mixing of intravesicular contents predominated over disruption of vesicles with leakage of contents. These data confirmed vesicular fusion and supplemented previous findings of QLS and EM studies.

**DISCUSSION**

The purpose of this work was twofold. One, to confirm and further explore what is so far a unique example of enzymatically controlled membrane fusion (13). The second purpose was to explore the possible relevance of this mechanism to destabilization of biliary lipid vesicles leading to cholesterol gallstone formation. The experimental results demonstrate that cholesterol-saturated model bile vesicles readily serve as a substrate for phospholipase C and that the time course of fusion is slow, on the order of minutes, under the conditions used. Due to technical constraints in QLS measurements, all experiments were performed at 25°C rather than at 37°C. Nevertheless, the phospholipase C activity used was similar in these experiments to the mean value of 1.2 nmol/min per ml found in gallbladder bile (1). This mean value is an approximation because the phase composition of the assay substrate can affect enzyme kinetics. Fusion results were also qualitatively similar to those found by Nieva, Goñi, and Alonso.
Transmission electron microscopy of phospholipase C-treated cholesterol-phosphatidylcholine model bile vesicles. Phospholipase C activity 4.3 nmol/min per ml added to 8.9 mM total lipid model bile vesicles. Left panel (A): no incubation with phospholipase C. Vesicles were fixed immediately after addition of enzyme. Vesicles are unaggregated, small, and relatively monodisperse. Right panel (B): 15 min incubation with phospholipase C. Vesicles are intact, but have enlarged markedly and show tertiary fusion intermediates. Note the slight difference in scale bars due to the small difference in magnification factors.

(13) at 37°C. An immediate increase in vesicle radius of 10 Å on addition of phospholipase C corresponds to rapid binding of phospholipase C to the vesicle surface, implying that vesicle binding of phospholipase C is not the rate-limiting step in phospholipid hydrolysis. Although phospholipase C activity is present in relatively small amounts in bile, QLS results demonstrate that physiologically relevant phospholipase C enzyme activity can result in model bile vesicle aggregation or fusion that can be self-sustaining over days after an initial exposure to phospholipase C activity for as little as 2–5 min (equivalent to 3–7 mol% diacylglycerol, respectively). These results are not consistent with vesicle fusion occurring due to a nonenzymatic interaction of the phospholipase C protein with Ca²⁺ and vesicles because the subsequent fusion process was dependent on the initial time of exposure to active phospholipase C.

EM experiments on phospholipase C-treated vesicles demonstrate fusion events between vesicles with resultant vesicle growth. The mean radius of 200 Å for untreated model bile vesicles as determined by EM was smaller than the measurement obtained by QLS (500 Å) due in part to the preferential increase in light scattering intensity of larger particles in the measured QLS samples thereby giving larger particles greater weight in the size distribution. Electron micrographs demonstrate a fusion intermediate in which model bile vesicles share a common membrane between them before complete internal mixing takes place. Although artifacts can be introduced during fixation for EM, each lipid sample served as its own control before and after treatment with phospholipase C. Striking and reproducible changes were observed in vesicle structure over time with phospholipase C treatment consistent with the results of the nonperturbing techniques.
low levels of DAG. They also noted maximum fusion in both phosphatidylethanolamine and cholesterol are required as components of PC vesicles to induce fusion at minutes or even hours, the amount of diacylglycerol required as components of PC vesicles to induce fusion is still very small, even at room temperature, and is similar to that required to induce fusion in pure phospholipid membranes. Because the vesicle fusion reaction may be self-sustaining after diacylglycerol has been released by phospholipase C, the instantaneous biliary activity of phospholipase C may not be a reliable indicator of phospholipase C-induced aggregation and fusion. Instead, these results suggest that the vesicular phase concentration of diacylglycerol, which has rarely been measured, may be a relevant predictor of vesicle instability. The effects of acyl chain length, degree of saturation, and position have been important factors in diacylglycerol-induced fusion in other lipid systems (20) but remain to be explored in biliary vesicles. Reduction in the bulk phase concentration of phosphatidylcholine which is a potent solubilizer of cholesterol has been posited as a possible mechanism by which cholesterol nucleation is promoted by phospholipase (1, 2). The additional mechanism, discussed here, of diacylglycerol-induced vesicle destabilization may also be important for cholesterol nucleation. This would require relatively small amounts of phospholipase C to initiate sustained vesicle fusion in bile. The mechanism of diacylglycerol-induced fusion has been extensively studied in pure phospholipid systems (19–21). It is clear from nuclear magnetic resonance data that an isotropic phase separation of diacylglycerol occurs in these systems that precedes the fusion event and likely leads to a hexagonal phase intermediate (19). A diacylglycerol-induced mechanism of vesicle fusion and destabilization would probably be slow, requiring hydrolysis and fusion of vesicles over hours, as in a poorly emptying gallbladder, to contribute significantly to cholesterol precipitation. The question of enzyme kinetics is likely the limiting factor with respect to the pathophysiologic importance of this mechanism. The concentration of phosphatidylcholine in bile is much higher (20–30 mM) than the phosphatidylcholine concentrations used in these experiments and hence the reaction time required to achieve a significant mole percentage of greater than 3 mol% diacylglycerol, assuming the phospholipase C activity in gallbladder bile of 1.2 nmol/min per ml reported by Pattinson (1), would be on the order of hours. It is well known, however, that in many cases human gallbladder stasis can be a critical adjunct to cholesterol gallstone formation (22–24) and that fasting gallbladder stasis occurs for several hours in normals. Apart from the possible relevance to biliary cholesterol precipitation, the fusion kinetics of the lipid system described herein are relatively slow and, therefore, may provide a system that could be useful to study other, potentially multistep, lipid–enzyme reactions designed to alter biological membrane structure and function.

In summary, using complimentary techniques, the enzymatic activity of phospholipase C has been shown to lead to the time-dependent aggregation and fusion of model bile vesicles. Once diacylglycerol had been generated at critical concentrations of 3–7 mol%, vesicle growth proceeded independently from continued phospholipase C activity and the growth process did not require calcium. Phospholipase C-induced vesicle fusion predominated...
over disruption. This mechanism of biliary lipid vesicle phase transition has important implications for the solubilization of cholesterol in bile where low levels of phospholipase C are known to be present.

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