Enzyme-containing liposomes can endogenously produce membrane-constituting lipids

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Background: 'Giant vesicles' are liposomes that have diameters of several micrometers. It is possible to microinject biochemicals into a single vesicle and follow the progress of a chemical reaction in real time by light microscopy. We have previously used this technique to inject phospholipase A_2 into giant vesicles; the vesicles disappeared as their components were hydrolyzed. Here we investigate whether the lipid components of a vesicle can be synthesized inside it.

Results: Giant vesicles composed of 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine (POPC) and palmitoyl-CoA were prepared in a solution containing *sn*-glycerol-3-phosphate. Microinjection of the enzyme *sn*-glycerol-3-phosphate-acyltransferase into the vesicle catalyzes the *in situ* production of the lipid membrane precursor 1-palmitoyl-*sn*-glycerol-3-phosphate, which remains incorporated in the membrane. The altered membrane chemistry causes shrinkage of the vesicle and formation of smaller liposomes on the inner surface at the site of injection. Similar transformations were seen when the enzyme was added to the outside of the vesicle.

Conclusions: We have used the first step of the 'salvage pathway' for synthesis of POPC to demonstrate that it is possible to localize the synthesis of a lipid membrane precursor inside a giant vesicle. In the future it may be possible to combine the necessary enzymes and substrates to carry out the reactions for a complete metabolic pathway within a liposome.

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Introduction

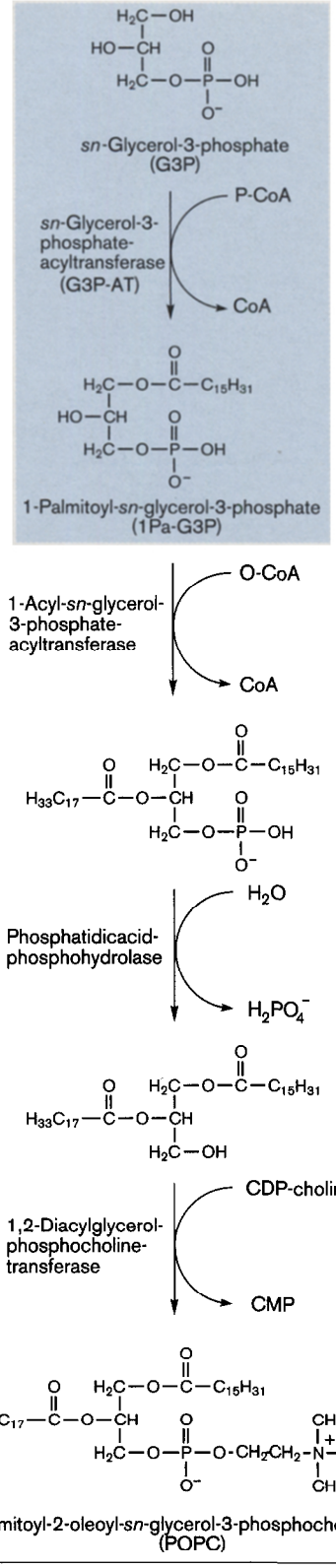
Liposomes are probably the prebiotic precursors of cells [1–5]; they are the simplest spherical structures in which an aqueous microenvironment is enclosed by a lipid bilayer membrane. Recently, a variety of chemical reactions have been carried out inside liposomes [6–28]. These studies have helped to determine the effects of a restricted compartment on biochemical reactions, including problems of trafficking, local concentration effects and the influence of concentration gradients across the surrounding membrane. The spherical microenvironment inside the liposome mimics the intracellular compartment, and it may be possible to construct a 'synthetic cell' in which the coupled reactions of a self-sustaining metabolic cycle are entrapped in a liposome.

In the past, entrapped reactions have been carried out in submicroscopic liposomes, which have diameters ranging from 50 to 400 nm and can only be observed by electron microscopy. These studies can be extended by using so-called 'giant vesicles' (or 'giant liposomes'), which reach the dimensions of microns and can be observed using normal light microscopy. Several recent papers have described the behaviour of giant vesicles, in particular the shape transformations that they can undergo [29–36].

We have recently developed a micromanipulation technique that allows the microinjection of biochemicals into a single giant vesicle [36]. In this way, it is possible to carry out a localized chemical reaction in an individual liposome and follow its progress in real time by light microscopy. We have used this technique previously to microinject phospholipase A_2 (PLA_2) into giant vesicles composed of 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine (POPC) [36]. Hydrolysis of POPC into the corresponding lysolecithin and oleic acid causes complex transformations in the shape of the liposome. Microinjection of PLA_2 onto the surface of the giant vesicle, or into the external medium a few microns away from the bilayer, also induces changes in the structure of the giant vesicle.

Here we investigate the possibility that the biochemical reaction(s) leading to the synthesis of the liposome membrane can be entrapped inside the vesicle. We have chosen the so-called 'salvage pathway' [37], which makes use of preformed choline for synthesis of POPC from *sn*-glycerol-3-phosphate (G3P), according to the reaction sequence illustrated in Figure 1. Four enzymes are involved in this pathway; here we describe the action of the first enzyme, which catalyzes the palmitoylation of water-soluble G3P, yielding the lecithin precursor

Figure 1



Pathway of POPC biosynthesis in four enzymatic reaction steps starting from G3P, acyl-CoA and CDP-choline [37]. The box highlights the reaction carried out inside giant vesicles in this study.

1-palmitoyl-*sn*-glycerol-3-phosphate (1P-G3P). This product is incorporated in the POPC membrane.

Results and discussion

Formation of giant vesicles

We prepared giant vesicles, according to a modification of the electroformation method of Angelova and Dimitrov [38,39], as described in detail elsewhere [36]. The vesicles were composed of POPC and had diameters of 10–60 μm. We previously developed a microinjection technique, based on the micromanipulation of biological cells [40,41], to inject substances into the interior of giant vesicles ([36], see Materials and methods).

The efficiency of the microinjection technique was demonstrated by injecting the water soluble polysaccharide Ficoll 70 (Mr ≈ 70 000) into giant vesicles. Phase contrast micrographs of a typical POPC giant vesicle (38 μm in diameter) before, during and after microinjection are shown in Figure 2. The darkening of the interior of the vesicle is due to the higher refractive index of the polysaccharide solution compared to the vesicle suspension medium. The injected giant vesicles are stable under the microscope for several hours, and no leakage can be detected.

Synthesis of 1P-G3P

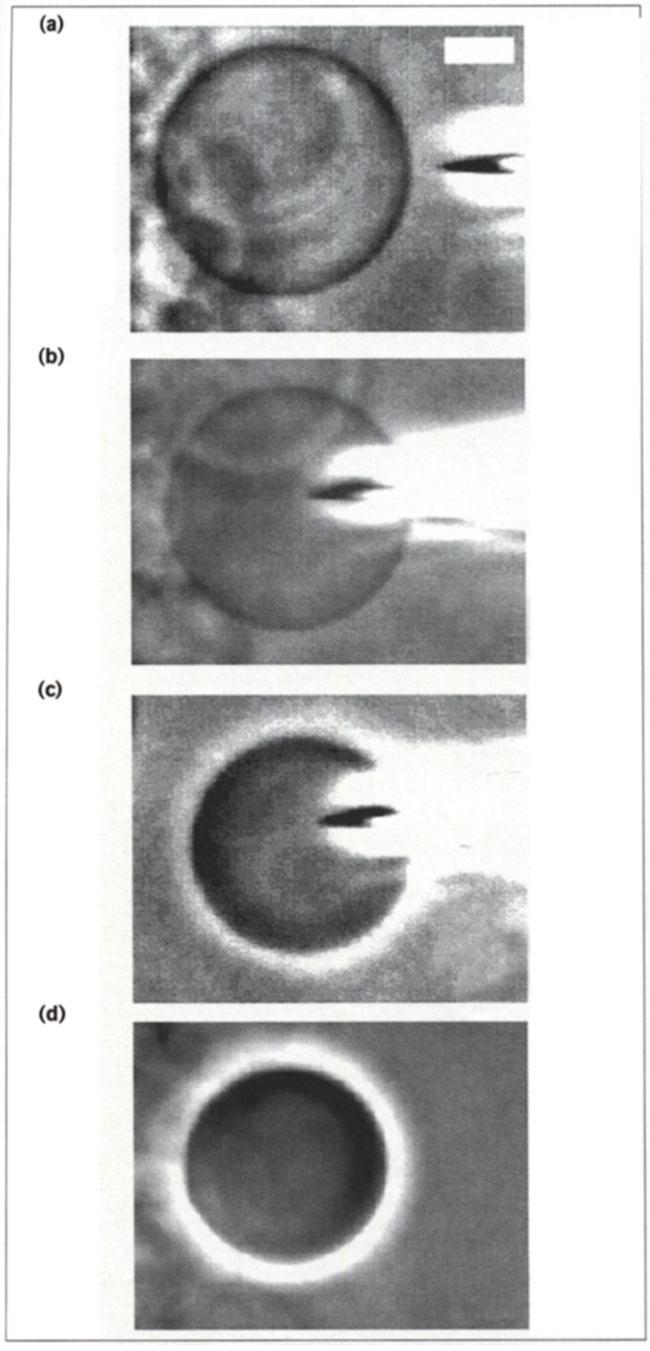
The 'salvage pathway' for biosynthesis of phosphatidylcholine makes use of preformed choline (Fig. 1). In this pathway, components are added to G3P from acyl coenzyme A (acyl-CoA) and CDP-choline [37]. The initial step of this synthesis is of particular interest in lipid biochemistry [42,43], because the lipid-membrane-soluble lyso-glycerophospholipid 1P-G3P is produced from two water-soluble precursors, G3P and palmitoyl-CoA (P-CoA). This reaction is catalyzed by the enzyme *sn*-glycerol-3-phosphate-acyltransferase (G3P-AT; EC 2.3.1.15), which is an integral membrane protein that is essentially inactive in water solution but regains its activity when reconstituted into phospholipid bilayers.

Injection of G3P-AT into mixed POPC/P-CoA giant vesicles

We prepared giant vesicles composed of a 9:1 (w/w) mixture of POPC and the substrate P-CoA. Based on the partition coefficient of P-CoA in a phosphatidylcholine bilayer [44], we estimate that all of the P-CoA is incorporated into the membrane of the giant vesicles (see Materials and methods). The vesicles were prepared in a solution containing G3P, resulting in encapsulation of this substrate. G3P-AT was microinjected into single vesicles (Fig. 3), and the ensuing transformations were observed in real time by light microscopy (Figs. 4–7).

We injected 180 fl (1 fl = 10^{-15} l) of enzyme stock solution (250 μg ml^{-1}), corresponding to about 3×10^5 enzyme molecules, into a single unilamellar giant vesicle (Fig. 4). Within 10 s the vesicle began to shrink and small liposomes

Figure 2

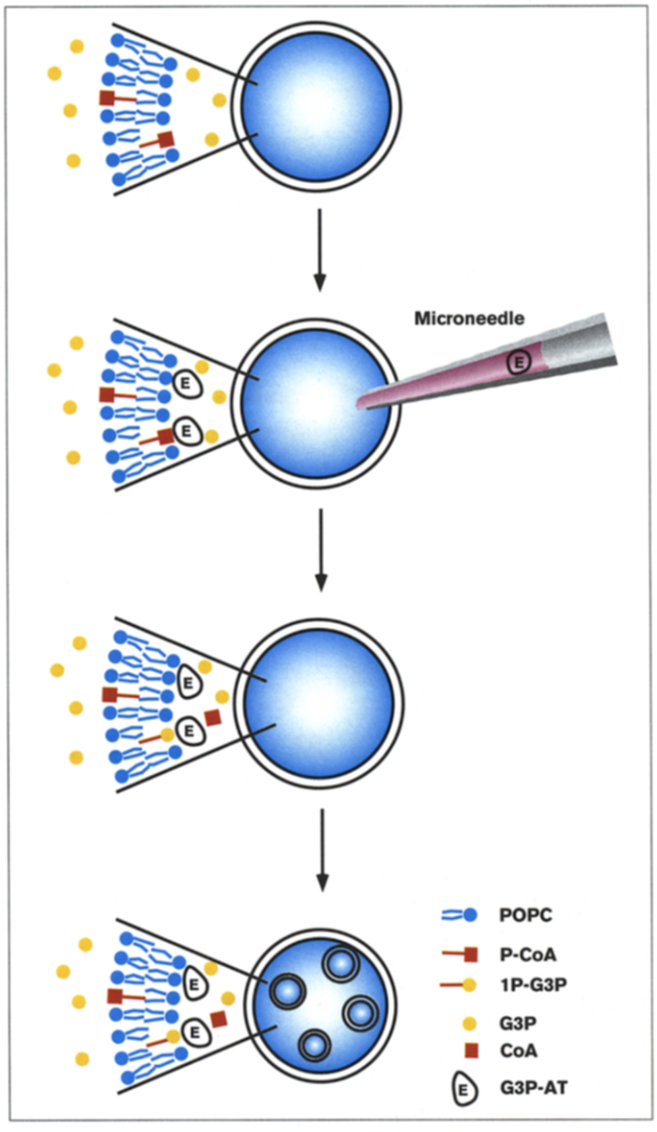


Microinjection into the aqueous interior of a single POPC giant vesicle can be visualized by injecting Ficoll 70. **(a)** The giant vesicle, with a diameter of 38 μm, before microinjection. The vesicle has an internal aqueous volume of 29 pl (1 pl = 10^{-12} l) and is composed of ~1.26 x 10^{10} molecules (assuming unilamellarity, a cross sectional area for one POPC molecule of 0.72 nm^2 [58] and a bilayer thickness of 3.7 nm [59]). **(b)** The tip of the microneedle 'type II' (see Materials and methods) has penetrated the vesicle and the addition of 1.8 pl of the polysaccharide is started. The vesicle is shown **(c)** 30 s and **(d)** 60 s after the start of the injection. In (d) the injection has been completed and the microneedle withdrawn. The maximum amount of liquid that can be injected inside a vesicle without bursting it is 6 % (v/v) of the encapsulated aqueous volume. The length of the bar corresponds to 10 μm.

began to form at the internal surface of the vesicle near the injection point (Fig. 4a). Five minutes after injection of the enzyme, the vesicle was dramatically reduced in size and was filled with smaller liposomes (Fig 4f).

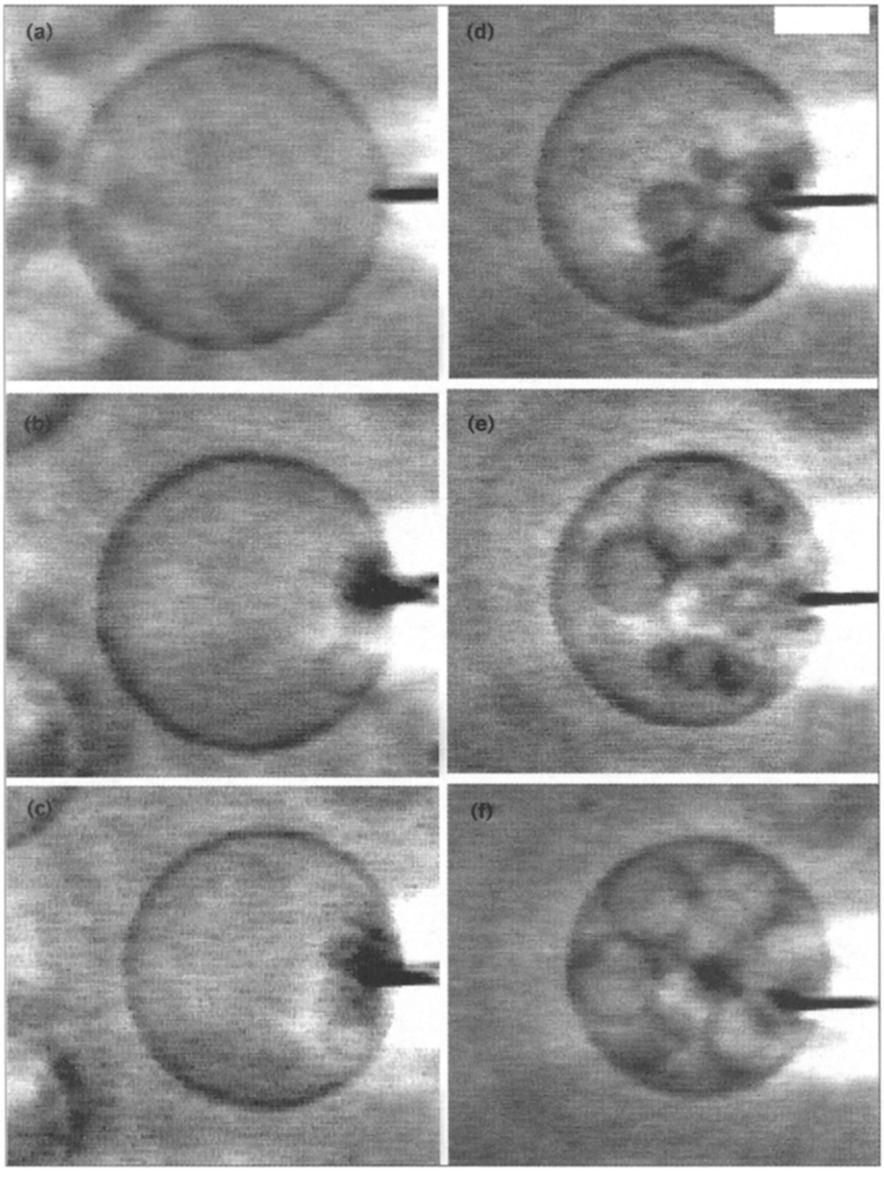
Based on the turnover number of the enzyme ([25], see Materials and methods), we estimate that ~4 x 10^8 P-CoA molecules were converted into 1P-G3P within five minutes after addition of G3P-AT (yield 40 %). We repeated the microinjection with the same amount of

Figure 3



Schematic representation of the synthesis of 1P-G3P from P-CoA and G3P in giant vesicles, catalyzed by G3P-AT (not to scale). **(a)** A single mixed POPC/P-CoA (9:1; w/w) giant vesicle prepared in the presence of G3P. **(b)** Microinjection of G3P-AT into the vesicle and binding of the enzyme to the substrate in the bilayer. **(c)** Conversion of P-CoA into 1P-G3P releasing CoA. **(d)** Morphological changes of the giant vesicle due to the enzymatic reaction (see Figs. 4–7).

Figure 4



Injection of G3P-AT into a POPC/P-CoA giant vesicle causes shrinkage of the vesicle and formation of smaller liposomes on the inner surface at the site of injection. The transformations of a single giant vesicle, induced by microinjection of 180 fl of G3P-AT solution (250 μg ml^{-1}), are shown. **(a–f)** Phase contrast micrographs of the giant vesicle taken 0, 10, 20, 50, 140 and 300 s, respectively, after microinjection. The length of the bar corresponds to 10 μm.

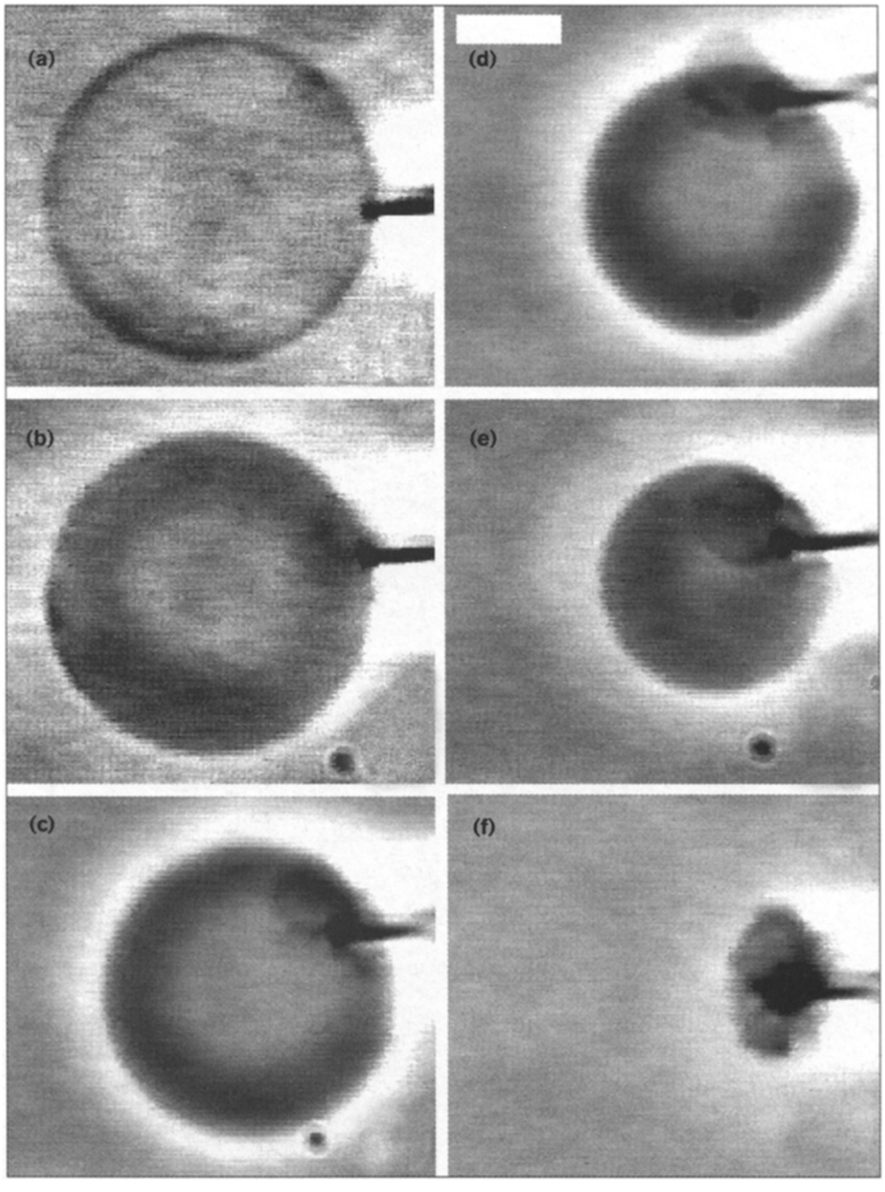
enzyme several times and did not observe any further transformations after 5 min, indicating that this was the maximum yield of the reaction. As the reaction is only taking place in the inner layer of the lipid bilayer, 80 % of the P-CoA in this layer is converted to 1P-G3P. In this reaction, the water-soluble CoA [45] is exchanged with G3P and released in the water phase, and the charged phospholipid product 1P-G3P stays in the membrane, replacing P-CoA. Because of the electrostatic and geometric variations in the head groups of the substrate and product, incorporation of 1P-G3P into the POPC liposomes brings about a significant change in the curvature of the membrane. The resulting energy destabilization can be relaxed by forming smaller liposomes.

Microinjection of twice as much enzyme into a giant vesicle also resulted in a reduction of vesicle size and formation of smaller liposomes inside (Fig. 5a–e). After 118 seconds, however, the giant vesicle disappeared completely, leaving several smaller liposomes associated with the tip of the microinjection needle (Fig. 5f). In either experiment, microinjection of G3P-AT into a giant vesicle composed of POPC and P-CoA produced lipid material (1P-G3P) and resulted in the formation of several smaller liposomes. This encapsulated reaction may be a primitive model for the multiplication of the shell of a cell-like structure.

External injection of G3P-AT

We microinjected 590 fl of G3P-AT solution onto the surface of a giant vesicle and observed the ensuing shape

Figure 5



Injection of 360 fl of G3P-AT solution (250 μg ml^{-1}, containing 8 % Ficoll 70 (w/w)) into a POPC/P-CoA giant vesicle causes the vesicle to disappear, leaving smaller liposomes at the site of injection. The injection of Ficoll 70 with the enzyme shows that the microneedle had indeed penetrated the bilayer. **(a–f)** Phase contrast micrographs of the giant vesicle taken 0, 30, 105, 110, 111 and 118 s, respectively, after microinjection. The length of the bar corresponds to 10 μm.

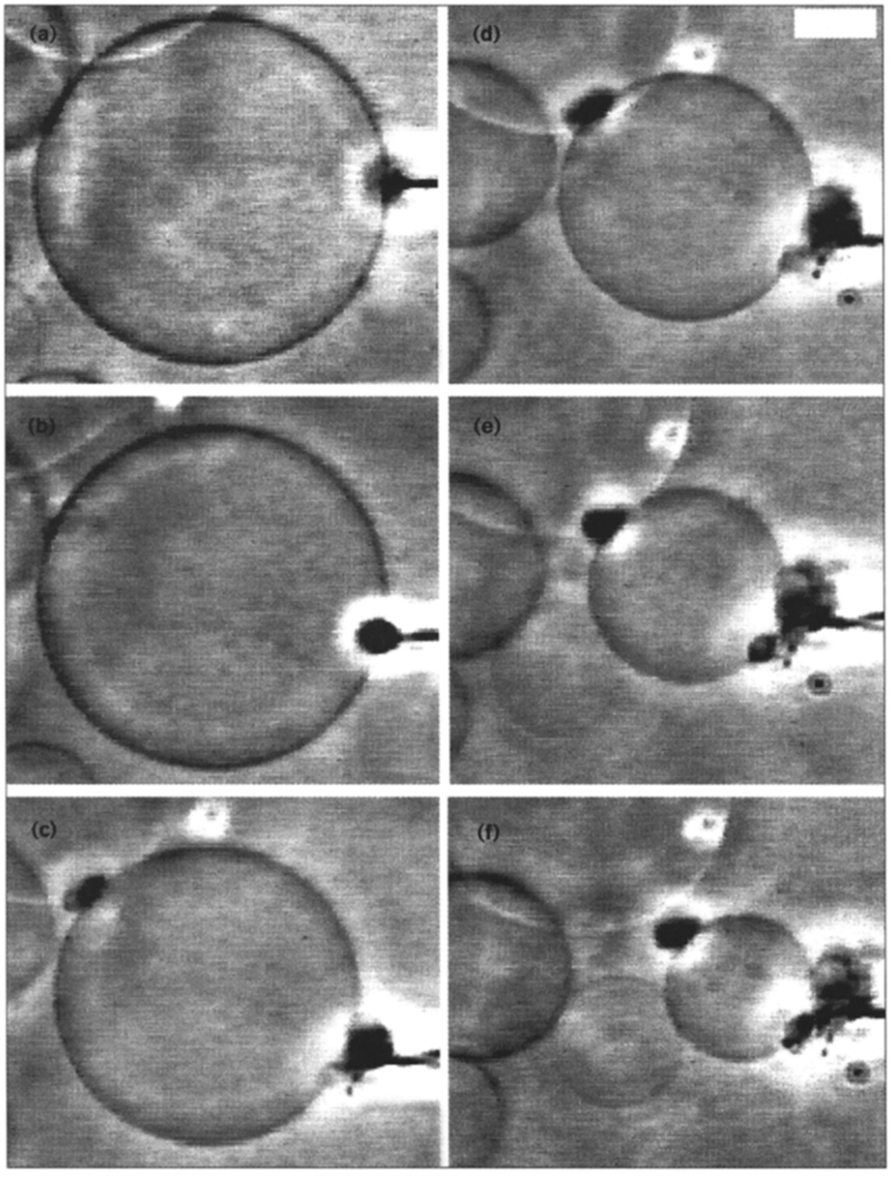
transformations (Fig. 6). Essentially the same behaviour was observed as when the enzyme was injected into the vesicle; a group of smaller liposomes formed at the contact point of the injection, and the giant vesicle, which initially had a diameter of 44 μm, shrunk to a final size of 19 μm.

We observed somewhat different behaviour when the enzyme solution was added at a distance of 10 μm from the vesicle surface (Fig. 7). The giant vesicle burst within 60 s (Fig. 7b) and a handful of smaller vesicles formed on its surface (Fig. 7e). Considerable bending of the surface of the vesicle ensued, converting the sphere into a kidney shape (Fig. 7f–h).

Two basic control experiments were performed: internal and external addition of G3P-AT to (1) POPC giant vesicles in the presence of G3P and to (2) POPC/P-CoA giant vesicles in the absence of G3P. In both cases, one substrate was missing and the addition of enzyme solution did not lead to changes in the size of the vesicle over several hours. The shape transformations of the giant vesicles are therefore observed only when the four components (POPC, enzyme, P-CoA and G3P) are present simultaneously.

Farge and Devaux [29] have extensively studied the incorporation of another lyso-glycerophospholipid into giant vesicles. These compounds, which have a single acyl

Figure 6



Addition of G3P-AT to the external surface of a single POPC/P-CoA giant vesicle causes shrinkage of the vesicle and formation of smaller liposomes at the contact point of the injection (the microneedle tip was touching the external vesicle surface). A 'type I' microneedle (see Materials and methods) was used. **(a–f)** Phase contrast micrographs of the giant vesicle taken 0, 45, 80, 90, 100 and 105 s, respectively, after addition of 590 fl of G3P-AT. The length of the bar corresponds to 10 μm.

chain, form micelles in aqueous solution instead of forming lamellar phases, but they can be incorporated into bilayers composed of other lipids [46,47]. When giant vesicles (diameter 60 μm) composed of egg phosphatidylcholine were submitted to a gentle stream of lysophosphatidylcholine in the vicinity of the outer envelope, ripples formed at the surface of the giant vesicles. After two minutes the ripples transformed into multiple protrusions; eventually small vesicles, with diameters smaller than 5 μm, budded off of the giant vesicle.

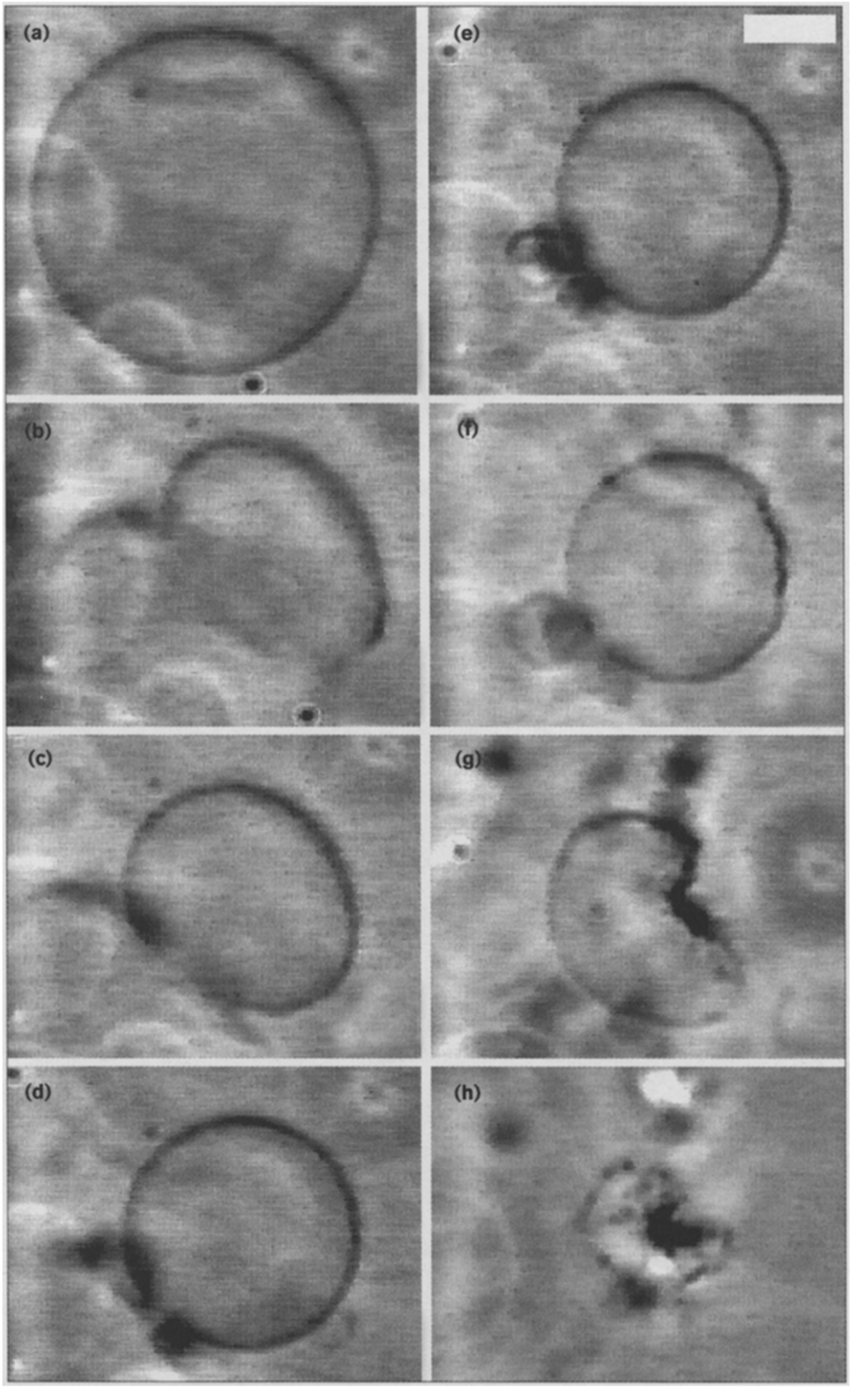
Significance

Here we report the formation of the lipid membrane precursor 1P-G3P within the bilayer membrane of giant vesicles. Production of lipid inside the vesicle causes specific effects on the bilayer membrane, including the formation of several smaller liposomes — a form of particle fragmentation.

In vivo, 1P-G3P would not be present for long enough to be incorporated into a membrane and destabilize it. 1P-G3P would immediately be converted to the next metabolite in the salvage pathway, 1,2-diacyl-*sn*-glycerol-3-phosphocholine, and eventually into a lecithin such as POPC [42,43] (Fig. 1). It may be possible to inject all four enzymes and the required substrates in this pathway and carry out the overall synthesis of POPC inside a giant vesicle; *in vitro* biosynthesis of lecithin has been reported [27]. Internal POPC production would cause the vesicle to grow in size or to multiply — a form

Figure 7

Addition of G3P-AT in the vicinity of a single POPC/P-CoA giant vesicle causes the vesicle to burst and leads to the formation of smaller liposomes on the vesicle surface. The distance between the vesicle surface and the microneedle tip was ~10 μm. **(a–h)** The giant vesicle is shown 0, 60, 61, 62, 63, 90, 120 and 130 s, respectively, after addition of 590 fl of G3P-AT with a 'type I' microneedle (see Materials and methods). The length of the bar corresponds to 10 μm.



of autopoietic self-reproduction [48–50]. Induction of changes large enough to be viewed by the light microscope, however, may require production of an amount of POPC molecules equivalent to that already present in the bilayer. This is presently not practicable because of the limited volume that can be injected. To overcome this limitation, a reaction catalyzed by lysophosphatidylcholine acyltransferase [51,52] could be used to synthesize a true membrane-forming lipid with substrates added from the outside. This enzyme could be incorporated into the membrane of a giant vesicle composed of POPC with acyl-CoA and lysophosphatide added. The vesicle should grow indefinitely as lipid is added to its membrane.

We are presently working on solving the technical limitations to carrying out biosynthetic reactions inside a liposome. An ultimate goal of this research is to construct a minimal cell model in which a liposome contains all of the components for a self-sustaining metabolic cycle and perhaps even the protein biosynthetic apparatus to produce the enzymes for that cycle.

Materials and methods

Isolation and purification of the enzyme G3P-AT

The enzyme G3P-AT was isolated from *Escherichia coli* VL3/plB3-4, which harbours a hybrid plasmid containing the plsB gene encoding G3P-AT and overproduces the enzyme [53,54]. The purification procedure was based on a modification of previously described protocols [55,56] and included a chromatographic separation on a Matrex Gel Green A (Amicon, USA) and on hydroxylapatite. Details of the procedure are given elsewhere [25]. The purified protein was analyzed by SDS polyacrylamide gel electrophoresis (10–15 % Phast gel on a PhastSystem (Pharmacia, Sweden)). A single band was stained with Coomassie blue, corresponding to a relative molecular mass of 83 000. The G3P-AT had a specific activity of 47.3 nmol mg^{-1} min^{-1} when measured using a 3 mM soybean phospholipid vesicular solution in 30 mM Tris (containing 2 mM G3P, 25 μM P-CoA, 4 mM Ca^{2+}, 70 mM NaCl, 1 mg ml^{-1} bovine serum albumin, 7.5 mM β-mercaptoethanol and <0.1 % (w/v) Triton X-100) at pH 7.4 and 30 °C [25]. To determine the kinetic parameters of the G3P-AT-catalyzed reaction, we measured the specific activity as a function of P-CoA concentration (1–10 μM) in excess G3P (2.2 mM) using a 3.6 mM soybean phospholipid vesicular solution in 30 mM Tris (containing 2 mM G3P, 25 μM P-CoA, 4 mM Ca^{2+}, 70 mM NaCl, 1 mg ml^{-1} bovine serum albumin, 7.5 mM β-mercaptoethanol and <0.1 % (w/v) Triton X-100) at pH 7.4 and 30 °C. The enzyme concentration was 25 μg ml^{-1}. The apparent values for $K_m = 12$ μM and $V_{max} = 75$ nmol mg^{-1} min^{-1} were calculated from a Lineweaver–Burk plot.

Micromanipulation of giant vesicles

Microinjection was carried out as previously described [36]. An 8 % solution of the water soluble polysaccharide Ficoll 70 (from Pharmacia, Sweden) was injected into a single POPC giant vesicle (prepared in 1 mM Tris, 1 mM Ca^{2+}, pH 7.4), to prove that the injection was indeed into the interior aqueous volume of the vesicle.

Transformations of single giant vesicles were studied after addition of G3P-AT. Each experiment (Figs 4–7) was carried out at room temperature, and a concentrated G3P-AT solution (250 μg ml^{-1} G3P-AT in 30 mM Tris pH 7.4, 0.1 % (w/v) Triton X-100) was added into, onto or near a particular mixed POPC/P-CoA (9:1; w/w) giant vesicle (prepared in 1 mM Tris, 1 mM G3P, 1 mM Ca^{2+}, pH 7.4). P-CoA forms micelles in water when present at a concentration above the 'critical micellar concentration' (cmc), which is in the low mM range [57]. The P-CoA molecules are located mainly in the membrane of the phospholipid vesicles. Requero *et al.* [44] determined an egg-phosphatidylcholine bilayer/water partition coefficient of 3.8×10^3, by adding [^{14}C]P-CoA and separating the liposomes by centrifugation. On the basis of this figure, which indicates a very high affinity of P-CoA for the lipid phase, we estimate that all of the P-CoA used in formation of the giant vesicles is bound to the vesicle membrane. Our own studies revealed that there exists a critical ratio of POPC:P-CoA of about 4:1, at which no more mixed giant vesicles but only mixed micelles are formed in solution. We therefore prepared mixed POPC/P-CoA giant vesicles starting from a POPC/P-CoA lipid film (9:1; w/w). This experimental setup insured a quantitative incorporation of the activated fatty acid in the membrane. The G3P, a water-soluble molecule, was located in the internal water pool of the vesicles and in the outer bulk, as it was added together with the buffer solution during the swelling of the lipid deposit.

Two types of microneedles were prepared from borosilicate tubes as previously described [36]. The 'type I' microneedle was used when reagents were added externally to the vesicles; the 'type II' microneedle was used for injection into the interior of the vesicles

The characteristics of the two microneedles used were: microneedle 'type I', inner diameter = 135 ± 5 nm, outer diameter = 245 ± 10 nm, injection volume = 590 ± 10 fl (2000 hPa, 1 s); microneedle 'type II', inner diameter = 100 ± 5 nm, outer diameter = 205 ± 6 nm, injection volume = 180 ± 10 fl (2000 hPa, 1 s).

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