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FUSION OF LIPOSOMES WITH PLANAR LIPID BILAYERS

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Summary

The fusion of liposomes with planar lipid bilayers was monitored by two different methods. (a) Liposomes consisting of phospholipids and cholesterol were added to the aqueous phase bathing the cholesterol-deficient planar lipid bilayers in the presence of nystatin. The resulting increase in the planar lipid bilayer's electrical conductance was considered indicative of fusion. (b) Trans-planar lipid bilayer injection of $^{35}SO_4^{2-}$ trapped inside the liposomes.

It is shown by both methods that fusion is specifically dependent on the presence of negatively charged phospholipids both in the liposomes and the planar lipid bilayers and on Ca^{2+} in the aqueous phase of the fusion system.

Introduction

Vesicle-membrane fusion is a common event in cell biology required, for example, for exocytotic discharge of neurotransmitters and cellular secretory products or for uptake by endocytosis. Evidence accumulated in recent years from model phospholipid systems and biological membranes indicate that, although the precise mechanism of fusion is yet unknown, it is undoubtedly mediated by the phospholipids themselves [1]. This presumption is strongly supported by studies on vesicle-vesicle fusion [2—7] and spherical bilayer fusion [8—11]. Therefore, lipid model membranes such as vesicles and planar bilayers are currently being used for studying the mechanism of membrane fusion at the molecular level.

Studies on fusion of phospholipid vesicles with planar bilayer membrane are especially warranted for the following reasons. As both sides of the planar bilayer are chemically and electrically accessible, this system provides a unique geometry for investigation of membrane transport. An understanding of

vesicle-planar bilayer fusion could lead the way towards successful incorporation of biologically functional proteins into planar bilayer, thus permitting thorough electrochemical characterization of these membrane preparations.

The interaction of phospholipid vesicles and biological microsomes with planar bilayer has now been reported several times [12—20], sometimes with some speculations regarding the occurrence of fusion. In most cases some kind of change in the properties of the planar bilayer (interacting with vesicles) has been observed, from which fusion has been inferred. However, there are several mechanism that could explain such changes: (1) transfer of single lipid molecules from vesicles to planar bilayers through the aqueous phase or by direct planar bilayer-vesicle contact, either long lived or transitory. (2) Permanent adhesion of vesicles to planar bilayer. (3) Semi-fusion in which adhering monolayers of vesicles and planar bilayer fuse but the back monolayers do not. (4) True full fusion. (5) Some combination of the above. Existing evidence could account only for mechanisms 1, 2, and 5 but not for 3 or 4. Clearly then, the most rigorous and incontestable evidence for fusion should involve injection of the vesicle content across the planar bilayer as well as the concomitant stochiometric mixing of the vesicle membrane and planar bilayer components. Both kinds of evidence are sought for in the present work.

The cholesterol-dependent nystatin-induced conductivity method

The conceptual basis for this indirect demonstration of fusion is as follows. Nystatin is a channel-former antibiotic which requires cholesterol for its action [21—24]. Thus, when cholesterol-containing liposomes fuse with planar bilayer and mixing of membrane constituents occurs, nystatin present in the aqueous phase can now form ionophoric channels in the planar bilayer and, consequently, planar bilayer electrical conductance should increase. Planar bilayer were formed without cholesterol, subsequently nystatin was added (from a stock solution in dimethylformamide) to a final concentration of 30—100 μg/ml, and a slight increase in the electrical conductance (G_m) was measured. Then liposomes, made up with phospholipids and cholesterol (at 3 : 1 (w/w) ratio), were added to the front chamber and the increase in G_m with time was monitored (Fig. 1). Table I summarizes the results obtained from such experiments with various lipids, either in the liposomes or in the planar bilayer-forming solutions.

Analyzing the results in Table I we arrive at the following conclusions: In the presence of pure neutral phospholipids such as phosphatidylcholine, either in the liposomes, in the planar bilayer, or, evidently, in both of them, no increase in G_m was observed. Using phosphatidylserine instead of phosphatidylcholine, no increase in G_m could be observed unless Ca^{2+} was added (10 mM final concentration). This result is consistent with the fact that Ca^{2+} has been shown to be indispensable for liposome fusion [1,2]. Non-purified soybean phosphatidylcholine, which may contain as much as 15% negatively charged lipids, was nearly as effective as phosphatidylserine. Liposomes prepared from soybean phosphatidylcholine interacted with phosphatidylserine membranes and vice versa (not shown). Thus, liposome-planar bilayer

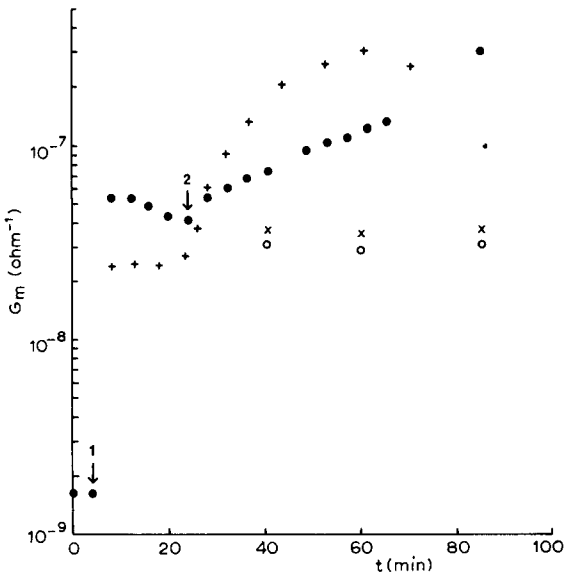

Fig. 1. Typical experiments showing the interaction between cholesterol-containing liposomes and planar bilayer in the presence of nystatin, as measured by membrane conductance G_m. Planar bilayer was formed by the air bubble technique from 1% phosphatidylserine (PS; Sigma) in *n*-decane. The aqueous medium consisted of 100 mM KCl, 10 mM Tris-HCl (pH 7.0), and 5 mM $CaCl_2$. $T = 22 \pm 1°C$. At the indicated time (arrow No. 1), nystatin was added to both sides of the membrane to a final concentration of 10 μg/ml. After G_m stabilized, 100 μl of liposome suspension, prepared from PS and cholesterol (3 : 1, w/w), were added (arrow No. 2) to the aqueous solution to give a final concentration of 50 μg/ml. G_m was monitored continuously. + and ○, liposomes added to front chamber only (○, liposomes without cholesterol); X, control, no liposomes added; ●, liposomes added simultaneously to both front and back chambers.

interaction was demonstrated to occur and to be dependent upon the presence of negatively charged phospholipids and Ca^{2+}. It has been suggested [25] that Ca^{2+} promotes fusion by including isothermal phase transition and phase separation in phospholipid membranes, conditions under which membranes become transiently susceptible to fusion as a result of changes in the molecular packing and creation of new phase boundaries.

At first sight our results do not exclude the possibility of hydrophobic transfer of cholesterol from liposomes (mechanism 1) to the planar bilayer, adhesion of vesicles to the planar bilayer (mechanism 2), or semi-fusion (mechanism 3), without requiring complete fusion. All three mechanisms are consistent with the immediate increase in G_m observed upon addition of liposomes to one side of planar bilayer. However, if mechanism 1 were operating, such an increase in G_m would have required a very rapid flip-flop of cholesterol across the planar bilayer, since cholesterol must be present on both monolayers of the planar bilayer in order to allow the formation of nystatin channels. The rate of cholesterol flip-flop across phospholipid membranes is still in debate, but values for the half-time vary from several hours to several days [26]. Such slow rates could not account for the half-time of 10—30 min for G_m increase in our experiments. The possibility that cholesterol-nystatin complexes (half-pores in the terminology of de Kruijff and Demel [23]) could flip across the

TABLE I

INTERACTION BETWEEN LIPOSOMES AND PLANAR BILAYER MEASURED BY THE NYSTATIN METHOD

Aqueous solutions: 120 mM KCl, 30 mM Tris, pH 7.

All membranes were formed from *n*-decane solutions of the appropriate phospholipid. EL, egg lecithin; chol, cholesterol; SBL, soy bean phosphatidylcholine; PS, phosphatidylserine. Liposomes were prepared from the appropriate phospholipid and buffer by vortexing for 1 min at room temperature; 10 min sonication in bath sonicator at 0°C under N_2; elution through Sephadex G-75 column and occasionally centrifugation (Fig. 2). Liposomes were added to a final concentration of 50—70 μg/ml (60—100 μM lipids). Basic conductivity (initial G_m) was measured on the blackened bilayer at the start of each experiment, at ±10 mV. Ca^{2+} was present only in the aqueous solutions bathing the planar bilayer, and absent from the liposome buffer.

No.	Membrane composition	Liposomes composition (per ml)	Initial G_m (10^{-10} $\Omega^{-1} \cdot cm^{-2}$)	Nystatin final concentration (μg/ml; both sides)	G_m increase due to nystatin (fold)	Further G_m increase after addition of liposomes	10 mM Ca^{2+}	$t_{1/2}$ (min)
1	1% EL *	1 mg EL	100—700	100	1— 2	1 — 2	±	
2	1% EL *	1 mg EL + 1 mg chol	30—400	100	1— 2	1 — 2	±	
3	2% SBL (n = 3)	1 mg PS + 1 mg chol	5— 20	30	1— 2	2 — 4	—	
4	2% SBL (n = 7)	1 mg PS + 1 mg chol	3— 60	30	1— 2	1.5— 7.5	+	10—40
5	1% PS (n = 3)	1 mg PS + 1 mg chol	2— 30	30	1— 2	1 — 2	—	
6	1% PS (n = 9)	1 mg PS + 1 mg chol	2— 30	30	2—400	4 —12	+	10—30

* The nystatin concentration has been raised to 100 μg/ml as no effect on G_m could be observed with 30 μg/ml.

planar bilayer can be excluded in view of the higher polarity of such complexes, compared with that of cholesterol alone. Marty and Finkelstein [27] have provided evidence against half-pore flip-flop. They have shown that nystatin added to one side of the planar bilayer induces a stable cationic conductance, as compared with larger anionic conductance which is observed when the antibiotic is added to both sides of the membrane.

All the above considerations lead us to the conclusion that actual fusion occurs in our system. If it were not fusion but rather hydrophobic exchange, adhesion, or semi-fusion, one would have expected a 2-fold reduction of $t_{1/2}$ for the increase of G_m by half when liposomes were added simultaneously on both sides of the planar bilayer. As a matter of fact, under these conditions, $t_{1/2}$ increased instead of decreasing. Further indirect evidence for fusion is as follows: Addition of large amounts of liposomes on one side of planar bilayer (in the presence of Ca^{2+}) creates an asymmetric distribution of Ca^{2+} across the planar bilayer due to adsorption of Ca^{2+} to the negatively charged liposomes. Such asymmetry as been suggested [25] to induce transient membrane instability and reorganization, which may render the membranes more susceptible to fusion. Concomitantly, Papahadjopoulos and Ohki [28] have shown that Ca^{2+} asymmetry produces large permeabilities in planar bilayer. The possibility that G_m increase resulted from the asymmetry in calcium concentration alone, is ruled out by the fact that addition of equal amounts of liposomes to both sides of the planar bilayer, in the presence of Ca^{2+}, also induced an increase in G_m with a final value comparable to that obtained by addition of the same total amount of liposomes to one side of the planar bilayer only. These results indicate that calcium asymmetry may affect the rate of fusion but not its extent.

In conclusion, the arguments presented above seem to exclude mechanisms 1—3. However, only direct determination of cholesterol flip-flop across planar bilayers, both in the presence and absence of liposomes, either in the presence or absence of nystatin, are required in order for this exclusion to be definitive.

Trans-planar bilayer injection of [^{35}S]sulfate

The only direct and unequivocal proof for vesicles' fusion with planar bilayer can be provided by a demonstration of the injection of intravesicular content across the bilayer. This content should consist of a molecule for which the planar bilayer is totally impermeable. Nevertheless, the quantitative aspects of such experiments impose inherent difficulties, as is exemplified by the following calculation: Suppose the radius of a spherical phospholipid vesicle is 150 Å, its volume would be $1.35 \cdot 10^{-14}$ μl and its area $2.8 \cdot 10^{-11}$ cm^2. Now suppose we have a given label at 1 Ci/ml (carrier free $^{35}SO_4^{2-}$, for example). In order to get 100 dpm across a planar bilayer, $4.5 \cdot 10^{-8}$ μl of vesicular volume will have to be injected across the planar bilayer or the equivalent of $3.4 \cdot 10^6$ vesicles. In other words, for a planar bilayer of 2 mm diameter, 0.3% of the planar bilayer phospholipids will have to be replaced by vesicular phospholipids. For practical purposes, one would require at least 10—100-fold more dpm to cross the planar bilayer. This would require either much higher radioactivities which may be radioactively harmful for the planar bilayer, much

larger vesicles (i.e., larger volume : surface ratios), or much greater extent of fusion (i.e., replacement of a considerable fraction of the planar bilayer phospholipids). This latter alternative may be plausible but would certainly prolong considerably the duration of an experiment, which is detrimental due to the generally poor stability of planar bilayers. Despite the above-mentioned difficulties, we succeeded in developing an experimental procedure whereby we were able to demonstrate trans-planar bilayer injection of the otherwise impermeant sulfate anion, with acceptable reproducibility.

A typical fusion protocol was as follows: A planar bilayer was formed by the air bubble technique from 1% phospholipid solution in decane across a 1—3 mm diameter hole in a Teflon rectangular cuvette positioned in a thermostated brass block. Thinning of the planar bilayer was followed optically, and membrane conductivity was measured by means of Ag/AgCl electrodes, a Keithley 610 electrometer as a voltmeter, a DC power supply and a second electrometer as an ammeter. Prior to the addition of liposomes (Fig. 2), both the front and the back chambers were sampled for radioactivity in order to assess any possible contamination from previous experiments. Then liposomes were added to the front chamber, and both chambers were sampled for radioactivity at fixed time intervals, usually 5—15 min, until the planar bilayers broke. Samples volumes were 50 μl from the front chamber and 1 ml from

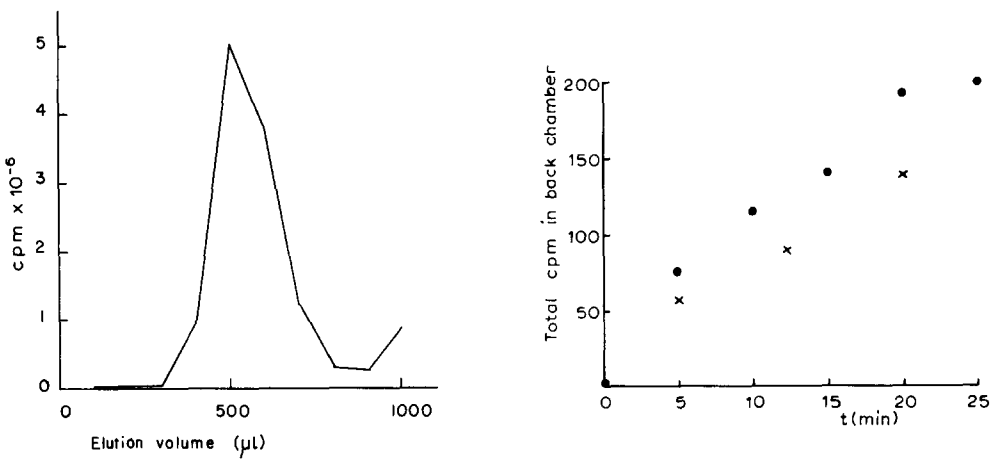

Fig. 2. Elution profile of [^{35}S]sulfate-loaded liposomes from Sephadex G-75 filtration column. Phospholipids dissolved in $CHCl_3$ (total weight 2 mg) were taken to dryness under a current of dry N_2. To this were added 10 μl of stock KCl and 10 μl of stock Tris-HCl solutions and 230 μl (1.25 mCi) of carrier-free [^{35}S]sulfate (Nuclear Research Center, Negev, Israel). The final concentrations of KCl and Tris were 10 mM and 1 mM, respectively. This mixture was vortexed at room temperature and then sonicated in a bath sonicator at maximum output, on ice, for 30 min. In order to discard the extravesicular radioactivity, this dispersion was overlayed on a Sephadex G-75 column (1 × 4 cm), preequilibrated with the appropriate buffer and eluted stepwise. A typical elution profile is shown. For subsequent elution experiments, the eluate was spun at 50 000 × g for 30 min, and the supernatant containing less than 20% of the total eluted counts was discarded.

Fig. 3. Rate of ^{35}S injection across planar bilayer. Planar bilayer was formed from 1% phosphatidylserine phosphatidylethanolamine (3 : 7, w/w) in decane. Liposomes prepared as detailed in the legend to Fig. 2 were added to the front chamber, and the back chamber was sampled for the appearance of ^{35}S. Two typical experiments are shown.

the back chamber out of 7 ml volume of each chamber. The back chamber was replenished with 1 ml unlabeled solution. Radioactivity was measured after addition of toluene/Triton X-100 scintillation fluor in a Packard Tricarb Liquid Scintillation Spectrometer. The amount of label that crossed the membrane was calculated according to the following equation:

$$C = \frac{C_{t+1} - \frac{6}{7}C_t}{\Delta t}$$

where C is the total cpm that crossed the planar bilayer per min; C_{t+1} is the total cpm present in the back chamber at the sampling time; C_t is the total cpm present in the back chamber at the previous sampling time, and Δt is the time lapse between the two sampling periods in minutes (see Fig. 3). Typical values of C were 5—15 cpm, while the total radioactivity in the front chamber was $2 \cdot 10^8$ cpm. This would mean that $1.5 \cdot 10^6$ vesicles with a mean equivalent radius of 150 Å fused into the planar bilayer, contributing about 0.1% of the total planar bilayer lipids every minute. To the best of our knowledge this is the first time that trans-planar bilayer injection of impermeant vesicular contents has been unequivocally demonstrated. No injection of radioactivity could be detected when either phosphatidylcholine vesicles were used or Ca^{2+} was absent from the aqueous medium.

Unfortunately, only about 50% success was achieved in these experiments. Lack of fusion could not be related to any specific factor. This, together with problems such as planar bilayer instability, further increased by the fusion process, and decontamination of chamber and electrodes from radioactivity, will probably prevent a wider use of this method in the future, and one must await for the development of more dependable techniques.

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