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RAPID TRANSBILAYER MOVEMENT OF PHOSPHOLIPIDS INDUCED BY AN ASYMMETRICAL PERTURBATION OF THE BILAYER

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Summary

Phospholipase D is used to convert egg phosphatidylcholine to phosphatidic acid in unilamellar vesicles. The transbilayer distribution of both lipids is determined by ^{31}P NMR using paramagnetic ions. Phosphatidic acid formed in the outer monolayer is translocated to the inner monolayer with a halftime of 30—40 min or less. This is accompanied by an equally fast movement of part of the phosphatidylcholine from the inner to the outer monolayer. During these fast transbilayer movements the barrier properties of the vesicle bilayer are maintained.

Introduction

It is now well documented that several biological membranes have an asymmetrical lipid distribution [1]. To understand this compositional asymmetry it is necessary to know the factors determining the rate of transmembrane movement of the lipids. The flip-flop of lipid molecules in single bilayer vesicles under equilibrium conditions is slow, with halftimes in the order of days [2—9]. In contrast, in biological membranes this process can be much faster, with halftimes in the order of minutes (bacterial membranes; ref. 10), hours (erythrocytes; refs. 11—13) and days (influenza virus; refs. 14,15). An obvious difference between the model and biological membranes is the presence of protein molecules in the latter. That proteins can dramatically increase the rate of phospholipid flip-flop was demonstrated recently for glycophorin, a membrane-spanning protein from the human erythrocyte membrane which, when incorporated in phosphatidylcholine bilayers, increased the rate of transbilayer movement of lysophosphatidylcholine by two orders of magnitude [16]. Furthermore, most biological membranes are highly dynamic structures in which lipid molecules are continuously renewed by metabolic processes or exchange.

At the sites of synthesis, degradation or exchange, transient states of local disorder must exist. For instance, the interfacial characteristics of precursor and product molecules are often different, causing a local inbalance in surface pressure. Such an imbalance in monolayer pressure could be a driving force for transbilayer movements of phospholipids, as was demonstrated recently in phosphatidylcholine vesicles [9]. To explore this possibility further we investigate in this study the phospholipase D-catalyzed formation of phosphatidic acid in sonicated phosphatidylcholine vesicles, where the transbilayer distribution of the lipids was measured by ^{31}P NMR. It is shown that the phosphatidic acid molecules enzymatically formed in the outer monolayer are rapidly translocated to the inner monolayer without loss of bilayer integrity.

Materials

Phosphatidylcholine was isolated from hen eggs according to standard procedures and was partially converted to phosphatidic acid with phospholipase D. (*N-methyl*-^{14}C)-labelled egg phosphatidylcholine was the kind gift of K.W.A. Wirtz. Phospholipase D was prepared from Savoy cabbage according to Davidson and Long [17] and was partially purified as described by Dawson and Hemmington [18]. The final preparation was stored in 1-ml aliquots at −20°C in 75 mM NaCl/25 mM sodium acetate/acetic acid, pH 5.5, buffer. Prior to use, the phospholipase D solution was centrifuged at 0°C for 30 min at 45 000 × *g* to remove some denaturated protein. The supernatant had an activity of 11—24 I.E.U./ml (assayed according to Comfurius and Zwaal; ref. 19) and a protein concentration [20] of 10—17 mg/ml. $Nd(NO_3)_3$ was obtained from Koch-Light (Colnbrook), Bucks, U.K.). All other chemicals were Analytical Grade.

Methods

Preparation of vesicles

Single bilayer vesicles were prepared in the indicated buffer by sonication at 0°C under nitrogen with a tip sonicator, as described before [21].

Action of phospholipase D on phosphatidylcholine vesicles

Phospholipase D is reported to require high concentrations of Ca^{2+} for its optimal activity in ether/water systems [18,19]. In view of the possibility of Ca^{2+}-induced vesicles aggregation or fusion during phosphatidic acid formation, pilot experiments were carried out to determine the minimal amount of Ca^{2+} needed in the incubation medium to give sufficiently high phosphatidic acid formation in the vesicles. These experiments demonstrated that in the absence of any added Ca^{2+}, and even in the presence of 0.2 mM EDTA, the phospholipase D preparation showed a considerable activity (60% of the activity in the presence of 50 mM Ca^{2+}) towards the phosphatidylcholine vesicles.

Three different incubation conditions were used.

a. Phosphatidylcholine vesicles (174 μmol in 3 ml 0.2 mM EDTA, 25 mM Tris/acetic acid, pH 5.5, containing 20% ^2H_2O) were incubated at 25°C with phospholipase D. The formation of phosphatidic acid was followed by ^{31}P NMR and after an appropriate time was stopped by cooling the mixture rapidly

to 0°C. This was followed by a 10 min centrifugation at 37 500 × *g* at 0°C to remove a protein precipitate formed during the incubation. The total intensity and linewidth of the narrow ^{31}P NMR signals of the vesicles remained constant when no more than 50 mol% of the phosphatidylcholine was converted to phosphatidic acid, demonstrating that no vesicle aggregation or fusion occurred under these conditions. To isolate the vesicles from the incubation mixture the supernatant was applied on a Sepharose 4B column (22 × 2 cm) equipped with a LKB 3300 Uvicord II registering the absorbance of the effluent at 280 nm. The column was eluted with the same buffer used for the incubation at a rate of 1.5 ml/min (Fig. 1). Phosphate analysis [22] revealed that 5—10% of the phospholipid was eluted in the void volume of the column (50 ml) as larger structures already present in the vesicle solution before the phospholipase D action. The unilamellar vesicles eluted between 70 and 120 ml. The peak at 175 ml indicated the hold-up volume of the column. The vesicle fractions between the arrows were pooled (total 20 ml) and contained 55—68% of the phospholipid. Since these vesicles were too dilute to study with ^{31}P NMR they were concentrated by vacuum dialysis using collodion bags SM 13200 (Sartorius). Using three collodion bags at the same time it was possible to concentrate the vesicle solution to a final volume of 4—6 ml in 60—90 min with a recovery of 66—78%. Centrifugation of these vesicles for 60 min at 37 500 × *g* sedimented no phospholipid phosphorus, demonstrating that no significant aggregation or fusion had occurred. 2 ml of the ^2H_2O analog of the buffer was added to the vesicle solution prior to analysis by ^{31}P NMR.

b. Phosphatidylcholine vesicles (175 μmol in 6 ml 0.2 mM EDTA/25 mM Tris/acetic acid, pH 5.5, containing 20% ^2H_2O) were incubated at 25°C with phospholipase D. After certain times, 1.2 ml aliquots were centrifuged at 0°C for 5 min at 37 500 × *g* and subsequently the supernatant was analysed by ^{31}P NMR.

c. Vesicles were prepared from 65 μmol phosphatidylcholine in 2.0 ml 0.2 mM EDTA/25 mM Tris/acetic, pH 5.5, buffer to which 30 μl of 100 mM paramagnetic shift reagent $Nd(NO_3)_3$ were added. The vesicles were dialysed at 0°C overnight against 500 ml 0.2 mM EDTA/25 mM Tris/acetic acid, pH 5.5, to remove the extra-vesicular Nd^{3+} ions. Phospholipase D was added at 25°C and the reaction was followed by ^{31}P NMR.

In most experiments [^{14}C]choline-labelled phosphatidylcholine was present in the vesicles to determine the extent of phosphatidic acid formation. 100-μl aliquots of the incubation mixture were extracted according to Bligh and Dyer

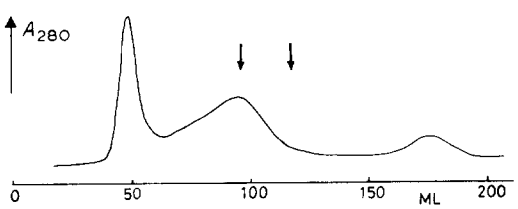

Fig. 1. Sepharose 4B column chromatography of phosphatidylcholine vesicles after phospholipase D incubation. For explanation of the arrows, see text.

[23]. The [^{14}C]choline product of the phospholipase D action was quantified in the aqueous phase by liquid scintillation counting. In some experiments the extent of the reaction was also characterized (with identical results) by a lipid determination exmploying phosphate analysis after thin layer chromatographic separation on silica gel H eluted with chloroform/methanol/water/ammonia, 90 : 54 : 5.5 : 5.5.

^{31}P NMR

36.5 MHz ^{31}P NMR spectra were recorded at 25°C on a Bruker WH-90 spectrometer. Typically, 500—1000 transients were recorded with a 1.7-s interpulse time using 90° pulses under conditions of broad band proton decoupling. The transbilayer distribution of the phospholipids was measured by broadening the signal from the outer monolayer beyond detection by the addition of 20 or 50 μl $CoCl_2$ (100 mM) [24] in the case of incubations of the type (a) or (b), respectively. By comparing the intensity of the remaining signal with the signal in the absence of Co^{2+} the transbilayer distribution of the phospholipids could be measured. The addition of Co^{2+} did not cause vesicle aggregation or fusion because (1) rechromatography of the vesicle on Sepharose 4B showed the same size pattern and (2) addition of an identical amount of Ca^{2+} did not cause any change in linewidth or signal intensity. In incubations of type c, Nd^{3+} is enclosed in the vesicles, causing a downfield shift of the resonance from the inner monolayer [21]. The appearance of an unshifted phosphatidic acid peak in the spectrum was followed with respect to time after the addition of phospholipase D to the vesicles. Some spectra were also recorded using gated decoupling with 5 T_1 waiting times between the pulses, and yielded similar transbilayer distributions of the phospholipids. Peak intensities were measured with respect to external triphenylphosphine by computer integration or by cutting out and weighing copies of the various peaks. Unless otherwise stated 90—100% of the phosphate nuclei of the phospholipids present in the sample were observed in the spectra. The error in the determination of the intensity of the phosphatidylcholine peak and the smaller phosphatidic acid peak was estimated as 5 and 10%, respectively. Chemical shifts are reported in ppm downfield from external triphenylphosphine.

Results

Phospholipase D action on phosphatidylcholine vesicles followed by isolation of the vesicles and subsequent ^{31}P-NMR analysis

The aim of these experiments was to introduce by phospholipase D action an amount of phosphatidic acid in phosphatidylcholine vesicles and to determine its transbilayer distribution by ^{31}P NMR after isolation of the vesicles from the incubation mixture. The results of such an experiment are shown in Fig. 2 and Table I. Egg phosphatidylcholine vesicles have a ^{31}P NMR spectrum as shown in Fig. 2A with a shoulder at the high field side which arises from a 0.10 ppm difference in chemical shift of the resonance from the outer and inner monolayer [21,24]. After phospholipase D action, chemical analysis revealed that 17% of phosphatidic acid was formed in the vesicles (Table I). ^{31}P NMR analysis of the vesicle solution (due to the incubation, gel chromatography and vesicle concen-

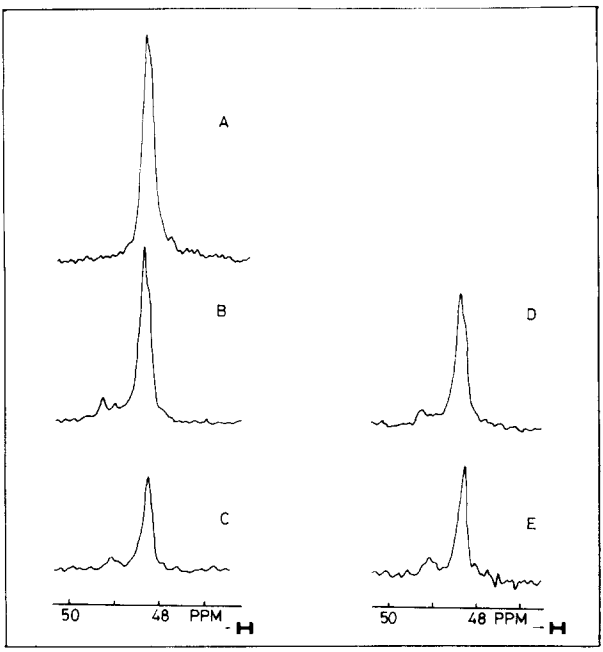

Fig. 2. 36.4 MHz ^{31}P NMR spectra of egg phosphatidylcholine vesicles after incubation with phospholipase D. Incubation conditions (a) as described in Methods. The vesicles were incubated at 25°C with 100 μl phospholipase D (2.4 I.E.U.; 0.93 mg protein) for 90 min which results in the formation of 17% phosphatidic acid (analysed by thin layer chromatography). The vesicles were passed over a Sepharose 4B column and were subsequently concentrated for ^{31}P NMR analysis. A, Control phosphatidylcholine vesicles; B, vesicles 7 h after the addition of phospholipase D; C, as B, $+Co^{2+}$; D, as B, after resonication; E, as D, $+Co^{2+}$.

trations these measurements were made 7 h after the addition of phospholipase D) demonstrated the presence of 18% of the intensity in two signals at 49.3 and 49.1 ppm, respectively (Fig. 2B), which are the chemical shifts of the signals of phosphatidic acid in the outer and inner monolayer of phosphatidic acid/phosphatidylcholine vesicles under these conditions. Addition of Co^{2+} broadens the resonance of the phospholipids in the outer monolayer beyond detection and reveals the spectrum of the inner monolayer in which 36% of the

TABLE I

TRANSBILAYER DISTRIBUTION OF PHOSPHATIDIC ACID IN PHOSPHATIDYLCHOLINE VESICLES AFTER THE ACTION OF PHOSPHOLIPASE D

Time (h) after incubation with phospholipase D	Percent phosphatidic acid formed *	Percent phosphatidic acid inner monolayer **	Percent of total phospholipid in monolayer
7	18	24	36
13.5	19	22	33
24	18	21	35
Vesicles resonicated	18	21	34

* Determined by NMR.

** Expressed as a percentage of the total phospholipids in the inner monolayer.

total phospholipids are present (Fig. 2C, Table I). 24% of the intensity of the inner monolayer spectra is in a phosphatidic acid peak at 49.1 ppm, whereas the phosphatidic acid peak at 49.3 ppm has disappeared. This unambiguously demonstrates that phosphatidic acid is present in the inner monolayer of these vesicles. Further incubation of the vesicles at 25°C for various times did not alter the transbilayer distribution of the lipids (Table I). The equilibrium distribution of phsphatidic acid in the vesicles was determined by resonication of an aliquot of the vesicle solution (Fig. 2D,E and Table I). It can be concluded that the transbilayer distribution of the resonicated lipids is very similar to that found 7 h after the addition of phospholipase D, demonstrating that in that time the phosphatidic acid formed in the outer monolayer by phospholipase D has redistributed across the bilayer to reach an equilibrium situation. Similar results were obtained in experiments where phospholipase D produced 12 and 28% of phosphatidic acid in the vesicle. The transbilayer distribution of phosphatidic acid at the first time of the NMR measurement (5—6 h after the addition of the enzyme) was similar to the distribution in the resonicated vesicles and did not change upon further incubation. High amounts of phospholipase D resulted in the rapid formation of up to 70% phosphatidic acid in the vesicle but this was always accompanied by vesicle aggregation or fusion as evidence by decreased intensities and increased linewidths of the ^{31}P NMR signals. Moreover, the 'vesicles' formed eluted in the void volume of the Sepharose 4B column.

^{31}P NMR analysis of the vesicles during phosphatidic acid formation

From the above experiments it can be concluded that the halftime of the rate of transbilayer distribution of phosphatidic acid is less than 6—7 h. Therefore, in order to be able to study the rate of this process, experiments were carried out in which the transbilayer distribution of the lipids was measured during phosphatidic acid formation. This was done in two ways. Firstly, aliquots of the vesicle solution were analysed by ^{31}P NMR during phospholipase D action using Co^{2+} to obtain the spectrum of the inside monolayer only. Fig. 3 shows that the rate of appearance of phosphatidic acid in the inner monolayer is similar to the rate of phosphatidic acid formation by phospholipase D. Furthermore, the number of phospholipid molecules in the inner monolayer decreases slightly. Freeze-etch electron microscopy of the vesicles 5 h after the addition of phospholipase D revealed no difference in the vesicle sizes as compared to vesicles incubated without the enzyme. The total intensity of the ^{31}P NMR signal of the vesicles remained constant during phospholipase D action. In the second approach, 1.5 mM Nd^{3+} was enclosed in the vesicles, causing a 9 ppm downfield shift of the inside resonance (see, for instance, ref. 21). From the appearance of an unshifted phosphatidic acid signal at 49.1 ppm the amount of phosphatidic acid in the outer monolayer could be measured. Since the outside-inside distribution of the total phospholipids and the total amount of phosphatidic formed was also measured, the inner monolayer composition could be calculated. The rate of appearance of phosphatidic acid in the inner monolayer parallels the rate of phosphatidic acid formation in the vesicle (Fig. 4). In these experiments the outside-inside distribution of the total phospholipids remained constant during the inward movement of phosphatidic

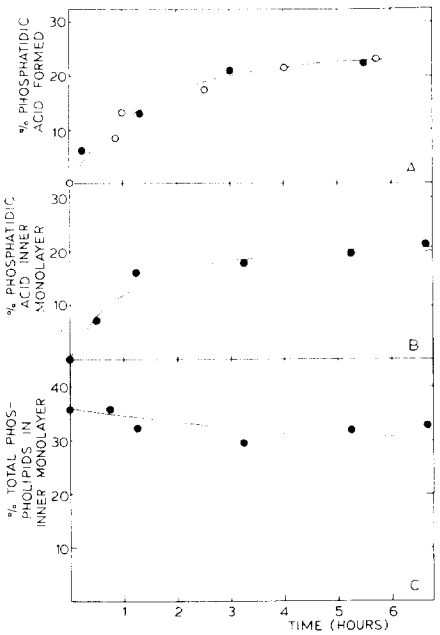

Fig. 3. Transbilayer distribution of phosphatidic acid formed during phospholipase D action. A, Percent of phosphatidic acid formed in the vesicle as determined by radioactivity (●) and ^{31}P NMR (○); B, percent of phosphatidic acid in inner monolayer, expressed as a percentage of the total phospholipids in the inner monolayer; C, percent of total phospholipids in the inner monolayer. Incubation conditions described in Methods (b). 75 μl of phospholipase D (1.8 I.E.U., 0.70 mg protein was added to the vesicles at 25° C and after the indicated times the amount of phosphatidic acid formed and the transbilayer distribution of the lipids was determined using Co^{2+} to resolve the resonance of the phospholipids in the inner monolayer.

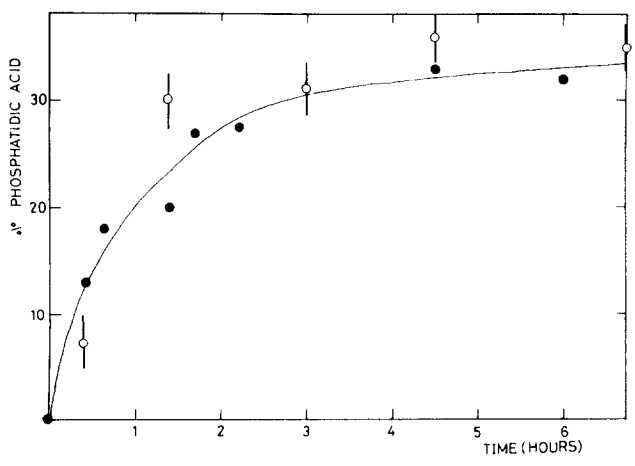

Fig. 4. Transbilayer distribution of phosphatidic acid during phospholipase D action. Incubation conditions described in the experimental section (c). 25 μl of phospholipase D (0.6 I.E.U., 0.23 mg protein) was added at 25°C to phosphatidylcholine vesicles in which $Nd(NO_3)_3$ was trapped. The percentage of phosphatidic acid in the inner monolayer (○) was calculated from the total amount of phosphatidic acid in the vesicle (●), the outside-inside distribution of the total phospholipids and the composition of the outer monolayer as determined by ^{31}P NMR. The error bars reflect the maximum error possible in determining the amount of phosphatidic acid in the inner monolayer from the integral of the ^{31}P NMR signal.

TABLE II

BILAYER INTEGRITY DURING PHOSPHOLIPASE D ACTION

Experimental details are as in the legend of Fig. 3 except that the vesicles were prepared in 400 mM Na_3PO_4/25 mM Tris/acetic acid pH 5.5 where extra-vesicular phosphate was removed by overnight dialysis at 4°C against 1 l 500 mM NaCl/25 mM Tris/acetic acid, pH 5.5. The amount of trapped phosphate was measured after the addition of 50 μl 100 mM $CoCl_2$, which eliminates the ^{31}P NMR signal from the extra-vesicular phosphate and does not affect the signal from the phosphate enclosed in the vesicles.

	Amount of phosphate trapped in the vesicles (mol phosphate/mol phosphatidylcholine)
Phosphatidylcholine vesicles after dialysis	7.0
incubated at 30°C for 6 h	6.2
incubated at 30°C for 6 h with phospholipase D	6.3

acid. The shifted inside resonance moved slowly upfield during phosphatidic acid formation. This most probably caused by a slow outward leak of Nd^{3+}. It was found in control experiments that, whereas pure phosphatidylcholine vesicles are extremely impermeable to Nd^{3+}, incorporation of small amounts of phosphatidic acid (5—15 mol%) made the bilayer much more permeable to Nd^{3+} as indicated by a downfield shift of the inside resonance with time after the addition of Nd^{3+} to the vesicles.

Integrity of the vesicle bilayer during fast transbilayer movement of phosphatidic acid

To ascertain whether during phosphatidic acid movement to the inner monolayer the permeability barrier of the vesicle was maintained, phosphate was enclosed in the vesicle and the amount of trapped phosphate was measured by ^{31}P NMR after the addition of phospholipase D. In the control incubation without phospholipase D 11% of the phosphate had leaked out of the vesicles in 6 h (Table II). A very similar amount of leakage was found for the vesicles incubated with phospholipase D in which the phosphatidic acid formed (total 20 mol%) was translocated to the inner monolayer similarly to the experiment shown in Fig. 3.

Discussion

The action of phospholipase D on unilamellar egg phosphatidylcholine vesicles results in the formation of phosphatidic acid in the outer monolayer which is rapidly translocated to the inner monolayer without affecting the barrier properties of the bilayer. This process does not result in an increased number of lipid molecules in the inner monolayer (see, for instance, Fig. 3). Therefore, an approximately equal number of phosphatidylcholine molecules have to move from the inner to the outer monolayer at a rate which is comparable to the rate of appearance of phosphatidic acid in the inner monolayer.

Since the inward movement paralleled the phosphatidic acid formation in the vesicles, only an upper limit of the rate of this process can be obtained. From several experiments a maximal value of 30—40 min was obtained. This

value is at least two orders of magnitude faster than the rate of transbilayer movement of lipids in protein-free bilayers under equilibrium vesicles [2—9].

Since phospholipase D is a water-soluble enzyme which acts on the polar head groups of the lipids on the exterior of the bilayer, it is unlikely that the enzyme itself (by penetrating the bilayer, for instance) is responsible for the phosphatidic acid translocation. Moreover, the counterflow of phosphatidylcholine molecules occurs from the opposite side, where phospholipase D is not present.

We propose the following mechanism. In the outer monolayer neutral phosphatidylcholine molecules are replaced by negatively-charged phosphatidic acid molecules which results in an expansion of the outer monolayer with respect to the inner monolayer and the formation of a concentration gradient of phosphatidic acid across the bilayer. To dissipate the resulting tension in the bilayer and to reduce the concentration gradient, phosphatidic acid molecules move to the inner monolayer. However, this leads to an increase in number of molecules in this layer which results in an outward movement of phosphatidylcholine molecules until finally the bilayer reaches a new equilibrium configuration in which each lipid is distributed in a manner similar to that in the resonicated vesicle. When the rate of phosphatidic acid formation in the outer monolayer is very fast (in the case of a high enzyme concentration) the phosphatidic acid cannot move fast enough to the inner monolayer creating an increasingly larger imbalance in surface pressure between the two monolayers, leading to vesicle disruption. By carrying out the phospholipase D incubations on the vesicles in the presence of 30% (v/v) glycerol, phosphotidylglycerol was formed which also rapidly translocated to the inner monolayer (de Kruijff, B and Baken, P., unpublished observations). The above process resembles the relatively fast inward movement of dioleoylphosphatidylcholine introduced into the outer monolayer of dimyristoylphosphatidylcholine vesicles by an exchange protein [9]. The halftime of 8 h reported for this movement is, however, much longer than the value for the rate of the phosphatidic acid translocation. Possibly this is caused by the larger stress on the bilayer imposed by the replacement of one phosphatidylcholine by a phosphatic acid molecule as compared to the replacement of one dimyristoylphosphatidylcholine molecule by a dioleoylphosphatidylcholine molecule.

These experiments suggest that lipids newly synthesized on one side of a biomembrane may be quickly redistributed across the membrane as a result of differences in surface pressure between the two monolayers. It is also possible, of course, that such processes may occur as a result of protein-mediated mechanism.

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