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PHOSPHOLIPID TRANSFER BETWEEN VESICLES

DEPENDENCE ON PRESENCE OF CYTOCHROME *P*-450 AND PHOSPHATIDYLCHOLINE-PHOSPHATIDYLETHANOLAMINE RATIO

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The rate of transfer of spin-labeled phospholipid from donor vesicles of sonicated 1-acyl-2-(10-doxylstearoyl)-*sn*-glycero-3-phosphocholine to other vesicles was determined as a function of content of cytochrome *P*-450 and the phosphatidylcholine/phosphatidylethanolamine ratio in the acceptor vesicles. The transfer rate was measured as an increase in intensity that resulted from a decrease in the line width in the EPR spectrum of the spin-labeled phospholipids as they were transferred to the nonspin-labeled acceptor vesicles. A lower transfer rate was observed for acceptor vesicles of pure egg phosphatidylcholine vesicles than for vesicles of a mixture of phosphatidylcholine and phosphatidylethanolamine. The presence of cytochrome *P*-450 in the acceptor vesicles further increased the transfer rate. Those alterations in the mole ratios of the protein and the two phospholipids that made the bilayer of the reconstituted vesicles more like the membrane of the endoplasmic reticulum resulted in an increase in phospholipid-transfer rate. The mole ratios of components that produce high phospholipid-transfer rates were similar to those that in an earlier study produced a ^{31}P-NMR spectrum characteristic of a nonbilayer phase. These findings suggest that, in the membrane of the endoplasmic reticulum, phospholipid exchange may be an important element in function and interaction with other intracellular organelles.

Introduction

The endoplasmic reticulum of liver cells contains phosphatidylcholine (PC) and phosphatidylethanolamine (PE) at an about 2:1 mole ratio [1] with a weight ratio of protein to phospholipid of 1.25 [2] even after removal of external and adsorbed proteins and ribosomes. The coexistence of both phospholipids is apparently important for optimum lateral organization of the membrane. In reconstitution experiments we have shown that use of a 2:1 mole ratio of PC to PE allows formation of stable, nonaggregated vesicles that contain a 2:1 phospholipid/protein weight ratio, whereas use of pure PC allows a minimum phospholipid/protein ratio of only 5:1 in reconstituted cytochrome *P*-450 vesicles. The PC/PE ratio has been shown to affect the activity of reconstituted K^+-ATPase [3], the function of reconstituted rhodopsin [4] and the activity of adenylate cyclase [5]. Also, it has been found that microsomal glucose-6-phosphate phosphohydrolase activity decreases when the PE content is decreased [6], whereas alterations in phosphatidylinositol or PC content produced little or no change in enzymatic activity. Moreover, recent ^{31}P-NMR experiments have

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; spin-labeled PC, 1-acyl-2-(10-doxylstearoyl)-*sn*-glycero-3-phosphocholine.

shown that incorporation of cytochrome *P*-450 into PC/PE (2:1, w/w) vesicles results in a change in the chemical shift anisotropy of the NMR spectrum [7]. This large change in the chemical shift anisotropy is not observed in the case of PC/PE vesicles without cytochrome *P*-450 unless PE content exceeds 70 mol% at 20°C. Both the reconstitution and the ^{31}P-NMR results suggest that the state of the phospholipids in PC/PE vesicles containing cytochrome *P*-450 may be very different from that in pure phospholipid vesicles.

In order to characterize further PC vesicle preparations that contain cytochrome *P*-450 or PE or both, we measured the rate of phospholipid exchange between vesicles. This technique provides further insight into the dynamics of the two-dimensional three-component vesicle system. We determined the rate of transfer of the spin-labeled phospholipid, 1-acyl-2-(10-doxylstearoyl)-*sn*-glycero-3-phosphocholine (spin-labeled PC), from vesicles containing a high percentage of this spin-labeled phospholipid to acceptor vesicles of various compositions. The rate of transfer was dependent on the lipid composition and on the presence of protein in the acceptor vesicles. Vesicles of high protein/lipid ratio, which could be prepared by the use of 33 mol% PE in PC, gave the highest rate of phospholipid transfer and also produced a ^{31}P-NMR spectrum with a chemical shift anisotropy that is not typical of spectra of pure PC bilayers. The appearance of these properties is discussed in terms of a structural model of membrane properties of such lipid mixtures in the presence of a protein.

Methods

Spin-labeled PC was prepared by adding 10-doxylstearic acid to egg lysophosphatidylcholine with the anhydride technique described by Hubbell and McConnell [8]. A suspension of spin-labeled vesicles was prepared by sonicating a homogeneous mixture of 0.1 mg spin-labeled PC and 0.1 mg egg PC in 1 ml of 20 mM potassium phosphate buffer, pH 7.5, with a microtip for 15 min under an N_2 atmosphere in an ice bath. Suspensions of acceptor vesicles were prepared in the same manner by adding 1 ml of the same buffer to a film of 20 mg total phospholipid in which the mol% of egg PE varied from 0 to 90% with the balance being egg PC. Cytochrome *P*-450 LM_2 was prepared from liver microsomes of phenobarbital-treated rabbits [9,10]. Suspensions of vesicles containing cytochrome *P*-450 LM_2 reconstituted with phospholipids were prepared by a slow cholate dialysis technique as previously described [11]. Briefly, the egg PC vesicles were prepared by suspending 10 mg egg PC [12] (prepared under an N_2 atmosphere) in a solution of 2% (w/w) sodium cholate, adding 1 mg cytochrome *P*-450 LM_2 in 0.1 M potassium phosphate buffer, pH 7.5, containing 20% (v/v) glycerol. The mixture, with a final protein concentration of 1 mg/ml and 2% sodium cholate, was allowed to stand at 4°C for 16 h and then dialyzed against the 20 mM potassium phosphate/glycerol buffer for 4 days with two changes of buffer daily to remove sodium cholate. All buffers were N_2 saturated to prevent lipid peroxidation that results in conversion of the doxyl spin labels to nonparamagnetic species. The resulting vesicles have been fully characterized as to diameter (400–700 Å), homogeneity of size, metabolic activity, protein/lipid ratio, and recovery of initial phospholipids and protein in the reconstituted vesicle. It was verified by electron microscopy that the size distribution of the various vesicles preparations was similar [11]. The egg PC/PE (2:1, w/w) vesicles containing cytochrome *P*-450 were prepared in an identical manner except that 5 mg cytochrome *P*-450 were used instead of 1 mg in the previous preparation.

For determination of the phospholipid-exchange rates, 50-μl (10 μg total phospholipid) aliquots of the suspension of spin-labeled PC vesicles were added to 50-μl (1 mg phospholipid) samples of the suspensions of unlabeled phospholipid acceptor vesicles that were varied in composition. After mixing, the ratio of spin-labeled phospholipid to unlabeled phospholipid was 1:200. A 30 μl aliquot of each mixture was placed in a quartz EPR sample cell and then the intensity increase of the center line was continuously measured on a Varian E-104A EPR spectrometer operating at 9.5 GHz at 25°C by locking the magnetic field. The spectrometer settings were microwave power 5 mW, modulation amplitude 1 G, scan range 100 G, and filter time constant 0.25 s.

The reaction amplitude after completion of the

phospholipid-exchange reaction is defined as 100% phospholipid exchange. It was identical in all samples. A small correction had to be applied for the PE-containing samples, because the small amount of lipoperoxides resulted in a measurable decrease in EPR intensity due to destruction of the paramagnetic species during the course of the experiment.

Results

The EPR spectrum of a suspension of vesicles that contains 50 mol% spin-labeled PC is very broad (Fig. 1, dashed line) due to spin-spin exchange broadening [13]. As the spin-labeled PCs are transferred to vesicles of nonspin-labeled phospholipids, the line width of the EPR spectrum progressively decreases (dotted line), the intensity increases, and the spectrum becomes identical to that of spin-labeled phospholipid in a phospholipid membrane containing less than 1 mol% of the spin label (solid line). The increasing intensity of the center line was continuously measured in order to obtain the phospholipid-transfer rates. The time course of the increase in the sharp hyperfine components at 37°C is seen in Fig. 2. A mixture of spin-labeled PC vesicles with vesicles containing cytochrome *P*-450 reconstituted in egg PC/PE (2:1 mol/mol) at a lipid/protein ratio of 2:1 (w/w) resulted in a rapid dilution of the spin label (upper trace) and in the appearance of a single, sharp EPR signal characteristic of anisotropic motion in phospholipid bilayers [8]. The lower trace

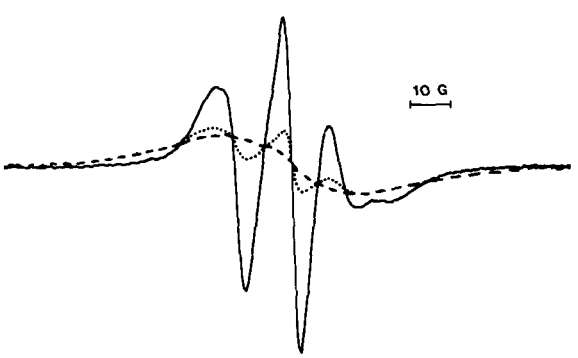

Fig. 1. EPR spectra of spin-labeled PC at 50 mol% in egg PC (— — —), 2 min after addition of a 100-fold excess of vesicles of egg PC/PE (2:1, w/w) (·····) at 30°C and after 5 h at 30°C (———).

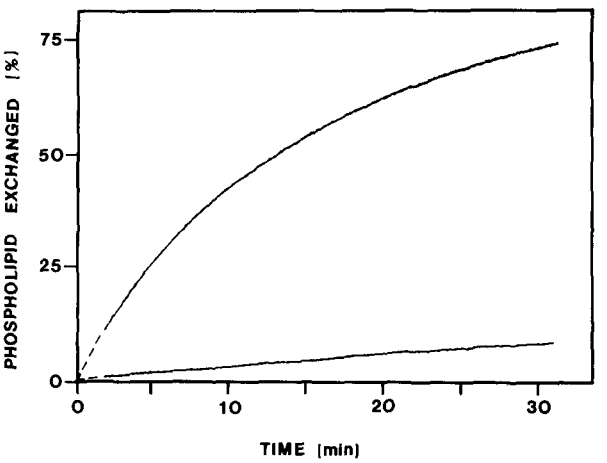

Fig. 2. Time course of the increase in intensity at the center hyperfine component measured at 37°C in 0.02 M potassium phosphate buffer, pH 7.5, containing 20% glycerol. The upper trace was obtained when cytochrome *P*-450 PC/PE vesicles (lipid/protein ratio 2:1, w/w; PC/PE 2:1, w/w) were added to spin-labeled PC vesicles. The lower trace was obtained when cytochrome *P*-450 PC vesicles (lipid/protein ratio 10:1, w/w) were added to the same amount of spin-labeled PC vesicles. The ratio of labeled to unlabeled phospholipid was 1:200 (w/w) after mixing.

in Fig. 2 resulted from phospholipid transfer from spin-labeled PC vesicles to vesicles containing cytochrome *P*-450 reconstituted with only egg PC at a lipid/protein ratio of 10:1 (w/w). It can be seen that, at the same total phospholipid concentration, the extent of phospholipid transfer is about one order of magnitude higher with the PE-containing vesicles during the first half-hour time period. When the phospholipid-transfer measurements in the two systems were repeated at 22°C, the transfer rate in each system was 7-times slower. When spin-labeled PC vesicles were mixed with liver microsomes from rabbits at 37°C, it was found that phospholipid transfer occurred at a rate similar to that with cytochrome *P*-450 PC/PE vesicles containing an identical amount of total phospholipid.

The two cytochrome *P*-450-containing vesicle systems that were used differed in two respects from each other: in the lipid/protein ratio and in the presence of PE. The reconstituted system of cytochrome *P*-450 in PC/PE (lipid/protein ratio 2:1, w/w) was chosen because of its similarity to microsomes. It could be prepared easily, whereas

only lipid-protein aggregates but no vesicles were formed when vesicle reconstitutions were attempted of cytochrome *P*-450 with only egg PC at low lipid/protein ratios such as 2:1 (w/w) [11]. Therefore, it was necessary to reconstitute cytochrome *P*-450 with egg PC at a lipid/protein ratio of 10:1. The observed higher rate of phospholipid transfer to vesicles of cytochrome *P*-450 reconstituted with PC/PE could be due to the presence of PE, the higher protein/lipid ratio, or both. Rates of phospholipid transfer to protein-free vesicles formed from either PC/PE or PC were measured to examine the effect of PE. Small unilamellar sonicated vesicles were mixed with vesicles that contained 50% spin-labeled PC and 50% egg PC. Fig. 3 shows the dependence of the half-times of the measured transfer rates on the PE/PC mole ratio at 30°C. It can be seen that the transfer occurred at about the same rate when up to 50 mol% PE was mixed in PC. At 60 mol% PE, the transfer rate became about 2.5-fold faster. No further significant change was observed at higher PE/PC ratios. The transfer rates obtained from these sonicated vesicles are higher than those obtained from vesicles formed by cholate dialysis. At the same total phospholipid concentration, the vesicle concentration is higher in the sonicated preparation because of their smaller size.

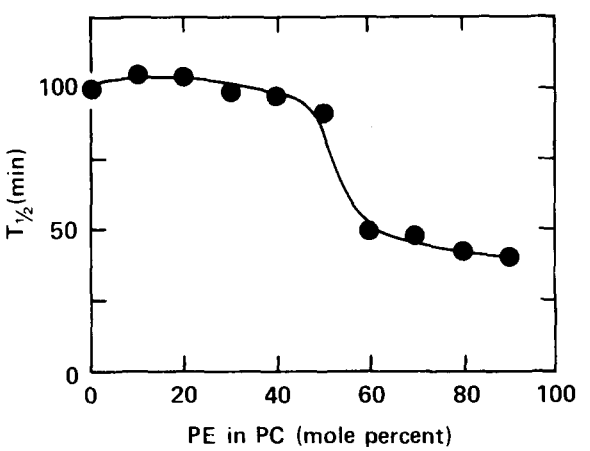

Fig. 3. Dependence of half-time of the transfer rate of spin-labeled PC on the composition of sonicated vesicles measured at 30°C. The ratio of spin-labeled to unlabeled phospholipid was 1:200 (w/w) after mixing. The total phospholipid concentration was 10 mg/ml in 0.02 M potassium phosphate buffer, pH 7.5, containing 20% glycerol. It can be seen that at about 60 mol% PE in PC the transfer rate became about 2.5-fold faster.

Discussion

Mixing of vesicles of spin-labeled PC with an excess of suspensions of vesicles of different defined composition resulted in dilution of the spin label and decrease in the line width of the EPR signal. Four possible mechanisms for dilution of the spin-labeled phospholipids from the donor vesicles into the large excess of phospholipids in the acceptor vesicles could be considered: Fusion of donor vesicles with acceptor vesicles; transfer of individual monomeric spin-labeled phospholipids in solution at the critical micelle concentration to acceptor vesicles; exchange of phospholipids between donor and acceptor vesicles during rapid collisional contacts; and exchange of phospholipids between donor and acceptor vesicles that exist as dimers or higher aggregates.

The possibility that fusion is responsible is unlikely because each fusion of a spin-labeled vesicle with a nonspin-labeled vesicle should reduce the concentration only by a factor of two. Many such fusions would be required to reduce the spin-label concentration below about 10 mol% where spin-spin exchange is diminished enough to begin to decrease the line width of the EPR spectrum. This series of fusion events should produce a time course of narrowing of the EPR spectrum that is of sigmoidal shape because the first few fusion events would not lead to a significant decrease in line width. It could be argued that PC vesicles with a high percentage of spin-labeled acyl chains do not form stable vesicles and fuse spontaneously. However, if 50 mol% is the spin-label concentration before the first fusion, after two fusion events the percentage of spin-labeled PC would be reduced to 12.5%. One might expect that vesicles with only 12.5% spin-labeled PC would not fuse spontaneously [14], but at that percentage of spin label the EPR spectrum still would be very broad [13]. In contrast to this prediction, it was observed that the spin-labeled PC was completely diluted into the acceptor vesicles as evidenced from narrow-line EPR spectra without broad components. Furthermore, the identical rate of transfer of the spin-labeled PC from the donor vesicles that con-

tain 100% spin-labeled phospholipid compared with those that contain only 50% provides evidence that the spin-labeled PC vesicles are not unstable and do not tend to form micelles or to fuse spontaneously with other vesicles in solution.

A second possible mechanism for the observed dilution of spin-labeled PC would be transfer of individual phospholipid molecules. This mode of transfer cannot be excluded but is unlikely to be important because of the extremely low monomer concentration of PC.

It is possible that collisional contacts are the primary events that lead to transfer of phospholipid molecules from vesicle to vesicle. However, the rate of transfer would be much higher in an aggregate where an increase in attractive forces or a decrease in repulsive forces would cause a decrease in the activation energy. Aggregation of pure PC vesicles is slow because of the dipolar repulsion of PC head groups and the resulting high energies that are required to bring PC bilayers together within a few angstroms of each other [15]. However, in a kinetic study of vesicle aggregation it has been shown [16] that vesicles aggregate only when the mole fraction of PE is greater than 0.6. In the present study, an increase in phospholipid transfer was observed when the acceptor vesicles contained a mole fraction of egg PE of 0.6.

In the present study, the presence of either cytochrome *P*-450 or more than 60 mol% PE in the acceptor vesicles resulted in an increase in the rate of transfer of spin-labeled PC. However, the protein-free phospholipid vesicles containing 30 and 40 mol% PE gave the same rate of phospholipid transfer as pure PC vesicles. The former two vesicle preparations encompass the 33 mol% PE that was used for reconstituting cytochrome *P*-450. Therefore, the presence of the protein in a reconstituted vesicle with 33 mol% PE causes some properties of the membrane to be like those of a vesicle with 70 mol% PE. It is possible that protrusion of cytochrome *P*-450 from the vesicles surface in combination with a nonplanar surface of the bilayer may allow direct contact between the spin-labeled PC vesicle and the protein-containing vesicle.

These results support a previous study [7] in which reconstitution of cytochrome *P*-450 into a vesicle with 33 mol% PE and 66 mol% PC and a 1:2 (w/w) protein/lipid ratio resulted in dramatic change in the ^{31}P-NMR chemical shift anisotropy compared to that observed in protein-free vesicles with an identical PC/PE ratio. Similar changes in the ^{31}P-NMR chemical shift anisotropy have been observed in PC/PE vesicles containing greater than 70 mol% PE [17]. These authors have proposed a model in which formation of an inverted micelle of PE at the surface of contact between two vesicles could facilitate phospholipid exchange [7,17].

Other techniques have been employed to study phospholipid transfer between membrane structures [18–20]. It has been shown that both cytochrome b_5 and NADH-cytochrome b_5 reductase are readily exchanged between vesicles [21]. It is a possibility, therefore, that a similar process occurs between vesicles that contain cytochrome *P*-450. Such a protein transfer might facilitate a phospholipid transfer by a mechanism similar to the one that is suggested for phospholipid-exchange proteins. However, there is no evidence for an exchange of the integral membrane protein cytochrome *P*-450 between vesicles. In fact, when a suspension of vesicles containing reconstituted cytochrome *P*-450 was mixed with a suspension of vesicles containing reconstituted NADPH-cytochrome *P*-450 reductase, very little reduction of the cytochrome *P*-450 occurred during a 1 h incubation at 37°C in the presence of NADPH. Moreover, after the incubation, the cytochrome *P*-450 vesicles could be completely separated from the NADPH-cytochrome *P*-450 reductase vesicles by a density gradient centrifugation.

Our results show that addition of spin-labeled PC vesicles to suspensions of biological membrane vesicles is an effective way of introducing a spin-labeled PC into a biological membrane even though model experiments performed with pure PC vesicles would suggest that this approach is not feasible. This study suggests that direct phospholipid exchange may be an effective mechanism for transfer of phospholipids between membranes of subcellular organelles, even in the absence of phospholipid-exchange protein. These results may also be useful in understanding the mechanism that leads to hepatic necrosis resulting from exposure of animals to some halocarbons. It is known that the lipids of the endoplasmic reticulum become peroxidized, increase in content of conju-

gated dienes, and have halogenated metabolites covalently bound to the fatty acid chains [22–24]. These altered phospholipids may have a much higher rate of exchange between organelles than has been measured for intact phospholipids in the absence of phospholipid-exchange proteins [18,19]. The ability of the endoplasmic reticulum to exchange phospholipids with other cellular membranes may mean that peroxidized or metabolite-bound phospholipids may be communicated throughout other organelles of the cell much more rapidly than previously thought.

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