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RETENTION OF LIPID ASYMMETRY IN MEMBRANES ON POLYLYSINE-COATED POLYACRYLAMIDE BEADS

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Summary

Phosphatidylcholine-specific exchange protein from calf liver was used to study the asymmetry and transmembrane movement of phosphatidylcholine in rat erythrocyte membranes isolated on polylysine-coated beads. While confirming previously published results for sealed ghosts, we found that for membranes attached to beads, where the cytoplasmic surface is exposed, about 36% of the total phosphatidylcholine is readily available for exchange, while the remaining 64% is exchangeable at a much slower rate. This indicates that the relative transbilayer asymmetry of phosphatidylcholine is largely maintained when red cell membranes are isolated on beads. On the other hand, transmembrane movement of phosphatidylcholine is decreased in membranes attached to cationized beads: the half-time for equilibration of phosphatidylcholine between the two monolayers of the membrane is 8 h for membranes on beads, compared to 1.5 h for sealed ghosts. Our results indicate that polylysine-derivatized beads are a useful tool for studying asymmetric properties of biological membranes.

Introduction

The plasma membranes of many cells can be rapidly isolated and purified if the intact cells are first bound to polylysine-coated beads [1,2]. In the case of erythrocyte membranes, biochemical and morphological criteria indicate that the bead-bound membranes are identical to ghost membranes [1,3]. Once bound, extracellular surfaces of the membranes remain firmly attached to the bead and the extracellular surface proteins become relatively inaccessible to

probing enzymes. In a complementary fashion, cytoplasmic surfaces of the membranes are selectively exposed to solution and cytoplasmic components become preferentially accessible to enzymatic digestion [1,3]. Thus, the asymmetric arrangement of the membrane proteins is retained in membranes bound to beads. However, it is well-known that the vectorial structure of the red cell membrane is also determined by the asymmetric distribution of membrane lipids [4,5]. We have therefore determined whether lipid asymmetry is maintained in membranes isolated on beads.

Phospholipid exchange proteins are suitable non-perturbing probes for studying membrane asymmetry and transbilayer movement of phospholipids [4—7]. Here, we investigate the protein-mediated phospholipid exchange between membranes on beads and lipid vesicles using the phosphatidylcholine-specific exchange protein from calf liver. We then use the exchange protein to determine the transmembrane distribution and movement of phosphatidylcholine in rat red cell membranes attached to beads.

Materials and Methods

Polylysine-coated polyacrylamide beads. Polylysine (molecular weight greater than 150 000) was covalently coupled to polyacrylamide beads (Bio-Gel P-2, 200—400 mesh, Biorad Laboratories) using carbodiimide as described by Cohen et al. [2]. The beads were stored in 10 mM Tris-HCl, pH 7.4, containing 0.2% sodium azide. Before use, they were washed extensively with 0.15 M Tris-HCl, pH 7.4, and twice with 20 vols. of a sucrose/phosphate buffer containing seven parts 310 mosM sucrose and three parts 310 mosM phosphate, pH 7.4 (7 : 3, v/v).

Lipids. L-α-Phosphatidylcholine (Type V-E) from egg yolk and cholesterol (99+% grade) were obtained from Sigma. Phosphatidyl[*methyl*-^{14}C]choline (50—60 Ci/mol) was obtaned from New England Nuclear. [2-^{3}H]Glycerol trioleate (2 Ci/mmol) was from ICN Pharmaceuticals. Radioactivity was counted with 'Aquasol', a xylene-based scintillation solution for aqueous samples (New England Nuclear).

Lipid-vesicles. For standard assays, sonicated phosphatidylcholine/cholesterol vesicles were prepared from phosphatidylcholine (100 μg), phosphatidyl-[^{14}C]choline (0.5 μCi), cholesterol (40 μg) and [^{3}H]triolein (5 μCi). Butylated hydroxytoluene (Sigma) was added as an antioxidant (molar ratio phosphatidylcholine:butylated hydroxytoluene = 1000 : 1) before the solvents were evaporated at 20° C under a stream of nitrogen. The lipids were redissolved in 1 ml of ether in a test tube, and the solvents again evaporated under nitrogen. The lipid mixture formed a thin film on the wall of the test tube. The lipids were immediately suspended in 1 ml of 5 mM phosphate, pH 7.6, using a Vortex mixer. The tube was then immersed in a Branson sonicating waterbath and was sonicated at 20° C for at least 30 min under nitrogen. For some experiments, large quantities of lipid vesicles were prepared from phosphatidylcholine (70 mg), cholesterol (30 mg), a trace of [^{3}H]triolein (0.1 μCi) and butylated hydroxytoluene (3.75 mg). The lipids were dried and dispersed in 25 ml of buffer as described above. The lipid mixture was then sonicated for 20 min with a probe-sonicator (Heat Systems-Ultrasonics Inc., model 185D) under a

N_2 atomosphere and the temperature held between 4 and 20° C. After addition of 2.5 ml of bovine serum albumin (20 mg/ml in 5 mM phosphate, pH 7.6), the dispersion was centrifuged 30 min at 25 000 × *g*. 80—85% of the lipids were recovered in the supernatant.

Phospholipid exchange protein. The phosphatidylcholine-specific exchange protein was precipitated at pH 3 from a calf liver supernatant fraction and purified essentially as described by Kamp and Wirtz [8] but omitting the last purification step (gel filtration). During purification exchange activity was assayed with the ghost-vesicle system described below but using 0.1 M KCl, 0.01 mM Tris-HCl, pH 7.4, as buffer. One unit of exchange activity was defined as the transfer of 1% of the labelled phosphatidylcholine during 1 min under standard conditions. The exchange protein was purified 720-fold and had a specific activity of 100 units/mg in our ghost-vesicle assay. The protein was stored at −20° C in 50% glycerol and, after the addition of 0.2 mg/ml bovine serum albumin, was dialyzed before use against 2 mM 2-mercaptoethanol, 5 mM phosphate buffer, pH 7.6.

Erythrocyte membrane preparations. Rat erythrocytes were labelled in vivo by injecting mature female rats intraperitoneally with $^{32}P_i$, obtained as orthophosphoric acid from New England Nuclear (1 mCi/100 g of body weight). 72 h after the injection, blood was drawn from CO_2 anesthetized rats by heart puncture and collected into evacuated blood collection tubes (Becton, Dickinson and Co.) containing 10 µl 0.2 M EDTA as anticoagulant/ml of blood.

All subsequent operations were performed at 0—4° C. Rat or blood bank human red blood cells were washed three times with phosphate-buffered saline (150 mM NaCl, 5 mM sodium phosphate, pH 7.6). The buffy coat was carefully aspirated each time from the surface of the pellet. Membranes were prepared by the method of Steck [9] as follows:

(a) Leaky ghosts were obtained from washed erythrocytes by hemolysis in 40 vols. of 5 mM phosphate, pH 7.6, and four additional washes with the same buffer. Part of the hemoglobin from rat erythrocytes precipitated in the chilled low ionic strength buffer and pelleted as a hard button below the ghosts. It was easily removed by aspiration.

(b) Sealed ghosts were prepared by hemolyzing washed erythrocytes with 100 vols. of 1 mM $MgSO_4$, 5 mM phosphate, pH 7.6. The ghosts were centrifuged at 25 000 × *g* for 10 min, washed three more times in 5 mM phosphate buffer containing magnesium, and then diluted approx. 5 : 1 in the same buffer. The diluted membrane suspension was layered upon an equal volume of a 8% (w/v) Dextran solution (Dextran D-5001, chemical grade, Sigma; containing 1 mM $MgSO_4$, 5 mM phosphate, pH 7.6). After centrifugation for 2 h at 200 000 × *g*, the floating band of sealed ghosts was carefully removed and washed three times with 40 vols. of buffer. The sealed ghosts were suspended at a concentration of 2—4 mg protein/ml (about $2 \cdot 10^4$ cpm/mg protein for ^{32}P-labelled rat ghosts) and stored at 0—4° C.

(c) Inside-out vesicles were prepared by suspending packed leaky ghosts in 40 vols. of 0.5 mM phosphate buffer, pH 7.6, and incubating 2 h on ice. The membranes were then pelleted at 25 000 × *g* for 30 min, stored overnight, resuspended in a small volume of 0.5 mM phosphate buffer, and passed eight times through a No. 27 gauge needle on a 1 ml syringe. The resultant vesicle

suspension was diluted with buffer and the sealed vesicles separated from unsealed vesicles by centrifugation onto a Dextran solution (8% (w/v) in 0.5 mM phosphate, pH 7.6) as described above. About 70% of the membrane protein loaded on the gradient was recovered in the floating band containing the sealed inside-out vesicles. The washed sealed inside-out vesicles were stored at a protein concentration of 2—4 mg/ml (for ^{32}P-labelled cells, about $4 \cdot 10^4$ cpm/mg protein) at 0—4° C. The quality of the inside-out vesicles was measured by comparing acetylcholinesterase activity in the presence and absence of detergent as follows. Vesicles (200 μg of protein) were incubated with and without phosphatidylcholine/cholesterol vesicles (2.5 mg phosphatidylcholine, 1 mg cholesterol) in 5 mM phosphate, pH 7.6 (total volume 2.5 ml). At time points 0, 2, and 4 h, 50 μl of 4 mM 5,5'-dithiobis(2-nitrobenzoic acid) and 50 μl of 12 mM acetylthiocholine chloride were added to 0.7 ml of the suspension, and the reaction followed spectrophotometrically at 412 nm. 5 μl of 10% Triton X-100 were then added, and the reaction followed in the presence of the detergent. Acetylcholinesterase activity was computed as the slope of the line obtained by plotting absorbance at 412 nm versus time. The latency of acetylcholinesterase activity, defined as 1.00 — (activity without Triton/activity with Triton) was taken as an index of the sidedness of the vesicles. The index was 0.85 in the absence of lipid vesicles and decreased to 0.75 in the presence of lipid vesicles. The latency of acetylcholinesterase was unchanged by incubation at 37° C.

Membranes on beads. Washed red cells (2.5 ml) were washed twice in 20 vols. of 310 mosM phosphate, pH 7.6, and twice in a sucrose-phosphate buffer containing seven parts 310 mosM sucrose and three parts 310 mosM phosphate buffer, pH 7.6 [3]. The cells were then suspended to a total volume of 5 ml in this sucrose/phosphate buffer. 10 ml of beads (suspended 1 : 1 (v/v) in sucrose/phosphate buffer) were added dropwise to 2.5 ml of the cell suspension in a 40 ml plastic tube and gently agitated. The cells and beads were incubated on ice and occasionally mixed by inverting the tube. The tube was then filled with sucrose/phosphate buffer, and the beads suspended by inverting the tube. After the beads settled (about 10 min), the supernatant containing unattached cells was removed by aspiration. The beads were washed twice more in the same way. The beads, which appeared completely covered by red cells when examined in a light microscope, were then vortexed and the tube immediately filled up with 5 mM phosphate, pH 7.6. The beads were suspended by vortexing and then sedimented in a clinical centrifuge for 10 s. The supernatant was removed by aspiration and the beads washed two more times in 5 mM phosphate, pH 7.6. The beads were then suspended in an equal volume of this buffer and sonicated with the microtip of a Heat Systems sonicator for 5 s (20 W, lowest setting). They were then washed twice or three times more until completely white and yielded about 30 μg of protein/100 μl of packed beads. The membrane-covered beads were washed twice with 0.2% bovine serum albumin (in 5 mM phosphate, pH 7.6) and finally suspended in an equal volume of this bovine serum albumin-containing buffer. The membranes on beads (containing $0.5—1 \cdot 10^3$ cpm/100 μl of packed beads for ^{32}P-labelled membranes) were immediately used for the exchange experiments.

Lipid extractions. The distribution of ^{32}P in rat erythrocyte lipids was deter-

mined by extracting ^{32}P-labelled membrane preparations with chloroform/methanol (2 : 1, v/v) according to Folch [10]. Non-labelled rat erythrocyte lipids were added as carrier to aliquots of the lipid extract and the phospholipids separated by two-dimensional thin-layer chromatography on Silica gel H plates with chloroform/methanol/NH_3/H_2O (16 : 10 : 1 : 1, v/v) in the first dimension and chloroform/methanol/acetic acid/H_2O (45 : 20 : 6 : 1, v/v) in the second dimension [11]. The spots were visualized by brief exposure to I_2 vapours. After evaporation of the I_2, the silicic acid was transferred to counting vials and counted in 10 ml of scintillation fluid (for phosphatidylcholine 1 ml of H_2O was added to assure complete extraction).

More than 92% of the total initial radioactivity was recovered in the phospholipid spots of the thin-layer chromatogram. Phosphatidylcholine accounted for 75 ± 1% of the radioactivity. The other labelled phospholipids were sphingomyelin (9%), phosphatidylethanolamine (6%), phosphatidylserine and phosphatidylinositol (4%), lysophosphatidylcholine (5%), and lysophosphatidylethanolamine (1%).

Exchange reactions. For standard exchange reactions, a modification of the procedure described by Rothman and Dawidowicz [12] was used. Sonicated phosphatidylcholine/cholesterol vesicles (1 μg phosphatidylcholine, 0.4 μg cholesterol containing 0.005 μCi of phosphatidyl[^{14}C]choline and 0.05 μCi of [^{3}H]triolein) were incubated with red cell membranes (100 μg of protein) and exchange protein (1—3 units) in a total volume of 0.5 ml (1 ml for membranes on beads) 5 mM phosphate, pH 7.6, containing 0.2% bovine serum albumin. The ratio of vesicle to ghost phosphatidylcholine was approximately 1 : 12.5. Incubations were carried out for 30 min (or as indicated) at 37° C with constant agitation. At the end of the incubation, exchange was stopped by chilling the mixture in ice. A 0.1 ml aliquot was removed for counting, and the ghosts were pelleted (10 min at 25 000 × *g*, 4° C). After centrifugation, a 0.1 ml aliquot of the supernatant was removed for counting. The aliquots were counted in 10 ml scintillation fluid. In the supernatant, the [^{3}H]triolein serves as a non-exchangeable marker so that the decrease in the ^{14}C/^{3}H ratio during incubation measures the fraction of the labelled vesicle phosphatidylcholine transferred to ghosts. The amount of exchange was calculated as follows. For supernatants, the fractions of phosphatidyl[^{14}C]choline remaining with the vesicles after the reaction was taken to be the supernatant ^{14}C/^{3}H ratio divided by the ^{14}C/^{3}H ratio for the starting vesicles. The small fraction of phosphatidyl[^{14}C]choline which was transferred in the absence of added exchange protein was subtracted as a blank.

For determination of exchange between lipid vesicles and ^{32}P-labelled red cell membranes, the incubation conditions described by Bloj and Zilversmit [13] were used modified as follows. Sealed ghosts (1—1.5 · 10^4 cpm) were incubated at 37° C with phosphatidylcholine/cholesterol vesicles (1 mg phosphatidylcholine/ml of incubation medium, ratio of ghost to vesicle phosphatidylcholine about 1 : 50) in the presence of exchange protein (18 units) in 4.5 ml of 5 mM phosphate, pH 7.6, containing 0.5 mM $MgSO_4$ and 0.2% bovine serum albumin. After 1 h, the ghosts were pelleted by centrifugation for 10 min at 25 000 × *g* and resuspended in a fresh supernatant containing phosphatidylcholine/cholesterol vesicles and exchange protein. At different time points, 0.5 ml of the incubation mixture was removed, of which 0.1 ml was transferred

to a counting vial and 0.4 ml was centrifuged for 10 min at 25 000 × *g*. Aliquots (0.1 ml) of the supernatant from this centrifugation were transferred to counting vials. An identical procedure was used for inside-out vesicles, except that the incubation medium contained no $MgSO_4$.

For membranes on beads, 2.5 ml membrane-covered beads ($0.8—1.2 \cdot 10^4$ cpm) were suspended in a total volume of 10 ml of 5 mM phosphate buffer, pH 7.6, containing 0.2% bovine serum albumin and phosphatidylcholine/cholesterol vesicles (1 mg phosphatidylcholine/ml incubation medium) in the presence of exchange protein (40 units). The suspension was incubated at 37°C with vigorous shaking to prevent settling of the beads. After 0, 15, and 30 min, 1 ml of the mixture was removed, of which 0.2 ml was transferred to a counting vial and 0.8 ml was centrifuged for 10 s in a clinical centrifuge to pellet the beads. Aliquants (0.2 ml) of the supernatant from this centrifugation were transferred to counting vials. After 1 h, the remaining incubation mixture was centrifuged and fresh supernatant was added to the beads. 1 ml portions of this suspension were transferred to small tubes and further incubated at 37°C with vigorous shaking. At different time points, the tubes were removed and 0.2 ml transferred to a counting vial. The remaining suspension (0.8 ml) was centrifuged, and 0.2 ml aliquots of the supernatant counted for radioactivity.

Exchange calculations. For all exchange experiments, control samples without exchange protein were included, and incubations were carried out in duplicate. Since phosphatidylcholine accounted for 75% of the ^{32}P radioactivity in the labelled rat erythrocytes, the ratio of $^{32}P/^{3}H$ in the starting suspension was corrected (multiplied by 0.75) to give the corrected [^{32}P]phosphatidylcholine/[^{3}H]triolein ratio in the starting suspension. The fraction of [^{32}P]-phosphatidylcholine removed from the ghost membranes after exchange was taken to be the supernatant $^{32}P/^{3}H$ ratio divided by the corrected $^{32}P/^{3}H$ ratio for the starting suspension. No corrections were made for contamination with lyso[^{32}P]phosphatidylcholine which is known to be spontaneously transferred to phosphatidylcholine/cholesterol vesicles in the presence and absence of phospholipid exchange protein [13].

The exchange values for inside-out vesicles were further corrected by assuming that the right-side-out vesicles (present as determined from measurements of acetylcholinesterase activity) exchange phosphatidylcholine at the same rate as sealed ghosts.

Analytical determinations. Protein was measured by the method of Lowry et al. [14] with bovine serum albumin as a standard. To determine the amount of protein on beads, the modification described by Cohen et al. [2] was used.

Results

Interaction of phospholipid exchange protein with membranes on beads

To determine if the lipid in membranes on beads is a substrate for the exchange protein, increasing amounts of non-labelled erythrocyte membranes on beads were incubated with labelled phosphatidylcholine/cholesterol vesicles in the presence or absence of exchange protein. Transfer of radioactive phosphatidylcholine from the lipid vesicles to membranes on beads was accelerated in the presence of exchange protein (Fig. 1), indicating that the exchange pro-

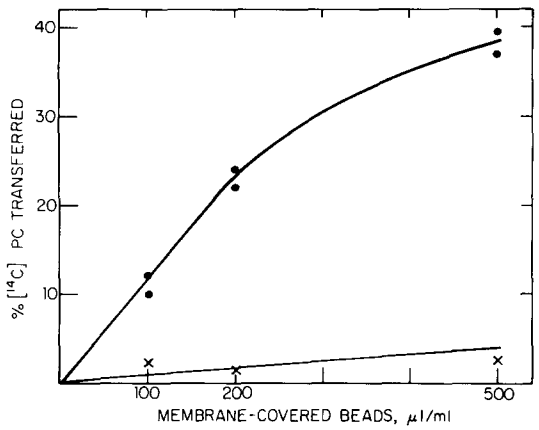

Fig. 1. Transfer of phosphatidyl[^{14}C]choline from lipid vesicles to membranes on beads. Human red cell membranes and lipid vesicles were incubated with (●———●) and without (X- - - - -X) exchange protein under standard conditions as described in Materials and Methods. Each point represents the average of two determinations from one incubation.

tein interacts with membranes attached to charged surfaces. Negligible transfer was observed in the absence of protein. Therefore, membranes on beads serve as a useful substrate for protein-catalyzed phospholipid exchange.

To determine if the beads affect vesicle recovery, vesicles were incubated with beads or membrane-covered beads under exchange assay conditions. After addition of plain beads to a suspension of phosphatidylcholine/cholesterol vesicles less than 10% of the lipid vesicles could be recovered in the supernatant, indicating that incomplete, variable recovery could be the result of binding of lipid vesicles to the beads. However, when bovine serum albumin was present in the assay system, the recovery of lipid vesicles in the supernatant was higher than 90% for both plain beads and membrane-covered beads (data not shown). Bovine serum albumin up to 0.5% had no effect on exchange activity (data not shown) and 0.2% bovine serum albumin was therefore used in all further exchange experiments.

It was important to know whether binding of the exchange protein to the beads or inactivation of the protein in the presence of beads occurred during long-term incubations. Therefore, phospholipid exchange protein was preincubated with beads in the presence of phosphatidylcholine/cholesterol vesicles (and, of course, 0.2% bovine serum albumin). The beads were then removed by centrifugation and the supernatant assayed for exchange activity. The recovery of exchange activity after preincubation with membrane-covered beads or plain beads was close to 100% (data not shown), indicating that there is no loss of exchange activity in the presence of beads.

In a comparative study, labelled lipid vesicles were incubated with membranes on beads or sealed ghosts in the presence of exchange protein. The time course of protein-catalyzed transfer of labelled phosphatidylcholine to membranes on beads was similar to transfer to ghosts (Fig. 2), indicating that under these conditions there was no difference in the interaction of the exchange protein with phosphatidylcholine in the two membrane systems. In both cases,

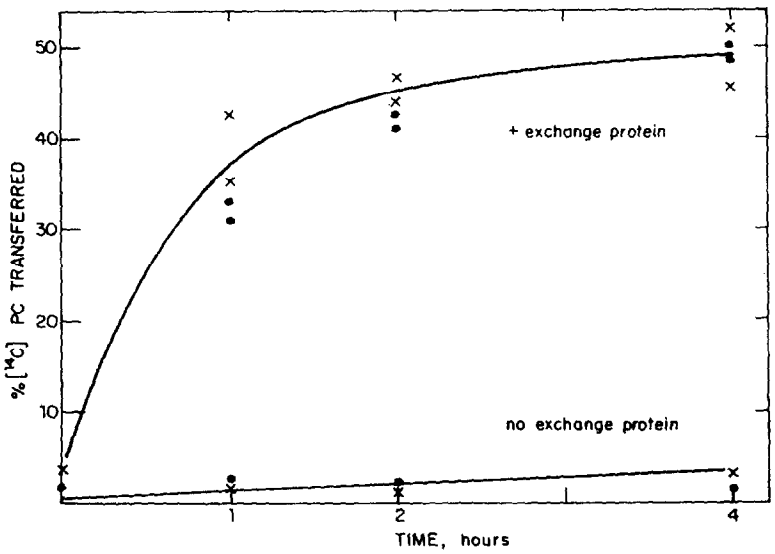

Fig. 2. Transfer of phosphatidyl[^{14}C]choline from lipid vesicles to erythrocyte ghosts (X- - - - -X) or membranes on beads (●——●) in the presence or absence of exchange protein. Lipid vesicles were incubated with sealed rat red cell ghosts (50 μg protein) or membranes on beads (300 μl) in the presence or absence of exchange protein under standard exchange conditions as described in Materials and Methods. Each point represents the average of two determinations from one incubation.

the ratio of vesicle phosphatidylcholine to membrane phosphatidylcholine accessible for exchange was estimated to be 1 : 10.

Exchange between membranes on beads and lipid vesicles

To measure retention of asymmetry and rate of transmembrane exchange, the transfer of phosphatidylcholine from labelled rat erythrocyte membranes on beads to lipid vesicles was measured. ^{32}P-labelled membranes attached to beads were incubated with an excess of lipid vesicles (membrane to vesicle phosphatidylcholine ratio about 1 : 50) with or without exchange protein. In a parallel experiment, the exchange of phosphatidylcholine between ^{32}P-labelled sealed ghosts and lipid vesicles was studied. At different time points, samples of the suspension were removed and the transfer of [^{32}P]phosphatidylcholine from membranes to lipid vesicles determined. As shown in Fig. 3, a saturating value of about 35% of the [^{32}P]phosphatidylcholine in membranes on beads was transferred to lipid vesicles in the presence of exchange protein, whereas about 80% of the [^{32}P]phosphatidylcholine could be removed from sealed ghosts. Significant exchange also took place in the absence of exchange protein. It amounted to about 10% for membranes on beads and, as reported previously [13], to about 25% for sealed ghosts. Determination of ^{32}P distribution in lipid vesicles by extraction and quantitative separation of phospholipids showed that phosphatidylcholine and lysophosphatidylcholine were preferentially lost from the membranes. For both sealed ghosts and membranes attached to beads, less than 5% of the total transferred radioactivity in the absence of exchange protein was found as labelled sphingomyelin, phosphatidylethanolamine or phosphatidylserine + phosphatidylinositol after 4 h of incubation.

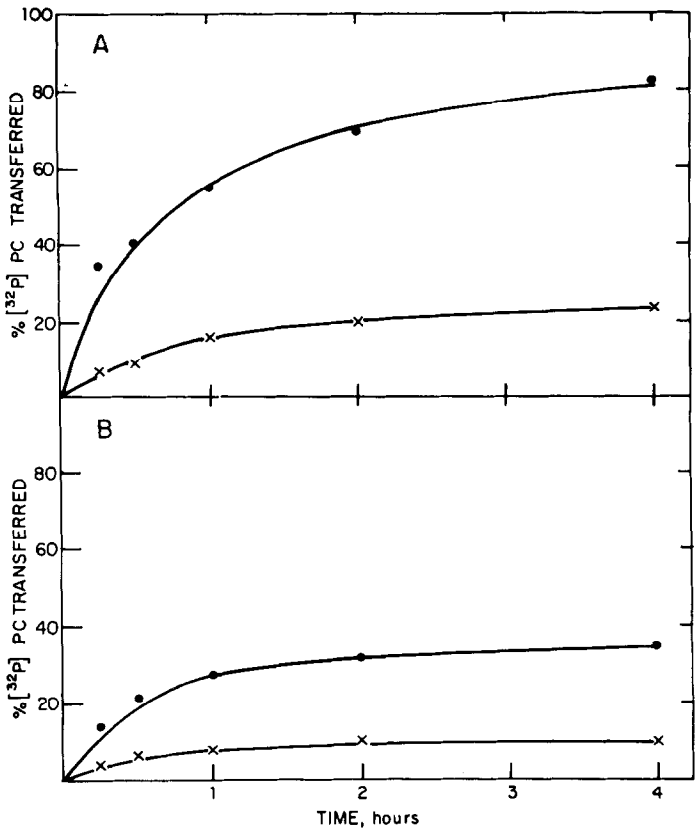

Fig. 3. Transfer of [^{32}P]phosphatidylcholine from sealed rat ghosts (A) or membranes on beads (B) to lipid vesicles. ^{32}P-labelled membranes were incubated with an excess of phosphatidylcholine/cholesterol vesicles in the presence (•———•) or absence (X- - - - -X) of exchange protein as described under Materials and Methods. Each point represents the average of four determinations, two from each of two separate incubations in one experiment.

The time course of a typical experiment is shown in Fig. 4. In this and all further experiments, the lipid vesicles were completely removed by centrifugation after 1 h of incubation and replaced by fresh supernatant in order to prevent reverse flow of labelled phosphatidylcholine during subsequent incubation. The exchange curves can be fitted by the sum of two exponentials indicating that the membrane phosphatidylcholine is distributed in two pools: a readily accessible pool which is easily exchangeable, and a relatively inaccessible pool which exchanges slowly. In sealed ghosts, the accessible pool is interpreted to be the fraction of phosphatidylcholine which is located in the extracellular monolayer. This pool should correspond to the phosphatidylcholine located in the inaccessible monolayer of the erythrocyte membrane which is appressed to the bead (Fig. 5). On the other hand, the relatively inaccessible pool is interpreted to be the phosphatidylcholine in the cytoplasmic monolayer [13]. This pool corresponds to the phosphatidylcholine in the accessible monolayer of membranes on beads. We used the two-pool model analysis described by Bloj and Zilversmit [13] to calculate the rate constants as follows. The slope

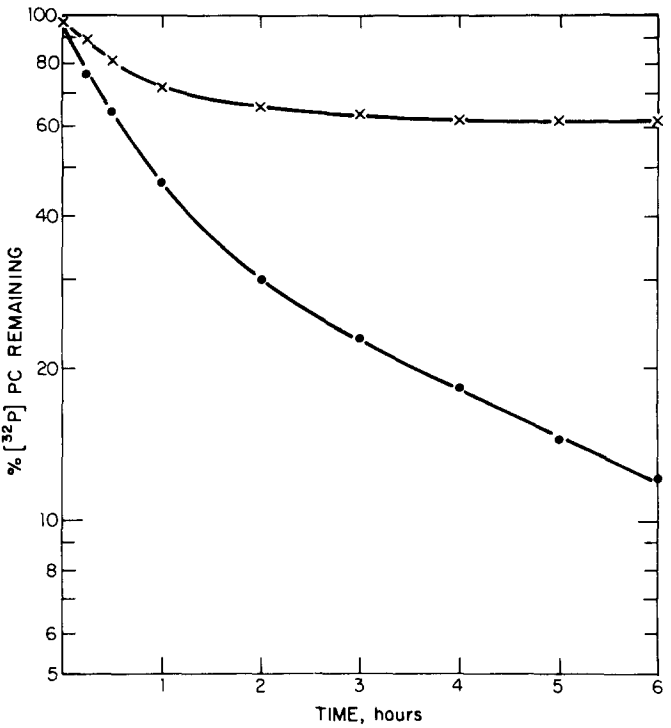

Fig. 4. [^{32}P]Phosphatidylcholine remaining in sealed rat erythrocyte ghosts (•———•) or membranes on beads (X- - - - -X) after exchange with unlabelled lipid vesicles. Labelled sealed ghosts or membranes on beads were incubated with an excess of phosphatidylcholine/cholesterol vesicles and exchange protein. After 1 h, the lipid vesicles were removed by centrifugation and replaced by fresh acceptor in order to prevent reverse flow of labelled phospholipid during subsequent incubation. For further details see Materials and Methods. Each point represents the average of four determinations, two from each of two separate incubations in one experiment.

of the fast (g_1) and slow (g_2) exchange and the intercept with the y-axis (C) of the final linear portion of the radioactivity curve was determined and the rate constants (K_{AB}, K_{BA}, and K_0) calculated with the relationships

$$K_{AB} + K_{BA} = \frac{g_1 \times g_2}{g_1 - (g_1 - g_2)C}$$

$$K_{AB} + K_{BA} + K_0 = g_1 + g_2$$

$$K_0 \times K_{BA} = g_1 \times g_2$$

The fraction of phosphatidylcholine mass present in pool A is then

$$r_A = \frac{K_{BA}}{K_{BA} + K_{AB}}$$

and the half-time of equilibration can be calculated as

$$t_{1/2} = \ln 2/(K_{AB} + K_{BA})$$

The values for the kinetic parameters we calculated are given in Table I. For sealed ghosts, we found that about 70% of the total phosphatidylcholine is

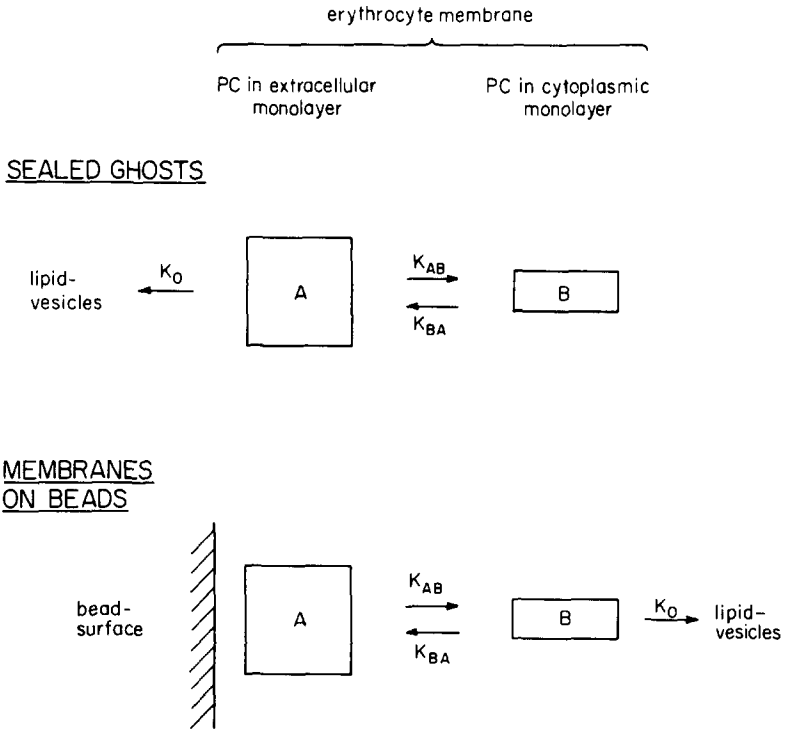

Fig. 5. Schematic illustration for exchange of phosphatidylcholine between lipid vesicles and membranes indicating the flow of labelled phosphatidylcholine and the phosphatidylcholine pools (A, pool of phosphatidylcholine in extracellular monolayer; B, pool of phosphatidylcholine in cytoplasmic monolayer).

located in the extracellular monolayer of the membrane and that the half-time for equilibration of phosphatidylcholine in the two monolayers is 1.5 h. This is close to the values determined by Bloj and Zilversmit [13], who found that the larger outside pool comprised 75% of the total phosphatidylcholine and calculated a half-time of equilibration of 2.3 h. For membranes on beads, the fast exchanging pool amounted to 36% of the phosphatidylcholine, and the half-time for phosphatidylcholine equilibration was 7.8 h. The sum of the forward

TABLE I

KINETIC PARAMETERS FOR THE EXCHANGE OF PHOSPHATIDYLCHOLINE (PC) BETWEEN SEALED GHOSTS AND PC/CHOLESTEROL VESICLES AND BETWEEN MEMBRANES ON BEADS AND PC/CHOLESTEROL VESICLES

The values are average ± S.D. of four separate experiments carried out in duplicate.

	Pool sizes (% of total PC)		$T_{1/2}$ (h)	Rate constants (h^{-1})		
	A (extracellular)	B (cytoplasmic)		K_0	K_{BA}	K_{AB}
Sealed ghosts	69.3 ± 4.2	30.7 ± 4.2	1.5 ± 0.2	1.9 ± 0.6	0.34 ± 0.1	0.15 ± 0.01
Membranes on beads	64.3 ± 1.5	35.7 ± 1.5	7.8 ± 1.4	0.9 ± 0.2	0.06 ± 0.1	0.03 ± 0.1

TABLE II

KINETIC PARAMETERS FOR THE EXCHANGE OF PHOSPHATIDYLCHOLINE (PC) BETWEEN INSIDE-OUT MEMBRANE VESICLES AND PC/CHOLESTEROL VESICLES

The values are average ± S.D. of four separate experiments carried out in duplicate.

	Pool sizes (% of total PC)		$T_{1/2}$ (h)	Rate constants (h^{-1})		
	A (extracellular)	B (cytoplasmic)		K_0	K_{BA}	K_{AB}
IOV	50.3 ± 5.1	49.7 ± 5.1	2.7 ± 0.6	1.5 ± 0.4	0.13 ± 0.02	0.14 ± 0.05
IOV [13]	63.1 ± 3.0	36.9 ± 3.9	5.3	2.3 ± 1.3	0.08 ± 0.02	0.05 ± 0.02

and backward transposition of phosphatidylcholine between the two monolayers of the membrane amounts to 4% of the total membranes phosphatidylcholine/h for bead-bound membranes as compared to 19% calculated for sealed ghosts.

Bloj and Zilversmit [13] have studied the distribution and dynamics of phosphatidylcholine in inside-out vesicles. They found that 37% of the phosphatidylcholine in these membranes was readily available for exchange. while 63% was exchanged at a slower rate. The half-time for equilibration of phosphatidylcholine in inside-out membrane vesicles was 5.3 h. We also performed exchange experiments between inside-out vesicles and phosphatidylcholine/cholesterol vesicles using the phosphatidylcholine-specific exchange protein. However, we were unable to find an asymmetric distribution of phosphatidylcholine in the membranes of our inside-out vesicles (Table II). The phosphatidylcholine in inside-out vesicles was found to be uniformly distributed, and about 50% was readily available for exchange. The orientation and impermeability of our inside-out vesicles was preserved during the incubation as indicated by the latency of acetylcholinesterase activity before and after exchange incubation (see Materials and Methods). The reason for the discrepancy between our results and those of Bloj and Zilversmit is at present unknown, although we note that Bloj and Zilversmit used the less specific exchange protein derived from beef heart, whereas we used the phosphatidylcholine-specific protein derived from calf liver.

Discussion

Our experiments show that the interaction of the exchange protein with phosphatidylcholine in the membrane interface is identical for ghost membranes and membranes on beads. Membranes on beads proved to be suitable both as acceptor and as donor membranes in phosphatidylcholine exchange reactions and allowed us to determine the extent to which the known lipid asymmetry of erythrocyte membranes was retained when the membrane was attached to a polylysine-coated bead. Confirming results using phospholipases [15,16], chemical labelling [17,18], and phospholipid exchange protein [13], our protein-mediated exchange measurements show that in sealed ghosts, about 70% of the phosphatidylcholine is readily exchangeable and thus is in the extracellular monolayer, while 30% is less readily exchangeable and thus in the cyto-

plasmic monolayer. Inversely, when membranes are isolated on beads so that the extracellular monolayer is tightly adherent to the bead surface, 36% of the phosphatidylcholine is readily exchangeable, while 64% is less readily exchanged. Because our experiments show that the exchangeability of phosphatidylcholine in membranes on beads is fundamentally similar to the exchangeability of lipids in ghost membranes, we interpret this inversion to indicate that the asymmetrical distribution of phosphatidylcholine is largely maintained when membranes are isolated on beads.

Transmembrane movement of phosphatidylcholine has been studied in different membrane systems. In artificials membranes, translocation of phosphatidylcholine across the bilayer is extremely slow, the half-time of equilibration being several days [12,19,20]. In biological membranes, on the other hand, transmembrane exchange of phosphatidylcholine is faster and occurs with half-times of hours in red cell membranes [13,16] and less than an hour in microsomal membranes [21,23]. Interestingly, it has been reported recently that transbilayer movement of phosphatidylcholine in lipid vesicles is stimulated by two orders of magnitude when glycophorin, a membrane-spanning protein, is incorporated in the bilayer [23]. All the findings support the idea that the transmembrane movement of phospholipids is enhanced in the presence of membrane proteins, and several possible mechanisms have been proposed. Thus, the membrane proteins could facilitate transbilayer exchange of phospholipids by forming discontinuities in the lipid bilayer [24], by providing an intramembrane surface with which the phospholipid headgroup could interact during passage through the membrane [4], or by offering specific mechanisms for translocation across the membrane [25]. Our studies show that the transmembrane movement of phosphatidylcholine is reduced in red cell membranes upon attachment to beads. Binding of the membranes to beads occurs through nonspecific adherence involving ionic interactions between the negatively charged membrane surface and the cationized bead surface. Under these conditions, transmembrane exchange of phosphatidylcholine is decreased to a half-time of equilibration of about 8 h, as compared to 1.5 h for sealed hosts. This decrease could be an indirect result of the bead affecting the protein or proteins which enhance transbilayer movement or a direct result of a bead-lipid interaction that promotes lipid bilayer stability. In either case, the decrease in phospholipid transbilayer movement when the membrane is attached to the bead should facilitate measurements in situations where transbilayer movement would otherwise obscure lipid asymmetry.

Our results show that polylysine-derivatized beads may be a useful adjunct to the available methods for measuring membrane lipid assymmetry. Because the extracellular monolayer of the plasma membrane is accessible in the intact cell and the cytoplasmic monolayer accessible in the bead-mounted membrane, the use of beads makes it possible to examine each of these monolayers separately with the same probe while avoiding the complications of standard and often lengthy preparation of inside-out plasma membrane vesicles.

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