Selective Extraction of Membrane-bound Proteins by Phospholipid Vesicles*

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Extraction of membrane proteins from erythrocytes into sonicated phosphatidylcholine vesicles is described. In a process involving phospholipid and neutral lipid exchange, cell membrane proteins associate with the vesicles and can be separated from the cells by centrifugation. The protein transfer appears to be reversible; phospholipid vesicles mediate the delivery of small amounts of previously extracted protein into cell membranes. Prior to extraction, all but one of the proteins are accessible to lactoperoxidase iodination, and lipid analysis indicates that primarily the outer monolayer of the cell is involved in phospholipid exchange. Among the extracted proteins is acetylcholinesterase which is removed much more efficiently by this procedure than by monolayer of the cell is involved in phospholipid exchange. The most abundant proteins of the erythrocyte membrane are not represented in the vesicle extract.

Interactions between phospholipid vesicles and intact cells give rise to several processes. Cholesterol (1, 2) and phospholipids (3) exchange between membranes of cells and vesicles, phospholipid vesicles "fuse" with some types of cells (4-6) and with one another (7), and still other cells may incorporate vesicles by endocytosis (8). The extent to which such events occur depends on the type of cell, the lipid composition of the vesicles, exposure time and temperature, and a number of other variables. In investigating cell-vesicle association between phosphatidylcholine vesicles and mammalian erythrocytes, we observed yet another phenomenon; the extraction of certain cell membrane proteins into the vesicle lipid fraction. The extraction process and its reverse, delivery of membrane proteins into cell membranes from vesicles, are described in this communication. The accompanying paper (9) discusses the effects of these processes on cell function.

EXPERIMENTAL PROCEDURES

Methods

Human erythrocytes were obtained from adult volunteers and used within 48 h of collection. Dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine were purchased from Calbiochem, and phosphatidyl[(U-14C)cholesterol, NaH][14C], and [14C]diphenylphosphoryl fluorophosphate were obtained from New England Nuclear. Bovine erythrocyte acetylcholinesterase was a product of Sigma Chemical Co.

Unless otherwise noted, all experiments were conducted in 310 mM NaCl of phosphate-buffered saline (NaCl/P/glucose) of the following composition: 140 mM NaCl, 5 mM KH2PO4, 2.5 mM Na2HPO4, 1 mM MgSO4, 10 mM glucose. pH was adjusted to 7.38 with 1 M NaOH. NaCl/P/glucose/sucrose used in some procedures was the above buffer containing 50 mM sucrose.

Materials

Preparation of Phospholipid Vesicles—Dimyristoyl- or dipalmitoylphosphatidylcholine (32 mg) was suspended in buffer (2 ml) by mixing on a vortex stirrer. Lipid mixtures were prepared by dissolution of the appropriate lipids in chloroform, followed by solvent evaporation under a nitrogen stream and suspension of the resultant mixture in the usual manner. The lipid suspensions were sonicated for 50 min at 50° in a test tube (13 x 100 mm) suspended in a Heat Systems Ultrasonics model 9H heated bath sonicator filled to a depth of 3.0 cm with a solution of a few grams of sodium dodecyl sulfate in water. Vesicles prepared in this manner are monodisperse (250 to 300 Å in diameter) and unilamellar. Thin layer chromatographic analysis showed that they contained less than 0.1% lysophosphatidylcholine. Liposomes were prepared by suspending warmed lipid/buffer mixtures with a vortex mixer.

Extraction of Erythrocyte Membrane Proteins—Freshly drawn human erythrocytes were separated from plasma by centrifugation, then washed three times by suspension in 4 volumes of 0.15 M NaCl, followed by centrifugation. A final wash in buffer followed. During supernatant removal after each wash, care was taken to remove all traces of "buffy coat."

Aliquots of packed cells were transferred to polycarbonate culture tubes and warmed to 37°. Phospholipid vesicles were added in a volume equal to that of the cells, making the final incubation medium ~11.5 ml in vesicle phospholipid and 4.3 ml in cell membrane phospholipid (calculated for 50% hematocrit). Incubation was carried out at 37° in an agitating water bath. At the specified times, aliquots of vesicle cell suspension were removed and chilled to 0°. Separation of cells and vesicles was accomplished by gentle centrifugation, accelerating the rotor until a force of 3000 x g was reached and then slowing it immediately. This procedure pelletted the cells but left most of the vesicles suspended. The supernatants were removed and stored at 4° for analysis.

Separation of Vesicles and Vesicle-bound Protein from Cells and Soluble Protein—Continuous gradients of 0 to 30% sucrose (w/v) were prepared above a 100 µl cushion of 50% sucrose in 4.7-ml cellulose nitrate tubes (11 x 60 mm). Samples of up to 100 µl of vesicle extract were layered on top. The gradients were spun at 50,000 rpm (246,000 x g) for 2½ h. Fractions (0.3 ml) were collected at once. Alternatively, vesicles could be separated from soluble protein by gel filtration through Sepharose 4B in the usual buffer.

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6759
Analysis of Lipid and Protein Composition of Cells and Vesicles -

Acetylcholinesterase activity in erythrocytes and vesicles was assayed by the colorimetric technique of Ellman (10). Total ATPase and ouabain-inhibitable ATPase were assayed by the procedure of Bouting et al. (11). Total protein was assayed by the technique of Lowry (12); cholesterol in whole cells and vesicles was assayed as described by Grunze and Deuticke (2). Phosphate assays were conducted by a modification of the technique of Ames (13). Thin layer chromatography of vesicle lipids was performed on Silica Gel G impregnated with sodium acetate, using a modification of the method of Skipski et al. (14).

Exchange of phospholipids and cholesterol were monitored using radioactively labeled lipids. Phosphatidyl[3-3H]choline or [3-3H]cholesterol was mixed with the dimyristoylphosphatidylcholine prior to sonication, as described above, and sonication and incubation with cells were carried out as usual. After removal of vesicle supernatant, cells were washed three times by 10-fold dilution into buffer, with careful removal at each step of any vesicle material deposited on the cell pellet. Aliquots of washed cells were lysed by addition to 500 μl of 1 M H2O in a liquid scintillation vial, bleached by addition of 400 μl of 30% H2O2, suspended in 10 ml of Aquasol, and counted in a Packard Tri-Carb liquid scintillation spectrometer. Samples of cells labeled with phosphatidyl[3-3H]choline in such an experiment were incubated with nonradioactive lipid vesicles for 20 min, and incorporation of 3H into the supernatant was measured by scintillation counting. Release of radioactively labeled lipid was determined in a supernatant containing no vesicles determined by the same procedure.

Delivery of Vesicle Contents - Vesicles containing [3H]sucrose were prepared by sonication in buffer containing the label and then transferred into unlabeled buffer by gel filtration through Bio-Rad P-4. After incubation with cells and separation by centrifugation, the vesicles were again subjected to gel filtration to determine the amount of label which had escaped and should be considered as "external." A parallel vesicle sample was prepared by sonication in standard buffer, and "external" [3H]sucrose was added subsequently quently. Incorporation of 3H into the thoroughly washed cells was determined by liquid scintillation counting.

Iodination of Erythrocyte Proteins - Lactoperoxidase iodination of cells was carried out by the procedure of Hubbard and Cohn (15). Iodinated cells were then incubated with vesicles and the extracted proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using the protocol of Weber and Osborn (16). Gels were sliced into 2-mm sections which were dissolved in hydroxide peroxide and subjected to liquid scintillation counting to determine distribution of 125I.

Incorporation of 125I-labeled protein into erythrocytes was achieved by incubating cells with the labeled protein in the presence of lipid vesicles. After 40 min at 37°C, cells were separated from vesicles and supernatant protein by centrifugation and repeated washing with buffer. 125I incorporation was measured by liquid scintillation counting. Trapping controls were conducted by adding vesicles incubated with labeled protein (40 min at 37°C) to cells, chilling immediately to 4°C, then separating and washing as described above.

To determine the extent of nonspecific trapping of protein, the above experiment was repeated exposing erythrocytes to [3H]diisopropyl fluorophosphate-labeled trypsin (17) in the presence and absence of lipid vesicles. Incorporation of [3H]trypsin into stroma was determined as described above.

RESULTS

Phospholipid Exchange - Incubation of erythrocytes with dimyristoyl- or dipalmitoylphosphatidylcholine vesicles resulted in phospholipid exchange. Fig. 1 shows incorporation of phosphatidyl[14C]choline from vesicles into cells as a function of incubation time. The phospholipid transfer was reversible; labeled cells transferred radioactive lipid to nonradioactive vesicles (Table I). No comparable release of 14C-lipid was observed in the absence of vesicles.

Analysis of Extracted Lipids - Thin layer chromatographic analysis of vesicles after exposure to cells revealed that sphingomyelin comprised approximately 4% of the total phospholipid and that cholesterol was present. Phosphatidylethanolamine and phosphatidylserine were not detected.

Cholesterol Exchange - As reported by Grunze and Deuticke (2), erythrocytes incubated with phosphatidylcholine vesicles lost cholesterol. In the 60-min time interval of these experiments, 15 to 20% of cell cholesterol was lost to the supernatant (Fig. 2). The reverse process, incorporation of 3H]cholesterol from labeled vesicles into cells, also is shown in Fig. 2.

Delivery of Vesicle Contents - Erythrocytes were incubated with dimyristoylphosphatidylcholine vesicles in the presence of [14C]sucrose, which was either entirely internal or entirely external to the vesicles. In Table II, cell incorporation of the internal and external sucrose is compared. Incorporation was not enhanced when the sucrose was inside the vesicles; indeed, uptake of internal sucrose was negligible when correction was made for sucrose released from vesicles during the experiment. Thus "fusion" of vesicles and erythrocytes, as described for other types of cells, did not occur.

Protein Extraction - Phosphatidylcholine vesicles contained associated proteins after incubation with erythrocytes. Shown in Fig. 3 are vesicles which had been exposed to cells for various time intervals, then separated from the cells and subjected to sucrose density gradient centrifugation at an average force of 248,000 × g. This procedure separated vesicles from soluble protein and, in samples incubated for longer times, separated dense, protein-containing vesicles from pure
Selective Membrane Protein Extraction Using Lipid Vesicles

Fig. 2. Cholesterol exchange between erythrocytes and dimyristoylphosphatidylcholine vesicles. Experimental procedure was as for Fig. 1. Total cholesterol in erythrocytes (A) and vesicles (Δ) as a function of incubation time using the assay described under “Methods.” Erythrocyte incorporation of [3-14C]cholesterol from labeled vesicles (●).

Table II

Incorporation of [14C]sucrose in erythrocytes

| Conditions                        | % of supernatant [14C]sucrose taken up |
|----------------------------------|--------------------------------------|
| a. Vesicles + internal sucrose   | 2.7 ± 1                               |
| b. Vesicles + external sucrose   | 3.1 ± 1                               |
| c. Buffer + external sucrose     | 7.0 ± 2                               |

Cells were incubated for 30 min at 37°C with (a) vesicles containing 2 × 10^5 cpm of [14C]sucrose (internal), (b) vesicles suspended in buffer with 2 × 10^5 cpm of [14C]sucrose (external), and (c) buffer with 2 × 10^5 cpm of [14C]sucrose.

lipid vesicles. Distributions of protein and lipid in vesicle samples centrifuged after 0 and 20 min of incubation are shown in Fig. 3b. Although the fine structure of the denser bands was lost in fractionation, it could be shown that protein was associated with those bands and not with the less dense lipid band.

Fig. 4 shows the result of an experiment designed to distinguish between membrane protein bound in some manner to vesicle membranes and soluble protein simply trapped in vesicle interstices. One of the extracted proteins is acetylcholinesterase, and its enzymatic activity was found almost exclusively in the dense vesicle fractions. In contrast, when a soluble preparation of the same enzyme (bovine erythrocyte acetylcholinesterase) was sonicated and incubated with vesicles under the same conditions less than 2% of the esterase activity was carried to the high density fractions. In addition, polyacrylamide gel electrophores of proteins from the vesicle and soluble fractions of supernatant showed different polypeptide distributions (Fig. 5). Thus, the proteins associated with vesicles were not representative of the proteins available in the supernatant.

Fig. 3. a, dimyristoylphosphatidylcholine vesicle suspensions centrifuged at 250,000 × g on a 0 to 30% sucrose density gradient, before (A) and after incubation with erythrocytes for (B) 0, (C) 5, (D) 10, (E) 15, and (F) 20 min. b, distribution of protein and phospholipid on sucrose density gradients after incubation times of 0 (protein, △; lipid, ○) and 20 (protein, ●; lipid, Δ) min. Protein distribution was determined by Lowry assay; phospholipid distribution was determined using [14C]phosphatidylcholine, BSA, bovine serum albumin.

Fig. 5 shows the protein patterns obtained for (a) washed stroma, (b) unfraccionated postextraction supernatant, (c) high density vesicle fractions, and (d) soluble protein fractions subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The high density vesicle fractions contained five major polypeptide species, of apparent molecular weights 91,000, 81,000, 68,000, 30,000, and 15,000. Minor bands with molecular weights of 42,000 and 20,000 appeared after long incubation times. Species with very high molecular weights (>200,000) appeared in the gels only after cell lysis became extensive. When lactoperoxidase-iodinated erythrocytes were incubated with vesicles, the [125I] distribution in the gels paralleled the Coomassie blue staining pattern except for the 15,000-dalton species (Fig. 6). Thus, the four higher molecular weight proteins evidently originated on the cell surface.

3 See accompanying paper (9).
Selective Membrane Protein Extraction Using Lipid Vesicles

FIG. 4. Distribution of acetylcholinesterase (ACHE) activity in phospholipid vesicles centrifuged as in Fig. 3. Vesicles incubated with erythrocytes (A) and vesicles sonicated and incubated with soluble commercial bovine erythrocyte acetylcholinesterase (Δ). Esterase activity is expressed as ΔA410/min/ml of supernatant.

FIG. 5. Densitometer scans of sodium dodecyl sulfate-polyacrylamide gels of (a) washed stroma, (b) total supernatant after vesicle extraction, (c) dense lipid-containing fraction of total supernatant, (d) soluble protein fraction of total supernatant. Arrows indicate positions of the major extracted protein species (from left to right) weighing 91,000, 81,000, 68,000, 59,000, 41,000, and 30,000. Separation was achieved by gel filtration through Sepharose 4B.

Of the seven polypeptide species found in lipid vesicle extracts, only one has been identified. That is the membrane acetylcholinesterase, more than 80% of which is transferred from cells to vesicles in 30 min at 37°. Molecular weights reported for this enzyme range from 80,000 to 91,000; thus, the esterase may migrate with the 81,000 or 91,000-dalton band on these gels (18). While the identities of the other extracted proteins are not yet known, a number of possibilities have been excluded. The molecular weights of the extracted species (with the exception of the acetylcholinesterase) did not correspond to the values known for the major protein components of erythrocyte membranes (Bands 1 and 2, Mr > 220,000; Band 3, Mr = 90,000; Band 4, Mr = 77,500; Band 5, Mr = 45,000; and Band 6, Mr = 36,200). In addition, the protein extracts were not stained by Schiff's reagent (19), indicating that the abundant glycoproteins of the red cell surface were not present. The extracts contained no ATPase or NADP reductase activities and no epinephrine-, tubocurarine-, atropine-, or tetrodotoxin-binding species. Efforts to characterize the extracted proteins are continuing, with particular attention paid to one or more species which appear to regulate membrane sodium ion flux (see accompanying paper (9)).

FIG. 6. 125I-labeled phospholipid vesicle extract total supernatant after polyacrylamide gel electrophoresis. Top, Coomassie blue staining; bottom, 125I incorporation. Arrows indicate positions of major extracted proteins; see Fig. 5. Note that the top does not exactly match Fig. 5b. The former represents extraction through 30 min whereas the latter shows protein pattern after 45 min extraction. See following paper (10) for details of protein extraction versus time.

TABLE III

Incorporation of 125I into erythrocytes

|                        | cpm above background in 50 μl cells |
|------------------------|-------------------------------------|
| Vesicles incubated with cells for 30 min | 47 ± 10                             |
| Vesicles added after 30 min          | 26 ± 5                              |

125I-labeled proteins were incubated with vesicles and cells, or the protein-vesicle mixture was incubated separately and added to cells after 30 min at 37°. Incorporation into cell membranes was determined by liquid scintillation counting after extensive washing. Data are the average of 10 samples in each case.
Transfer of Extracted Proteins from Vesicles to Erythrocytes—When cells were incubated with vesicles in the presence of extracted proteins labeled with $^{35}$S, the cells incorporated a small fraction of the label (Table III). In the absence of vesicles, uptake of the labeled protein could not be detected. Vesicles did not mediate uptake of soluble protein (disopropyl fluorophosphate-inhibited trypsin) into red cell membranes.

Interaction of Liposomes with Erythrocytes—Incubation of cells with suspension of large, multimamellar vesicles (liposomes) for 1 h at 37° resulted in no detectable extraction of acetylcholinesterase and no cell lysis.

DISCUSSION

Incubation of human erythrocytes with sonicated phosphatidylcholine vesicles results in exchange of cholesterol, phospholipids, and certain proteins between their membranes. The molecular details of these processes are not understood at present, although it is known that small vesicles (~250 Å) rather than liposomes are required. The protein transfer is selective to the extent that many abundant proteins of the cell surface are not extracted, but the basis for this selectivity is not evident.

With one exception, the extracted species are cell surface proteins, since they are accessible to lactoperoxidase-catalyzed iodination in intact cells. Moreover, sphingomelin and phosphatidylethanolamine of the erythrocyte exists in the inner membrane monolayer, whereas phosphatidylcholine and sphingomelin are localized mostly in the outer monolayer. These observations suggest a superficial interaction between cell and vesicle in which neither loses its integrity. Present evidence suggests that the process is reversible; phospholipids, cholesterol, and small amounts of protein originating in the vesicles associated strongly with the cells during incubation. It is conceivable that a highly specific budding process occurring only in particular membrane regions could yield vesicles of the observed composition. However, to account for the reversibility of lipid and protein transfer, such budding would have to be coupled to the reverse process—fusion of vesicles with the cells. Attempts to deliver contents of the vesicle lumen into cells were unsuccessful, and detectable amounts of intracellular proteins (hemoglobin and NADP-reducing enzymes) did not appear in the supernatant until well after protein extraction was underway (see accompanying paper (9)).

Since lipid and protein transfer occur without mixing of cell and vesicle contents, a true fusion and budding mechanism appears unlikely.

Vesicle extraction of membrane proteins differs from other extraction processes in several respects. Extraction of erythrocytes with detergents, low or high ionic strengths, urea, and CaCl$_2$-free buffers all yield classes of proteins presumed to associate with the cells by specific electrostatic, hydrophobic, or cation-dependent forces (21, 22). The proteins extracted by these methods bear little resemblance to the vesicle extract, except for one or two common bands. Moreover, vesicle-extracted proteins are not technically "solubilized." They are associated with the vesicle membranes to the extent that physiological concentrations of salts do not dissociate them, but they are solubilized by 1 M saline or detergents which disrupt the vesicles. This association is not simply trapping in collapsed vesicle interstices since (a) protein species found in lipid fractions separated from soluble protein by density centrifugation are not representative of those in the soluble fractions, as would be the case for solubilized proteins partially trapped, and (b) the extracted acetylcholinesterase is equally active in intact vesicles and in vesicles disrupted by concentrated salt solutions, indicating full exposure to the solvent.

There is no conclusive evidence that extracted proteins associate with the vesicle membrane in a manner analogous with their native state, but their behavior is consistent with such an inference.

Continuing investigations are aimed at determining the roles of lipid charge, acyl chain length, cell surface charge and composition, cation concentrations, and other variables in the selectivity of protein extraction. Any mechanistic insight gained from these studies is of interest, not least because alteration of the selectivity could expand the field of proteins which could be partially purified by this approach. Other investigations are underway to improve the efficiency of protein transfer from vesicles back to cells since this delivery could provide a useful avenue to selective modification of membranes.

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