A Possible Role of Cholesterol in Membrane Adhesion†

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ABSTRACT: Calcium phosphate induced membrane aggregation was studied for erythrocyte vesicles and lipid membrane vesicles. The later lipid membrane components were similar in composition to those of erythrocyte membranes. The presence of an appropriate amount of cholesterol is an important factor in the production of the calcium phosphate dependent membrane aggregation.

Cholesterol is an important component of many biological membranes. Several roles of cholesterol have been elucidated such as the modification of membrane structure and its fluidity and molecular exchanges (Houslay & Stanley, 1982). We have found a new role of cholesterol related to membrane adhesion. Experimental evidence to support this proposal is presented.

Recently, it has been demonstrated that nascent calcium phosphates induce aggregation and fusion of human erythrocyte ghosts (Zakai et al., 1976, 1977), fresh erythrocytes (Baker & Kalra, 1979; Majumdar & Baker, 1980), and phospholipid vesicles (Fraley et al., 1980). Electron microscopic study shows that the fusing area of these membranes is an apposition of smooth surfaces that may consist of two apposed lipid membranes free from membranous particles (Zakai et al., 1977). These results indicate that lipid membrane interaction may be an essential part in the actual membrane fusion process in such a system.

In order to investigate further the molecular processes involved in these membrane aggregation and fusion events, we have produced a simple model membrane from erythrocytes, specifically cytoskeleton-free erythrocyte membrane vesicles (Leonards & Ohki, 1983), and studied the aggregation and fusion of these vesicles. It was observed that these vesicles aggregated in the presence of calcium phosphate and that aggregation was not affected by modifying various protein residues of the membrane surface with enzyme treatments (Leonards & Ohki, 1984). This suggested to us that membrane lipids are responsible for this aggregation phenomenon. We therefore investigated this possibility to determine which lipid in the membrane is responsible for the aggregation events observed.

In this report, we have performed a study of calcium phosphate induced aggregation of vesicles having different lipid compositions using the components found in the erythrocyte membrane: (a) erythrocyte vesicles with and without cholesterol partially depleted from the membranes; (b) lipid vesicles made of the total lipids from erythrocyte membranes with and without cholesterol depleted; (c) lipid vesicles made of various mixtures of the lipids consisting of the outer monolayer of erythrocytes [phosphatidylcholine (PC), sphingomyelin (SM), phosphatidylethanolamine (PE), and cholesterol].

Experimental Procedures

The cytoskeleton-free erythrocyte vesicles were prepared from fresh erythrocytes by titrating with ethylenediaminetetraacetic acid (EDTA) and Ca^{2+} at 45 °C together with the use of sucrose density gradient centrifugation techniques. The details of the procedure used to produce this preparation and the protein and lipid compositions of the resulting vesicles are described in an earlier paper (Leonards & Ohki, 1983). Cholesterol-depleted erythrocyte ghosts were prepared according to published methods (Pal et al., 1981). The cholesterol was determined according to Kates (1972) and the phosphate according to Bartlett (1959). For cholesterol and phospholipid determination, samples of erythrocyte vesicles were washed 3 times in distilled water to eliminate water-soluble components. The Bio-Rad protein assay (Bradford, 1976) was employed for determination of equivalent proteins in the vesicles. All organic solvents used (chloroform, methanol, and acetone) were redistilled from reagent-grade stocks. Lipids used for making lipid vesicles of type c were obtained from Avanti Polar Lipids (>99% pure). Cholesterol was obtained from Fisher Chemical Co. and was recrystallized from ethanol before use. Lipid vesicles using lipids of types b and c were small unilamellar vesicles and were made in the presence of 0.1 M NaCl and 2 mM Hepes, pH 7.4. The details of vesicle preparation have been published elsewhere (Ohki et al., 1982). The supernatant of the vesicle suspensions, centrifuged at 100000*g* for 1 h, was used in the experiments as the stock vesicle suspension. The vesicle preparation was conducted at 37 °C.

Calcium phosphate induced vesicle aggregation was monitored turbidimetrically. For a detailed discussion concerning the turbidity assay, its limitations, and the optimization of the experimental conditions for studying the aggregation of these vesicles, see Leonards & Ohki (1984). In brief, an aliquot (see figures) of the stock erythrocyte or lipid vesicles was suspended in 0.1 M NaCl and 2 mM phosphate at pH 7.6. After the vesicles were incubated for 10 min in the vesicle suspension solution at 30 °C, Ca^{2+} was added to the solution to give a final Ca^{2+} concentration of 10 mM. The time-dependent turbidity changes were monitored continuously at 400 nm (A_{400}) with a double-beam spectrophotometer (Hitachi 100-60), equipped with a temperature-controlled cell housing connected to a water bath/pump (Neslab Instruments). In some experiments, the amounts of phosphate and vesicle concentrations were varied. The experimental temperature was kept at 30 °C throughout.

It should be noted that the experimental conditions employed in the present study were specifically chosen to avoid membrane fusion (Leonards & Ohki, 1984). One of the advantages of using calcium phosphate is that turbidity changes due to vesicle aggregation are fully reversible (OD_{400} returns to 0) by acidifying the medium with acetic acid, while those due to membrane fusion are not (Zakai et al., 1976). In the present experiments all turbidity increases were fully reversible by acetic acid titration.

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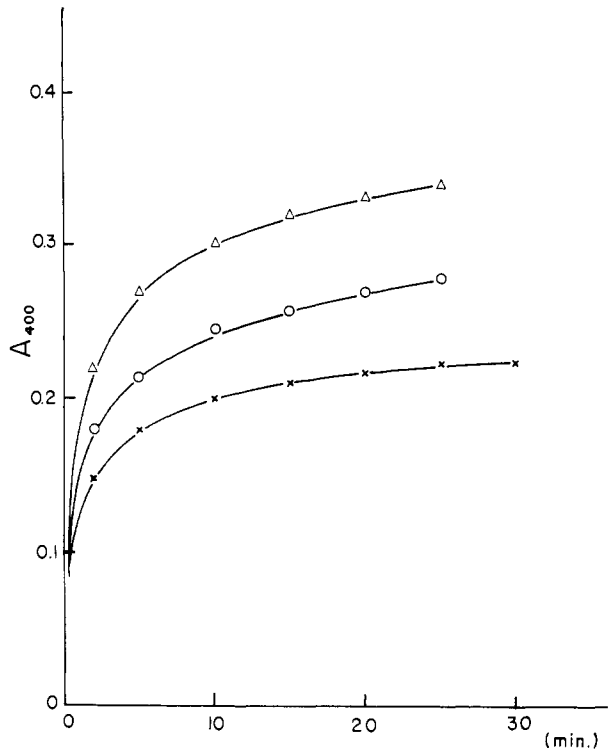

FIGURE 1: Turbidity (A_{400}) of human erythrocyte vesicle (Heves) suspension by calcium phosphate as a function of time (pH 7.6; 30 °C): (△) Heves (not cholesterol-depleted) suspension [2 mM phosphate + 10 mM $CaCl_2$; cholesterol/phospholipid = 0.94/1.0 (mol/mol)]; (○) cholesterol-depleted (~40%) Heves suspension [2 mM phosphate + 10 mM $CaCl_2$; cholesterol/phospholipid = 0.56/1.0 (mol/mol)]; (×) control (no vesicles; 2 mM phosphate + 10 mM $CaCl_2$). The vesicle concentration is ~24 μg of protein/mL of 0.1 M NaCl buffer solution. In order to deplete cholesterol from erythrocyte vesicle membranes, Heves was incubated in polyvinylpyrrolidone (PVP)–bovine serum albumin (BSA)–PC complex buffer solution (Pal et al., 1981) for 5 h under gentle stirring. For cholesterol-nondepleted erythrocyte vesicles, Heves was incubated under the same condition as the above except that it was incubated in PVP–BSA complex buffer solution. Then, both vesicles were washed and centrifuged with the buffer solution 2 times. The experiments were done by using these washed vesicles.

Results and Discussion

In the first series of experiments, the calcium phosphate dependent aggregation of erythrocyte vesicles with and without cholesterol depleted from the membrane was examined by measuring the turbidity changes (A_{400}) of the vesicle suspension with time. The cholesterol/phospholipid ratio of these isolated erythrocyte vesicles (nondepleted) is 0.94/1.0 (mol/mol), which is slightly higher than that found for Dodge ghosts (0.83/1.0). The phospholipid composition of the vesicles is the same as that found in Dodge ghosts (Leonards & Ohki, 1983). Previous experiments have also indicated no change in the size distribution of the erythrocyte vesicles as a consequence of membrane modification by a variety of agents (K. S. Leonards et al., unpublished results). In all experiments of this series, both the vesicle concentration (~24 μg of protein/mL) and Ca^{2+} concentration (10 mM) were kept constant. Vesicle aggregation is defined as the difference in turbidity between the calcium phosphate solutions with and without suspended vesicles. The experimental results are shown in Figure 1. Erythrocyte vesicles in which cholesterol was not depleted (normal) showed a greater degree of aggregation than those where the cholesterol content had been depleted 40% [cholesterol/phospholipid = 0.56/1.0 (mol/mol)]. The turbidity of 3 mM phosphate incubations was about twice that of the 2 mM phosphate incubations, but the net turbidity change due to vesicle aggregation (corrected for calcium phosphate aggregates) was approximately the same for the two cases.

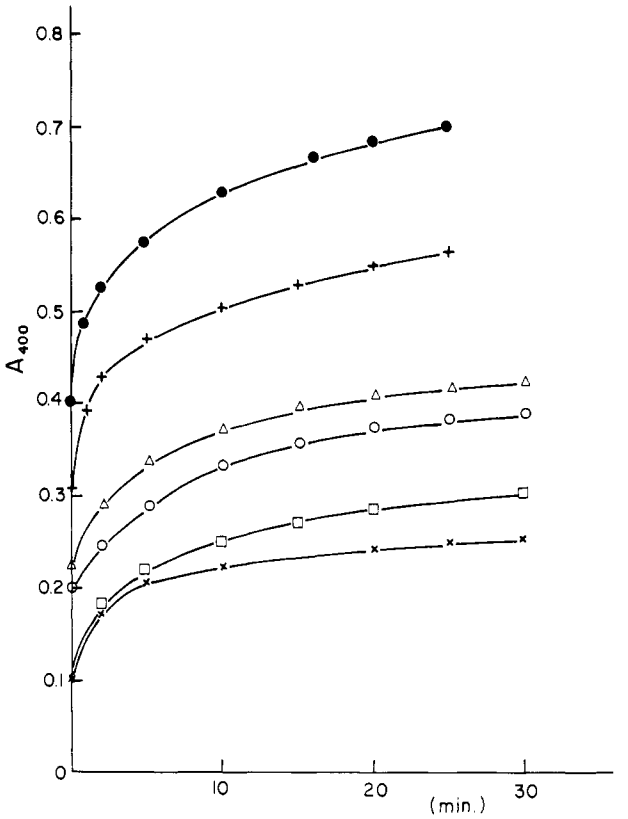

FIGURE 2: Calcium phosphate induced aggregation of erythrocyte lipid vesicles as a function of time. The vesicles were made of either the total lipid extracts of Dodge ghosts obtained from human erythrocytes or the cholesterol-depleted lipids from the total lipids by a silicic acid column as acetone and methanol elution fractions (Radin, 1969). It was found that the content of glycolipids was very small compared to that of phospholipids. (×) Control (no vesicles; 2 mM phosphate + 10 mM $CaCl_2$); (□) the total phospholipid vesicle (30 nmol of phospholipid/mL) suspension (2 mM phosphate + 10 mM $CaCl_2$); (○) the total lipid vesicle (20 nmol of phospholipid/mL) suspension (2 mM phosphate + 10 mM $CaCl_2$); (△) the total lipid vesicle (30 nmol of phospholipid/mL) suspension (2 mM phosphate + 10 mM $CaCl_2$); (+) control (no vesicles; 3 mM phosphate + 10 mM $CaCl_2$); (●) the total lipid vesicle (20 nmol of phospholipid/mL) suspension (3 mM phosphate + 10 mM $CaCl_2$). The suspension solution was 0.1 M NaCl, pH 7.6 at 30 °C.

In the second series of experiments, the aggregation of vesicles made from the total lipids extracted from erythrocyte membranes with and without cholesterol was studied in a way similar to the first experiments. The experimental results are shown in Figure 2, where vesicle and phosphate concentrations were varied while the Ca^{2+} concentration was kept constant at 10 mM. In this case, the vesicle membrane that contained cholesterol also showed the greatest turbidity change as observed in the intact erythrocyte vesicles. In order to ascertain that the increase in turbidity is due to cholesterol and not to different concentrations of lipid vesicles, the following two cases were examined: (1) the same phospholipid concentration for two vesicle suspensions (total lipid vesicles and cholesterol-depleted total lipid vesicles); (2) the same lipid weight for two vesicle suspensions (total lipid vesicles and cholesterol-depleted total lipid vesicles). In both cases, cholesterol-containing lipid vesicle suspensions (Figure 2, △ and ○) showed greater turbidities than did cholesterol-depleted lipid vesicle suspensions (Figure 2, □). The vesicles without cholesterol showed slightly more turbidity than the control experiment (calcium phosphate only). The reason for this slight increase in turbidity by

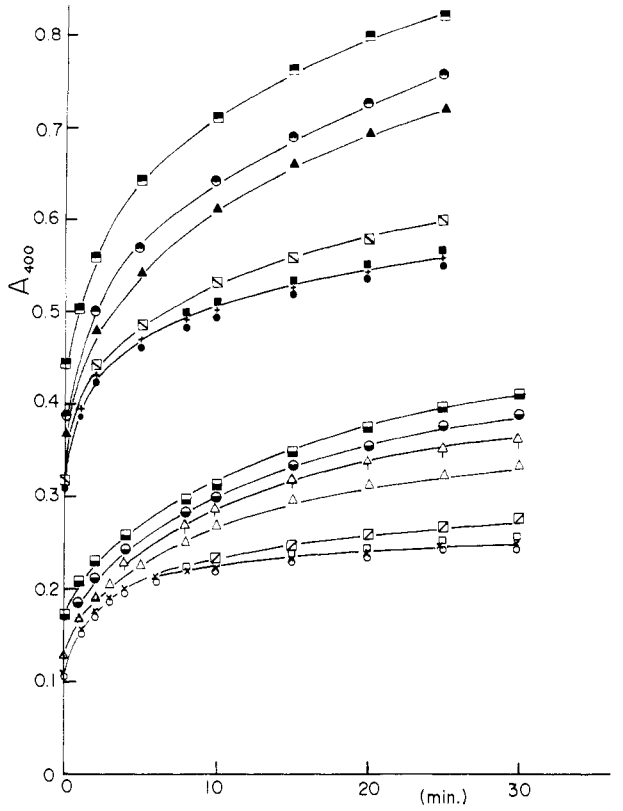

FIGURE 3: Calcium phosphate induced aggregation of lipid vesicles as a function of time. The vesicles were made of various mixtures of phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), and cholesterol (Chol) and suspended in 0.1 M NaCl buffer solution, pH 7.6 at 30 °C. The following were at 2 mM phosphate + 10 mM $CaCl_2$: (×) control (no vesicles); (○) PC (30 nmol of PL/mL); (□) PC/SM (1:1) (30 nmol of PL/mL); (right-slashed □) PC/PE/SM (1:1:1) (30 nmol of PL/mL); (△) PC/Chol (1:1) (20 nmol of PL/mL); (tailed △) PC/PE/Chol (1:1:2) (20 nmol of PL/mL); (bottom-filled ○) PC/PE/SM/Chol (1:1:1:3) (20 nmol of PL/mL); (bottom-filled □) PC/PE/SM/Chol (1:1:1:3) (30 nmol of PL/mL). The following were at 3 mM phosphate + 10 mM $CaCl_2$: (+) control (no vesicles); (●) PC (30 nmol of PL/mL); (■) PC/SM (1:1) (30 nmol of PL/mL); (left-slashed □) PC/SM/PE (1:1:1) (30 nmol of PL/mL); (▲) PC/Chol (1:1) (20 nmol of PL/mL); (top-filled ○) PC/PE/SM/Chol (1:1:1:3) (20 nmol of PL/mL); (top-filled □) PC/PE/SM/Chol (1:1:1:3) (40 nmol of PL/mL).

calcium phosphate may be due to the presence of acidic phospholipids (e.g., phosphatidylserine) in the total lipids of the erythrocyte membrane. The change in turbidity depended on vesicle concentrations.

In the third series of experiments, a similar aggregation study was made on lipid vesicle suspensions where vesicles were made having defined phospholipid compositions with and without cholesterol (Figure 3). The vesicles made of phosphatidylcholine only showed no aggregation in the presence of calcium phosphate. Phosphatidylcholine/sphingomyelin (1:1 mol/mol) vesicles also showed no aggregation. The vesicles made of PC/SM/PE (1:1:1) showed slight aggregation by calcium phosphate. Aggregation was greatly enhanced when cholesterol was incorporated into the vesicle membrane at a ratio of PC/SM/PE/cholesterol of 1:1:1:3; this enhancement was observed whether PC/SM/PE/cholesterol vesicle aggregation was compared to PC/SM/PE vesicle aggregation on an equal mole basis with respect to the phospholipids or on an equal mass basis with respect to the total lipids. When the phosphate concentration was increased from 2 to 3 mM, the turbidity of the vesicle suspension increased more than twice but the time-dependent net change in turbidity was approximately the same for both. When the vesicle concentration was increased, the initial slope of the turbidity curve was steepened with respect to time but the net turbidity change did not increase linearly with vesicle concentration.

It appeared that there was an optimum value for vesicle concentration to exhibit the largest turbidity change at a given calcium phosphate concentration for all the series of experiments mentioned above. This may be due to the adsorption of phosphate molecules on the vesicle surfaces. The contribution of the calcium phosphate complex to the total turbidity may therefore be slightly reduced. The increase in the steepness of the initial slope of the turbidity curve as the vesicle concentration was increased may indicate a greater rate of vesicle aggregation due to an increased rate of vesicle collision, which depends on the concentration of vesicles. Calcium or phosphate ions alone did not produce the vesicle aggregations mentioned above.

Our experimental results show clearly that the incorporation of cholesterol into neutral phospholipid membranes such as PC, PE, etc., at a molar ratio of 1:1, alters calcium phosphate dependent aggregation. It is known that when a lipid membrane is composed of equimolar cholesterol and neutral phospholipids, it forms a closed-pack molecular arrangement of the complete mixture (Copeland & McConnell, 1980). Also, NMR studies (Yeagle et al., 1975) suggest that when the cholesterol content in a phosphatidylcholine membrane was increased to 33–40% (molar ratio), the phospholipid head group structure is altered in such a way that the intermolecular complex formation between a phosphate and choline methyl group is inhibited; a cholesterol molecule may be located in between the two phospholipids and may disengage such intermolecular interaction between two head groups of the two phospholipid molecules. This is not observed when the cholesterol content is less than 30%. Probably the 1:1 mixture of phospholipid and cholesterol may just fit in the geometry to form molecular configurations such that the membrane surface may form a cavity where the hydroxyl head group of a cholesterol molecule can be situated at the center of the cavity and is surrounded by several phospholipid polar groups (Copeland & McConnell, 1980). It is considered that this cavity may be a place where a phosphate molecule can be adsorbed and onto which Ca^{2+} may bind and form a calcium–phosphate–lipid complex.

As has been suggested (Zakai et al., 1977), this type of calcium–phosphate–lipid complex surface may be responsible for the observed vesicle aggregation. The cell membrane adhesion found in myelin membrane systems may also be related to formation of such membrane surface complexes. Also, such calcium–phosphate–lipid complexes may be related to the site of fusion for two interacting erythrocyte membranes (Zakai et al., 1976; Majumdar & Baker, 1980; Hoekstra et al., 1983).

In this paper, we have stressed the role of cholesterol in lipid membrane adhesion, which may, in turn, play an important role in cell membrane adhesion, such as in erythrocytes, myelin membranes, etc. However, membrane-associated proteins may also contribute to membrane adhesion when exposed to phosphate and calcium ions. Such membrane protein involvement has been demonstrated for fusion by such agents as Sendai virus (Bachi et al., 1972), calcium ionophore (Ahkong et al., 1975), dimethyl sulfoxide and glycerol (McIntyre et al., 1974), and calcium phosphate in human erythrocyte ghosts (Zakai et al., 1976, 1977) and in intact human erythrocytes (Majumdar & Baker, 1980).

We suggest that the degree of each of the above two factors (membrane proteins and lipid membrane surface) that con-

tributes to cell adhesion and fusion events should be examined in the future.

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Occurrence of Novel Antigenic Phosphoinositol-Containing Sphingolipids in the Pathogenic Yeast *Histoplasma capsulatum*†

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ABSTRACT: Five alkali-stable lipids from the yeast phase of *Histoplasma capsulatum* have been purified and analyzed. Each compound has equimolar amounts of hydroxysphinganine (phytosphingosine) and a hydroxy or nonhydroxy 24:0 fatty acid. All yield inositol phosphate after acid hydrolysis, and several are novel in that they also yield dimannosylinositol (compound V) and isomeric galactosyldimannosylinositols (compounds VI and VIII) after strong ammonolysis. The foregoing as well as other data suggest that compound V is a dimannosylinositolphosphoceramide and compounds VI and VIII are galactosyldimannosylinositolphosphoceramides with isomeric head groups. The chromatographic behavior of compounds II and III indicates that they are similar to the inositolphosphoceramides previously observed in *Saccharomyces cerevisiae*. Compounds V and VI are virtually absent from the mycelial phase of *H. capsulatum*. Antibodies that react with compounds V, VI, and VIII have been detected in sera from patients with histoplasmosis.

Sphingolipids have been implicated in the regulation of mammalian cell growth and communication. Some specific cell surface components, such as the ABH and Lewis blood group antigens, stage-specific embryonic antigens, and some tumor antigens, have been identified as glycosphingolipids (Hakomori, 1981).

Higher plants (Laine et al., 1980) and fungi (Brennan & Lösel, 1978) contain a group of sphingolipids, not found in animals, with a ceramide consisting of a long-chain base, usually hydroxysphinganine (phytosphingosine), N-acylated with hydroxy and nonhydroxy fatty acids and with polar head groups consisting of inositol, phosphate, and carbohydrate. Although no function has been ascribed to these charged molecules, the demonstration of a mutant strain of *Saccharomyces cerevisiae* with an absolute requirement of a long-chain base for growth and synthesis of sphingolipids suggests that one or more of these lipids plays a vital role (Wells & Lester, 1983).

Histoplasma capsulatum, the causative agent of histoplasmosis, is a pathogenic, dimorphic fungus. In the soil, *H. capsulatum* grows as a filamentous mycelium, but it converts to a yeast-like form in the tissues of infected animals. The mycelial and yeast phases can be maintained in the laboratory by incubation at 25 and 37 °C, respectively (Pine, 1954). The biochemical basis of morphogenesis is unknown, although observed phase transition related changes in cAMP concentrations (Maresca et al., 1977) and cell wall glycans (Domer et al., 1967; Domer, 1971) have implicated surface components in the transition.

The possible involvement of sphingolipids in phase-transition phenomena and the possibility that such lipids might evoke antibodies of diagnostic significance in histoplasmosis led us to purify five major alkali-stable phospholipids from the yeast phase of *H. capsulatum*. Two of these, designated as compounds II and III, are similar to the inositol phosphoceramides isolated from *S. cerevisiae* (Smith & Lester, 1974). Glyco-

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