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Effect of cell shape, membrane deformability and phospholipid organization on phosphate-calcium-induced fusion of erythrocytes

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Fusion of bovine and goat erythrocytes was studied using the phosphate-calcium protocol. Both bovine and goat red cells are resistant to fusion with phosphate and calcium, under conditions that promote fusion of normal human erythrocytes. Fusion resistance is not related to decreased (5%) membrane deformability of erythrocytes of these species, since chicken erythrocytes which are 40% less deformable than human erythrocytes undergo fusion with efficiency similar to human red blood cells. Incorporation of either phosphatidylcholine or phosphatidylserine into bovine erythrocytes mediated by lipid exchange/transfer protein, caused fusion of these erythrocytes. Fluorescence analysis of merocyanine 540 dye labeled erythrocytes, by flow cytometry, showed that the frequency of cells which exhibit dye binding was much less (35%) in dimyristoylphosphatidylcholine (DMPC) incorporated compared to untreated bovine erythrocytes (80%), indicating that incorporation of DMPC caused closed packing of lipids in the external leaflet of the bilayer. These studies show that fusion of bovine erythrocytes, mediated by phosphate and calcium, has a requirement for either specific phospholipids such as phosphatidylcholine, phosphatidylserine, or closed packing of lipids in the external leaflet of the bilayer.

Introduction

The fusion of cellular plasma membranes is an essential step in a variety of biologically important processes, such as mitosis, phagocytosis, fertilization, exocytosis and endocytosis. The fusion in vitro can be experimentally induced by treatment with viruses, chemicals or by an electric field [1–3]. Numerous studies have revealed the role of specific surface proteins in the virus-induced fusion process [3–7]. However, relatively little is known of the biochemical and biophysical events involved in the membrane fusion process. During the past few years we and others [8–10] have studied the fusion of human red blood cells by the combined action of phosphate and calcium. The order of addition is critical since calcium followed by phosphate is ineffective, as is addition of a premixture of phosphate and calcium. Studies also

Abbreviations DMPC, dimyristoylphosphatidylcholine, DMPS, dimyristoylphosphatidylserine, DMPE, dimyristoylphosphatidylethanolamine, PC, phosphatidylcholine, PE, phosphatidylethanolamine, PS, phosphatidylserine, PI, phosphatidylinositol, Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

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showed that some of the rare-earth metals could substitute for calcium in the fusion of human red blood cells [10]. It has been observed that the fusogens cause aggregation of membrane-associated proteins and it is thought that fusion is initiated in the protein-free areas of lipids, where cells are in close juxtaposition to each other.

Since phospholipids are asymmetrically distributed in normal human red blood cells and these cells undergo fusion, we sought to determine whether altered organization and composition of membrane phospholipids would affect the fusion process, mediated by phosphate and calcium The results presented in this study show that bovine and goat erythrocytes, which lack phosphatidylcholine in their membranes, were resistant to fusion. Addition of phosphatidylcholine into bovine erythrocyte membranes mediated by phosphatidylcholine exchange protein renders them fusible. Moreover, studies reveal that the incorporation of phosphatidylcholine into bovine erythrocyte membranes results in closed packing of lipids in the external leaflet of the bilayer, as determined by steady-state fluorescence and flow cytofluorography of merocyanine 540 dye-labeled ghosts and intact red blood cells, respectively.

Part of this work was presented at XIth International Symposium on Structure and function of erythroid cells, August 31–September 3, 1986, Berlin, Germany.

Experimental Procedures

Merocyanine 540 was obtained from Molecular Probes, Junction City, OR; phosphatidic acid, cholesterol, DMPC, DMPS, DMPE, phospholipase A_2 and dipicolinic acid were obtained from Sigma. Terbium chloride was purchased from aldrich Chemicals (Milwaukee, WI). Silica gel TLC plates were obtained from J.T. Baker Chemical Co., NJ, U.S.A. All other reagents were of reagent or AR quality.

Red blood cells and ghost preparation. Human blood in heparinized tubes was obtained from normal individuals. Fresh bovine blood was collected into acid/citrate/dextrose at the slaughter house (Globe Meat Packing Co., Vernon, CA). Goat and chicken blood was obtained by venipuncture and collected in Alsevers media (7 5 g/l NaCl, 20 g/l D-glucose and 7.5 g/l sodium citrate) (Mission Laboratory Supply, Rosemead, CA). The blood was washed three times in phosphate-buffered saline (145 mM NaCl and 10 mM sodium phosphate buffer (pH 7.4)) by centrifugation at $800 \times g$ for 10 min. The buffy coat was removed by aspiration after each centrifugation. Erythrocyte ghost membranes were prepared using 40 vol of 5 mM phosphate buffer (pH 8.0) as hemolyzing buffer. This procedure was repeated three times with 40 vol. of 5 mM phosphate buffer (pH 8). The ghosts were sealed in this buffer containing 1 mM $MgSO_4$ at 37°C as described [11].

Assay of red blood cell fusion The fusion of red blood cells by phosphate and calcium was monitored by phase contrast microscopy, as described earlier by Baker and Kalra [9] and Majumdar et al. [10]. Briefly, red blood cells were washed three times in isotonic NaCl (300 mosM, pH 7 0) and resuspended in 40 mM phosphate-buffered saline (33 mM Na_2HPO_4/7 mM KH_2PO_4/90 mM NaCl (pH 7.4), 300 mosM) to a 10% hematocrit After 10 min at room temperature the cells were centrifuged at $300 \times g$ for 5 min and the supernatant was discarded. To the pellet, 20 mM $CaCl_2$ in Hepes-buffered saline (16 mM Hepes and 142 mM NaCl (pH 7.0), 300 mosM) was added to give a 10% hematocrit. After 10 min at room temperature, the cell suspension was incubated at 37°C for various lengths of time. An aliquot was withdrawn at various time intervals and examined by phase contrast microscopy [10].

Measurement of ghost fusion by fluorescence assay The fluorescence of the Tb-dipicolinic acid complex was measured by mixing equal amounts (40–60 g protein/2 ml) of terbium-containing and dipicolinic acid-containing ghosts in the reconstitution buffer, along with 0 1 mM EDTA, in a cuvette at 30°C, essentially according to the procedure of Hoekstra et al. [12]

Membrane deformability assay The membrane deformability of red blood cells was measured by a Nuclepore aspiration technique [13]. Briefly, red blood cells were aspirated into the pores of a Nuclepore filter (pore diameter 0.6 μm, Nuclepore Corporation, Pleasanton, CA) by hydrostatic pressure followed by glutaraldehyde fixation and examination by scanning electron microscopy. The average ratio, length/radius (L/r), of pressure-

induced pseudopods was determined on approx. 30 cells per sample to calculate membrane deformability.

Composition and orientation of phospholipids Lipids were extracted from washed erythrocytes using chloroform and isopropanol by the method of Rose and Oklander [14]. Phospholipid orientation was determined utilizing bee venom phospholipase A_2 (Sigma) as described [15]. Briefly, washed erythrocytes (0.5 ml packed cells) suspended in 5 vol of glycylglycine buffer [15] were treated with phospholipase A_2 (40 IU/5 ml) for 3 h at 37 °C. The reaction was terminated by the addition of EDTA (5 mM).

The extent of hemolysis caused by phospholipase treatment was ascertained by comparing the hemoglobin content of the supernatant to that of a 100% hemolyzed control. The lipids were extracted and quantified by two-dimensional TLC on silica gel G plates using $CHCl_3/CH_3OH/HOAc/H_2O$ (50 : 25 : 8 : 4, v/v) in the first direction and $CHCl_3/CH_3OH/H_2O$ (5 : 10 : 1, v/v) in the second direction as described earlier [15]. The phospholipids were also separated by one-dimensional TLC on silica gel G plates utilizing chloroform/ methanol/ formic acid (65 : 25 · 5, v/v) solvent system [16] wherein one can separate PE and PS easily. The extent of phospholipid degradation in phospholipase-treated cells was ascertained by calculating the decrease in the ratio of unhydrolized phospholipid to sphingomyelin. Phospholipase A_2 employed in these studies does not hydrolyze sphingomyelin. The phospholipid phosphorus in individual lipid spots was determined after $HClO_4$ digestion [17].

Phosphatidylcholine-transfer protein and non-specific phospholipid-transfer protein. The phosphatidylcholine (PC) transfer protein was isolated from bovine liver and purified essentially by the procedure of Westerman et al [18]. The non-specific lipid-transfer protein was purified from bovine liver following the method of Bloj and Zilversmit [19]. The lipid transfer proteins were stored in 50% glycerol at −20 °C Before use, the glycerol was removed by overnight dialysis against 5 mM phosphate buffer containing 10 mM β-mercaptoethanol.

Preparation of phospholipid vesicles Phospholipid vesicles were prepared containing phosphatidylcholine, cholesterol and phosphatidic acid (47 : 47 : 6, mol/mol) as described earlier [20]. Where indicated, phosphatidylcholine was replaced by equimolar amount of phosphatidylserine or phosphatidylethanolamine for the preparation of PS or PE liposomes. The lipids in chloroform were dried under reduced pressure to a thin film in a 15 ml Corex tube. 2 ml of 10 mM Tris buffer (pH 7.4) containing 150 mM NaCl, 25 mM glucose, 1 mM EDTA and 3 mM NaN_3 were added and the contents sonicated for 15–20 min in a Bransonic 220 Sonifier bath (125 watts) above the phase transition temperature. The vesicles were then centrifuged at $20000 \times g$ for 20 min to remove lipid aggregates.

Incubation of erythrocytes with exchange protein. 5 ml of bovine or goat erythrocytes (10% hematocrit in normal saline) were incubated with 0.5 ml of liposomes (5 μmol of phospholipid) in the presence or absence of exchange protein (120–150 μg protein), in a 15-ml Corex tube. The contents were shaken at 20 rpm on a Speci-mix (Thermolyn-Sybron) for 2 h at room temperature. The sample was centrifuged at $500 \times g$ for 10 min and washed three times with ice-cold phosphate-buffered saline to remove adhering liposomes.

Measurement of merocyanine 540 labeling to human and bovine erythrocyte ghosts and cytofluorography. The binding of merocyanine to the erythrocyte ghosts was done according to the procedure of McEvoy et al. [21]. Ghosts (500 μg protein/ml) were incubated with the dye (5 μg/ml) in 200 mM Tris-acetate buffer (pH 8.1), containing 100 mM NaCl and 0.2 mM EDTA in a total volume of 2 ml. After washing the unbound dye from the incubation medium, the fluorescence was measured in a Perkin Elmer MPF-4 Spectrofluorometer at 25 °C, with excitation and emission wavelengths set at 570 and 590 nm.

For flow cytometric analysis washed bovine erythrocytes ($5 \cdot 10^6$ cells/ml) were suspended in phosphate-buffered saline containing 0.1% bovine serum albumin. The merocyanine dye was added to a final concentration of 5 μg/ml and incubated for 4 min at room temperature. After washing three times with phosphate-buffered saline/ bovine serum albumin the cells were resuspended at 0.5% hematocrit in the same buffer. Flow cytometry was performed on a cytofluorograph 50H (Ortho

Diagnostic, Raritan, NJ). The cell sorter was equipped with an argon ion laser, operated at 400 mW. The relative fluorescence intensities were recorded by using excitation BP 545/20 and emission LP > 580 filters. The percentage of cells which exhibited fluorescence intensity above background was determined by FACS interface computer integration [22]

Phase contrast and scanning electron microscopy. Fusion of red blood cells was monitored in slide-cover slip preparations which were photographed using phase contrast microscopy. Gutaraldehyde-fixed cells were prepared for scanning microscopy by dehydrating in graded alcohol, critical point drying in liquid CO_2 and then sputter coating with gold and platinum [9,10]. Specimens were examined in a AMR-1000 scanning microscope (Advanced Metal Research Corp., Bedford, MA).

Analytical methods. SDS-gel electrophoresis was carried out on a 7.5% acrylamide gel according to the procedure of Fairbanks et al. [23]. Protein was estimated by the method of Lowry et al. [24], using bovine serum albumin as standard.

Results

Effect of red blood cell shape and membrane deformability on the fusion efficiency

In our previous studies [9], we showed that normal human red blood cells could be fused by the combined action of phosphate and calcium. Agglutination and fusion were contingent on phosphate treatment prior to calcium addition. Goat erythrocytes, which appear morphologically triangular/irregular in shape (Fig. 1(A)) [25], did not undergo fusion up to 3 h with the phosphate-calcium protocol. Fusion resistance of goat erythrocytes could be due to its non-biconcave shape. However, bovine erythrocytes, which are biconcave in shape (Fig. 1(B)), also did not fuse with the above protocol. In contrast, chicken erythrocytes, which are ellipsoidal and nucleated, have been observed to fuse [26]. As shown in Table I, chicken erythrocytes, with 40% reduced membrane deformability compared to human erythrocytes, fused at a rate comparable to human erythrocytes. Goat and bovine red blood cells were only 4–5% less deformable than human red blood cells (Table I), but still could not fuse. Thus, it appears that membrane deformability and cell shape of erythrocytes may not be determining factors for the phosphate-calcium fusion process.

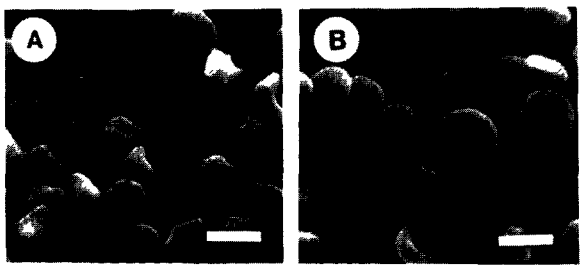

Fig 1 Scanning electron microscopy of erythrocytes Scanning electron micrograph of (A) goat erythrocytes and (B) bovine erythrocytes Bar, 5 μm.

Kinetics of human and bovine erythrocyte ghosts fusion as monitored by mixing of aqueous contents

Since intact bovine and goat erythrocyte did not fuse it was of interest to determine whether ghost membranes derived from bovine erythrocytes were capable of undergoing fusion induced by phosphate and calcium. As shown in Fig. 2, the addition of 1.75 mM Ca^{2+} to 1:1 mixture of terbium- and dipicolinic acid-loaded human

TABLE I

EFFECT OF CELL SHAPE AND MEMBRANE DEFORMABILITY ON THE FUSION EFFICIENCY

The efficiency of fusion was monitored by phase contrast microscopy at 600× and scored −, no fusion; +, 1–2 fusion vesicles/field, + + +, 4 or more fusion vesicles per field The membrane deformability was measured by nuclepore aspiration technique as described in Materials and Methods L/r is the ratio of length to radius of pseudopod of red blood cell extruded through Nuclepore filter and examined by scanning electron microscopy as described in Materials and Methods

Red blood cell species	Cell shape	Pseudopod (L/r)	Relative deformability (% of human red blood cell)	Fusion
Human	biconcave	3 21 ± 0 64	100	+ + + +
Bovine	biconcave	3 07 ± 0 47	96	−
Goat	triangular	3 05 ± 0 53	95	−
Chicken	ellipsoidal	1 87 ± 0 27	58	+ + + +

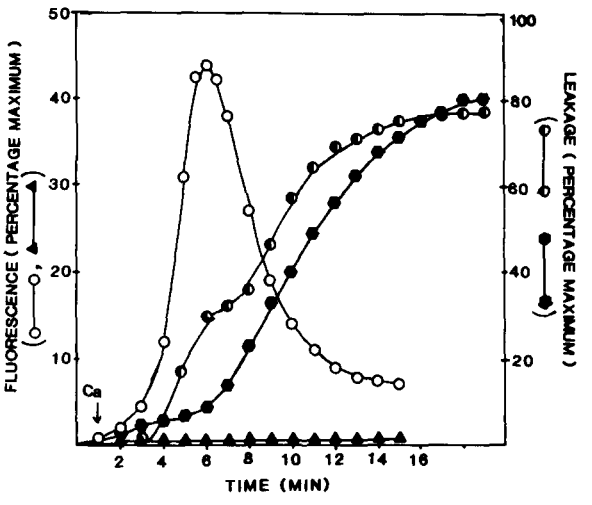

Fig. 2 Calcium and phosphate induced fusion of bovine and human erythrocyte hosts and release of their contents. Equal amounts of Tb-containing and dipicolinic acid-containing ghosts were mixed (approx 50 μg protein/ml) in the fusion buffer consisting of 120 mM KCl, 30 mM NaCl, 0 1 mM EDTA and 10 mM sodium phosphate buffer (pH 7 4) at 30 °C. The fusion was initiated by the addition of 1 75 mM Ca^{2+} (arrow) and fluorescence was measured continuously as described in Materials and Methods. Release of contents were determined in parallel experiments by monitoring the release of encapsulated fluorescent Tb-dipicolinic acid complex from Tb dipolinic acid-loaded erythrocyte ghosts The release of encapsulated materials was monitored by following the decrease in the fluorescence intensity since EDTA and Ca^{2+} present in the medium will result in instantaneous disappearance of fluorescence ○, Fusion of human erythrocyte ghosts; ▲, fusion of bovine erythrocyte ghosts, ◐, leakage of encapsulated Tb-dipicolinic acid complex from bovine erythrocyte ghosts, ◆, leakage of terbium-dipicolinic acid complex from human erythrocyte ghosts

erythrocytes ghosts, in the presence of 10 mM phosphate, resulted in an increase in fluorescence, an index of formation of terbium-dipicolinic acid complex, as a result of fusion of vesicles. The extent of fusion was 5% at 1.0 mM Ca^{2+} and 40% at 1.75 mM Ca^{2+} (data not shown), in agreement with studies of Hoekstra et al. [12]. Under similar conditions (1.0–2.0 mM Ca^{2+}), bovine erythrocyte ghosts did not exhibit an increase in fluorescence (Fig. 2), indicating absence of fusion.

Since the fluorescence intensity decreased after 6 min following addition of Ca^{2+} to a mixture of terbium- and dipicolinic acid-containing human erythrocyte ghosts, the decrease in fluorescence could have occurred as a result of leakage of contents into the external EDTA-containing media. The leakage of contents was monitored by the release of terbium-dipicolinic acid complex into the external EDTA-containing medium from Tb · dipicolinic acid-entrapped ghosts. As shown in Fig. 2, the addition of Ca^{2+} (1.75 mM) caused 8 and 50% release of contents after 6 and 10 min, respectively, in human erythrocyte ghosts. However, the extent of release of aqueous contents was much faster, 50% at 8 min, in the case of bovine erythrocyte ghosts upon the addition of Ca^{2+} (1.75 mM). Since bovine erythrocyte ghosts exhibited rapid leakage of entrapped fluorescent dye, this assay could not be employed in subsequent studies to evaluate fusion efficiency of bovine erythrocytes.

Composition and orientation of phospholipids in bovine and goat erythrocytes

To investigate specific cellular properties in erythrocytes of bovine and goat which could play a role in their resistance to fusion, their membrane lipid composition was determined. As shown in Table II, bovine and goat erythrocytes show a striking absence of phosphatidylcholine, which otherwise is present to the extent of 26–30% of total phospholipids in normal human erythrocytes. Our results confirm the earlier finding of Nelson [27], who also showed the absence of phosphatidylcholine in red blood cells from these ruminant species. The amount of sphingomyelin was 45–50% of total phospholipid in erythrocytes from goat and cow as compared to 25–27% in human erythrocytes. The organization of phospholipids in erythrocytes membranes of these species was determined by using phospholipase A_2 as a probe. Phospholipase A_2 treatment of bovine erythrocytes (Table II) resulted in the hydrolysis of 17.8 $\pm$ 3.1% phosphatidylethanolamine and 9.8 $\pm$ 2.1% of the phosphatidylserine, while the content of sphingomyelin and phosphatidic acid remained unaltered. Phospholipase A_2 treatment caused a minimal cell lysis (1–2%) as determined by hemoglobin release. When goat erythrocytes were treated with phospholipase A_2 approx. 18.7 $\pm$ 2.24% of the phosphatidylethanolamine and 12.7 $\pm$ 2.1% of phosphatidylserine was degraded (Table II)

TABLE II

COMPOSITION AND DEGRADATION OF PHOSPHOLIPIDS BY PHOSPHOLIPASE A_2 IN BOVINE AND GOAT ERYTHROCYTE MEMBRANE

Bovine or goat erythrocytes (10% hematocrit) were incubated in the presence or absence of phospholipase A_2 (40 units) in glycylglycine buffer as described in Materials and Methods After 3 h, the reaction was terminated by the addition of EDTA (5 mM) Red blood cells were centrifuged at $300 \times g$ for 10 min and washed three times with 154 mM NaCl and hemolyzed with 500 μl of water Lipids were extracted and analyzed The percentage of phospholipid degraded by phospholipase A_2 was calculated and expressed as mean ± S D of three independent observations n.d , not determined

Phospho-lipids	Bovine		Goat	
	total phospho-lipids (%)	phospholipids hydrolyzed by phospholipase A_2 (%)	total phospho-lipids (%)	phospholipids hydrolyzed by phospholipase A_2 (%)
Sphingo-myelin	47 48 ± 1 58	0	45 76 ± 5 16	0
PC	< 0 2	0	< 0 2	0
PE	28 23 ± 1.42	17 88 ± 3 12	28 51 ± 4.46	18 72 ± 2 19
PS	17 25 ± 5.46	9 87 ± 1 63	13 03 ± 3 72	12 69 ± 2 11
PI	3 70 ± 0 28	n d	4 60 ± 0 39	n d
Phosphatidic acid	6 70 ± 0 49	n d	5 89 ± 0 63	n.d

Incorporation and orientation of phosphatidylcholine into bovine erythrocytes

Since bovine and goat erythrocyte membranes do not contain phosphatidylcholine, exogenous phosphatidylcholine was incorporated into these membranes utilizing purified phosphatidylcholine-transfer protein. Erythrocytes were incubated with phosphatidylcholine liposomes (DMPC/cholesterol/phosphatidic acid, 47 : 47 : 6, mol/mol) in the presence and absence of exchange protein at room temperature for 2 h. As shown in Table III, phosphatidylcholine was incorporated to the ex-

TABLE III

EFFECT OF INCUBATION OF DMPC LIPOSOMES WITH BOVINE ERYTHROCYTES ON THE LIPID COMPOSITION

Phospholipid vesicles (approx 5 μmol phospholipids prepared from dipalmitoylphosphatidylcholine/cholesterol/phosphatidic acid (47 . 47 6, mol/mol) were incubated with 5 ml of bovine red blood cells (10% hematocrit) in 10 mM Tris buffer containing 1 mM EDTA, 25 mM glucose, 150 mM NaCl and 3 mM NaN_3 (pH 7 4) in the presence or absence of 25 μg/ml phosphatidylcholine-exchange protein for 2 h at 25 °C After incubation, liposomes were separated from erythrocytes by centrifugation at $500 \times g$ for 10 min, followed by three washes with 10 ml of phosphate-buffered saline Lipids from the erythrocyte pellet were extracted and quantified as described in Materials and Methods The values are mean ± S.D of six determinations. Hydrolysis of phospholipids by phospholipase A_2 was carried out as described in the legend to Table II. n d , not determined

Phospholipids	Total phospholipids (%)			% of individual phospholipids hydrolyzed by phospholipase A_2	
	Untreated	After incubation			
		− PC-transfer protein	+ PC-transfer protein	− PC-transfer protein	+ PC-transfer protein
Sphingomyelin	50 30 ± 2 63	49 40 ± 4 75	40 77 ± 6 93	0	0
PC	< 0 2	< 0 2	11 69 ± 1 16	0	55 87 ± 7 98
PE	25 67 ± 3 45	26 11 ± 4 32	22 42 ± 6 48	16 52 ± 3 83	14 26 ± 4 65
PS	16 14 ± 1 55	15 85 ± 3.47	16 68 ± 3 78	10 12 ± 1 39	8 09 ± 2 98
Total phospholipids [a]	363 30 ± 56.49	345 21 ± 7.04	348 20 ± 9 40	n d	n d
Cholesterol phospho-lipid molar ratio	0 93 ± 0 03	0 98 ± 0.04	0 92 ± 0 02	n d	n d

[a] Expressed as μg/mg protein

tent of 12% of total phospholipids present in bovine erythrocytes. Under these conditions and utilizing an increased amount (2–3-fold) of exchange protein there was an insignificant incorporation of phosphatidylcholine into goat erythrocytes (data not shown).

Incubation of bovine erythrocytes with phosphatidylcholine liposomes (DMPC/cholesterol/phosphatidic acid) in the presence of exchange protein showed that 8–12% of sphingomyelin was removed from the membrane, while the content of phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol remained unchanged The molar ratio of cholesterol to phospholipid remained unchanged upon transfer of phosphatidylcholine into bovine erythrocyte membranes (Table III).

Phospholipase A_2 treatment of phosphatidylcholine-incorporated bovine erythrocytes showed 55% degradation of phosphatidylcholine, while the extent of hydrolysis of other phospholipids, phosphatidylethanolamine and phosphatidylserine remained unchanged (Table III). These results indicate that incorporated phosphatidylcholine is distributed symmetrically in the lipid bilayer of these membranes.

Since incubation of human erythrocytes with phosphatidylcholine liposomes has been shown [28] to result in the removal of specific proteins from membranes, it was of interest to determine whether this was also true for bovine erythrocytes. Incubation of either bovine or goat erythrocytes with DMPC liposomes (DMPC/cholesterol/phosphatidic acid) in the presence of exchange protein did not exhibit removal of any of the major membrane proteins (data not shown), as determined by SDS-gel electrophoresis.

Effect of incorporated phospholipids in bovine erythrocytes on the fusion efficiency

Bovine erythrocytes upon treatment with phosphate and calcium, fusion protocol did not exhibit fusion (Fig. 3(A)). However, phosphatidylcholine-incorporated bovine erythrocytes exhibited significant fusion after 30–45 min (Fig. 3(B)). Bovine erythrocytes which were incubated with PC liposomes in the absence of exchange protein did not show fusion activity up to 2 h (Fig. 3(A)). The time required to undergo fusion was delayed up to 1 h when the extent of incorporation of PC was 4% of total phospholipids. Goat erythrocytes did not show fusion with phosphate and calcium (data

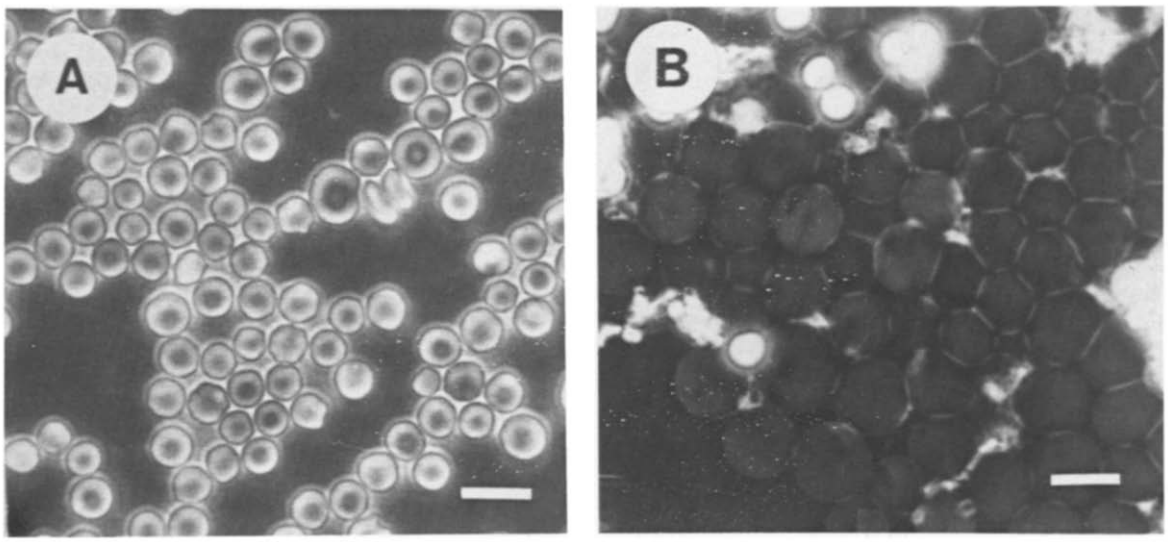

Fig 3 Fusion of PC-incorporated bovine erythrocytes induced by phosphate and calcium Phosphatidylcholine-incorporated bovine erythrocytes were prepared as described in Materials and Methods. Phase contrast micrographs of phosphate- and calcium-treated bovine erythrocytes (A) and (B) PC-incorporated bovine erythrocytes, incubated at 37 °C for 60 min In B, unfused erythrocytes appear dense, while fused vesicles are translucent Bar, 10 μm

not shown). Similarly, goat erythrocytes incubated with phosphatidylcholine liposomes in the presence of exchange protein, in which PC could not be incorporated, were also unable to fuse by the addition of phosphate and calcium (data not shown). Studies were carried out to determine whether the incorporation of other phospholipids into bovine erythrocytes membranes affected the fusion efficiency. Incubation of bovine erythrocytes with PS containing liposomes (DMPS/ cholesterol/phosphatidic acid, 47 . 47 : 6, mol/ mol), in the presence of non-specific lipid transfer protein, resulted in an increase in PS content from 14 to 18% of total phospholipids (Table IV). These PS-incorporated erythrocytes also underwent fusion with phosphate and calcium, though the extent of fusion after 2 h was 10–15% less compared to PC-incorporated bovine erythrocytes (data not shown). However, bovine erythrocytes incubated with phosphatidylethanolamine-containing liposomes (DMPE/ cholesterol/ phosphatidic acid, 47 : 47 : 6, mol/mol), in the presence of non-specific lipid-transfer protein, did not incorporate PE into membranes and also these erythrocytes failed to fuse.

Assessment of the lipid packing in erythrocytes

Since bovine erythrocytes in which either phosphatidylcholine or phosphatidylserine was incorporated underwent fusion by phosphate and calcium, it was of interest to determine how the phospholipid incorporation induced fusion. It has been shown recently [29,30] that the fluorescence of Merocyanine 540 dye is sensitive to the packing of lipids in the outer leaflet of the membrane. At a lipid/dye ratio of 1 : 20 (mol/mol) we compared the fluorescence in untreated, PC-incorporated, PE-incorporated and PS-incorporated bovine erythrocytes (Table V). The fluorescence of merocyanine 540 was reduced by 36% upon incorporation of PC, 18% by PS and 11% when PE was incorporated into bovine erythrocyte membranes compared to untreated membranes (Table V). However, when PC liposomes were incubated with bovine erythrocytes, in the absence of PC-transfer protein, the fluorescence intensity was similar to that of untreated bovine erythrocytes (data not shown).

Merocyanine 540-staining profile of untreated and PC-incorporated bovine erythrocytes was also ascertained by flow cytometry (FACS) technique. The fluorescence intensity profiles (Fig. 4(A)) show that a majority of merocyanine 540-labeled bovine erythrocytes exhibit fluorescence higher than the background. The median fluorescence intensity, as determined by FACS analysis, was similar (390 arbitrary units of fluorescence in log scale along the abscissa) for both untreated (Fig. 4(A)) and

TABLE IV

EFFECT ON LIPID COMPOSITION IN BOVINE ERYTHROCYTES UPON INCUBATION WITH PE- OR PS-CONTAINING LIPOSOMES

Phosphatidylethanolamine and dimyristoylphosphatidylserine vesicles were prepared by sonication with PE/- or PS/cholesterol/ phosphatidic acid (47 47 6, mol/mol), respectively Aggregates of multilamillar liposomes were removed by centrifugation at 20000 × *g* for 20 min Bovine erythrocytes (10% hematocrit) were incubated with PE or PS liposomes (5 μmol phospholipid) in 5 ml of 10 mM Tris-HCl buffer containing 1 mM EDTA, 25 mM glucose, 150 mM NaCl and 3 mM NaN_3 (pH 7 4), in the presence or absence of non-specific lipid-exchange protein (100 μg/ml). After 2 h of incubation, the cells were washed three times with phosphate-buffered saline and lipids were analyzed as described in Materials and Methods [14] The values (% of total phospholipids) are mean ± S.E of three independent observations

Phospholipids	PE liposomes		PS liposomes	
	non-specific lipid-exchange protein		non-specific lipid-exchange protein	
	−	+	−	+
Sphingomyelin	40 71 ± 3 10	40 52 ± 1 09	41.82 ± 1 07	40.73 ± 2 81
PC	< 0.2	< 0 2	< 0 2	< 0 2
PE	21 1 ± 0 82	20 7 ± 1 27	22 11 ± 1 70	20 41 ± 3 20
PS	16.82 ± 0 70	16 91 ± 0 21	14 11 ± 0 71	18 72 ± 1 20
Phosphatidic acid	6.70 ± 0 50	6 71 ± 0 69	6 71 ± 0 19	5 71 ± 0 18

TABLE V

MEROCYANINE 540 FLUORESCENCE OF BOVINE ERYTHROCYTES INCUBATED WITH PHOSPHOLIPIDS IN THE PRESENCE AND ABSENCE OF PC-TRANSFER PROTEIN

Bovine erythrocytes were incubated with either PC, PS or PE liposomes in the presence or absence of lipid transfer protein Sealed erythrocyte ghosts (approx. 1–2 mg protein) were incubated with merocyanine 540 dye (1.75 μM) in 200 mM Tris-acetate buffer (pH 8.1) 100 mM NaCl 0 2 mM EDTA in a total volume of 2 ml Steady-state fluorescence was measured in spectrofluorometer as described in Materials and Methods Merocyanine 540 dye binding and frequency of cells exhibiting fluorescence was determined by fluorescence-activated cell sorter (FACS). n d , not determined. Relative change refers to percentage change in fluorescence of liposome-treated red blood cells in the presence of lipid transfer protein compared to untreated red blood cells (100%)

Red blood cells incubated with	Steady-state in ghost membrane (arbitrary units) lipid-transfer protein		Relative change (%)	% cells positive for merocyanine 540 lipid-transfer protein	
	–	+		–	+
–	28	28	100	80	80
PC	27.5	18	64	77	35
PS	26	23	82	n d	n d
PE	26	25	89	n d	n d

bovine erythrocytes incubated with DMPC liposomes in the absence of phosphatidylcholine-exchange protein (Fig. 4(B)). However, incorporation of DMPC into bovine erythrocytes, utilizing PC-transfer protein, resulted in a lowered median fluorescences (Fig. 4(C)) compared to control bovine erythrocyte sample. Approx. 80% of bovine erythrocytes exhibited fluorescence above the background. This percentage of cells did not change significantly (77%) when bovine erythrocytes were incubated with DMPC liposomes in the absence of exchange protein (Table V). However, when bovine erythrocytes were incubated with DMPC liposomes in the presence of PC-transfer protein, which results in the net incorporation of PC, there was a significant decrease (2.7-fold) in the frequency (35%) of cells which exhibited merocyanine 540 dye binding (Fig. 4 and Table V).

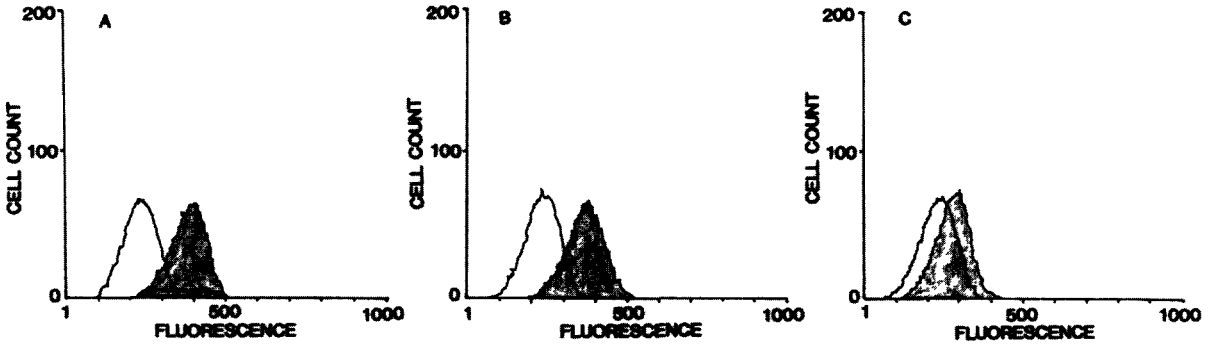

Fig 4 Flow cytometry analysis of the merocyanine 540-labeled bovine erythrocytes Washed bovine red blood cells were incubated with merocyanine 540 and the cell population which exhibited fluorescence was analyzed by a fluorescent-activated cell sorter (FACS) as described in Materials and Methods FACS generated fluorescence histograms for (A) bovine erythrocytes, (B), bovine erythrocytes incubated with DMPC liposomes in the absence of PC transfer protein, and (C) bovine erythrocyte incubated with DMPC liposomes in the presence of PC-transfer protein The abscissa shows the arbitrary units of logarithmically increasing fluorescence Unshaded area under the curve shows unstained cells while shaded area corresponds to dye labelled cells

Discussion

The present study shows that bovine and goat erythrocytes did not fuse by the combined action of calcium and phosphate under standard conditions that induce fusion of normal human erythrocytes [8–10]. The fusion incompetence of goat erythrocytes could have been due to its different morphology (triangular/irregular shape) [25]. However, bovine erythrocytes, which are discoidal in shape like normal human erythrocytes, also did not undergo fusion, although chicken erythrocytes, which are ellipsoidal in shape, undergo fusion by the phosphate-calcium protocol. Thus, it appears that cell shape may not be a determinant in the fusion process.

Since bovine and goat erythrocytes were slightly (4–5%) less deformable than normal human red cells, it would appear that the fusion incompetence might be due to reduced membrane deformability of these cells. However, erythrocytes of chicken are 40% less deformable than normal human red blood cells, yet the former fuse with equal efficiency. These results indicate that there is no correlation of membrane deformability of erythrocytes to fusion efficiency.

To investigate the specific cellular properties in the bovine and goat erythrocytes which may have been responsible for their resistance to fusion, we determined the lipid composition of these erythrocyte membranes The sphingomyelin content of bovine and goat erythrocytes was significantly high (45–48%) as compared to human erythrocytes [31]. The contents of phosphatidylethanolamine and phosphatidylserine in both bovine and goat erythrocytes are similar to that present in the normal human erythrocytes. Both bovine and goat erythrocytes lack phosphatidylcholine [27], which is a normal constituent of human erythrocytes [32,23]. Analysis of phospholipid orientation, using phospholipase A_2 as a probe, revealed that 18% of PE and 10% of PS in the case of bovine erythrocytes and 18% of PE and 12% of PS for goat erythrocytes were localized in the external leaflet of the bilayer of these membranes. The fusion incompetence of both bovine and goat erythrocytes may not be due to the altered fluidity of these membranes, since the cholesterol to phospholipid molar ratio was found to be similar to that of normal human erythrocytes. However, other factors, such as the ratio of unsaturated to saturated fatty acyl group affecting fluidity, have not been ruled out in the present study. The resistance to fusion could not be attributed to differences in the protein composition since SDS-gel profiles of erythrocyte membranes of bovine and goat were similar to humans. Thus, the only apparent difference between goat and bovine erythrocytes compared to human erythrocytes was the absence of phosphatidylcholine in these ruminant species.

The results show that the incubation of bovine erythrocytes with phosphatidylcholine-containing liposomes (DMPC/ cholesterol/ phosphatidic acid, 47 . 47 : 6, mol/mol) in the presence of phosphatidylcholine-transfer protein results in the incorporation of phosphatidylcholine (12% of total phospholipids), while in the absence of phosphatidylcholine-transfer protein PC is not incorporated into bovine erythrocyte membranes However, using a similar protocol, we were unable to incorporate phosphatidylcholine into goat erythrocyte membranes. It is pertinent to mention that the extent of exchange of PC in intact human or rat erythrocytes, mediated by phosphatidylcholine-transfer protein, was observed to be significantly slow compared to exchange reaction between phospholipid vesicles and rat liver microsomes [32]. Moreover, studies by Van Meer et al. [33] have shown that the exchange reaction in intact human erythrocytes was facilitated by utilizing a higher concentration of exchange protein. Thus, the inability to incorporate PC into goat erythrocyte membranes could be due to the stringent conditions required, which have not been established in the present study.

The phosphatidylcholine incorporated bovine erythrocytes underwent fusion in the presence of phosphate and calcium, as revealed by phase contrast microscopy. The fusion of bovine erythrocytes was also observed in PS-incorporated cells, although fusion efficiency was lower than that of PC-incorporated erythrocytes. Fusion was not observed in bovine erythrocytes which were incubated with PE-containing liposomes, irrespective of the presence or absence of non-specific phospholipid-exchange protein. In agreement with studies of Hoekstra et al. [12], we observed the

fusion of human erythrocyte ghosts, using terbium-dipicolinic acid fusion assay, while under similar conditions the fusion of PC-incorporated bovine erythrocyte ghosts was not observed, since encapsulated material leaked out within 8 min, prior to the time (30 min) necessary to undergo fusion. The resistance of fusion of bovine erythrocytes could not be due to the high content of sphingomyelin since incubation of these red blood cells with phosphatidylserine liposomes did not result in the removal of sphingomyelin from the membrane and yet these PS incorporated cells fused.

Since incorporation of phosphatidylcholine (PC) and phosphatidylserine (PS) into bovine erythrocyte membranes promoted fusion in the presence of phosphate and Ca^{2+}, we measured the lipid packing in the external leaflet of the bilayer with merocyanine 540 dye, which has been shown to be sensitive to the packing of lipids [30]. These studies also demonstrated that merocyanine 540 binds strongly to fluid-phase bilayers and not to gel-phase membranes. It has been observed that the fluorescent probe merocyanine 540 does not bind to normal human erythrocytes but binds strongly to human spherocytic erythrocytes [34], which have been shown to exhibit altered organization of phospholipids. Our studies also show that normal human erythrocyte membranes yield marginal fluoresccence with merocyanine 540, while bovine erythrocyte membranes exhibit a 5-fold increase in fluorescence when the dye to lipid molar ratio was more than 1 : 20. The fluorescence of merocyanine 540 in PC-incorporated bovine erythrocytes was 30% less compared to untreated erythrocytes at an equivalent dye to lipid molar ratio. Flow cytometry analysis showed that incorporation of PC into bovine erythrocytes results in a lower percentile of cells (35%) exhibiting fluorescence than untreated bovine red blood cells (80%).

Our results indicate that the incorporation of phosphatidylcholine into bovine erythrocyte membranes lead to an ordered packing of phospholipids in the external leaflet of the bilayer, similar to the situation prevalent in normal human erythrocytes The ordered packing of lipids may cause expulsion of water from the outer leaflet of the bilayer, a situation which is conducive for fusion to occur, as has been indicated for fusion of phospholipid vesicles [35,36]. The steps involved in fusion mediated by phosphate and calcium are not exactly clear, except that we know that entry of phosphate into cell is not essential [9] and the formation of crystalline hydroxyapatite on membrane surface is a pre-requisite [12] for fusion initiation. The addition of phosphate and calcium creates protein-free lipid areas wherein the apposed membranes come in contact and fusion may be initiated [9].

It appears that a disordered arrangement of phospholipids in the external leaflet of the bilayer results in the resistance to fusion of bovine erythrocytes. It should be pointed out that the presence of acidic phospholipids, such as phosphatidylserine, has been shown to promote fusion of phospholipid vesicles in the presence of calcium [37]. It has been suggested that Ca^{2+} induces phase separation of acidic lipids concomitant with the formation of crystalline domains. The fusion occurs between closely apposed membranes at the boundaries between crystalline domains and the surrounding non-crystalline lipids [38,39]. However, in normal human erythrocytes, negatively charged phospholipids are oriented on the inner leaflet of the bilayer, indicating that the presence of acidic phospholipids in the external leaflet may not be absolutely necessary in the fusion event of erythrocytes induced by phosphate and calcium. This is also supported by the observation that chicken erythrocytes, which lack phosphatidylserine [40] in their membranes, fuse with the same efficiency as human erythrocytes [26]. Studies are being carried out to delineate the molecular details of the fusion induced by phosphate and calcium.

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References

1 Poste, G and G L. Nicholson, (1978) Cell Surface Review, Vol 5, Elsevier/North-Holland, Amsterdam
2 Zimmerman, U , and Vienken, J (1982) J Membrane Biol 67, 165–182
3 Strittmatter, W J , Couch, C B and Mundy, D I (1985) Membrane Fluidity in Biology, Vol 4 (Aloia, R C and Boggs, J M , eds), pp. 259–291, Academic Press, New York
4 Scheid, A and Choppin, P W (1974) Virology 57, 475–490
5 Ozawa, M and Asano, A (1981) J Biol Chem 256, 5954–5956
6 Chejanovsky, N and Loyter, A (1985) J Biol Chem 260, 7911–7918
7 Doms, R W , Helenius, A. and White, J (1985) J Biol Chem 260, 2973–2981
8 Zakai, N , Kulka, R G and Loyter, A (1976) Nature 263, 696–699
9 Baker, R F and Kalra, V K (1979) Biochem. Biophys Res Commun 86, 920–928
10 Majumdar, S , Baker, R F and Kalra, V.K (1980) Biochim Biophys. Acta 61, 411–416
11 Steck, T L (1974) Methods Membrane Biol 2, 245–281
12 Hoekstra, D , Wilschut, J and Scherphof, G (1985) Eur J Biochem 146, 131–140
13 Baker, R F (1981) Blood Cells 7, 551–558
14 Rose, H G and Oklander, M (1965) J Lipid Res 6, 428–431
15 Jain, S K (1984) J Biol Chem 259, 3391–3394
16 Daleke, D.L and Huestis, W H (1985) Biochemistry 24, 5406–5416
17 Marinetti, G V (1962) J Lipid Res 3, 1–20
18 Westerman, J , Kamp, H H and Wirtz, K W A. (1983) Methods Enzymol 98, 581–586
19 Bloj, B and Zilversmit, B (1983) Methods Enzymol 98, 574–581
20 Sikka, S C , Green, G A , Chauhan, V P.S and Kalra, V K (1982) Biochemistry 21, 2356–2366
21 McEvoy, L , Williamson, P and Schlegel, A (1986) Proc Natl Acad Sci USA 83, 3311–3315
22 Green, G A , Rehn, M M and Kalra, V K. (1985) Blood 65, 1127–1133
23 Fairbanks, G , Steck, T L and Wallach, D F H (1971) Biochemistry 10, 2606–2026
24 Lowry, O H , Rosebrough, N.J , Farr, A L and Randall, R J (1951) J Biol Chem 193, 265–275
25 Jain, N C , Kono, C S , Myers, A and K Bottom, L Y (1980) Res Vet Sci 28 (1), 25–35
26 Majumdar, S and Baker, R F (1980) Exp Cell Res 126, 175–181
27 Nelson, G.J. (1967) Biochim. Biophys Acta 144, 221–232
28 Bouma, S R , Drislane, F.W and Huestis, H W (1977) J Biol. Chem 252, 6759–6753
29 Williamson, P , Mattock, K and Schlegel, R (1983) Biochim. Biophys Acta 732, 387–393
30 Williamson, P., Algarin, L , Bateman, J , Choe, H.R and Schlegel, R.A. (1985) J Cell Physiol 123, 209–214
31 Op den Kamp, J.A F (1979) Annu Rev Biochem 48, 47–71
32 Zwaal, R F A , Roelofsen, B , Comfurius, P and Van Deenen, L L.M (1975) Biochim Biophys Acta 406, 83–86
33 Van Meer, G., Poorthuis, B J H.M , Wirtz, K W A , Op den Kamp, J A F and Van Deenen, L L M (1980) Eur. J Biochem 103, 283–288
34 Williamson, P , Bateman, J , Kozarsky, K , Mattock, K , Hermanowicz, N , Choe, H R and Schlegel, R A (1982) Cell 30, 725–733
35 Pullman, B., Berthod, H and Gresh, N (1975) FEBS Lett 53, 199–204
36 Rupert, L.A M , Engberts, J B F N. and Hoekstra, D. (1986) J Am Chem Soc 108, 3920–3925
37 Fraley, R , Wilschut, J , Düzgünes, N , Smith, C and Papahadjopoulos, D. (1980) Biochemistry 19, 6021–6029
38 Papahadjopoulos, D., Poste, G and Vail, W J (1979) Methods Membrane Biol. 10, 1–119
39 Duzgünes, N and Papahadjopoulos, D. (1983) in Membrane Fluidity in Biology, Vol 1, General Principles (Aloia, R.C., ed), pp 187–216, Academic Press, New York
40 Kleinig, H , Zentgraf, H , Gomes, P and Stadler, J (1971) J Biol Chem 246, 2996–3000