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PHOSPHOLIPID ASYMMETRY IN THE MEMBRANES OF INTACT HUMAN ERYTHROCYTES AND IN SPECTRIN-FREE MICROVESICLES DERIVED FROM THEM

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Phospholipase A_2 from bee venom and *Naja naja* has been used to study the orientation of phospholipids present in the membrane of intact human erythrocytes and in spectrin-free microvesicles derived from the cells by treatment with Ca^{2+} and A23187. Little difference between the cells and microvesicles was observed in the apparent accessibility of phospholipids to the enzyme, suggesting that the original lipid asymmetry was maintained in the absence of spectrin. However, incubation of the microvesicles for 16 h at 37°C did lead to partial loss of asymmetry in the transmembrane distribution of phosphatidylcholine and phosphatidylethanolamine but not of phosphatidylserine. Despite the similarity of lipid asymmetry in cells and fresh microvesicles, the latter were about 40-fold more sensitive to phospholipase treatment than were cells. Although they retained the lipid asymmetry of intact cells, the microvesicles resembled ghosts in their great sensitivity to phospholipase A_2 attack, suggesting that the lipid packing in microvesicles and ghosts was similar. This conclusion was supported by the results of experiments with a fluorescent probe Merocyanine 540.

Introduction

One of the best-established features of the human erythrocyte membrane is the asymmetric distribution of phospholipids between the two leaflets of the lipid bilayer [1–4]. Virtually all of the PE and PS of the membrane is found in the inner leaflet of the bilayer, whereas PC and sphingomyelin are preferentially located in the outer leaflet. The origin of this asymmetry and the reason for its maintenance during the life of the cell are still open to conjecture but some recent studies have raised the possibility that interactions between spectrin, the major skeletal protein and the anionic phospholipids (especially PS) may help to maintain the asymmetry of these phospholipids in the inner leaflet of the membrane bilayer [5–8]. The lipid asymmetry, according to this hypothesis, is thus at least partially a consequence of the asymmetric distribution of spectrin across the membrane. Experiments supporting the above idea show that removal or denaturation of spectrin in the native erythrocyte membrane leads to a loss in lipid asymmetry, with the migration of the anionic phospholipids to the outer leaflet of the bilayer [5,6]. However, the possibility exists that the procedures used to remove or denature spectrin may affect lipid accessibility independently and the conclusions would be more convincing if similar results were obtained using membranes freed of spectrin by alternative methods. We therefore decided to study the orientation of phospholipids in the spectrin-free microvesicles which are released

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; Mops, 3-(*N*-morpholino)propanesulphonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

from human erythrocytes on treatment with Ca^{2+} and A23187 [9,10]. The membrane of these microvesicles has a similar lipid and protein composition to the bilayer portion of the cell membrane, and is devoid of skeletal proteins. Their ease of isolation, homogeneity and well-defined topology make such microvesicles a good system in which to examine phospholipid orientation in the absence of spectrin. In addition to experiments with phospholipase A_2, the fluorescent probe Merocyanine 540 [11] was also employed since there has been a suggestion [7] that this probe shows enhanced fluorescence in the presence of anionic phospholipids which might become exposed on external membrane faces following loss of phospholipid asymmetry.

Materials and Methods

Phospholipase A_2 from bee venom and *Naja naja*, standard phospholipids, Mops and Hepes were obtained from Sigma Chemical Company. A23187 was supplied by Calbiochem. Merocyanine 540 was a product of Eastman Kodak.

Production and isolation of microvesicles. Fresh blood samples obtained from various donors were sedimented and washed four times with 0.15 M NaCl with removal of the buffy coat. Microvesicles were isolated from erythrocytes treated with Ca^{2+} plus ionophore A23187 [12]. The microvesicles were resuspended in 20 mM Mops/NaOH buffer, pH 7.1 containing 0.15 M NaCl (Mops-saline) and were quantified by measurement of their phospholipid content after extraction with methanol/chloroform (2 : 1, v/v) [12].

Preparation of ghosts. Haemoglobin-free ghosts were prepared from fresh erythrocytes by lysis at 4°C in 20 volumes of 10 mM Hepes/NaOH buffer, pH 7.4 containing 0.1 mM EDTA, followed by four washes in the same medium.

Treatment with phospholipase A_2. Washed erythrocytes were resuspended at approx. 25% haematocrit in Mops-saline containing 1 mM $CaCl_2$ and preincubated at 37°C for 5 min. Phospholipase A_2 (10 $\mu g \cdot ml^{-1}$) from bee venom or *N. naja* was added and at timed intervals, 0.5 ml samples were added to 1.8 ml of methanol/chloroform (2 : 1, v/v) containing 2.5 mM EDTA in order to inactivate the enzyme and extract the lipids. Controls were incubated in the absence of added phospholipase A_2. Individual phospholipids were separated and quantified as previously described [12]. Haemolysis was determined by withdrawing 0.2 ml samples into 1.8 ml of Mops-saline containing 2.5 mM EDTA. After spinning at $16000 \times g_{av}$ for 20 min, the haemoglobin released into the supernate was quantified by measurement at 410 nm.

Ghosts and microvesicles at concentrations of lipid phosphorus equivalent to those in the intact red cell samples, were digested similarly with phospholipase A_2 (0.25 $\mu g \cdot ml^{-1}$).

Treatment of aged microvesicles with phospholipase A_2. Microvesicles obtained from fresh erythrocytes were kept at 37°C for 16 h in Mops-saline containing penicillin (200 $U \cdot ml^{-1}$) and streptomycin sulphate (100 $\mu g \cdot ml^{-1}$). Lysis measured by quantifying the haemoglobin released in the supernatant liquid after sedimenting the microvesicles was less than 20%. The 'aged' microvesicles were resuspended in Mops-saline and treated with phospholipase A_2 as described for fresh microvesicles.

Treatment of erythrocytes, ghosts and microvesicles with Merocyanine 540. Various concentrations of (a) erythrocytes, (b) ghosts, (c) ghosts plus haemoglobin or (d) microvesicles were incubated at 20°C in Mops-saline containing 0.5 μg Merocyanine 540. Emission spectra at 590 nm of these samples and of Merocyanine 540 alone were measured using a Perkin-Elmer LS-5 luminescence spectrometer. Cell, ghost and microvesicle concentrations were determined from their lipid phosphorus contents [12].

Results and Discussion

The results of our experiments in which intact cells or ghosts were exposed to phospholipase A_2 from bee venom (Figs. 1a, b) closely resemble those of previous investigators [1,2]. Under conditions where lysis was less than 2%, whole cells lost more than 50% of their PC, less than 5% of their PE and no PS, whereas ghosts lost more than 80% of their PC and PE and more than 60% of their PS. These results confirm the well-attested phospholipid asymmetry of the intact cell membrane and demonstrate the free access of the phospholi-

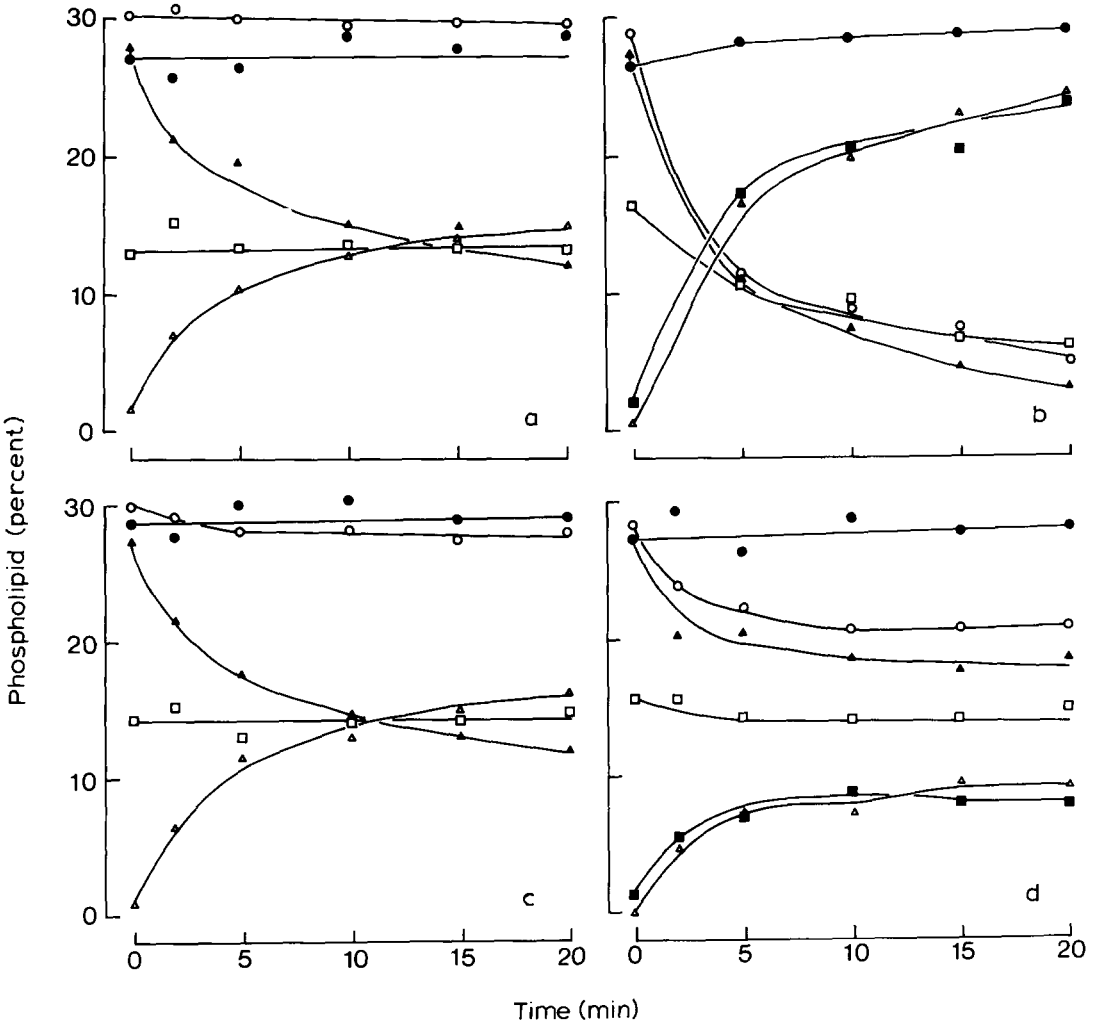

Fig. 1. Degradation of phospholipids in human erythrocytes, ghosts and microvesicles by phospholipase A_2 from bee venom. Washed erythrocytes (a), ghosts (b), fresh microvesicles (c) or 'aged' microvesicles (d) were resuspended in Mops-saline containing 1 mM $CaCl_2$ and preincubated at 37°C for 5 min. Phospholipase A_2 from bee venom (10 $\mu g \cdot ml^{-1}$ for intact cells and 0.25 $\mu g \cdot ml^{-1}$ for ghosts and microvesicles) was added and at timed intervals, the enzyme was inactivated and the lipids extracted. Enzyme-dependent lysis was less than 2% in intact cells and fresh microvesicles but increased to approx. 10% on treatment of 'aged' microvesicles with phospholipase A_2. The amounts of each lipid class (○, PE; ●, sphingomyelin; ▲, PC; □, PS; △, LPC and ■, LPE) were expressed as % of total phospholipid. The results shown here are derived from a single sample of cells but closely similar results were obtained in three other experiments using different cell samples under the same conditions.

pase to phospholipids in the ghost membrane. Essentially identical results were obtained using phospholipase A_2 from *N. naja* but the bee venom enzyme was preferred since it produced significantly less lysis that the enzyme from snake venom.

Microvesicles resembled ghosts in their extreme susceptibility to phospholipase A_2; maximum breakdown of phospholipids was achieved in both cases with amounts of enzyme which were only about 2% of those required for maximal phospholipid breakdown in cells (Fig. 1c). Despite this, the pattern of phospholipid degradation and enzyme-dependent lysis in microvesicles was extremely similar to that in whole cells, suggesting that the original phospholipid asymmetry was conserved even in the absence of cytoskeletal proteins. It appeared, however, that on prolonged incuba-

tion ('ageing') of microvesicles at 37°C (Fig. 1d), there appeared to be a significant loss of the original asymmetrical distribution of phospholipids because not only was more PE (28%) and PS (11%) degraded by phospholipase A_2 but PC become less accessible (35% breakdown). Since under these conditions, enzyme-dependent lysis after 20 min incubation was approx. 10%, these figures are likely to be overestimates of the amounts of each lipid which is broken down in the intact microvesicles.

Allowing for this lysis, a simple interpretation would lead to the conclusion that after 16 h at 37°C, there is virtually no transmembrane migraton ('flip-flop') of PS but that 15–20% of the PE has migrated to the outer leaflet of the microvesicle membrane. On the basis of recent work [13], demonstrating a slow 'flip-flop' of animal-derived PC across human erythrocyte membranes with a half-time of 11–13 h for trans-bilayer equilibration it might be anticipated that more than 50% of the PC in the aged microvesicles would still be present in the outer membrane leaflet after 16 h at 37°C. Our results on the hydrolysis of PC (Fig. 1d) present a problem since the accessibility of this phospholipid seems to be much lower than would be expected. It seems rather unlikely that PC actually reverses its original asymmetric distribution although it is possible that for some unknown reason reorientation of PE makes PC on the external leaflet less accessible to the phospholipase.

The fluorescent probe Merocyanine 540 was employed following reports that this compound was sensitive to changes in either lipid asymmetry [7] or surface pressure [14]. Haemoglobin-free ghosts showed no fluorescence at 590 nm in the absence of Merocyanine 540; however on addition of Merocyanine 540, a strong fluorescence was observed, consistent with a partition of the probe into a non-polar phase (Fig. 2). It was not possible to obtain meaningful results with intact cells since the large amount of haemoglobin present attenuated the excitation beam at 540 nm to such an extent that emission at 590 nm could not be measured (Fig. 2).

The fluorescence signal from Merocyanine 540 in the presence of fresh and 'aged' microvesicles was substantially weaker than that from ghosts but

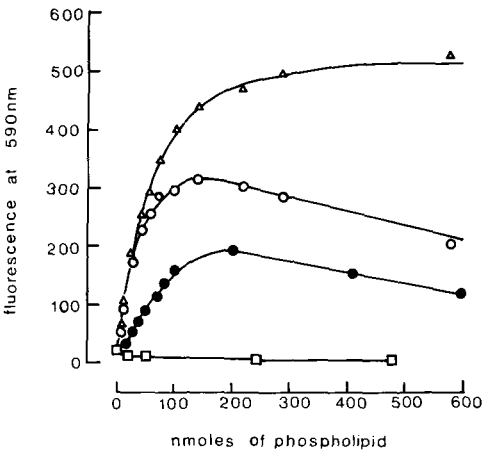

Fig. 2. Titration of Merocyanine 540 by cells, ghosts and microvesicles. Merocyanine 540 (0.5 μg) was added to increasing concentrations of intact cells (□), ghosts (△), ghosts plus haemoglobin (○) or fresh microvesicles (●). Fluorescence intensity at 590 nm was measured from emission spectra taken after each addition. The (ghosts + haemoglobin) and microvesicle samples contained approximately equivalent amounts of haemoglobin. Closely similar results were obtained in four further experiments using different preparations of cells, ghosts and microvesicles.

showed a similar saturation concentration (160 nmol of phospholipid/0.5 μg Merocyanine 540). Part of the decreased fluorescence with microvesicles compared with ghosts is no doubt accounted for by the absorption of the excitation beam by haemoglobin present inside the microvesicles but this seems unlikely to account for all the differences between ghosts and microvesicles since ghosts with sufficient added haemoglobin to mimic the proportions of membrane and haemoglobin in the microvesicles still showed about 1.5–2-times the Merocyanine 540 fluorescence exhibited by microvesicles (Fig. 2). It thus appears that there is a significant but unexplained disparity between the Merocyanine 540 signal at 590 nm with ghosts compared with that from microvesicle membranes. This disparity may be related to the obvious differences in the topological features of ghosts and microvesicles. Regardless of this point, our data seems to contradict the suggestion of Williamson et al. [7] that Merocyanine 540 does not readily enter a non-fluid lipid phase such as that presumed to exist in the outer leaflet of the red cell membrane. Spectrin-free microvesicles are shown here to have a lipid orientation very similar

to that of intact cells and yet they are able to take up Merocyanine 540 as judged by the enhanced fluorescence of this probe at 590 nm.

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