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Phospholipid outside-inside translocation in lymphocyte plasma membranes is a protein-mediated phenomenon

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We have measured the transbilayer diffusion of spin-labeled analogs of sphingomyelin, phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine in pig lymphocyte plasma membrane. At 4°C and 37°C the aminophospholipids are rapidly transported from the outer to the inner leaflet of the membrane, whereas the choline-containing phospholipids experience a slower diffusion. This selectivity is abolished after cell treatment by SH-group reagents indicating that the aminophospholipid translocation is protein-dependent and must be driven by a system analogous to the one existing in the human red cell membrane. The fact that the selectivity exists at low temperature, that it does not depend on cytoskeleton integrity and that there is a competition between the two aminophospholipids show that this translocation is not purely an endocytic process.

It has been recently demonstrated that, in the human erythrocyte, the transverse diffusion of aminophospholipids (i.e., phosphatidylserine and phosphatidylethanolamine) is dependent on cytoplasmic ATP [1–3] which is utilized by a transport system [4] to translocate these molecules from the outer to the inner membrane leaflet. On the other hand phosphatidylcholine and sphingomyelin diffuse passively, without such an ATP requirement, and at a much lower rate [1–3,5]. This selective transmembrane movement of lipids is related to the known asymmetrical distribution of these molecules between both halves of the erythrocyte membrane [6,7]. Although preliminary experiments have suggested the existence of a similar transport in platelets [2,8], the question remains whether the same system would exist in other cells, in particular in cells which are not from the red cell lineage. In this report we characterize phospholipid translocation in a lymphocyte plasma membrane: we present evidence for a selective and fast translocation of aminophospholipids from the membrane outer layer, which shares some of the characteristics of the system existing in erythrocytes which indicates that the putative 'aminophospholipid translocase' could exist in a wide variety of cells.

We have studied the phospholipid relocation in lymphocytes in suspension by using spin-labeled analogs as in our previous studies [1,4,5]. Lymphocytes were obtained from pig mesenteric lymph node cell suspensions by sequential re-

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moval of red blood cells by Ficoll-Hypaque density gradients. Lymphocyte suspension of 10^9 cells/ml were supplemented with phospholipid paramagnetic analogs which have a short β-chain (5 carbons) and bear an oxazolidine ring at the 4th carbon position. The analog final concentration was 30 μM representing approximately 1% of the plasma membrane phospholipids knowing that 10^9 lymphocytes contain 5 mg of plasma membrane proteins [9] with 730 nmoles of phospholipids per mg of protein [10]. Previous experiments have shown that such nitroxides can be chemically reduced by reducing compounds present in the aqueous phase (cytoplasmic content or externally-added ascorbate for instance), and that only those spin labels incorporated in the leaflet directly exposed to the reducing agents are modified. This family of paramagnetic molecules have proven to be reliable reporters of phospholipid behavior as the ATP requirement for aminophospholipid transport was later confirmed by other laboratories using long-chain diacyl phospholipids [2,3]. Fig. 1 shows the spontaneous reduction of the labels at 37°C after their insertion in the membrane outer leaflet. Since this reduction occurs at the cell interior, it reflects the outer to inner leaflet translocation of the phospholipids. It is clear that aminophospholipids, phosphatidyl-serine and -ethanolamine, are transported at a much higher rate than the other phospholipids, phosphatidylcholine, sphingomyelin and phosphatidic acid. This selectivity belongs to the cellular system and does not reflect a difference in the physical properties of the probes, since in pure lipid model membrane the five probes diffuse at the same slow rate [5]. In addition the chemical resistances of these spin labels to ascorbate and cell cytoplasm reducing system have always been found identical. The apparent sigmoid shape of the curves displayed in Fig. 1 is likely to be the consequence of the two sequential phenomenons taking place, translocation and reduction, the time constants of which are of the same order of magnitude. The same experiment can be performed at 4°C. In contrast to red cells, lymphocytes exhibit sufficiently high intracellular reducing properties at low temperatures that spontaneous reduction of the paramagnetic analogs at the cell interior can be monitored (Fig. 2). Here also a selective inward transport of aminophospholipids is evidenced, phosphatidyl-serine and -ethanolamine being reduced at a higher rate than phosphatidylcholine and sphingomyelin. Overall, these results are in agreement with those obtained in human red blood cells, although the kinetics were significantly slower in the latter case [1,11]. The increased

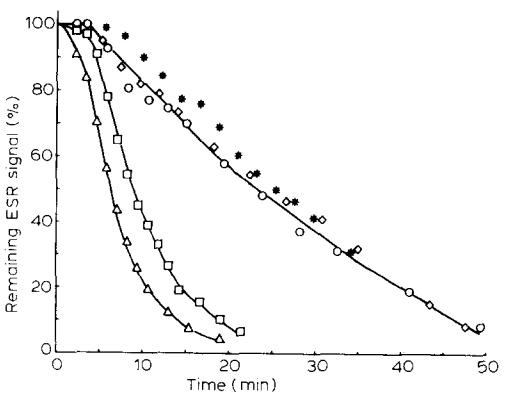

Fig. 1. Spontaneous reduction at 37°C of various spin-labeled phospholipid analogs incorporated in lymphocyte plasma membrane. After labeling of the cells by the analog, the evolution of the ESR signal is followed by repetitive scans in a Varian E-9 spectrometer the cavity of which was thermostated at 37°C. In order to minimize any phospholipase A_2 activity, lymphocytes were pretreated with 4 mM diisopropylfluorophosphate. △, phosphatidylserine; □, phosphatidylethanolamine; ○, phosphatidylcholine; ◇, sphingomyelin; *, phosphatidic acid.

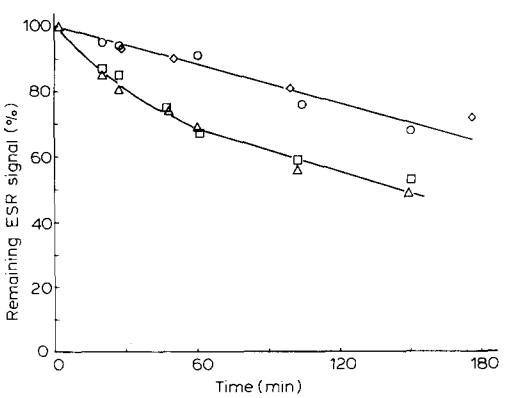

Fig. 2. Spontaneous reduction at 4°C of the paramagnetic phospholipid analogs incorporated in lymphocyte plasma membrane. Experimental conditions were identical to the ones described in Fig. 1 with the exception of temperature. For symbols, see also Fig. 1.

velocity in lymphocytes might be due to the presence of intracellular membranes which suck the probes from the cytoplasmic half of the plasma membrane. This phenomenon would lead to an over-estimation of the translocation rates but by no mean can explain the differences between the aminophospholipids and the choline derivatives.

The following experiments were aimed to show the involvement of a protein in the translocation process. Vanadate ions, which are known to prevent ATPases from forming a phosphorylated intermediate, inhibit aminophospholipid reorientation in erythrocytes. With lymphocytes, addition of 2 mM vanadate at 37°C increased the apparent half-time of reduction of aminophospholipids 1.5-times, without affecting the slow transport of phosphatidylcholine (not shown). Higher concentrations of vanadate led to a partial cell lysis. In order to further assess the proteic nature of the specific transport of aminophospholipids, lymphocytes were treated by a SH-group reagent, namely *N*-ethylmaleimide. This treatment abolished the reducing capability of the cell cytoplasm. Accordingly the phospholipid reorientation had to be tested at 4°C by using the classical ascorbate assay [1], i.e. by using an exogenous reducing agent. Results obtained are displayed in Fig. 3, which shows that there is no more difference between phosphatidyl-serine and -choline. Lymphocytes were also treated by iodoacetamide. This glycolysis inhibitor lowers the ATP content of the cell and reacts with all SH-groups available, thereby suppressing the reducing power of the cytoplasm. Results obtained by the ascorbate assay, on cells treated with iodoacetamide are included in Fig. 3. The data obtained are essentially identical to the ones resulting from *N*-ethylmaleimide treatment: namely the discrimination between aminophospholipids and choline phospholipids is lost.

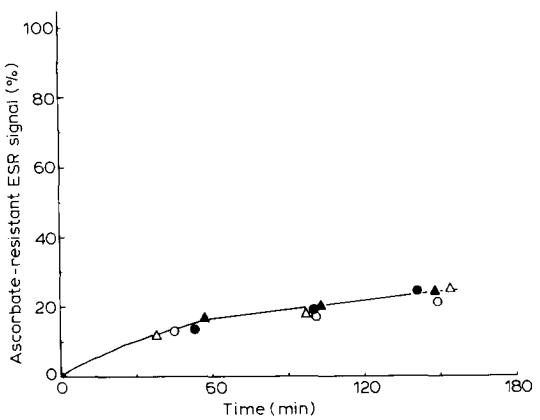

Fig. 3. Reorientation of phosphatidylserine (△, ▲) and phosphatidylcholine (○, ●) after cell treatment by *N*-ethylmaleimide (open symbols) and iodoacetamide (closed symbols). Lymphocytes were incubated at 37°C for 5 min in the presence of 10 mM *N*-ethylmaleimide or for 60 min with 1 mM iodoacetamide. Then cells were washed three times and cooled down to 4°C before addition of the spin-labeled analogs. As these treatments abolished the reducing capacity of cell cytoplasm, the estimation of the amount of probe which has moved from the membrane outer layer is done by adding to the cell suspension 20 mM of buffered sodium ascorbate which reduces the molecules in the membrane outer half.

One may question whether this selective internalization of aminophospholipids might be due to endocytosis of some membrane domains rather than to selective inward translocation by the putative aminophospholipid translocase [4]. Several arguments lead to the conclusion that endocytosis does not play an important role here. First of all, the experiment can be performed at 4°C (Fig. 2), a temperature at which endocytosis does not take place to any appreciable degree in lymphocytes [12]. Another argument against involvement of endocytosis in this selective uptake comes from the treatment of the cells at 37°C by a mixture of cytochalasin B (acting on microfilaments) and of colchicine (acting on microtubules), each at 7 μM final concentration. It has been shown that, in lymphoid cells, the internalization of the coated pit to form a coated vesicle is sensitive to cytochalasin [13] and thus dependent on some properties of the cytoskeleton [14]. Subsequent measurement of the spontaneous reduction of paramagnetic phosphatidylserine at 37°C showed no difference when compared to the control experiment in the absence of drugs (not shown). Finally, at 37°C, we assayed the simultaneous reduction of the two aminophospholipids when they were added together to lymphocytes. To distinguish between phosphatidyl-serine and -ethanolamine, the former probe was labeled by a ^{14}N-containing doxyl ring and the latter by a ^{15}N-containing one which gives rise to composite ESR spectra where

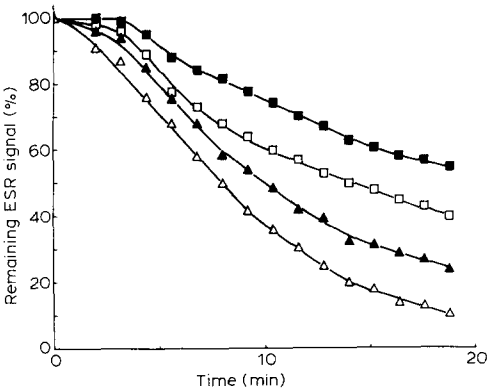

Fig. 4. Spontaneous reduction at 37°C of phosphatidylserine (▲, △) and phosphatidylethanolamine (■, □) incorporated independently (open symbols) or simultaneously in equimolar amounts (closed symbols) in lymphocyte plasma membranes. Each phospholipid is individualized by the nitrogen isotope of the nitroxide residue (see Ref. 4).

each contribution can be separately determined [4]. Results (Fig. 4) indicate a mutual slowing down of each reduction rate by the simultaneous presence of the two probes in the membrane: when we used an equimolar mixture of the analogs the half-time of phosphatidylserine reduction increased by 35% and that of phosphatidylethanolamine reduction by 50%. This can be interpreted as a competition for a putative aminophospholipid translocase of the two analogs as has been observed in erythrocytes [4].

These observations lead us to postulate that an amino-phospholipid translocation system exists not only in cells from the red cell lineage (erythrocyte, see Refs. 1–5; platelets, Refs. 2, 8), but also in other cells and might be a very general phenomenon of plasma membranes. Some of the physiological implications of lipid asymmetry in plasma membranes have been discussed elsewhere: they include cell shape modulation [15], macrophage recognition [16], attachment to endothelial surfaces [17], cell ageing [18] and blood coagulation [19,20]. We wish to suggest speculatively that it could also be involved in the early stage of endocytosis since this phenomenon requires the local formation of membrane invagination with a very low radius of curvature and hence implies a difference in outer- and inner-membrane surfaces, for which a requisite is a local redistribution of phospholipids (for instance in a vesicle which has a diameter of 120 nm and a bilayer thickness of 4 nm the inner leaflet area is equal to 87% that of the outer leaflet). This speculation is not in disagreement with the present observations that lipid translocation is not purely an endocytic phenomenon.

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