Biochimica et Biophysica Acta 862 (1986) 273–277
Elsevier

BBA 72969

The transbilayer distribution of phosphatidylethanolamine in erythroid plasma membranes during erythropoiesis

W. Nijhof [a], P.H. van der Schaft [b], P.K. Wierenga [a], B. Roelofsen [b], J.A.F. Op den Kamp [b] and L.L.M. van Deenen [b]

[a] *Laboratory of Physiological Chemistry, State University Groningen, Bloemsingel 10, 9712 KZ Groningen (The Netherlands) and* [b] *Laboratory of Biochemistry, State University Utrecht, Padualaan 8, P.O. Box 80.054, 3508 TB Utrecht (The Netherlands)*

(Received 15 May 1986)

Key words: Phosphatidylethanolamine; Fluorescamine; Transbilayer distribution; Erythropoiesis; (Friend cell)

Fluorescamine was used to assess the transbilayer distribution of phosphatidylethanolamine in the plasma membrane of murine erythroid progenitor cells, CFU-E (colony-forming unit erythroid), at different stages of their differentiation pathway. Intact cells were exposed to increasing concentrations of fluorescamine and the amount of labeled phosphatidylethanolamine was determined by measuring the fluorescence intensity of its fluorescamine derivative. A semilogarithmic plot of the dose–response curve revealed three different pools of phosphatidylethanolamine, representing its fractions in, respectively, the inner- and outer monolayers of the plasma membrane and subcellular membrane systems. These results show that 9–11% of the total cellular phosphatidylethanolamine is present in the outer leaflet and 9–10% of it is located in the inner leaflet of the plasma membrane in early as well as late erythroblasts. This symmetric distribution of phosphatidylethanolamine over the two halves of the bilayer in the plasma membrane of CFU-E is very similar to that observed earlier in the plasma membrane of friend erythroleukaemic cells (Rawyler, Van der Schaft, Roelofsen and Op den Kamp (1985) Biochemistry 24, 1777–1783). These observations imply that the characteristic asymmetric distribution of phosphatidylethanolamine, as is found in mature erythrocytes, is accomplished at a very late stage of erythropoiesis and possibly during enucleation of the cells or shortly thereafter.

Introduction

It is well known that phospholipids in the erythrocyte membrane are asymmetrically distributed over the two leaflets of the lipid bilayer [1]. The choline containing phospholipids are mainly located on the outside, and the aminophospholipids on the inside of the bilayer. An important question related to phospholipid asymmetry in erythrocytes, is that of its biogenesis. The differentiation pathway leading to the erythrocyte starts in the bone marrow, where cycling pluripotential stem cells become committed to erythropoiesis. They differentiate into orthochromatic erythroblasts, which extrude their nucleus. A reticulocyte is formed which enters into the bloodstream, where it completes its differentiation to the final mature erythrocyte [2].

In a recent study, the distribution of phospholipids in the plasma membrane of Friend cells was determined [3]. Friend cells are erythro-

Correspondence; Dr. J.A.F. Op den Kamp or Dr. P.H. van der Schaft, Laboratory of Biochemistry, State University Utrecht, Padualaan 8, P.O. Box 80.054, 3508 TB Utrecht, The Netherlands.

leukaemic cells, derived from susceptible mouse spleens infected with the Friend virus complex [4]. They are blocked at an early stage of their normal differentiation pathway, presumably between the BFU-E (burst-forming unit erythroid) and CFU-E (colony-forming unit erythroid) stages.

Two of the major phospholipids were found to be arranged in the plasma membrane of the Friend cell in a similar way as in the murine erythrocyte membrane; sphingomyelin is mainly present in the outer leaflet and phosphatidylserine in the inner layer. In contrast, phosphatidylcholine and phosphatidylethanolamine are arranged in a symmetric way over the two leaflets of the plasma membrane of Friend cells.

The question then to be solved is, at what stage during differentiation the ultimate distribution of phosphatidylcholine and phosphatidylethanolamine will be obtained. It is possible to induce differentiation in Friend cells by adding a variety of agents to the culture medium, of which dimethylsulfoxide and hexamethylenebisacetamide are most commonly used [5]. However, differentiation is only initiated but not completed. Therefore, we initiated studies using another in vitro differentiation system. Early erythroid progenitor cells were isolated from the spleens of thiamphenicol pretreated mice [6]. The different stages of erythropoiesis up to the reticulocyte stage can be sequentially followed by in vitro culture of these cells [7].

Since the number of cells obtained by this procedure was far too small to apply the usual phospholipid localization techniques, which are based on chemical analysis of phospholipids, a recently developed approach [14] (making use of fluorescence measurements) was applied.

Fluorescamine, which reacts rapidly with free aminogroups giving fluorescent products has been used in the present studies to determine the localization of phosphatidylethanolamine in the plasma membrane of differentiating erythroid progenitor cells.

Materials and Methods

Isolation of cells

Friend erythroleukaemic cells (clone 745) were cultivated as described previously [9].

CFU-E were isolated from spleens of thiamphenicol-pretreated anaemic mice as described before [6]. In short: groups of 15 female DBA-2 mice were treated with thiamphenicol in a dialysis bag implanted in the neck. The mice were bled on the second and third day after implantation of the dialysis bag, which was then removed. After another $3\frac{1}{2}-4$ days, the spleens were removed, weighed and disrupted into α-medium (GIBCO). The cells were filtrated through gauze and applied into the bypass chamber of the Beckman elutriator system (centrifuge J2-21 + JE-6 rotor) running at 2000 rpm ($2 \cdot 10^9$ cells per run) with a counterflow of 20 ml/min. A CFU-E rich cell population was collected between a counterflow of 20 and 30 ml/min. These cells (up to $5 \cdot 10^8$ cells) were loaded on a Percoll density gradient (54% Percoll, Pharmacia Fine Chemicals, Uppsala, Sweden). Separation of cells was performed at 13000 rpm in a Beckman J2-21 centrifuge, rotor JA-20 for 30 min at room temperature. The peak fractions at a density of 1.070 g/ml were diluted with α-medium and the cells were collected. CFU-E numbers were assayed with the method of Iscove and Sieber [8]. The different stages of erythropoiesis up to the reticulocyte stage can be sequentially followed by in vitro culture of these cells [7].

Erythrocytes

Mouse blood was drawn from DBA/2J or BALB/c mice, and collected in acid citrate/dextrose.

After three times washing with 150 mM NaCl/5 mM KCl/1 mM $MgCl_2$/1 mM $CaCl_2$/5 mM glucose/5 mM Tricine (pH 7.4) cells were resuspended in 0.3 ml labeling buffer (150 mM NaCl/5 mM KCl/1 mM $MgCl_2$/1 mM $CaCl_2$/5 mM glucose/20 mM Tricine (pH 8.0)) using, respectively, $25 \cdot 10^6$ erythrocytes and $1 \cdot 10^6$ of either Friend cells or CFU-E. The pH of this labeling buffer was adjusted at 20°C with NaOH, taking into account a ΔpK_a/Cdeg of -0.021 for Tricine [10] in order to obtain pH 8.0 at the chosen temperature for the labeling reaction.

Fluorescamine labeling of phosphatidylethanolamine in intact cells

CFU-E and Friend erythroleukaemic cells were labeled at 0–4°C and erythrocytes at room temperature.

Increasing amounts of fluorescamine (up to 150 nmol in 0.015 ml dimethylsulfoxide/acetone (22.5 : 62.5, v/v)) were added to 0.3 ml cell suspension, which was vortexed for exactly 15 s. The reaction was terminated by adding 0.2 ml of 0.5 M ammonia and vortexing for 10 s followed by lipid extraction according to Reed et al. [11]. In experiments in which erythrocytes were labeled, extraction was done according to Rose and Oklander [12].

After washing the total cellular lipid extracts three times according to Folch et al. [13], they were divided into two equal portions. After drying under a stream of nitrogen, one of these portions was dissolved in 0.2 ml chloroform/triethylamine (40 : 0.2, v/v), and an excess of fluorescamine (100 nmol fluorescamine in 0.01 ml dimethylsulfoxide) was added to derivatize all of the aminophospholipids to completion.

Fluorescence measurements were made on a Perkin Elmer MPF-3 spectrophotometer at an excitation wavelength of 390 nm and an emission wavelength of 465 nm.

After correction for the fluorescence intensity of the fluorescamine derivative of phosphatidylserine in the completely derivatized extract, the ratio of the fluorescence intensities of the original extract and this completely derivatized extract was considered to represent the percentage of phosphatidylethanolamine labeling.

Fluorescamine was from Sigma Chemical Company. Solvents from J.T. Baker Chemicals were redistilled before use. Other chemicals were analytical grade products from Merck, or Janssen Chimica, Belgium.

Results and Discussion

Fluorescamine was introduced as a permeant probe to localize phosphatidylethanolamine in intact Friend cells as described earlier [12]. By making use of the fluorescent properties of the fluorescamine derivatives of the aminophospholipids, it appeared to be possible to determine the localization of phosphatidylethanolamine, in particular in small amounts of cells. Aliquots containing $1 \cdot 10^6$ cells were incubated in the presence of fluorescamine as described in Materials and Methods. After reaction, the percentage of derivatized phosphatidylethanolamine was determined by measuring the fluorescence intensities in the lipid extract before and after the complete conversion of the aminophospholipids. Corrections at this stage were made for the fluorescence, due to the presence of phosphatidylserine. The corrections were based on the data obtained in similar experiments with Friend erythroleukaemic cells, since the amounts of CFU-E were too small to allow these experiments. As shown before [12], fluorescamine labeling of phosphatidylserine in Friend cells is very slow and less than 5% of the fluorescence intensity in the original lipid extracts originates from the fluorescamine-PS derivative. After complete conversion of all aminophospholipids, 18% of the fluorescence comes from the PS derivative and both values have been used to make the corrections in the present experiments.

As shown before for Friend erythroleukaemic cells, the labeling of phosphatidylethanolamine in the various pools in CFU-E occurs in a concentration dependent way. The actual data are presented

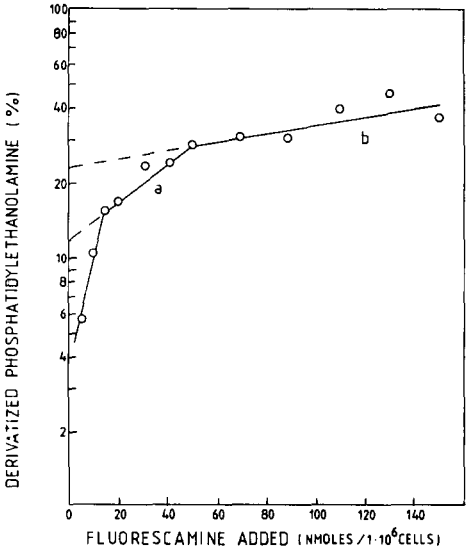

Fig. 1. Semi-logarithmic plot of the appearance of the fluorescamine derivative of phosphatidylethanolamine in CFU-E, labeled at 0–4°C with increasing amounts of fluorescamine. The results shown were derived from a typical experiment. Phosphatidylethanolamine pools attributable to the outer and inner plasma membrane monolayer and to the intracellular membranes were derived by extrapolating respectively, lines (a) and (b).

in Fig. 1. At low concentrations of fluorescamine only the phosphatidylethanolamine in the outer layer of the plasma membrane is reacting; an increasing fluorescamine concentration converts the phosphatidylethanolamine in the inner layer of the plasma membrane and finally, the phosphatidylethanolamine in the intracellular membranes becomes accessible for the reagent. For freshly isolated CFU-E, extrapolation of line (a) in Fig. 1 shows that 9–11% of the total cellular phosphatidylethanolamine is present in the outer monolayer of the plasma membrane. Extrapolation of line (b) indicates that 19–22% of the total cellular phosphatidylethanolamine belongs to the entire plasma membrane. The difference between these two values, which is 10–11% of the total cellular phosphatidylethanolamine, can be assigned to the inner monolayer of the plasma membrane.

In the same way, the phosphatidylethanolamine localization was studied in CFU-E, which were incubated in the appropriate media for 14 and 28 h. After 28 h of incubation, when the cells are in a late erythroblast stage, the distribution of phosphatidylethanolamine is not changed (Table I). 9–11% of the total cellular phosphatidylethanolamine is present in the outer monolayer of the plasma membrane, 19–21% of the total cellular phosphatidylethanolamine is localized in the entire plasma membrane, and the difference, which is 9–10% of the total cellular phosphatidylethanolamine, is present in the inner monolayer of the plasma membrane. As was reported already [14], labeling of Friend erythroleukaemic cells with increasing amounts of fluorescamine also revealed three pools of phosphatidylethanolamine. These three pools represent the phosphatidylethanolamine in, respectively, the outer and inner monolayer of the plasma membrane, and in the intracellular membranes. They contain 16–18%, 16–20% and 64–66% of the total cellular phosphatidylethanolamine, respectively (Table I).

TABLE I

LOCALIZATION OF PHOSPHATIDYLETHANOLAMINE IN THE PLASMA MEMBRANES OF CFU-E, FRIEND CELLS AND MOUSE ERYTHROCYTES, AS DETECTED BY TREATMENT OF INTACT CELLS WITH FLUORESCAMINE

	Percentage of total cellular phosphatidylethanolamine present in plasma membrane monolayers			
	Outer + inner	Outer	Inner	Outer/Inner
CFU-E				
0 h	19–22	9–11	10–11	49/51
28 h	18–21	9–11	9–10	49/51
Friend cell	34–36	16–18	16–20	48/52
Erythrocyte	100	20	80	20/80

The phosphatidylethanolamine distribution in the plasma membrane of mouse erythrocytes, which do not contain any intracellular membranes, was studied before [3], and appeared to be completely different from the values shown above for the plasma membranes of CFU-E and Friend erythroleukaemic cells; 20% of the phosphatidylethanolamine is present in the outer and 80% in the inner leaflet of the erythrocyte membrane (Table I). The latter data were obtained using the regular localization techniques and were confirmed by the approach used in this report. Again, phosphatidylserine in the erythrocytes reacted very slowly with fluorescamine, so the contribution to the fluorescence intensity of the fluorescamine derivative of this phospholipid in the original total cellular lipid extract was ignored.

Comparing the results obtained with Friend cells and erythroid progenitor cells, it is striking to observe that a smaller fraction of the total cellular phosphatidylethanolamine is present in the plasma membrane of CFU-E than in that of Friend cells. However, it should be noted that the values for CFU-E were obtained from calculations in which the assumption was made that 18% of all the aminophospholipid consisted of phosphatidylserine, as was found for Friend cells. This value could not be verified for the CFU-E, due to the small amount of cells available. This assumption, however, has no effect on the observation that phosphatidylethanolamine is symmetrically divided over the two monolayers of the plasma membrane of CFU-E.

It is of interest to note that there is no change in phosphatidylethanolamine localization between freshly isolated CFU-E and CFU-E cultured for 28 h. The latter cells have reached the late erythroblast stage just before enucleation. Preliminary data recently obtained indicate that also in di-

methylsulfoxide induced Friend cells, which undergo a partial differentiation, phosphatidylethanolamine localization is not changing. Furthermore, it has been observed that the overall phospholipid composition hardly changes during dimethylsulfoxide treatment of Friend erythroleukaemic cells, at least under the conditions applied routinely in our studies [9].

It is obvious, that in the early stages of differentiation (proerythroblast, basophilic erythroblast, polychromatophilic erythroblast and orthochromatic erythroblast), changes in the phospholipid organization do not occur. The large alterations which are necessary to end up with the phospholipid composition and distribution typical for the mature erythrocyte, must take place, therefore, after the late erythroblast stage has been reached. To investigate the possibility that phospholipid rearrangements do occur during or after enucleation, attempts were made to determine the phosphatidylethanolamine localization in the plasma membrane of reticulocytes. In the in vitro system developed by Nijhof and Wieringa [7], it is possible to continue the differentiation up to a point where 30–60% of the cells has been converted into reticulocytes and so-called naked nuclei, i.e., nuclei surrounded by some residual cytoplasm and part of the original plasma membrane. This mixture of normoblasts, naked nuclei and reticulocytes in a ratio of about 1 : 1 : 1, was labeled with increasing amounts of fluorescamine. Approximately the same pattern as shown in Fig. 1 was obtained. This indicates that after 48 h of growth, the remaining normoblasts and naked nuclei still have a symmetric distribution of phosphatidylethanolamine over the two leaflets of the plasma membrane. It is not possible to determine the phosphatidylethanolamine localization in the reticulocytes derived from this in vitro system. The amount of phospholipid in the plasma membrane of reticulocytes is 5–10-times less than that in the nucleated precursor cells. The experiments carried out in the way described here with the CFU-E, are at the limit of detection; the extension to a 10-times larger scale will be very hard to perform.

References

1 Op den Kamp, J.A.F. (1979) Annu. Rev. Biochem. 48, 47–71
2 Harrison, P.R. (1976) Nature (London) 262, 353–356
3 Rawyler, A., Van der Schaft, P.H., Roelofsen, B. and Op den Kamp, J.A.F. (1985) Biochemistry 24, 1777–1783
4 Friend, C., Scher, W., Holland, J.G. and Sato, I. (1971) Proc. Natl. Acad. Sci. USA 68, 378–382
5 Marks, P.A. and Rifkind, R.A. (1978) Annu. Rev. Biochem. 47, 419–448
6 Nijhof, W. and Wierenga, P.K. (1983) J. Cell Biol. 96, 386–392
7 Nijhof, W. and Wierenga, P.K. (1984) Exp. Hematol. 12, 115–120
8 Iscove, N.N. and Sieber, F. (1975) Exp. Hematol. 3, 32–43
9 Storm, G., Bosman, G.J.C.J.M., Boer, P., Roelofsen, B., Op den Kamp, J.A.F. and Van Deenen, L.L.M. (1982) Biochem. Int. 5, 169–176
10 Good, N.E., Winget, G.D., Winter, W., Connolly, T.N., Izawa, S. and Singh, R.M.M. (1966) Biochemistry 5, 467–477
11 Reed, C.F., Swisher, S.N., Marinetti, G.V. and Ede, G.V. (1969) J. Lab. Clin. Med. 56, 281–289
12 Rose, H.G. and Oklander, M. (1965) J. Lipid Res. 6, 428–431
13 Folch, J., Lees, M. and Sloane Stanley, G.H. (1957) J. Biol. Chem. 226, 497–509
14 Rawyler, A., Roelofsen, B. and Op den Kamp, J.A.F. (1984) Biochim. Biophys. Acta 769, 330–336