Biochimica et Biophysica Acta 815 (1985) 455–460
Elsevier

BBA 72579

Influence of cholesterol and prostaglandin E_1 on the molecular organization of phospholipids in the erythrocyte membrane. A fluorescent polarization study with lipid-specific probes

Efim M. Manevich [a,*], Kapiton M. Lakin [a], Alexander I. Archakov [b], Vladimir S. Li [b], Julian G. Molotkovsky [c], Vladimir V. Bezuglov [c] and Lev D. Bergelson [c,*]

[a] *N.A. Semashko Medical Stomatological Institute, 117433, Moscow, ul. Delegatskaya, 20/1,* [b] *N.I. Pirogov Medical Institute, 117437, Moscow, ul. Ostrovitianova, 1, and* [c] *M.M. Shemyakin Institute of Bioorganic Chemistry USSR Academy of Science, 117988, Moscow, Ul. Vavilova, 32. (U.S.S.R.)*

(Received May 28th, 1984)
(Revised manuscript received December 6th, 1984)

Key words: Prostaglandin; Cholesterol; Membrane lipid; Fluorescence depolarization; (Rabbit erythrocyte)

Anthryl-labeled fluorescent probes closely mimicking phosphatidylcholine and sphingomyelin were applied to study the state of these phospholipids in the rabbit erythrocyte membrane. At normal cholesterol levels both probes exhibited higher fluorescence polarization values in the membranes than in phospholipid vesicles of similar lipid composition, indicating a decreased fluidity of the probe environment in erythrocyte ghosts. In ghosts prepared from normal erythrocytes no evidence of lateral separation of phosphatidylcholine and sphingomyelin was found. At higher cholesterol levels, however, these lipids appear to segregate. Probably the effect of cholesterol on the erythrocyte membrane lipids involves lipid-protein interactions. At physiological concentrations, prostaglandin E_1 only weakly affects the state of phosphatidylcholine and sphingomyelin in erythrocyte membranes. Cholesterol enrichment amplifies the effect of prostaglandin E_1. Although the prostaglandin E_1-induced changes depended much upon whether the ghosts were enriched with cholesterol in vitro or in vivo, with both types of ghosts effects of prostaglandin E_1 were seen at extremely low effector concentrations that may have presented a few molecules of prostaglandin per ghost. The structural and functional significance of these findings is discussed.

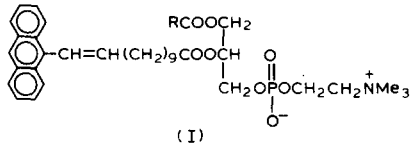
Introduction

The erythrocyte membrane is probably the most widely studied biological membrane. Nevertheless, the surface topography of the erythrocyte lipids is still not fully understood. Particularly, little is known about the existence of lipid domains within the erythrocyte membrane. Co-existence of different lipid phases in the erythrocyte membrane has been hypothesized by some authors [1–5] whereas others were not able to confirm this suggestion [6,7].

We are investigating the state of individual phospholipid species in biological membranes with the help of anthryl-labeled fluorescent probes (I) and (II) that mimic natural phosphatidylcholine and sphingomyelin.

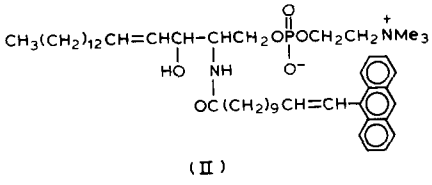
In comparison with the commonly used non-lipid apolar fluorescent probes such as diphenylhexatriene or pyrene, anthryl-labeled lipid-specific probes have additional advantages in that: (i) the orientation of the chromophore relative to the

* To whom correspondence should be addressed.



(I)

Scheme I. Structure of probe (I).



(II)

Scheme II. Structure of probe (II).

bilayer is known (for probes (I) and (II) it has been demonstrated that their anthryl groups reside near the center of the bilayer and are oriented perpendicular to the surface [8]); (ii) the lipid-specific probes appear to be less perturbant than nonlipid molecules (probes (I) and (II) were shown to induce no alterations in the conformation of the phospholipid head groups and only minor perturbations in the packing of the hydrocarbon chains [8]). Moreover, because the fluorescent probes (I) and (II) are identical to natural phosphatidylcholine and sphingomyelin with respect to head-group structure and polarity, they may be expected to reflect differentially the behavior of their natural prototypes in biological membranes.

An important parameter relating to the structure and functional state of biological membranes is the so-called 'membrane fluidity' (for a review see Ref. 9). For a given fluorescent probe the emission polarization (P) is sensitive to the fluidity of the environment: in fluid systems the values of these parameters are lower than in less-fluid phases. Steady-state polarization of nonlipid apolar or amphiphilic fluorescent probes has been used extensively to assess the fluid state of biological membranes. However, being unspecific in respect to different lipid classes, such probes permit the measurement of only total membrane fluidity or of the fluidity of some unspecified regions within the membrane and are not able to provide information about the existence of lipid-specific domains. In contrast, the phospholipid-specific probes (I) and (II) should discriminate between putative phosphatidylcholine-rich and sphingomyelin-rich domains within the membrane.

Indeed, in biological membranes the P values of the probes (I) and (II) have been shown to differ considerably more than in homogeneous environments (e.g., egg phosphatidylcholine vesicles [8,10]). We assume that differences in the P values of the two probes ($P_{II} - P_I$) significantly exceeding those exhibited by the probes in homogeneous environments are caused by differences in the fluidity of their host lipids. Consequently, sufficiently high differences between the P values of the two probes in biological membranes should allow the detection of nonrandom distributions of phosphatidylcholine and sphingomyelin in the membranes. In the present study we utilized the two phospholipid probes (I) and (II) to investigate changes in the molecular organization of phosphatidylcholine and sphingomyelin in rabbit erythrocyte membranes induced by cholesterol and prostaglandin E_1.

Materials and Methods

Lipids and lipid probes. 1-Acyl-2-(12-(9-anthryl)-11-*trans*-dodecenoyl)-*sn*-glycero-3-phosphocholine (I) [11] and *N*-(12-(9-anthryl)-11-*trans*-dodecenoyl)sphingosyl-1-phosphocholine (II) [12] were prepared as described earlier. Egg phosphatidylcholine and cattle brain sphingomyelin were from Sigma (U.S.A.) and cholesterol from Serva (F.R.G.). Prostaglandin E_1 (crystalline form) was a generous gift from Dr. J.E. Pike (Upjohn Co., U.S.A.).

Vesicles. Unilamellar phospholipid vesicles were prepared by ultrasonic treatment as described in Ref. 12.

Animals. Adult male chinchilla rabbits weighing 2.5–3.0 kg were used and fed a standard rabbit chow. The cholesterol content of erythrocytes was altered in vivo by the addition of 0.25 g cholesterol per kg weight to the diet for 10–12 weeks.

Membrane preparation and cholesterol loading. Blood samples of about 20 ml were withdrawn in heparin from the marginal vein of the ear. Plasma and cells were separated by centrifugation (2000 × g for 10 min). Plasma and white blood cells were discarded and the erythrocytes were washed three times with 10 vol. of isotonic Tris-saline buffer (10 mM Tris and 150 mM NaCl, adjusted to pH 7.4 with HCl). Packed cells were hemolyzed with 10

vol. of 10 mM histidine buffer (pH 7.4) [13]. The membranes were pelleted by centrifugation at 28 000 × *g* for 20 min and were then washed with 5 mM Tris buffer, 1 mM Na_2EDTA (pH 7.4) until a white preparation was obtained (3–5 washes). Ghost-membranes were suspended in isotonic Tris-saline buffer.

In vitro cholesterol loading of the erythrocyte membranes was carried out by incubation of about 1 ml packed cells/10 ml of cholesterol-rich medium at 37°C for 24 h. Control and cholesterol-rich media were prepared as described by Shinitzky [14] using bovine serum, previously heated to 57°C for 30 min. Membrane preparation was performed as described above. Protein content was determined by the method of Lowry et al. using bovine serum albumine as standard.

Lipid analyses. The erythrocyte membrane lipids were extracted with chloroform/methanol (2 : 1, v/v) [15]. The cholesterol content of the membrane lipid extracts was determined as described by Webster [16]. Phospholipids were determined by phosphorus analysis according to Bartlett [17] after separation by two-dimensional thin-layer chromatography on Silica gel G (Merck) using chloroform/methanol/water (65 : 25 : 4, v/v) and chloroform/methanol/7 N NH_4OH (14 : 6 : 1, v/v) as solvents. Identification of phospholipids was performed using the molybdenum reagent [18].

Fluorescent measurements. Fluorescence spectra (uncorrected) were registered with an Amino SPF-1000 spectrofluorimeter equipped with a thermostated cell in quartz cuvettes (10 × 10 mm) with continuous stirring of the samples. The fluorescence polarization values were calculated from excitation spectra (λ_{ex} 370 nm, λ_{em} 440 nm; bandwidths: for excitation, 2 nm, for emission, 20 nm) according to the equation

$$P = \frac{(I_{\parallel} - I^o_{\parallel}) - (i_{\perp} - I^o_{\perp})}{(I_{\parallel} - I^o_{\parallel}) + (I_{\perp} - I^o_{\perp})}$$

where I is the fluorescence intensity of erythrocyte membranes with (I) or without (I^o) probes, with parallel ($\parallel$) or cross ($\perp$) polarizers and analyzers. In all experiments a depolarizer was used.

Ethanol solutions of fluorescent probes were added to the suspension of erythrocyte ghosts to a final ratio of probe to phospholipids of 1 : 100 (mol/mol). All measurements were made after 3.5 h incubation (36.5°C) of the probes with erythrocyte ghosts. Ethanol solutions of prostaglandin E were added to the membranes suspensions. The total amount of ethanol was always less then 0.5% of the sample volume. Such amounts of ethanol did not induce detectable perturbations in the membranes.

Results and Discussion

Phospholipid vesicles

In egg phosphatidylcholine vesicles the fluorescent polarization of the sphingomyelin probe (II) was only slightly higher than that of the phosphatidylcholine probe (I) (Tables I and II). In egg phosphatidylcholine–brain sphingomyelin (2 : 1) vesicles the polarization of both probes increased. At the temperature of our experiments the two phospholipids are completely miscible at the macroscopic level [18] and addition of small amounts (≈ 1%) of the fluorescent probes hardly can be expected to induce phase separation. Probably the $P_{II} - P_I$ difference reflects differences in the molecular structure of phosphatidylcholine and sphingomyelin as well as differences in molecular translocations caused by the ability of sphingomyelin to form hydrogen bonds, although the possibility that small amounts of gel-phase lipids are present cannot be excluded. The large increase in P_{II} and moderate increase in P_I upon addition of sphingomyelin to the phosphatidylcholine vesicles apparantly reflects the increased amount of gel phase in the vesicles. Introduction of 25 mol% cholesterol into the phosphatidylcholine-sphingomyelin vesicles had only a small effect on the fluorescence polarization of the two probes; however, introduction of 40 mol% cholesterol caused the P values to shift in opposite directions (Tables I and II): the P value of the phosphatidylcholine probe (I) increased, while that of the sphingomyelin probe (II) decreased considerably. The different response of the two probes to addition of cholesterol indicates that the sterol causes the host lipids to seggregate. However, differential scanning calorimetry shows no phase separation in vesicles of similar composition at 36°C [19,20]. This discrepancy may be explained by assuming the existence of transient clusters (do-

TABLE I

FLUORESCENCE POLARIZATION (P)($\times 10^{-2}$) OF PROBES I AND II IN LIPID VESICLES AND RABBIT ERYTHROCYTE GHOSTS

EYP, egg yolk phosphatidylcholine; BSM, brain sphingomyelin; PG, prostaglandin.

Fluorescent probe	Effector	Lipid vesicles [a]			Erythrocyte ghosts [a,c]		
		EYP	EYP-BSM (2:1)	EYP-BSM-cholesterol (2:1:2)	Controls	Cholesterol--loaded	Hypercholesteremic animals
I	–	3.8±0.2	5.2±0.2	6.3±0.2	8.8±0.3	6.8±0.2	7.3±0.3
II	–	4.6±0.2	8.3±0.2	5.0±0.2	11.0±0.5	12.6±0.5	13.8±0.5
I	PG E_1 [b]	3.7±0.2	5.1±0.2	6.2±0.2	8.0±0.3	8.1±0.2 [d]	15.8±0.6
II	PG E_1 [b]	4.5±0.2	8.3±0.2	5.1±0.3	11.3±0.3	7.6±0.3	13.0±0.3

[a] Averaged results of three independent experiments.
[b] Approx. 10^{-9} M.
[c] The statistical significance of changes in fluorescence polarization in comparison with control evaluated by the paired t test.
[d] Not statisticaly significant ($P > 0.05$).

mains) that can be detected by fluorescent probes with short lifetimes of the exited state, but are not detectable by differential scanning calorimetry, which permits observation of only the stationary (averaged) state of bilayer. If P_I preferentially samples fluid-phase domains and P_{II} is preferentially sampling solid-phase domains, then the differential effect of cholesterol on the P values is readily explained by the well-known ability of cholesterol to 'fluidize' and disorder lipid apolar chains below the phase-transition temperature and to decrease chain flexing above this temperature.

TABLE II

THE DIFFERENCE BETWEEN THE FLUORESCENCE POLARIZATIONS OF THE SPHINGOMYELIN (II) AND PHOSPHATIDYLCHOLINE (I) PROBES ($P_{II} - P_I$) IN LIPID VESICLES AND ERYTHROCYTES WITH DIFFERENT CHOLESTEROL CONTENT

Abbreviations and statistical significance are as indicated in Table I.

Sample	($P_{II} - P_I$) ($\times 10^{-2}$)
1. EYP vesicles	0.8
2. EYP-BSM (2:1) vesicles	3.1
3. EYP-BSM-cholesterol (2:1:2) vesicles	−1.3
4. Normal rabbit erythrocyte ghosts	2.2
5. Sample 4 after in vitro cholesterol loading	5.8
6. Hypercholesteremic rabbit erythrocyte ghosts	6.5

Erythrocyte membranes

Influence of cholesterol. The cholesterol content of the rabbit erythrocyte membrane can be greatly increased by in vitro incubation of the cells with cholesterol or by prolonged cholesterol feeding of the animals. In the present study we used both approaches to increase the cholesterol content in rabbit erythrocytes. The chemical composition of the ghosts is shown in Table III.

After injection of the phospholipid probes (I) or (II) into aqueous medium their fluorescence was negligible due to self-quenching of the anthryl fluorophores. However, when the probes were injected into suspensions of erythrocyte ghosts, their fluorescence intensity increased rapidly with time and reached a maximal value after 150 min. This shows that the probes were dispersed as they became associated with the ghosts.

As can be seen from Table I the P values of the two probes associated with the control (low cholesterol) ghosts were in all samples higher than in liposomes made from phospholipids and cholesterol. Increasing the cholesterol : phospholipid ratio of the ghosts from 0.4 to 0.7–0.9 caused the fluorescence polarization of the two probes to change in different directions: the P value of the phosphatidylcholine probe (I) decreased, whereas that of the sphingomyelin probe (II) increased with increasing amounts of cholesterol (Table I). These data suggest that cholesterol induces or

TABLE III

COMPOSITION OF RABBIT ERYTHROCYTE GHOSTS

Average results from three independent experiments; S.E. ± 5%.

Erythrocyte sample	Components (mg/ml)			Phospholipids composition (%)			
	Protein	Cholesterol	Phospholipids	Phosphatidyl-choline	Spingomyelin	Phosphati-dylethanol-amine	Others
Normal control	5.0	0.9	2.2	43.2	14.8	31.3	10.7
Cholesterol-loaded	3.9	1.3	1.8	45.4	14.0	32.6	8.0
Normal control	4.4	0.9	2.2	41.6	17.4	24.1	16.9
Hypercholesteremic animals	5.5	1.4	1.6	42.0	21.3	22.7	14.0

increases segregation of the two probes in erythrocyte ghosts.

In erythrocyte ghosts from hypercholesterolemic animals the fluorescent polarization of the sphingomyelin probe (II) is about 2-times higher than that of the phosphatidylcholine probe (I). Since both probes contain the same fluorophore, these data demonstrate that the observed fluorescent polarization differences are not due to fluorophore-protein interaction and suggest strongly that in cholesterol-rich erythrocytes phosphatidylcholine and sphingomyelin are, at least partly, segregated.

Significantly, cholesterol enrichment has oppositely directed effects on the fluorescent polarization of the probes in lipid vesicles and erythrocyte ghosts. In the ghosts the difference $P_{II} - P_I$ increases dramatically upon introduction of cholesterol, whereas in vesicles that difference decreases (Table II). this suggests that cholesterol-protein and/or phospholipid-protein interactions must be involved.

Such a proposition was further supported by study of the effect of prostaglandin E_1 on the fluorescence polarization of the two phospholipid probes in normal and cholesterol-enriched erythrocytes.

Influence of prostaglandin E_1. Mammalian red blood cells are responsive to physiological and subphysiological concentrations of prostaglandins. For example prostaglandin E_1 decreases the degree of hemolysis, changes the size of the rapidly exchangeable calcium pool and increases the single cell flexibility of human erythrocytes [21–23]. Using ESR data, Kury and McConnel [24] arrived at the conclusion that these phenomena are due to alterations in the lipid fluidity. However, the probe used by them (a spin-labeled fatty acid) could provide only general information about the lipids in toto and did not allow probing of the fluidity state of different phospholipid components of the erythrocyte.

Here we present data suggesting that physiological concentrations of prostaglandin E_1 induce oppositely directed changes in the fluidity of two major phospholipids of the erythrocyte membrane. Addition of prostaglandin E_1 to normal erythrocyte ghosts causes only insignificant changes in P_I and P_{II}. However, much more pronounced effects were observed with cholesterol-enriched ghosts. In this case addition of prostaglandin E_1 resulted in a significant increase in the fluorescence polarization of the phosphatidylcholine probe (I) and a concomitant decrease in the P value of the sphingomyelin probe (II) (Table I). It should, however, be noted that the extent of these changes was quite different for ghosts enriched with cholesterol in vivo or in vitro, demonstrating that the two types of ghosts may be regulated by different mechanisms. Nevertheless, in each case the effects of prostaglandin E_1 were seen already at concentrations of 10^{-12}–10^{-9} M prostaglandin E_1. This may have presented from 0.1 to 100 molecules of prostaglandin per ghost. At such concentrations prostaglandin E_1 had no effect on the two phospholipid probes in phosphatidylcholine-sphingomyelin (2 : 1) vesicles independently of their cholesterol content. Hence the observed changes in phospholipid fluidity probably are brought about by prostaglandin E_1-induced changes in the state

of membrane proteins. Which of the erythrocyte proteins is responsible for the observed effects remains unknown at the moment.

Summarizing, the data of the present work provide strong evidence that both cholesterol enrichment and addition of small amounts of prostaglandin E_1 induce rearrangements of phospholipids in the erythrocyte membrane. That prostaglandin E_1 induces membrane changes in human erythrocytes has already been postulated by some investigators; however, the data obtained so far have been contradictory. Thus, Kury and McConnel [24] reported that prostaglandin E_1 increased the lipid chain flexibility in human erythrocytes, whereas Taniguchi et al. [23] found that prostaglandin E_1 caused the erythrocyte lipids to become more compactly arranged. The present study demonstrates for the first time that prostaglandin E_1 affects different phospholipid species in the erythrocyte membrane in a different manner. Enrichment of the membrane with cholesterol amplifies and reverses the rearrangement effects of prostaglandin E_1. This may be important for a better understanding of prostaglandin–red cell interactions under conditions of atherosclerosis.

Another important finding of this study is that both cholesterol enrichment and prostaglandin E_1 treatment appear to increase lateral heterogeneity in the erythrocyte membrane, and to induce a reorganization of the erythrocyte lipids into domains which probably are enriched in phosphatidylcholine or sphingomyelin. Whether the domains consist of protein-free or protein-containing lipid regions must be ascertained. Moreover, correct evaluation of the physiological significance of the data presented requires investigation of larger amounts of samples and an appropriate statistical analysis.

References

1 Cullis, P.R. (1976) FEBS Lett. 68, 173–176
2 Zimmer, G. and Shirmer, H. (1974) Biochim. Biophys. Acta 345, 314–320
3 Gottlieb, M.H. and Eanes, E.D. (1974) Biochim. Biophys. Acta 373, 519–522
4 Verma, S.P. and Wallach, D.F. (1976) Biochim. Biophys. Acta 436, 307–318
5 Cullis, R.P. and Grathwohl, C. (1977) Biochim. Biophys. Acta 471, 213–226
6 Maraviglia, B., Davis, J.H., Bloom, M., Westerman, J. and Wirtz, K.W.A. (1982) Biochim. Biophys. Acta 686, 137–140
7 Karnovsky, M.J., Kleinfeld, A.M., Hoover, R.L. and Klansher, R.D. (1982) J. Cell Biol. 94, 1–6
8 Molotkovsky, Jul.G., Manevich, Y.M., Gerasimova, E.N., Molotkovskaya, I.M., Polessky, V.A. and Bergelson, L.D. (1982) Eur. J. Biochem. 122, 573–579
9 Shinitzky, M. and Hencart, P. (1979) Int. Rev. Cytol. 60, 121–147
10 Moltokovsky, Jul.G., Manevich, E.M., Shvets, V.I., Krylova, V.N., Vorkunova, G.K., Bukrinskaya, A.G. and Bergelson, L.D. (1983) Voprosy virusolog. 1, 182–187
11 Molotkovsky, Jul.G., Dmitriev, P.I., Nikulina, L.F. and Bergelson, L.D. (1979) Bioorg. Chim. 5, 588–594
12 Molotkosvky, Jul.G., Dmitriev, P.I., Molotkovskaya, I.M., Bergelson, L.D. and Manevich, E.M. (1981) Bioorg. Chim. 7, 586–600
13 Kolchinskaya, L.I., Lichko, V.K. and Malycheva, M.K. (1976) Biochimiya 41, 933–938
14 Shinitzky, M. (1978) FEBS Lett. 85, 317–320
15 Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497–509
16 Webster, D. (1962) Clin. Chim. Acta 7, 277–284
17 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468
18 Vaskovsky, V.E., Kostetsky, E.Y. and Vasendin, I.M. (1975) J. Chromatogr. 114, 129–141
19 Untracht, S.H. and Shipley, G.G. (1977) J. Biol. Chem. 252, 4449–4457
20 Calhoun, W.I. and Shipley, G.G. (1979) Biochemistry 18, 1717–1722
21 Ramussen, H., Lake, W.V. and Allen, J.E. (1975) Biochim. Biophys. Acta 411, 63–73
22 Kury, P.G., Ramwell, P.W. and McConnel, H.M. (1974) Biochem. Biophys. Res. Commun. 56, 478–483
23 Taniguchi, M., Aikava, M. and Sakagami, T. (1982) J. Biochem. 91, 1173–1179
24 Kury, P.G. and McConnel, H.M (1975) Biochemistry 14, 2798–2803