Biochimica et Biophysica Acta, 470 (1977) 382—388

BBA 77836

THE EFFECT OF PHOSPHATIDYLCHOLINE TO SPHINGOMYELIN MOLE RATIO ON THE DYNAMIC PROPERTIES OF SHEEP ERYTHROCYTE MEMBRANE

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(Received January 14th, 1977)

Summary

Sheep red blood cells are shown to incorporate phosphatidylcholine when incubated in human plasma in the presence of EGTA. This treatment results in up to a 5-fold increase in mol ratio of phosphatidylcholine to sphingomyelin. By replacing EGTA with Ca^{2+} the increase of phsphatidylcholine content is completely inhibited, due to the activation of the membrane bound lecithinase which rapidly degrades the incorporated phosphatidylcholine. Analogous treatments of the isolated erythrocyte membranes resulted in similar phosphatidylcholine incorporation but in the presence of Ca^{2+} a residual phosphatidylcholine uptake was still observed. These results suggest that in the isolated membranes small amounts of phosphatidylcholine can be incorporated into an additional region which is unavailable for the membrane lecithinase. The increase in the phosphatidylcholine to sphingomyelin mol ratio in sheep red blood cells is concomitant with an increase in lipid fluidity, as well as increase in osmotic fragility.

Introduction

Phosphatidylcholine and sphingomyelin constitute more than 50% of the phospholipids in most mammalian membranes [1]. These phosphorylcholine phospholipids impart on lipid regions markedly different dynamic qualities, because of inherent structural differences. The high degree of saturation of the acyl chains, as well as the *trans* double bond and the amide bond in the hydrophilic region of sphingomyelin contribute to its rigidifying nature [2]. Natural

* Abbreviations used: PC/S, phosphatidylcholine to sphingomyelin mol ratio; EGTA, ethyleneglycol bis (2-aminoethyl ether)*N,N'*-tetraacetic acid.

occurring phosphatidylcholine thus forms highly fluid lipid regions, whereas natural sphingomyelin induces high rigidity within membrane lipid layers [2]. It is therefore apparent that the mol ratio of these two phospholipids (PC/S) may play a significant role in regulating the dynamic properties of biological membranes. The PC/S varies widely in cells from different tissues of the same organ and in membranes of the same cell from different species [1]. Both aging and arteriosclerosis are characterized by a substantial decrease in membrane PC/S [3], whereas maturation of the fetus lung is associated with an increase in PC/S of the pulmonary surfactant [4].

In this study we present a method for changing the PC/S of sheep erythrocyte membranes. The effect of the modified PC/S on membrane fluidity and on osmotic fragility was examined. The sheep erythrocyte membrane was chosen since its PC/S is unusually low [1,5] and it exhibits a relatively high membrane-associated lecithinase activity [5,6]. This activity can be readily blocked by chelating agents and reactivated by Ca^{2+} [6].

Materials and Methods

Heparinized sheep blood drawn from a young sheep was used within 1 h after drawing. The red cells were sedimented at $1000 \times g$ and were then washed three times and resuspended in phosphate-buffered saline-EGTA (0.15 M NaCl + 5 mM phosphate + 2 mM EGTA), pH 7.4.

Leaky-ghost-membranes from sheep erythrocytes were isolated by the method of Dodge et al. [7], resuspended in phosphate-buffered saline-EGTA, pH 7.4, and stored at −20°C until use. Protein concentration was determined according to Lowry et al. [8], using bovine serum albumin as a standard.

Lipid analysis. Lipids were extracted by the method of Renkonen et al. [9] and separated by two-dimensional thin layer chromatography following the technique of Broekhuyse [10]. The mol content of the phospholipids was determined by phosphorus analysis after conversion to phosphoric acid with 70% $HClO_4$ at 190°C by a modification of the Fiske-SubbaRow procedure [11]. The cholesterol level in the membrane lipid extracts was determined by the method of Chiamori and Henry [12].

Incubation of sheep erythrocytes with plasma. Cell suspensions (approximately 10^9 cells/ml) were mixed with equal volumes of human plasma previously inactivated by incubation at 56°C for 30 min, supplemented with glucose (1 mg/ml) penicillin (500 μ/ml) and either 2 mM EGTA or 5 mM Ca^{2+}. This incubation was carried out at 37°C with constant shaking during which samples were taken out at different time intervals. After three washings with phosphate-buffered saline-EGTA, pH 7.4, the osmotic fragility of the cells was determined (see below). The membranes of the tested erythrocytes were then isolated and used for protein determination, lipid analysis and membrane fluidity measurements.

Incubation of sheep erythrocyte-ghost-membranes with plasma. Membrane suspensions (approximately 5 mg protein per ml) were mixed with equal volumes of heat-inactivated human plasma (see above), containing 1 mM sodium azide and either 2 mM EGTA or 5 mM Ca^{2+}. The incubation was performed at 37°C with constant shaking. After different periods of time aliquots of the in-

cubating mixtures were washed three times with phosphate-buffered saline-EGTA, pH 7.4, and then used for protein determination, lipid analysis and membrane fluidity measurements.

Membrane fluidity measurements. The fluidity of the membrane lipid core was measured by a fluorescence polarization technique with the lipid probe 1,6-diphenyl-1,3,5-hexatriene (Fluka AG) as outlined by Shinitzky and co-workers [2,13] *. The method is based on the Perrin equation:

$$\frac{r_0}{r} = 1 + C(r)\frac{T\tau}{\eta}$$

where r_0 and r are the limiting and the measured fluorescence anisotropies, respectively, $C(r)$ is a structural term which changes slightly with r, T is the absolute temperature, τ is the excited state lifetime and η is the apparent microviscosity. At the temperature range of 0—40°C the product $C(r) \cdot T \cdot \tau$ is approximately constant and the term $(r_0/r-1)^{-1}$ can serve as a relative scale for microviscosity [13].

A suspension of $2 \cdot 10^{-6}$ M diphenyl hexatriene in phosphate-buffered saline-EGTA (pH 7.4) was freshly prepared before each measurement by injecting 10^{-3} M diphenyl hexatriene in tetrahydrofuran into the aqueous medium under vigorous stirring. Membrane suspensions containing 0.05 mg protein/ml were mixed with equal volumes of the diphenyl hexatriene suspension and incubated at 37°C for 15 min. The samples were then brought to room temperature and the degree of the fluorescence anisotropy, r, was determined. Excitation was performed with polarized 360 nm beam and the emitted light was detected in two independent cross-polarized channels after passing through 380 nm cut-off filters ($r_0 = 0.362$, ref. 2).

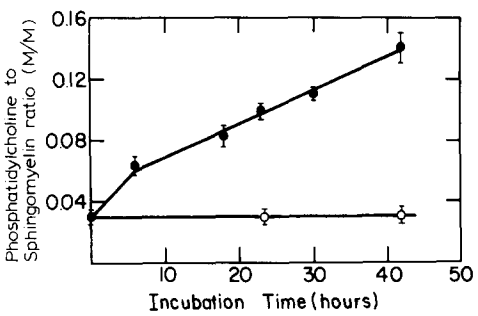
Osmotic fragility measurements. 20 µl of a cell suspension of approximately 30% haematocrit were rapidly mixed at 23°C with 3 ml of 2 mM sodium phosphate, pH 7.4, containing NaCl at various concentrations within the range 70—120 mM. After 10 min at 23°C samples were centrifuged at 2000 × *g* for 5 min. Percent of haemolysis was determined by measuring the haemoglobin absorbance in the supernatant at 540 nm.

Results

The PC/S of sheep serum is similar in magnitude to that of human serum [14], but the latter is much richer in total phospholipid content. Hence the use of human serum as a phosphatidylcholine source seemed more appropriate. When intact sheep erythrocytes were incubated with inactivated human plasma containing 2 mM EGTA an up to 5-fold increase in the PC/S was observed. Treatments were carried out for up to 42 h where the fractional haemolysis never exceeded 0.5%. The increase in the membrane PC/S with time of incubation with plasma-EGTA is shown in Fig. 1.

Along with the observed increase in the PC/S, the osmotic fragility of the treated cells increased by about 10 mM NaCl units (Fig. 2, Table I). Concomi-

* A review of the method is in preparation and will be submitted for publication to Biochim. Biophys. Acta (Reviews on Biomembranes).



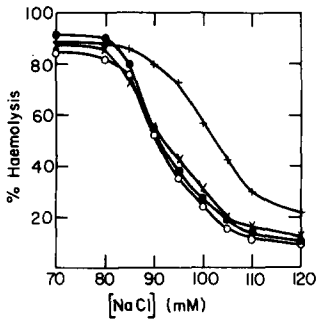


Fig. 1. The rate of increase in membrane PC/S followed by treatment of intact sheep erythrocytes with human plasma containing 2 mM EGTA (•) and human plasma containing 1.5 mM free Ca^{2+}, (○).

Fig. 2. Typical set of haemolysis profiles for sheep erythrocytes after 42 h treatment at 37°C. Untreated cells X———X, phosphate-buffered saline-EGTA treatment, •———•; human plasma-Ca^{2+} treatment, ○———○; human plasma-EGTA treatment, +———+.

tantly, an increase of almost 25% in membrane fluidity, presented by $(r_0/r-1)^{-1}$ at 25°C, was recorded (Table I). No detectable change in any of the above parameters occurred in analogous treatments of the erythrocytes with either plasma-Ca^{2+} or with phosphate-buffered saline-EGTA (Fig. 2, Table I).

We have further applied the above treatments to sheep erythrocyte ghost membranes. Incubation of membranes with inactivated human plasma containing 2 mM EGTA resulted in an increase of up to an order of magnitude in the measured PC/S (see Fig. 3). After about 36 h the increase in PC/S leveled off at PC/S = 0.20 ± 0.01. Only a slight increase in the rate of phosphatidylcholine uptake could be detected when the incubation was performed at 42°C (Table II). In the analogous experiments in the presence of 1.5 mM free Ca^{2+}, the uptake of phosphatidylcholine was markedly reduced but not completely inhibited (see Fig. 3). No appreciable changes in the protein concentration or the cholesterol content in the treated membranes could be detected. In addition, no change in the contents of lysolecithin, phosphatidylserine or phosphatidylethanolamine was observed in the plasma-EGTA treated membranes.

TABLE I

THE CORRELATION BETWEEN MEMBRANE PC/S, MEMBRANE FLUIDITY AND THE OSMOTIC FRAGILITY OF SHEEP ERYTHROCYTES

Treatment	μg Phosphatidylcholine per mg lipid	μg Sphingomyelin per mg lipid	PC/S (M/M)	$(r_0/r-1)^{-1}$ (25°C)	Osmotic fragility (mM NaCl at 50% haemolysis)
Plasma-EGTA, 37°C, 42 h	85 ± 5	610 ± 5	0.14 ± 0.01	2.22 ± 0.04	102 ± 2
Plasma-Ca^{2+}, 37°C, 42 h	20 ± 3	670 ± 5	0.030 ± 0.005	2.58 ± 0.04	91 ± 2
Phosphate-buffered saline-EGTA, 37°C, 42 h	17 ± 2	570 ± 8	0.030 ± 0.005	2.66 ± 0.04	94 ± 2

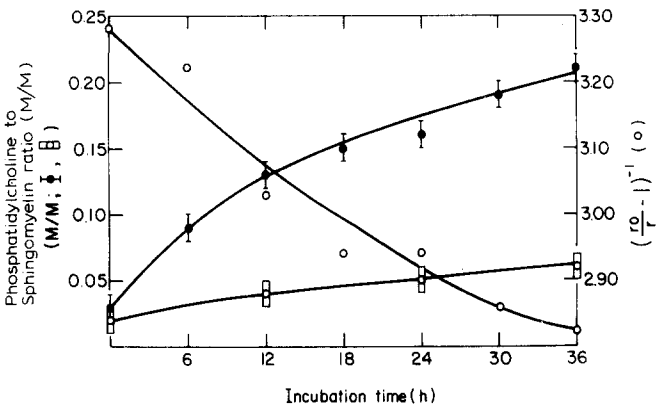


Fig. 3. The rate of increase in phosphatidylcholine to sphingomyelin mol ratio (PC/S) of isolated sheep erythrocyte membranes incubated at 37°C in human plasma containing 2 mM EGTA (●) and in human plasma containing 1.5 mM free Ca^{2+} (■). The concomitant changes in membrane microviscosity at 20°C, presented by the parameter $(r_0/r-1)^{-1}$, following the plasma-EGTA treatment, are also presented (○———○).

Membrane microviscosity, presented by the parameter $(r_0/r-1)^{-1}$, was found to decrease with increase in PC/S as shown in Fig. 3. It seems reasonable to attribute the observed decrease in membrane microviscosity to the increase in PC/S. Indeed, a plot of $(r_0/r-1)^{-1}$ vs. PC/S yields a straight line (Fig. 4). Membranes incubated with phosphate-buffered saline-EGTA showed no change in either lipid composition or membrane fluidity (Table II).

Discussion

The important role of lipid fluidity in controlling different membrane functions is now well established [13,15–19]. The main factors which grossly de-

TABLE II

CHANGES IN LIPID COMPOSITION AND MEMBRANE FLUIDITY OF SHEEP ERYTHROCYTE GHOST MEMBRANES UPON VARIOUS TREATMENTS

For details, see text.

Treatment	Incubation time (h)	Lysophosphatidylcholine/phospholipids (M/M)	Cholesterol/phospholipids (M/M)	Phosphatidylcholine/sphingomyelin (M/M)	$\left(\frac{r_0}{r}-1\right)^{-1}$ (25°C)
Plasma-EGTA 37°C	0	0.01	0.9	0.02	3.36
	6	0.03	1.0	0.06	3.23
	12	0.02	0.9	0.10	3.10
	18	0.03	0.9	0.14	3.00
	24	0.02	0.9	0.16	2.95
Plasma-EGTA 42°C	0	not determined	0.9	0.02	3.38
	12	not determined	1.0	0.12	3.05
	24	not determined	0.9	0.19	2.85
	36	not determined	1.1	0.21	2.80
Plasma-Ca 37°C	0	0.01	0.9	0.02	3.36
	24	0.08	1.0	0.05	3.30

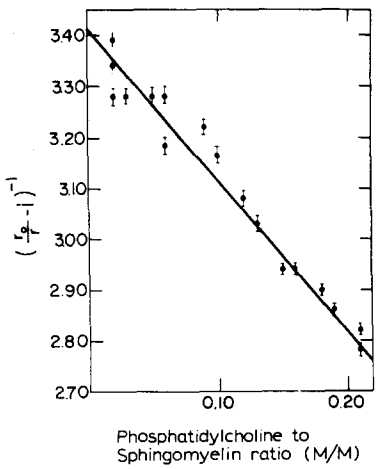


Fig. 4. The correlation between PC/S and $(r_0/r-1)^{-1}$, measured at 20°C, in sheep erythrocyte membranes.

termine the fluidity of membrane lipids are: (a) the cholesterol to phospholipid mol ratio, (b) the degree of unsaturation of the phospholipid acyl chains, and (c) the PC/S. In several studies the effect of the first two parameters on the dynamic properties and function of various artificial and biological membranes were thoroughly investigated [13,17—19]. However, the amount of data now available on the effect of PC/S on membrane properties is still scarce. Human erythrocyte membranes from patients with abetalipoproteinemia have an abnormal level of sphingomyelin with PC/S ≈ 0.65. This low PC/S, which is about half the normal PC/S, induces an appreciable increase in membrane microviscosity [20]. The increase in PC/S of the fetus lung surfactant with maturation [4] is also concomitant with reduction in microviscosity [21]. These two sole findings are in accord with Shinitzky and Barenholz [2] who have established how PC/S may affect membrane fluidity properties.

The value of PC/S in sheep erythrocytes is much below the PC/S in the sheep serum (PC/S = 5.4, ref. 14), or in all other mammalian erythrocytes [1]. At least part of this abnormality is due to the presence of lecithinase in the outer surface of the sheep erythrocyte membrane [5,6]. These unique properties presumably facilitate the exchange and incorporation of phosphatidylcholine into the sheep erythrocyte membrane, processes which are of negligible rates in human erythrocytes [22]. As indicated in Fig. 3, the rate of phosphatidylcholine incorporation into the isolated sheep erythrocyte membranes depends on Ca^{2+} concentration. However, even in the presence of 1.5 mM free Ca^{2+} the phosphatidylcholine uptake is not completely inhibited, which suggests that phosphatidylcholine can intercalate independently in two distinct regions. One region is probably at the outer surface in the vicinity of the lecithinase and is available for its activity, and the other region is unavailable for the lecithinase activity and is presumably located at the inner half of the membrane [5,6]. This suggestion is supported by the finding that the phosphatidylcholine uptake by intact sheep erythrocyte (see Fig. 1) is slower than by the isolated membrane, and can be completely blocked by Ca^{2+} (see Fig. 1).

The maximal rate of phosphatidylcholine uptake by intact sheep erythrocyte is observed within the first 6 h of incubation and is approximately 1 μg phosphatidylcholine/mg membrane protein/h. This value is only ≈20% of the lecithinase activity of sheep erythrocyte membrane-proteins [6]. Hence, the complete inhibition of phosphatidylcholine incorporation into intact sheep erythrocyte membranes in the presence of Ca^{2+} can be explained by an immediate degradation of the phosphatidylcholine which is taken up. The modulation of lecithinase by EGTA and Ca^{2+} could serve as a tool for studying the rate of flip-flop of phosphatidylcholine in sheep erythrocytes. Experiments along this line are now in progress.

The phosphatidylcholine level in intact sheep erythrocyte and in its isolated membrane can be increased at least 5-fold by the method described here. A direct correlation between PC/S and osmotic fragility was observed, which presumably results from the accompanied changes in membrane fluidity rather than changes in surface to volume ratio [23]. It therefore seems that this system could serve as a convenient model for investigation of the role of the phosphatidylcholine-sphingomyelin balance in regulation of various membrane functions.

Acknowledgements

We are grateful for the technical assistance of Mr. Rudolf Fluckiger. This work was supported by a grant from the Roche Research Foundation and by the Swiss National Science Foundation Grants No. 3.341-0.74 and No. 3.297.74.

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