THE EXCHANGE OF PHOSPHOLIPIDS BETWEEN RAT ERYTHROCYTES AND PLASMA, AND THE TRANSLOCATION OF PHOSPHATIDYLCHOLINE ACROSS THE RED CELL MEMBRANE, ARE TEMPERATURE DEPENDENT PROCESSES

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1. Introduction

Increasing evidence suggests an asymmetrical distribution of phospholipids between the inner and outer layer of the erythrocyte membrane [1–8] and other biological membranes [9–11]. The phospholipid composition of the outer layer of the human [4,7] and rat [8] erythrocyte membrane reflects the plasma phospholipid composition [12]. This is comprehensible, because one of the major pathways for renewal of phospholipids in the mature erythrocyte is the exchange of phospholipids between serum lipoproteins and the red cell membrane [13–17]. This exchange is thought to take place at the outer surface layer of the erythrocyte membrane [8,17–20].

Various reports discuss the possibility of a translocation process, by which phosphatidylcholine, the main phospholipid component in both plasma and the outer membrane layer, can migrate between inner and outer layer of the red cell membrane (a flip-flop process) [8,17–20]. In the present study we report the temperature dependence of phospholipid exchange between plasma and the rat erythrocyte membrane and a similar temperature dependent transfer of phosphatidylcholine across the membrane.

2. Methods and materials

2.1. *Incubation conditions for exchange of phospholipids*

Radioactively labelled phospholipids in rat plasma were obtained by injecting a rat intraperitoneally with 1 mCi $^{32}P_i$ [8]. Exchange of phospholipids between ^{32}P-labelled rat plasma and non-radioactive rat erythrocytes was measured as follows: samples of 0.15 ml unlabelled packed cells were incubated with 0.25 ml ^{32}P-labelled plasma with gentle stirring for 2 h at different temperatures. After the incubation the cells were spun down and washed 4 times with 10 ml ice-cold 0.9% NaCl. The lipids were extracted according to the method of Reed et al. [21]. The phospholipids were separated via silica thin-layer chromatography, using chloroform/methanol/glacial acetic acid/water (70:35:8:4, by vol.) as developing solvent. The radioactivity of the individual phospholipids was assayed using a Packard liquid scintillation counter, model 2425 B, and a scintillation system as described by Fricke [22].

2.2. *Incubation conditions to measure translocation of phosphatidylcholine across the red cell membrane*

Unlabelled rat erythrocytes (7.5 ml) were incubated with 9.0 ml ^{32}P-labelled rat plasma with gentle stirring for 2 h at 37°C. After the incubation the cells were spun down and washed successively with 70 ml 0.9% NaCl (2 times), 70 ml of a saline solution of 1% defatted serum albumin (2 times), 9.0 ml unlabelled plasma and finally 3 times with 70 ml 0.9% NaCl. Aliquots of 0.25 ml packed cells were subsequently reincubated in 5 ml 0.85% NaCl, 10 mM Tris–HCl (pH 7.4) for 0, 1 and 2 h at different temperatures. After this reincubation the combined action of phospholipase A_2 from *Naja naja* venom and sphingomyelinase from *Staphylococcus aureus* was used to discriminate between the phospholipids of the outer

and inner layer of the rat erythrocyte membrane [8]. The cells were spun down after the reincubation and the supernatant was replaced by 5 ml of a solution containing 0.87% NaCl, 10 mM $CaCl_2$, 0.25 mM $MgCl_2$ (adjusted to pH 7.4 with Tris–HCl) and 20 I.U. of phospholipase A_2 purified from *Naja naja* venom. After 1 h incubation at 37°C 2 I.U. of sphingomyelinase were added. For each incubation time and temperature control samples were run without the enzymes. In parallel incubations the degree of degradation of the membrane phospholipids was measured. One hour after the addition of sphingomyelinase, the cells were centrifuged down and the reaction was terminated by adding EDTA in a final concentration of 10 mM. Hemolysis never exceeded 3–4%. The lipids were extracted [21] and separated via 2-dimensional silica thin-layer chromatography as described by Broekhuyse [23]. The radioactivity of the individual phospholipid constituents was measured as described above and the amount of phosphorus was determined according to the procedure described by Bartlett [24]. Purified phospholipase A_2 from *Naja naja* venom and sphingomyelinase from *Staphylococcus aureus* were a generous gift from Dr R. F. A. Zwaal (Biochemisch Laboratorium, State University of Utrecht, Utrecht, The Netherlands).

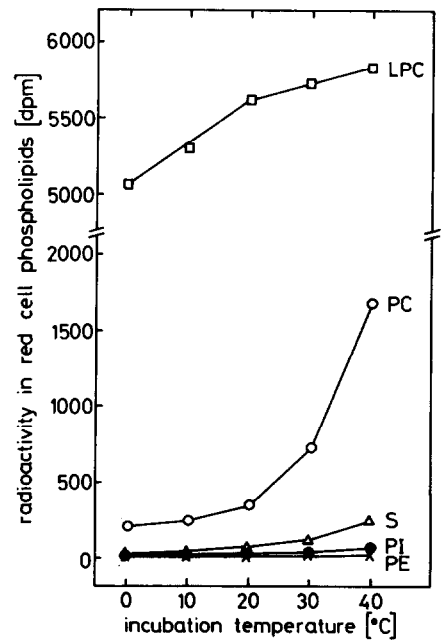

Fig.1. Radioactivity of red cell phospholipids after incubation in ^{32}P-labelled plasma. Non-radioactive rat erythrocytes were incubated in ^{32}P-labelled rat plasma as mentioned in detail under methods. *Abbreviations:* LPC, lysophosphatidylcholine; PC, phosphatidylcholine; S, sphingomyelin; PI, phosphatidylinositol; PE, phosphatidylethanolamine.

3. Results

3.1. *Temperature dependence of phospholipid exchange*

Figure 1 shows the radioactivity of rat erythrocyte phospholipids after incubation in ^{32}P-labelled plasma at different temperatures. At all temperatures there is hardly any labelling of erythrocyte phosphatidylethanolamine and phosphatidylinositol. Although there is some exchange of sphingomyelin, phosphatidylcholine and its lyso-derivative are the main exchangeable phospholipids.

It is known from other experiments (not shown here) that the exchange of phosphatidylcholine is linear for more than 2 h under these conditions. The radioactivity of this red cell phospholipid can, therefore, be used as an indication of the exchange rate.

The rate of exchange of phosphatidylcholine is hardly influenced by a temperature increase from 0–20°C, but there is a considerable increase in exchange rate at higher temperatures. As described by several authors [8,14–17] exchange of lysophosphatidylcholine is very rapid. Even at low temperature 2 h incubation is enough to approach exchange equilibrium. This explains why increase in temperature has such a relatively small effect on the labelling of red cell lysophosphatidylcholine.

3.2. *Temperature dependence of translocation of phosphatidylcholine across the red cell membrane*

Rat erythrocytes were incubated in ^{32}P-labelled plasma for 2 h. After this incubation the majority of the labelled red cell phosphatidylcholine is found at the outside of the membrane (see fig.2 and [8]). After removal of the plasma and washing of the erythrocytes the cells were reincubated in a buffer at 3 different temperatures and successively treated with phospholipase A_2 and sphingomyelinase to discriminate between

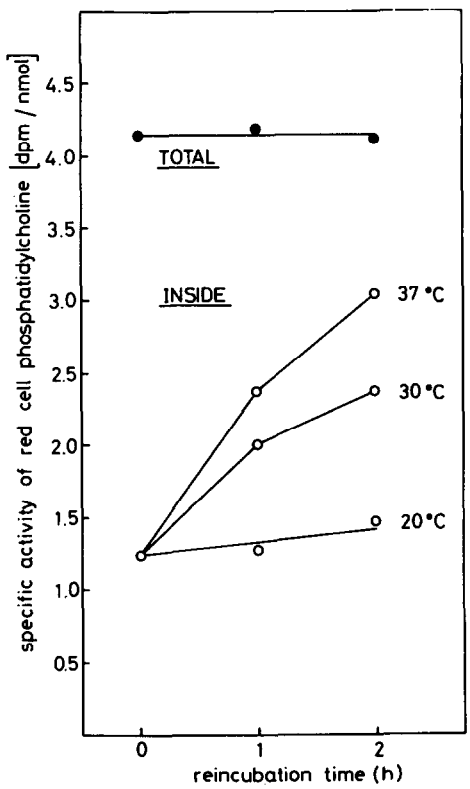

Fig.2. Transfer of [^{32}P] phosphatidylcholine across the rat erythrocyte membrane at different temperatures. Unlabelled erythrocytes were incubated in ^{32}P-labelled plasma (45%, by vol.) for 2 h at 37°C. After the incubation the cells were spun down and washed extensively (see methods). Samples of 0.25 ml labelled packed cells were subsequently incubated in a salt buffer for 0, 1 and 2 h at the indicated temperatures, followed by a treatment with phospholipase A_2 and sphingomyelinase at 37°C to discriminate between the inner and outer layer of the membrane. The degree of degradation of phosphatidylcholine was 60%.

the inside and outside of the membrane.

Figure 2 shows that the total specific activity of phosphatidylcholine remains constant upon reincubation at different temperatures, whereas the radioactivity at the inside of the membrane increases depending on the reincubation temperature. The increase in radioactivity of phosphatidylcholine is very slow at 20°C, which suggests that there is hardly any transmembrane migration of phosphatidylcholine at 20°C. This translocation process is considerably accelerated at higher temperatures.

4. Discussion

Figure 1 shows that the exchange of phosphatidylcholine between plasma lipoproteins and the erythrocyte membrane is temperature dependent. The exchange rate increases considerably at temperatures above 20°C. Erythrocytes cannot use water-soluble ^{32}P-labelled components for phospholipid synthesis, since they lack the enzymes for de novo phospholipid synthesis (see e.g. [14]). Nevertheless, we must be aware that under the conditions of the experiment in fig.1 part of the labelled phosphatidylcholine might be derived from acylation of membrane lysophosphatidylcholine, which receives a high specific radioactivity after exchange with plasma lysophosphatidylcholine.

The amount of phosphatidylcholine, derived from acylation of its lyso-derivative, however, is by far exceeded by the exchanged amount of phosphatidylcholine [13,15].

Figure 2 shows that the translocation of phosphatidylcholine from the outside to the inside of the membrane is hardly measurable at 20°C and is considerably enlarged at higher temperatures. In this respect it is interesting to mention that Zimmer et al. [25,26] reported a change in the viscosity of the human erythrocyte membrane around 20°C.

Summarizing the results of fig.1 and fig.2 suggests that both exchange and transmembrane migration of phosphatidylcholine in the rat erythrocyte membrane are temperature dependent processes which become relevant at temperatures higher than 20°C.

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References

[1] Bretscher, M. (1972) J. Mol. Biol. 71, 523–528.
[2] Gordesky, S. E. and Marinetti, G. V. (1973) Biochem. Biophys. Res. Commun. 50, 1027–1031.

[3] Zwaal, R. F. A., Roelofsen, B. and Colley, C. M. (1973) Biochim. Biophys. Acta 300, 159–182.
[4] Verkleij, A. J., Zwaal, R. F. A., Roelofsen, B., Comfurius, P., Kastelijn, D. and Van Deenen, L. L. M. (1973) Biochim. Biophys. Acta 323, 178–193.
[5] Kahlenberg, A., Walker, C. and Rohrlick, R. (1974) Can. J. Biochem. 52, 803–806.
[6] Gordesky, S. E., Marinetti, G. V. and Love, R. (1975) J. Membrane Biol. 20, 111–132.
[7] Zwaal, R. F. A., Roelofsen, B., Comfurius, P. and Van Deenen, L. L. M. (1975) Biochim. Biophys. Acta 406, 83–96.
[8] Renooij, W., Van Golde, L. M. G., Zwaal, R. F. A. and Van Deenen, L. L. M. (1976) Eur. J. Biochem. 61, 53–58.
[9] Tsai, K. H. and Lenard, J. (1975) Nature 253, 554–555.
[10] Depierre, J. W. and Dallner, G. (1975) Biochim. Biophys. Acta 415, 411–472.
[11] Rothman, J. E., Tsai, D. K., Dawidowicz, E. A. and Lenard, J. (1976) Biochemistry 15, 2361–2370.
[12] Nelson, G. J. (1967) Lipids 2, 323–328.
[13] Lovelock, J. E., James, A. T. and Rowe, C. E. (1960) Biochem. J. 74, 137–140.
[14] Mulder, E. and Van Deenen, L. L. M. (1965) Biochim. Biophys. Acta 106, 348–356.
[15] Sakagami, T., Minari, O. and Orii, T. (1965) Biochim. Biophys. Acta 98, 356–364.
[16] Soula, G., Valdiguié, P. and Douste-Blazy, L. (1967) Bull. Soc. Chim. Biol. 49, 1317–1330.
[17] Reed, C. F. (1968) J. Clin. Invest. 47, 749–760.
[18] Bloj, B. and Zilversmit, D. B. (1976) Biochemistry 15, 1277–1283.
[19] Rousselet, A., Guthmann, C., Matricon, J., Bienvenue, A. and Devaux, P. F. (1976) Biochim. Biophys. Acta 426, 357–371.
[20] Steck, T. L., Wackman, N. and Tavlov, A. R. (1976) J. Supramol. Struct. 4, 169–180.
[21] Reed, C. F., Swisher, S. N., Marinetti, G. V. and Ede, E. G. (1960) J. Lab. Clin. Med. 56, 281–289.
[22] Fricke, U. (1975) Anal. Biochem. 63, 555–558.
[23] Broekhuyse, R. M. (1969) Clin. Chim. Acta 23, 457–463.
[24] Bartlett, G. R. (1959) J. Biol. Chem. 234, 466–468.
[25] Zimmer, G. and Schirmer, H. (1974) Biochim. Biophys. Acta 345, 314–320.
[26] Zimmer, G., Schirmer, H. and Bastian, P. (1975) Biochim. Biophys. Acta 401, 244–255.