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NUCLEAR MEMBRANES AND PLASMA MEMBRANES FROM HEN ERYTHROCYTES

III. LOCALIZATION OF ACTIVITIES INCORPORATING FATTY ACIDS INTO PHOSPHOLIPIDS

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SUMMARY

1. Long chain fatty acid (oleic and palmitic acids) incorporation into phospholipids was studied *in vitro* in the two membrane systems of the mature hen erythrocyte. In the presence of CoA, ATP, and $MgCl_2$ the nuclear membrane had about a 100-fold higher incorporating activity than the plasma membrane. Fatty acid incorporation into neutral lipids (triglycerides and cholesterol esters) was negligibly low. The molar incorporation into phosphatidylcholine was about 10 times higher than that into phosphatidylethanolamine.

2. The incorporation of labelled palmitic acid from [^{14}C]palmityl-CoA into the membranous phospholipids was almost identical in both plasma and nuclear membranes, thus demonstrating that it is the activation of the offered fatty acid by acyl-CoA synthetase (EC 6.2.1.3) which is selectively reduced in the plasma membrane, and not the activity of the enzyme esterifying the lysophosphatides or the concentration of the lyso-derivatives.

3. The results are discussed in relation to the questions of the use of fatty acid incorporating activity as an enzyme marker for membranes of the endoplasmic reticulum system, in comparison with plasma membranes.

INTRODUCTION

The existence of enzymatic activities which catalyse the incorporation of free fatty acids into phospholipids is well known in a variety of cell systems[1–6]. In such reactions both the fatty acid incorporating enzymes and the lysophosphatide acceptor molecules can be membrane bound, as has been reported for various membrane types (rat liver mitochondrial membrane[5,7–11], microsomal membrane of rat liver[1,5,7,9–14], plasma membrane of rat liver[10,15], and plasma membrane of mammalian erythrocytes[3,16–21]). It is obvious that this enzymatic mechanism counteracts the formation of lyso-derivatives from the membrane phospholipids and, therefore

must have importance for the structure and function of the specific membrane[22], in the sense of a "phospholipid repair mechanism".

The aim of the present study was to clarify whether the ability to esterify lysophosphatides is different for the different kinds of membranes in the same cell, and secondly, to elucidate the enzymatic step whose activity correlates with any possible differences. To answer this question we have chosen the mature avian erythrocyte for the following reasons. (i) The cellular membrane pattern is greatly simplified since endomembranes (endoplasmic reticulum, Golgi apparatus, secretory vesicles) and mitochondria[23–25] are greatly reduced during erythropoiesis so that the mature erythrocyte contains less than 1% of membrane which does not belong to either the nuclear envelope or the plasma membrane[25]. These membranes, the plasma membrane and the (duplicate) nuclear membrane, are present in an almost 1:1 area ratio. (ii) Examination of the literature suggests that the fatty acid incorporating capacity of the mammalian erythrocyte plasma membranes[3,20,21] is far below the corresponding activities reported for various mammalian liver endomembrane fractions[6,11]. In order to confirm this it was necessary to prepare both classes of membranes from the same cell to eliminate possible intercellular differences.

MATERIALS AND METHODS

Isolation procedures

Nuclei, nuclear membranes and plasma membranes were isolated from purified hen erythrocytes as described elsewhere[25–27]. The purity, structural preservation, and chemical composition of these fractions have been documented in our earlier publications[24–26]. Rat (Wistar II, 150 g body weight) erythrocyte plasma membranes were isolated exactly in parallel with the nucleated avian erythrocytes. The plasma membranes obtained by this method showed about the same fatty acid incorporating activity as "ghosts" prepared from the same blood sample by the method of Dodge *et al.*[28].

Enzyme assays

Acyl-CoA synthetase assay was performed by a slight modification of the methods of Oliveira and Vaughan[18], Mulder and van Deenen[3], and Donabedian and Karmen[19]. The complex of fatty acid-free albumin (prepared according to Goodman[29]) and the radioactive fatty acid was prepared in the presence of antibiotics (0.4 mg penicillin G, 0.4 mg streptomycin and 0.04 mg chloramphenicol per ml). The final enzymatic reaction mixture consisted of 4 mg NaCl, 40 mg albumin complexed with 3 μCi of [^{14}C]oleic acid, (spec. act. 60 Ci/mole, Radiochemical Centre, Amersham, Great Britain) or [^{14}C]palmitic acid (spec. act. 58 Ci/mole, same source), 1 μmole CoA, 4 μmoles ATP, 8 μmoles $MgCl_2$, the antibiotics indicated above, 2–5 mg protein of the specific biological fraction, in a total volume of 2 ml 0.1 M potassium phosphate buffer. The resulting pH was 7.2. Incubation was carried out at 37 °C for 3 h under gentle magnetic stirring. After the incubation the nuclear or membranous fractions were washed three times with an excess of ice-cold buffer solution (0.1 M potassium phosphate, 5 mM $MgCl_2$, pH 7.2) by centrifugation at $3000 \times g$ for 10 min. ^{14}C-labelled palmityl-CoA (spec. act. 53 Ci/mole) was purchased from New England Nuclear. The incubation was carried out with the same

concentration of serum albumin as used for the formation of the fatty acid–albumin complex in the corresponding experiments with the non-activated fatty acid. Protein determinations were carried out by the method of Lowry *et al.*[30].

Lipids: extraction, separation, and determination of radioactivity

The lipids were extracted and washed free from protein according to Folch *et al.*[31]. Neutral lipids were separated by thin-layer chromatography on silica gel G (Merck, Darmstadt, Germany) with light petroleum (60–70 °C)–ether–acetic acid (90:10:1,v/v/v) as solvents and radioactivity was scanned on the plate with a gas-flow proportional counter (Berthold/Frieseke GmbH, Wildbad, Germany). In addition the phospholipids were separated two-dimensionally with the solvent systems chloroform–methanol–acetic acid–water (40:20:0.5:2,v/v/v/v) and chloroform–methanol–25% aqueous ammonia (50:20:2.5,v/v/v), and were visualized by gently spraying fluorescent dye[32] under ultraviolet light (350 nm). The individual spots were quantitatively scraped off and their radioactivity was counted after addition of 1 ml NCS (Nuclear Chicago Solubilizer, Amersham/Searle, U.S.A.) and 10 ml toluene-based scintillation liquid. Counting was performed in a liquid scintillation spectrometer at an efficiency of 60–70%, as monitored using internal and external standards. Aliquots of the phospholipid containing spots were used for determination of phosphate according to Gerlach and Deuticke[33]. The samples for the radiochemical analysis of fatty acids were processed as described in a previous publication [34].The methyl esters were analysed in a Varian 1800 gas chromatograph equipped with a thermal conductivity detector. The gas chromatograph was coupled to a Perkin–Elmer gas-flow proportional counter which had been modified to improve the resolution, as follows: the diameter of the reactor tube was reduced to the diameter of the separation column (1/8 inch) and prolonged to a total length of 1 m. For oxidation we used, instead of the usual granulated CuO, a thin copper wire which was continuously reoxidized by a gentle stream of O_2 at a rate of 2 ml/min. Using a helium: methane ratio of 1:2 we could work at a counting efficiency of approximately 10%.

In pilot experiments we found that the commercially available radioactive fatty acids contained minor, but significant contaminations of other fatty acids and, in addition, that such fatty acid impurities can be preferentially (up to 50% of total radioactivity) incorporated into the phospholipids during the assay; see also refs 16, 19. Therefore we routinely purified the fatty acids by preparative gas chromatography of the corresponding methyl esters, followed by alkaline saponification.

RESULTS

When purified hen erythrocytes were incubated with [^{14}C]acetic acid, incorporation into phospholipids was extremely low compared to that of whole blood cells. This suggests that our purified erythrocytes are tolerably free from leucocytes and reticulocytes, which are known to be primarily responsible for the incorporation of acetate into blood cell lipids[35–39]. The small amount of acetate incorporation into the phospholipids of fowl blood erythrocytes reported by Webb *et al.*[40] could well be explained by contamination with other blood cells. On the other hand, there is a remarkable incorporation of fatty acids into the phospholipids of the mature avian erythrocytes (Table I). An incorporation of fatty acids into phospholipids is

TABLE I

IN VITRO INCORPORATION OF FATTY ACIDS INTO THE PHOSPHOLIPIDS IN ERYTHROCYTES AND ERYTHROCYTE FRACTIONS

Unless otherwise indicated the incubation was carried out with the addition of all cofactors.

Precursor	*Erythrocytes from*	*Fraction*	*Incorporation (dpm) in*			
			Phosphatidylcholine (PC)		*Phosphatidylethanolamine (PE)*	
			per μmole PC	*per mg protein*	*per μmole PE*	*per mg protein*
Oleic acid	hen	whole erythrocytes	255000	2100	33000	190
		nuclei	535000	8350	73000	570
		nuclei, *minus* cofactors	50500	800	9200	72
		nuclear membranes	890000	115000	120000	7650
		nuclear membranes, *minus* cofactors	37500	7700	5100	320
		plasma membranes	685	98	163	23
		plasma membranes, *minus* cofactors	360	52	300	43
	rat	plasma membranes	23500	5500	2400	183
Palmitic acid	hen	nuclei	850000	13200	66000	515
		nuclear membranes	1400000	182000	137000	8720
		plasma membranes	7000	1000	500	98
	rat	plasma membranes	30500	7150	2700	203

also well documented in the (mitochondria-free) non-nucleated mammalian erythrocytes[3,16,18–21,41,42]. It is obvious that this fatty acid incorporation has to be preceded by the activation of the offered fatty acid by an acyl-CoA synthetase[1], which is dependent on the addition of ATP, CoA and $MgCl_2$. The further transfer of the CoA-activated fatty acid to a lysophosphatide molecule does not require addition of other energy-rich compounds. Incorporation of radioactive oleic acid is reduced to less than 9% in nuclei and nuclear membranes when the cofactors are omitted (*cf.* Farstadt *et al.*[9] who describe this effect on rat liver microsomes), but is influenced by the added cofactors only to a slight extent in the corresponding plasma membranes. The reaction is heat sensitive (total inactivation by preheating for 30 min at 60 °C). Most of the radioactivity incorporated into the phospholipids is stable to treatment with 1% sodium deoxycholate. The incorporating activity was relatively stable during storage at −20 °C and lost only about 10% activity per month. Table I shows marked differences in the subcellular distribution of fatty acid incorporating activity between the two membrane systems present in hen erythrocytes, the nuclear membrane and the plasma membrane. This is observed with both precursors, oleic acid and palmitic acid. For instance, the specific activity (on a protein basis) of oleic acid incorporation by nuclear membranes was about 1000 times greater than that by plasma membranes. Correspondingly, there was a 200-fold difference with palmitic acid. On a molar basis, the incorporation of both oleic acid and palmitic acid is about 10 times higher into phosphatidylcholine than into phosphatidylethanolamine (Table I). Table I also demonstrates that the nuclear fatty acid incorporation into phospholipids is located in and firmly associated with the nuclear membranes, as shown by the 13- to 17-fold enrichment of specific activity (per mg protein) from nuclei to nuclear membranes. Unlike our nuclei and nuclear membrane preparations, the plasma membranes showed much more variability of incorporation from one experiment to another, ranging, for instance, from 5 to 50 μmoles oleic acid incorporated per mole lecithin (after 3 h of incubation), perhaps depending on the purity of the membrane fraction. It must be emphasized, however, that even in the most active plasma membrane preparations the activities are still below 1% of that of the corresponding nuclear membranes. Our rat erythrocyte plasma membrane ("ghost") preparations are more active in incorporating fatty acids than the rat erythrocyte ghost preparations reported by other authors[3,18]. Marked differences between plasma membrane preparations from different vertebrates are well known, in particular for various mammals[17,18]. Again, it has to be emphasized that the activity of the rat erythrocyte ghosts is an order of magnitude lower than that of the hen erythrocyte nuclear membranes. The incorporation of fatty acids into the neutral lipids of nuclei, nuclear membranes, and plasma membranes under assay conditions was very low compared to the incorporation into the phospholipids (Fig. 1), and was not specifically investigated. In order to distinguish which reaction step in the incorporation of fatty acids into a lysophosphatide (*i.e.* the activation with CoA or the esterification) is responsible for the pronounced difference between the activities of the two membrane types, we have examined the incorporation of previously activated fatty acid by using [^{14}C]palmityl-CoA. The results (Table II) show that the incorporation of fatty acids into the phospholipids is then essentially the same in plasma membranes from the mammalian and the avian erythrocyte and avian nuclear membranes. This makes it clear that the extremely low fatty acid incorporation

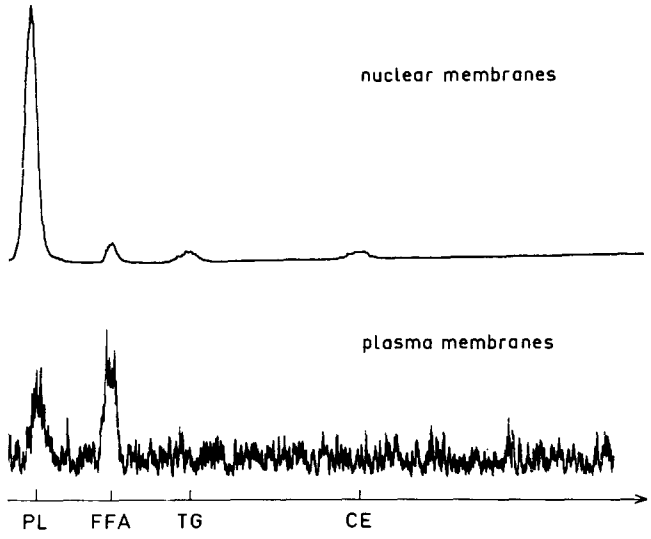

Fig. 1. Distribution of radioactivity in thin-layer chromatograms (silica gel G, light petroleum (60–70 °C)–ether–acetic acid (90:10:1, v/v/v) in lipid extracts of nuclear membranes and plasma membranes isolated from hen erythrocytes, after incubation *in vitro* with purified [^{14}C]oleic acid. In both chromatograms comparable amounts of lipids were applied. The nuclear membrane lipid scan is shown with a scale reduced by 100:1 relative to the plasma membrane lipid scan. PL, phospholipids (remaining at the start); FFA, free fatty acids; TG, triglycerides; CE, cholesterol esters.

TABLE II

COMPARISON OF THE INCORPORATION OF CoA-ACTIVATED AND NON-ACTIVATED PALMITIC ACID INTO PHOSPHOLIPIDS IN DIFFERENT ERYTHROCYTE MEMBRANE FRACTIONS

Fraction	*Incorporation of precursor (sum of radioactivity incorporated into phospholipids as μmoles/mole phospholipid)*		*Factor*
	[^{14}C]*Palmitic acid*	[^{14}C]*Palmityl-CoA*	
Rat erythrocytes, "ghosts"	180	15200*	85
Hen erythrocytes			
plasma membranes	34.5	7050	204
nuclear membranes	4800	11700	2.4

* This value represents a turnover rate of 1.5% during 3 h of incubation.

activities of erythrocyte plasma membranes, in particular of the avian ones, is limited by the acyl-CoA synthetase activity. Both sorts of membranes are almost equally capable of transferring CoA-activated fatty acids to the lysophosphatide acceptor molecule. The activities of the fatty acid incorporating enzymes is not grossly altered by the homogenization and centrifugation procedures involved. The total number of fatty

acid molecules incorporated in phospholipids was about the same (±16%) whether cell disruption, centrifugation, and membrane isolation were performed before or after the incorporation assay. We cannot yet rule out the possibility that fatty acid incorporating enzyme activities might also occur in places other than membranes, since we consistently find such incorporating activity (to an extent of 13–35% of the total recovered radioactivity, depending on the specific preparation scheme) in the material not sedimented at $100000 \times g$. The specific activity of incorporation per phospholipid mass is slightly (1.5-fold) higher in the non-particulate (or smaller or lighter particle containing) material. This increase is more pronounced (approx. 3 times) after extensive use of cellular disruption procedures before the incubation. This could simply reflect a better substrate accessibility. The present observations of fatty acid incorporating activities in $100000 \times g$ supernatants parallel the similar observations in rat and guinea pig liver by Kornberg and Pricer[1], Farstadt *et al.*[9], and Lands[12] but contrast to other reports from the mammalian liver system, such as those of Pande and Mead[10] and Creasey[13]. Therefore, further studies on the separation and characterization of the fatty acid incorporating moieties present in such supernatants are required before a decision as to their exclusive membrane-bound localization *in vivo* can be made.

DISCUSSION

The present study shows that the membranes isolated from a cell system, which has no fatty acid synthesizing capacity, are capable of incorporating fatty acids into membrane phospholipids, probably by coupling to lysophosphatide. Our finding of a marked difference of the two chief membrane types present in the hen erythrocyte is compatible with the general interpretation that fatty acid incorporating activity is characteristically high in the endoplasmic reticulum system and is low in plasma membranes. From the well-known morphological continuity and biochemical relationship of the nuclear envelope with the endoplasmic reticulum[43–50] it is reasonable to regard the nuclear envelope of the mature avian erythrocyte as representing the only quantitatively important part of the endoplasmic reticulum system[25]. However, more detailed investigations are needed before a conclusion can be made as to the use of fatty acid incorporating activity as a relative enzyme marker for endomembrane fractions in general, or if this holds only for the avian erythrocyte. The greatly reduced activity of the avian erythrocyte plasma membranes in coupling the fatty acid to a membrane lysophosphatide in principle could be explained in two ways: (i) The plasma membranes contain less lyso-derivatives. This is not the case, as demonstrated by the comparison of the phospholipid patterns of our hen erythrocyte nuclear and plasma membrane preparations[24]. The low and almost identical contents of lyso-derivatives in the nuclear and plasma membrane fractions were also examined and confirmed in the course of the present study. Another argument is that the accepting lyso-derivatives are not present in high steady-state concentrations but are rapidly generated at low concentrations via the "monoacyl–diacyl phospholipid cycle"[51] at different rates in the two membranes. Such a limitation seems to be ruled out by the complete restoration of the fatty acid incorporating activity in the plasma membrane when the fatty acid is added in its activated form. (ii) One or both of the enzymes involved in the fatty acid incorporation are not present or

are inactive in the plasma membrane. Our results clearly show that only the first step of the fatty acid incorporating reaction is selectively reduced, which means that the acyl-CoA synthetase (EC 6.2.1.3) is either much less concentrated or is inactivated in the plasma membrane.

The observed increase in fatty acid incorporation in the hen erythrocyte plasma membrane when the CoA-activated form is offered parallels the data from human erythrocytes[21]. The data available at present do not allow us to make a conclusion as to the meaning of the quantitative differences for specific fatty acids and the accepting membrane lysophosphatides (compare also the articles of van den Bosch and van Deenen[51], Lands and Merkl[2], and Scherphof and van Deenen[7], which describe the rat liver system; for mammalian erythrocytes see refs 3, 7, 16, 52). It has been proposed by various workers that the capacity of a membrane to take up fatty acids from its environment for re-esterification of its lysophosphatides is criterial for the control of the level of the lyso-products[20,21,52,53]. Consequently, this capability is important for the structure and the life time of the membrane[18]. So the high fatty acid incorporating capacity of endoplasmic reticulum-type membranes might contribute to their stability as well as to other properties depending on the specific lipid composition.

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