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Clustering in coated vesicles of polyunsaturated phospholipids segregated from plasma and Golgi membranes of adrenocortical cells

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In bovine adrenocortical cells, the fatty acyl chains of the phospholipids have been identified in the membranes of the different cell compartments: plasma membranes, Golgi complex and coated vesicle membranes. An increase in the total number of unsaturation in the fatty acid is demonstrated in the coated vesicle membranes as compared with the plasma and Golgi membranes. Furthermore, it appears that phosphatidylcholine and phosphatidylethanolamine are both enriched in polyunsaturated fatty acyl chains, namely arachidonic and adrenic acids in both types of coated vesicles. Only two of the fatty acids are characteristic of Golgi complex and small coated vesicles, 22:5 (*n*–6) in PC and 22:6 (*n*–3) in PE, suggesting that the SCV could originate from the Golgi stacks. A high value of the ratio 22:5 (*n*–3)/22:6 (*n*–3) is observed which is, as far as we know, characteristic of adrenal cells.

Phospholipid; Fatty acid; Endocytosis; Steroidogenesis; Coated vesicle; (Bovine adrenocortical cell)

1. INTRODUCTION

In mammalian adrenal cortex, the receptor mediated endocytosis of LDL carrying the cholesterol involves the formation of coated pits from specific areas of the plasma membranes where ligand bound or free receptors are clustered. These coated pits invaginate to form coated vesicles which carry the LDL-receptor complex through endosomes. From lysosomes the free cholesterol is delivered to the cell. This step of cholesterol internalisation through the LDL carrier is one of the key processes of steroidogenesis in adrenals which is under acute ACTH control.

The coated pit formation appears to be induced by the clathrin network coating specific areas on the cytoplasmic side of the plasma membrane. It has been suggested that under the influence of clathrin a segregation of certain lipids from the membrane bilayer could occur, leading to a modification of the membrane fluidity [1,2]. Such a lipid reorganisation could possibly initiate the local change in the radius of curvature of the plasma membrane. The relationship between the mechanism of action of steroid hormones and the membrane organisation and dynamics, has been reviewed and discussed [3]. Different steps of adrenal steroidogenesis have been shown to be in relation with phospholipid metabolism in the membranes: Farese et al. [4] have demonstrated

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Abbreviations: PL, phospholipid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; LDL, low density lipoprotein; ACTH, adrenocorticotropic hormone; CV, coated vesicle; SCV, small coated vesicle; LCV, large coated vesicle; TLC, thin layer chromatography; GLC, gas liquid chromatography

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the involvement of phospholipids in the steroidogenic action of ACTH. More recently Igarashi and Kimura [5] have evidenced an increased content in adrenic acid for PE in the rat adrenal mitochondria under ACTH stimulation, leading in vitro to an increased formation of pregnenolone from endogenous cholesterol.

In a previous study [2] of the very first step of steroidogenesis in adrenals, i.e. cholesterol internalisation, we have investigated the possible role of phospholipids. An enrichment in PE in the coated vesicles was demonstrated by comparison with plasma membrane from which they should derive. The present work is a comparative study of the fatty acyl chain content of the phospholipids from different cellular membranes: plasma, Golgi and membranes from the different coated vesicles characterized in the adrenocortical cells. The segregation, which is evidenced in coated vesicle membranes, of PE and PC enriched in different polyunsaturated fatty acyl chains raises the question of their possible role in steroidogenesis and its regulation by ACTH.

2. MATERIALS AND METHODS

DC Kiesel gel platten 60 were from Merck. All other chemicals were Merck products of analytical grade. GLC was performed on a Gierdel series 30 gas chromatograph equipped with a flame ionization detector.

2.1. *Adrenal cell membrane preparation*

The enriched plasma membrane fraction was obtained according to Schlegel and Schwyzer [6] in 1 mM $NaHCO_3$, 0.25 M sucrose, pH 7.4 buffer. A sucrose density discontinuous gradient was used for further purification, and the fraction collected at the 26–30% sucrose interface was characterized as plasma membranes. For the enriched Golgi membrane fraction the same gradient was used and the fraction was collected at the 22.5–26% interface.

2.2. *Coated vesicle preparation*

Coated vesicles were isolated according to a described method [2]. In all cases the two consecutive ^2H_2O sucrose gradients were made and two different classes of vesicles were collected at the 1.15–1.18 (SCV) and 1.18–1.21 (LCV) density interfaces. Each fraction has been controlled by electron microscopy.

2.3. *Protein content*

The protein content of samples was determined by the method of Peterson [7].

2.4. *Lipid analysis*

The lipid analysis of cell membranes and coated vesicles was carried out as described [2]. The analysis of the different classes of fatty acids was made on the lipid extract of the cell membranes and coated vesicles and also on the whole cell extract. In some cases the fatty acids of PC and PE were measured on GLC after separation by TLC using the following solvent system: a first migration was run up to half the plate with chloroform/methanol/acetic acid/water (75:45:12:6, v/v). The plates were dried and run in the same direction all the way to the top with petroleum ether/diethyl ether/acetic acid (90:30:1, v/v). Spots were scraped off by comparison with the standards revealed by iodine vapor. Hydrolysis and methylation were performed on the dry extract for the whole phospholipid fatty acid analysis and directly on the silica gel for TLC separated PC and PE, according to Van Wijngaarden [8]. Fatty acids were analysed by means of GLC using a 25 m capillary column filled with 20 M Carbowax, operating between 180°C and 200°C at 0.5°C per min. Detection was by flame ionization.

3. RESULTS

The phospholipid analysis has been performed on the different lipid extracts of plasma membranes, Golgi membranes and each type of coated vesicle membranes. As previously reported, a relative increase in the PE percent of PL is observed in both types of CV. Therefore, an analysis of PL fatty acyl chain content has been undertaken in order to support the hypothesis of a segregation of PL enriched in unsaturated fatty acids in coated pits and coated vesicles. As shown on table 1, a significant increase in unsaturated fatty acids is observed in SCV and LCV comparatively to plasma and Golgi membranes. It appears that it is mainly arachidonic acid 20:4 ($n-6$) present at a relatively high percentage (20%) and adrenic acid

Table 1

Fatty acid content of whole lipidic extract of different adrenal cell membranes

Fatty acid	Plasma membrane	Golgi membrane	LCV membrane	SCV membrane
14:0	0.8 ± 0.5	0.7 ± 0.3	0.9 ± 0.6	0.6 ± 0.3
16:0	16.5 ± 2.7	17.1 ± 3.4	15.6 ± 2.9	14.6 ± 2.7
16:1	1.8 ± 1.8	1.2 ± 0.4	1.5 ± 0.5	2.1 ± 2.3
18:0	23.4 ± 2.4	23.0 ± 4.0	17.7 ± 3.0	18.6 ± 3.4
18:1$n-9$	16.1 ± 2.0	17.3 ± 3.3	15.5 ± 1.1	16.4 ± 2.5
18:1$n-7$	3.3 ± 0.5	3.7 ± 0.7	3.7 ± 1.0	3.6 ± 0.3
18:2$n-6$	8.9 ± 2.8	7.7 ± 1.7	8.3 ± 1.7	8.4 ± 1.5
18:3$n-3$	0.8 ± 0.3	0.4 ± 0.3	0.6 ± 0.2	0.6 ± 0.2
20:3$n-9$	0.7 ± 0.1	0.6 ± 0.2	0.8 ± 0.3	0.8 ± 0.2
20:3$n-6$	1.6 ± 0.4	1.4 ± 0.3	1.4 ± 0.2	1.5 ± 0.3
20:4$n-6$	18.7 ± 1.9	18.3 ± 3.8	23.0 ± 2.0	22.6 ± 4.5
20:5$n-3$	2.7 ± 1.7	2.8 ± 2.0	3.3 ± 2.1	3.1 ± 1.6
22:4$n-6$	0.6 ± 0.2	1.0 ± 0.4	1.5 ± 0.5	1.4 ± 1.0
22:5$n-3$	4.2 ± 1.4	4.5 ± 1.8	6.1 ± 1.6	5.9 ± 1.6
22:6$n-3$	0.3 ± 0.3	0.3 ± 0.3	0.3 ± 0.1	0.2 ± 0.2
Total no. of unsatur.	160 ± 18	160 ± 31	192 ± 27	189 ± 28

Values are expressed as mol% of total fatty acids and are the average over 6 preparations

22:4 ($n-6$) which are increased. A smaller increase of docosapentaenoic acid 22:5 ($n-3$) is also observed. Each of the two main classes of PL, i.e. PE and PC, of the different membranes has been analysed separately for its fatty acyl chain content. As shown in tables 2 and 3, the differences between CV (LCV and SCV) and plasma and Golgi membranes are found again, namely in the 20:4 ($n-6$) and the 22:4 ($n-6$) fatty acids. It is worth noting that two polyunsaturated fatty acids are characteristic of Golgi and SCV membranes, namely the 22:5 ($n-6$) in PC and the 22:6 ($n-3$) in PE. The whole adrenal cell lipidic extract, used as a control, evidences a high ratio 22:5 ($n-3$)/22:6 ($n-3$) (table 4) when compared with other cells (Bereziat, G., personal communication).

4. DISCUSSION

The present data have been obtained on homogeneous preparations of coated vesicles from bovine adrenal cortex, which after purification and homogeneity control by electron microscopy, have been separated in two different types according to their size and density: the large CV and the small CV.

The characterization of each type has been undertaken [2] and the lipid analysis was made in order to get further insights on their membrane origin, comparatively with preparations enriched in plasma membranes and Golgi complex membranes from the same adrenocortical cells. A very interesting result emerges from the present data: there is a significant difference between the polyunsaturated fatty acid content of both types of CV on the one hand and of plasma and Golgi membranes on the other. This difference is clear for the 20:4 arachidonic acid, the 22:4 adrenic acid and the 22:5 ($n-3$) fatty acid.

Such a difference between CV and the cell membranes in the polyunsaturated fatty acyl chain content, could account, at least in part, for changes in the lateral diffusion coefficient observed [1,2] in the CV compared to plasma membranes lipid

Table 2

Fatty acid content of PE from the different adrenal cell membranes

Fatty acid	Plasma membrane	Golgi membrane	LCV membrane	SCV membrane
14:0	0.35 ± 0.15	0.2 ± 0.1	–	0.3 ± 0.1
ald. 16	1.30 ± 0.14	1.5 ± 0.9	1.5 ± 1.5	2.6 ± 0.4
16:0	5.55 ± 0.05	5.2 ± 0.4	4.3 ± 2.5	5.1 ± 1.1
16:1	0.55 ± 0.05	0.6 ± 0.1	0.7 ± 0.5	0.4 ± 0.2
ald. 18	1.90 ± 0.4	2.9 ± 1.5	2.3 ± 1.6	3.0 ± 0.9
18:0	23.5 ± 1.5	22.3 ± 1.8	24.7 ± 0.8	19.3 ± 0.7
18:1*n* – 9	10.0 ± 0.8	9.6 ± 1.3	7.1 ± 0.7	7.0 ± 0.1
18:1*n* – 7	2.4 ± 0	2.5 ± 0.4	1.9 ± 0.4	2.2 ± 0.3
18:2*n* – 6	3.5 ± 0	2.8 ± 0.05	2.6 ± 0.7	2.6 ± 0.2
20:3*n* – 9	0.8 ± 0.8	0.8 ± 0	–	0.7 ± 0.2
20:3*n* – 6	1.25 ± 0.25	1.2 ± 0.1	1.0 ± 0.2	1.0 ± 0
20:4*n* – 6	30.3 ± 1.3	28.8 ± 0.1	32.2 ± 1.9	35.3 ± 1.8
20:5*n* – 3	8.5 ± 0.4	8.8 ± 0.4	6.3 ± 0.1	7.1 ± 0.4
22:4*n* – 6	0.95 ± 0.05	1.2 ± 0.1	2.1 ± 0.8	1.5 ± 0.2
22:5*n* – 3	9.6 ± 0.5	10.5 ± 0.25	13.7 ± 3.8	10.5 ± 0
22:6*n* – 3	–	1.4 ± 0.9	–	1.7 ± 1.4
Total no. of unsatur.	240 ± 9	249 ± 3	255 ± 38	265 ± 3

Table 3

Fatty acid content of PC from the different adrenal cell membranes

Fatty acid	Plasma membrane	Golgi membrane	LCV membrane	SCV membrane
14:0	0.2 ± 0.14	0.4 ± 0.1	0.25 ± 0.05	0.15 ± 0.05
16:0	18.9 ± 0.15	19.2 ± 0.4	19.6 ± 0.7	17.5 ± 3.7
16:1	0.7 ± 0.3	0.8 ± 0.1	0.8 ± 0.2	0.4 ± 0.3
18:0	19.6 ± 0.25	18.4 ± 2.5	16.6 ± 1.9	20.3 ± 5.0
18:1*n* – 9	21.6 ± 1.6	23.7 ± 1.5	20.1 ± 2.4	20.7 ± 2.3
18:1*n* – 7	3.5 ± 0.7	4.1 ± 0.8	3.7 ± 0.9	3.5 ± 0.7
18:2*n* – 6	8.0 ± 0.8	6.6 ± 0.9	9.3 ± 1.2	8.3 ± 1.9
18:3*n* – 3	0.8 ± 0.2	1.0 ± 0.3	0.9 ± 0.1	0.6 ± 0.05
20:3*n* – 9	1.1 ± 0.3	0.5 ± 0.5	0.9 ± 0.4	0.7 ± 0.2
20:3*n* – 6	1.9 ± 0.5	2.2 ± 0.3	1.7 ± 0.1	1.7 ± 0.1
20:4*n* – 6	16.7 ± 0.8	13.9 ± 0.6	18.2 ± 3.0	17.5 ± 2.5
20:5*n* – 3	3.9 ± 0.4	4.2 ± 0.4	4.0 ± 0.4	3.4 ± 0.1
22:4*n* – 6	0.3 ± 0.05	–	0.4 ± 0.0	0.2 ± 0.2
22:5*n* – 6	–	2.5 ± 0.1	–	3.2 ± 0.1
22:5*n* – 3	2.6 ± 0.5	2.9 ± 0.7	3.4 ± 0.2	2.2 ± 0.1
22:6*n* – 3	0.2 ± 0.1	–	0.2 ± 0.0	–
Total no. of unsatur.	155 ± 6	156 ± 6	166 ± 9	164 ± 6

Table 4

Fatty acid content of the lipidic extract from the whole adrenal cell

Fatty acid	Adrenal cell
14:0	0.4
ald. 16	0.3
16:0	14.4
16:1	0.6
ald. 18	1.1
18:0	17.0
18:1$n-9$	13.9
18:1$n-7$	4.5
18:2	13.1
18:3$n-3$	0.9
20:3$n-9$	0.6
20:3$n-6$	1.7
20:4$n-6$	20.6
20:5$n-3$	2.8
22:4$n-6$	1.2
22:5$n-6$	–
22:5$n-3$	6.9
22:6$n-3$	0.1
Total no. of unsatur.	191

bilayer. However, it is only when analysing separately all detectable fatty acids from the two main classes of PL namely PE and PC, that interesting features can be demonstrated. Both PE and PC are enriched in polyunsaturated fatty acids. However, the total increase in unsaturation in CV phospholipids compared with cell membranes (~20%) cannot be accounted for only by its increase in PE and PC in CV. Therefore, the observed segregation of PE in coated pits and CV could be the required feature which can explain the difference [2]. It can be pointed out that the PE is generally known, in all types of cells, as esterified mainly by arachidonic and adrenic acids [9].

Other interesting data emerge from the fatty acid analysis, i.e. the presence common to SCV and Golgi membranes of the 22:5 ($n-6$) acid in PC and the 22:6 ($n-3$) acid in PE. It is the unique data obtained from the lipid analysis which could indicate that SCV originate from Golgi stacks.

It should be remembered that adrenal mitochondria, which are adrenal organelles rich in PE and plasmalogenes [10], are isolated from cell extract by low speed centrifugation, (10000 × g) and therefore are not likely to contaminate the CV preparation obtained after repeated high and low speed centrifugations (105000 × g and 10000 × g), and density gradients. Therefore, the significant increase of PE with unsaturated fatty acyl chains in CV cannot arise from contaminating mitochondria which could be moreover easily detected by the electron microscopy controls performed on each preparation.

The present data demonstrate that at the level of the plasma membrane, not only proteins but also phospholipids with a high content in polyunsaturated fatty acyl chains are selected and clustered in the coated pits. Their internalisation and recycling through coated vesicles leads to a specific distribution among the different compartment membranes.

McGookey et al. [11] have described the detection of cholesterol in membranes derived from coated vesicles by filipin technique. This author suggests that coated membranes could be enriched in cholesterol due to a rapid movement of lipids from the membrane into endocytic vesicles. Such a problem was also discussed in our previous paper [1]. More recently, Pagano and Sleight [12] reviewing this problem of lipid biology in the cell, enumerate three general mechanisms for lipid movement. The role of lateral diffusion and vesicle budding and fusion, the transport of molecules from one organelle to another is emphasized and further supported by the present results.

The question which arises is whether the segregation of phospholipids plays a role in the formation of coated vesicles by inducing an asymmetry in the lipid bilayer and on the lipid distribution among the cell organelles, or if there is a relationship between steroidogenesis and phospholipid content of the CV membranes. In the adrenal cells the hypothesis of a specific phospholipid metabolism is supported by the high value of the 22:5 ($n-3$)/22:6 ($n-3$) ratio. Moreover, a characteristic distribution of phospholipids in the coated vesicles could be related to the fact that both phospholipid metabolism and steroidogenesis are under ACTH control for its acute stimulating effect [4,13]. The possible relation between the specific polyunsaturated fatty acids, namely arachidonic acid, segregated in the CV and the prostaglandin biosynthesis which could be initiated

at their level is currently under study in the laboratory.

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